University of Dundee

University of Dundee

DOCTOR OF PHILOSOPHY

Targeting Opioid Receptor Signal Transduction to Produce Sustained Analgesia

Bull, Fiona A.

Award date:
2015

Awarding institution:
University of Dundee

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 20. Apr. 2025

Targeting Opioid Receptor Signal Transduction to Produce Sustained Analgesia

Fiona A Bull

BSc(Hons), MBChB, FRCA

Thesis submitted in candidature for

the degree of Doctor of Philosophy,

University of Dundee, November 2015.

Table of Contents

List of Tables ---------- vi

List of Figures ---------- vii

Abbreviations ---------- xii

Acknowledgements ---------- xvii

Candidates declaration ---------- xix

Supervisors Declaration ---------- xx

Abstract ---------- xxi

Chapter 1: Introduction ---------- 1

1.1 Opium ---------- 2

1.2 Opioid receptors ---------- 8

1.3 Constitutive activity ---------- 11

1.4 The endogenous opioid system ---------- 13

1.5 The genetic manipulation of the opioid receptor system ---------- 16

1.6 MOP knockout mice ---------- 17

1.7 DOP receptor knockout mice ---------- 19

1.8 KOP receptor knockout mice ---------- 20

1.9 Structural models of the opioid receptors ---------- 20

1.10 The arrestin system ---------- 22

1.11 Beta-arrestin2 knockout mice ---------- 25

1.12 Pain and nociception ---------- 26

1.13 Genetic influences on rodent nociceptive responses ---------- 30

1.14 Tolerance---31

1.15 The RAVE hypothesis---34

1.16 Opioid receptor desensitisation in tolerance---36

1.17 The role of DOP receptors and BAR2 in tolerance---37

1.18 Pharmacokinetics of morphine in rodents---38

1.19 Mesocorticolimbic reward pathway---38

1.20 Psychomotor effects of morphine---43

1.20.1 Locomotor activation---43

1.20.2 Reinforcement---45

1.21 c-Src and the opioid receptors---46

1.22 Aims and Hypotheses---49

Chapter 2: Methods---52

2.1 Breeding Colony Maintenance and Housing---53

2.2 Genotyping---54

2.3 Behavioural Studies---59

2.3.1 Assessing morphine analgesia---59

2.3.2 Morphine tolerance---60

2.3.3 Psychomotor testing: Locomotor activation and CPP---61

2.4 Dorsal Root Ganglion neurone culture preparation---64

2.5 Immunohistochemistry---66

2.6 Slice Electrophysiology---68

2.7 SW620 cell culture---71

2.8 Western Blotting---71

2.9 Statistical Analysis ---73

Chapter 3: Analgesia and Tolerance ---74

3.1 Introduction---75

3.2 Analgesia---79

3.2.1 The role of MOP receptors ---79

3.2.2 The Role of DOP receptors ---83

3.2.3 The role of β-arrestin2 --85

3.3 Tolerance --89

3.3.1 The role of MOP receptors ---89

3.3.2 A role for DOP receptors in morphine tolerance --------------------------92

3.3.3 A role BAR2 in morphine tolerance--94

3.4 Summary---97

Chapter 4: Psychomotor effects of morphine--------------------------------------- 103

4.1 Introduction-- 104

4.2 Locomotor effects of morphine --- 108

4.2.1 The role of MOP receptors -- 108

4.2.2 The role of DOP receptors -- 115

4.2.3 The role of β-arrestin2 --- 119

4.3 Conditioned place preference -- 125

4.3.1 The role of MOP receptors in reinforcement------------------------------ 125

4.3.2 The role of DOP receptors in reinforcement------------------------------- 130

4.3.3 The role of β-arrestin2 in reinforcement ---------------------------------- 133

4.4 Summary-- 138

Chapter 5: Opioid receptor signalling within the VTA ------------------------------ 144

5.1 Introduction -- 145

5.2 VTA cell type identification -- 150

5.3 The influence of opioid drugs on IPSC events within the VTA -------------- 154

5.4 DOP receptors within the VTA -- 163

5.5 The role of β-arrestin2 in signalling within the VTA--------------------------- 167

5.6 Effects of naloxone on sIPSC frequency within the VTA --------------------- 171

5.7 Summary -- 174

Chapter 6: The effects of c-Src on opioid receptor signalling --------------------- 181

6.1 Introduction -- 182

6.2 PP2 and Dasatinib inhibit c-Src *in vitro* and *in vivo*---------------------------- 184

6.3 The role of c-Src in morphine tolerance -- 187

6.4 The effects of c-Src inhibition on locomotor activation by morphine ---- 194

6.5 The effects of c-Src inhibition on conditioned place preference to morphine -- 200

6.6 The effects of c-Src inhibition on sIPSC frequency in the VTA -------------- 203

6.7 The effects of MEK inhibition on sIPSC frequency within the VTA -------- 206

6.8 Summary -- 209

Chapter 7: Discussion and Conclusions --- 212

7.1 Discussion --- 213

7.2 Analgesia and Tolerance -- 218

7.2.1 MOP receptors in analgesia and tolerance -------------------------------- 218

7.2.2 DOP receptors in analgesia and tolerance -------------------------------- 221

7.2.3 The role of BAR2 in analgesia and tolerance------------------------------ 223

7.2.4 The effects of c-Src inhibition within the pain pathway ---------------- 224

7.2.5 Summary analgesia and tolerance --- 226

7.3 Genetic polymorphisms and opioid activity ------------------------------------ 231

7.4 The psychomotor effects of morphine--- 233

7.4.1 The role of MOP receptors --- 233

7.4.2 DOP receptors in the psychomotor effects of morphine---------------- 239

7.4.3 The role of BAR2 in the psychomotor effects of morphine------------- 240

7.4.4 The role of DOP receptors and BAR2 in VTA signalling ----------------- 243

7.4.5 The effects of c-Src inhibition on the psychomotor effects of morphine

--- 246

7.5 Conclusions-- 249

References --- 251

List of Tables

Table 1.1: The differing selectivity's of the endogenous and exogenous opioid receptor agonists.-- 8

Table 2.1: The PCR master-mix recipe for each of the mouse genotypes.---------57

Table 2.2: The forward, reverse and middle primer sequences required for genotyping each genetically modified mouse line. -------------------------------------57

Table 2.3: The PCR thermo-cycler conditions --58

Table 3.1: Summary of morphine ED_{50}--- 102

Table 5.1: Characteristics of action potentials recorded from spontaneously firing WT VTA neurones in current clamp mode. -- 153

Table 5.2: TTX does not significantly affect IPSC parameters within the VTA.-- 159

List of Figures

Figure 1.1: Opioid receptor signalling. ---11

Figure 1.2: The dopaminergic component of the mesocorticolimbic reward pathway. ---42

Figure 1.3: Signalling within the mesocorticolimbic reward pathway. -------------42

Figure 1.4: The involvement of BAR2, c-Src and MOP and DOP receptors in analgesia, tolerance and reinforcement. --51

Figure 2.1: CPP apparatus. ---64

Figure 3.1: MOP receptors are required for morphine analgesia and inhibition of VACCs in DRG neurones. --82

Figure 3.2: Morphine is less potent as an analgesic in DOP-/- mice. ----------------84

Figure 3.3: A lack of BAR2 has no significant effect on the dose-response relationship for morphine analgesia but causes basal analgesia. --------------------87

Figure 3.4: Mice that lack DOP receptors and BAR2 exhibit basal analgesia. -----88

Figure 3.5: Development of morphine tolerance after repeated daily dosing in mice. --91

Figure 3.6: Mice lacking DOP receptors exhibit reduced morphine analgesic tolerance. ---93

Figure 3.7: BAR2+/- and BAR2-/- mice exhibit reduced tolerance to morphine analgesia. --95

Figure 3.8: BAR2-/-//DOP-/- mice do not become tolerant to morphine. ---------96

Figure 3.9: The roles of MOP and DOP receptors and BAR2 in basal analgesia and the development of morphine tolerance.-- 102

Figure 4.1: Morphine causes a dose dependent locomotor activation in WT mice. -- 111

Figure 4.2: MOP-/- mice do not exhibit locomotor activation or sensitisation to morphine.-- 112

Figure 4.3: MOP+/- mice show a significant dose-dependent locomotor activation by morphine without sensitisation.--- 113

Figure 4.4: Locomotor activation by morphine is dependent on the MOP receptors.-- 114

Figure 4.5: DOP-/- mice show significant locomotor activation following both 3 mg/kg and 10 mg/Kg morphine.--- 117

Figure 4.6: The absence of DOP receptors does not affect locomotor activation following morphine 10 mg/Kg. --- 118

Figure 4.7: BAR2-/- mice exhibit locomotor activation by morphine. ------------ 122

Figure 4.8: BAR2-/-//DOP-/- mice exhibit locomotor activation following morphine administration.--- 123

Figure 4.9: Removing β-arrestin2 significantly reduced locomotor activation following morphine 10 mg/Kg administration.--- 124

Figure 4.10: MOP receptors are required for the reinforcing effect of morphine. -- 128

Figure 4.11: Morphine has a lower potency for reinforcement in MOP+/- mice. -- 129

Figure 4.12: DOP receptors are not required for morphine reinforcement. ---- 132

Figure 4.13: BAR2-/- mice exhibit increased sensitivity to morphine reinforcement.-- 136

Figure 4.14: BAR2-/-//DOP-/- mice exhibit morphine reinforcement.------------ 137

Figure 5.1: The identification of dopaminergic cells within the VTA. ------------- 151

Figure 5.2: Current clamp recordings from WT VTA neurones in the presence of morphine (10 µM) and naloxone (10 µM).-- 153

Figure 5.3: sIPSC events within the VTA are $GABA_A$ receptor mediated.--------- 158

Figure 5.4: Morphine inhibits sIPSC activity in a concentration dependent manner in WT neurones.-- 158

Figure 5.5: TTX does not affect IPSC frequency or inhibition by morphine. ----- 159

Figure 5.6: Morphine inhibits sIPSCs in a concentration dependent manner in WT and MOP+/- neurones; it does not cause significant inhibition in MOP-/- neurones. --- 160

Figure 5.7: Addition of the $5\text{-}HT_3$ receptor antagonist ondansetron results in inhibition of sIPSC frequency within the VTA.-- 161

Figure 5.8: DAMGO inhibits sIPSC frequency in a concentration dependent manner in WT neurones within the VTA.-- 162

Figure 5.9: DOP receptors are functional and can signal independently of MOP receptors within the VTA. -- 164

Figure 5.10: The absence of DOP receptors in the VTA significantly reduces the ability of morphine to inhibit sIPSC frequency. --------------------------------------- 165

Figure 5.11: DOP receptors are not involved in signalling within the VTA in response to DAMGO. -- 166

Figure 5.12: The absence of BAR2 reduces inhibition of the frequency of sIPSC events in the VTA by opioid drugs.--- 169

Figure 5.13: BAR2 and the DOP receptor are involved in the response of VTA neurones to morphine. -- 170

Figure 5.14: Deletion of MOP receptors, DOP receptors or BAR2 does not affect the sIPSC frequency in the absence of drug. -- 170

Figure 5.15: Naloxone causes an inhibition of IPSC frequency that is independent of MOP receptors. -- 173

Figure 5.16: Naloxone does not cause significant inhibition of sIPSC events in DOP-/-, BAR2-/- or BAR2-/-//DOP-/- neurones.--------------------------------------- 173

Figure 6.1: PP2 and dasatinib inhibit the phosphorylation of c-Src.--------------- 186

Figure 6.2: The c-Src inhibitor dasatinib inhibits the development of morphine tolerance in WT mice.--- 192

Figure 6.3: c-Src inhibition inhibits morphine tolerance in MOP+/- mice. ------- 193

Figure 6.4: Dasatinib has no consistent effect on locomotor activation by morphine.--- 198

Figure 6.5: Dasatinib has no direct locomotor effect and does not differ from vehicle in its effect of locomotor stimulation by morphine. ------------------------ 199

Figure 6.6: Dasatinib does not affect morphine conditioned place preference. 202

Figure 6.7: PP2 significantly reduces the inhibition of sIPSC frequency in VTA neurones by morphine.-- 205

Figure 6.8: MEK inhibition by SL327 has no significant effect on morphine inhibition of sIPSC frequency within the VTA.-- 208

Figure 7.1: The role of MOP and DOP receptors, BAR2 and c-Src in opioid receptor signalling in the pain and reward pathway.--------------------------------- 217

Abbreviations

5-HT	5 hydroxytryptamine
7TM	seven transmembrane spanning receptors
aCSF	artificial cerebrospinal fluid
AKT	also called protein kinase B
ANOVA	analysis of variance statistical test
AP	action potential
ATCC	American type culture collection
ATP	adenosine triphosphate
BARs	beta-arrestins
BAR1	beta-arrestin1
BAR2	beta-arrestin2
BBB	blood brain barrier
BCA	bicinchoninic acid
Bend	beta-endorphin
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
C_{brain}	brain concentration
CFA	complete Freund's adjuvant
C_{max}	maximum concentration
CNS	central nervous system
CPA	conditioned place aversion
CPP	conditioned place preference
CREB	cAMP response element binding protein
c-Src	Proto-oncogene tyrosine-protein kinase Src
DA	dopamine
DAMGO	[D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin

DAR	dopamine receptor
DAS	dasatinib
DH	dorsal horn
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOP	delta opioid receptor
DPDPE	[D-Pen^2, D-Pen^5] enkephalin
DRG	dorsal root ganglion
EC_{50}	concentration of drug that gives a half maximum response
ECS	extracellular fluid
ED_{50}	median effective dose
EPSC	excitatory postsynaptic current
ERK	extracellular signal related kinase
FBS	fetal bovine serum
GABA	γ-Aminobutyric acid
GAD	glutamate decarboxylase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanine diphosphate
GFP	green fluorescent protein
GH_3	rat pituitary cell line
GIRKs	g protein activated inwardly rectifying potassium channels
GPCRs	g protein coupled receptors
GRKs	g protein coupled receptor kinases
GTP	guanine triphosphate
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hsp	heat shock protein

IASP	International Association for the Study of Pain
ICD-10	International Statistical Classification of Diseases and Related Health Problems 10th edition
ID	inner diameter
I_h	hyperpolarisation current
IHC	immunohistochemistry
IP	intraperitoneal
IPSCs	inhibitory postsynaptic currents
IUPHR	International union of basic and clinical pharmacology
JAX	Jackson laboratories
JNK	c-Jun N terminal Kinase
K_d	dissociation constant
KO	knockout
KOP	kappa opioid receptor
M6G	morphine 6 glucuronide
M3G	morphine 3 glucuronide
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
mIPSC	miniature postsynaptic current
MOP	mu opioid receptor
MOPS	3-(*N*-morpholino)propanesulfonic acid
MPE	maximum possible effect
mRNA	messenger ribonucleic acid
NA	nucleus accumbens
NGF	nerve growth factor
NG108-15	neuroblastoma/ glioma cell line
NOP	nociceptin/orphanin receptor
N-Src	neuronal c-Src
OD	outer diameter

Oprd	gene encoding the delta opioid receptor
Oprk	gene encoding the kappa opioid receptor
Oprm	gene encoding the mu opioid receptor
ORL-1	opioid receptor like receptor
PAG	periaqueductal gray
PB	phosphate buffer
PBP	parabrachial pigmented area
PBT	phosphate buffer with triton x
PCR	polymerase chain reaction
Pc-Src	phosphorylated c-Src
PDGFRβ	platelet derived growth factor receptor beta
PDYN	prodynorphin
PENK	proenkephalin
PFA	paraformaldehyde
PFC	prefrontal cortex
PKA	protein kinase A
PKC	protein kinase C
PN	postnatal day
POMC	pro-opiomelanocorntin
PP2	3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine
PP3	1-Phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine
RAVE	relative activity versus endocytosis
R_{input}	input resistance
RIPA	Radioimmunoprecipitation assay buffer
RMTg	rostromedial tegmental nucleus
SC	subcutaneous
SDS	sodium dodecyl sulfate
SEM	standard error of the mean

SFKs	Src family non-receptor tyrosine kinases
sIPSC	spontaneous inhibitory postsynaptic current
SL327	α-[Amino[(4-aminophenyl)thio]methylene]-2-(trifluoromethyl)benzeneacetonitrile
SNc	substantia nigra pars compacta
SNC-80	(+)-4-[(α*R*)-α-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide
SNP	single nucleotide polymorphism
SW620	Human metastatic colon cancer cells
T70	time taken for IPSC to decay by 70%
TAE	buffer solution containing a mixture of Tris base, acetic acid and EDTA
TAT	transactivator of transcription
TH	tyrosine hydroxylase
Th-/-	dopamine deficient mice
THC	delta9-tetrahydrocannabinol
TTX	tetrodotoxin
Tω	weighted decay time constant
UV	ultraviolet light
VACCs	voltage activated calcium channels
VGlut2	vesicular glutamate transporter 2
VP	ventral pallidum
VTA	ventral tegmental area
WT	wild type C57Bl/6 mice

Acknowledgements

I would like to acknowledge and thank a number of people who have encouraged, assisted and supported me over the last few years. There have been a lot of people involved in this project and I have been very encouraged and hugely grateful to the people who have taught me and supported me through this process. Firstly, Tim Hales my supervisor and the members of his lab. Without Tim and the help of Lisa Wright, Daniel Baptista-Hon, Mike Gallagher and Sharon King many of the experiments would not have been done. Thank you all for your patience. Thank you to Professor Jerry Lambert, Dr Delia Belelli and Dr Ed Maguire for all of your help and advice but particularly with the slice electrophysiology technique. Thank you to Dr Janet Lowe who travelled from University of Bristol and who was very encouraging in the early days of my recording in the VTA. The advice of Professor John Peters and Professor Keith Matthews resulting from thesis monitoring committee discussions has also been invaluable.

The transgenic mice were very kindly transferred to us by Dr Wendy Walwyn, without whom it would not have been possible to complete this work. Thank you also for accepting me into your lab at UCLA and teaching me how to perform conditioned place preference. The set-up for these experiments in Dundee has involved Dr Stephen Martin and Dr Lianne Strachan at the behavioural neuroscience core facility. I am also very grateful to the staff at the MSRU who have provided ongoing advice and support throughout the project with regards

to the management of the animal colony. We have also utilised the imaging core facility at the medical school, thank you to Dr Alison Milne for all the help and support with the confocal imaging. I would also like to thank everyone at the department of anaesthesia in Ninewells Hospital. Thank you for all of your support, without which it would not have been possible to consider embarking on this journey never mind complete it. With special thanks to Dr Cameron Weir, Dr William McClymont and Dr Pamela Johnston. I would also like to thank Professor Sara Marshall for all of her help and support, which has been very much appreciated. As a clinician contemplating time out of training to complete a PhD the advice and support of Sara, Professor Doreen Cantrell and Tim has been invaluable. This work has been funded by the Wellcome Trust and taken place at the University of Dundee. I am very grateful to both organisations for their continued support. Thank you also to all of the support staff within the department who have answered questions, assisted and supported me over the last few years. Last but definitely not least, thank you to all of my friends and family, without your continued encouragement and support this would not have been possible. Thank you especially to my now husband, Matthew, you were not involved in the initial decision for me to start this process. Despite that you have continually supported and encouraged me, I could not have completed this work without you. This time has involved continuous learning of both new information and techniques, it has been very demanding but hugely enjoyable and I am very much looking forward to the challenges that are ahead.

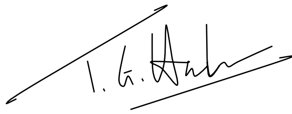
Candidates declaration

I declare that I am the author of this thesis, and that it is a true record of the work performed by myself. This thesis has not previously been submitted in application for a higher degree. All of the references used in the preparation of this thesis have been consulted and are cited appropriately. This work was carried out in the Division of Neuroscience and the Institute of Academic Anaesthesia at the University of Dundee, supervised by Professor Tim Hales and funded by the Wellcome Trust.

Fiona A Bull, BSc(Hons), MBChB, FRCA

Supervisors Declaration

I certify that Fiona A Bull has completed nine terms of experimental research and that she has fulfilled the conditions of Ordinance 39, University of Dundee, such that she is eligible to submit the following thesis in application for the degree of Doctor of Philosophy.



Professor Tim G. Hales, BSc (Hons), PhD, FRCA (Elect)

Abstract

Mu opioid receptors (MOPs) in the pain pathway contribute to morphine analgesia. Morphine also stimulates reward/reinforcement through disinhibition of dopaminergic (DA) neurones in the ventral tegmental area (VTA), an effect implicated in its abuse and dependence. We hope to develop approaches to achieve sustained analgesia without affecting reward by exploiting differential MOP signalling mechanisms in the pain and reward pathways. MOPs, delta opioid receptors (DOPs) and β-arrestin2 (BAR2) are all necessary components of the signalling complex in nociceptive neurones for morphine analgesic tolerance; c-Src (a tyrosine kinase), thought to couple to MOP receptors through BAR2 has also been implicated.

To investigate opioid receptor signalling in response to morphine we used a variety of different techniques that included behavioural measures of nociception, reinforcement and locomotion and electrophysiological methods to study DRG neurones from the pain pathway and brain slices containing VTA neurones.

This study in mice confirms that morphine administered subcutaneously (SC) causes analgesia, analgesic tolerance, and has psychomotor effects leading to enhanced locomotion and reinforcement. In VTA neurones morphine and the selective MOP receptor agonist DAMGO caused concentration-dependent inhibition of the frequency of IPSCs. All these actions of morphine were absent

from MOP-/- mice. Morphine exhibited reduced potency as 1) an analgesic, 2) stimulator of locomotion, 3) a reinforcer in CPP and 4) an inhibitor of sIPSC frequency, when applied to MOP+/- mice or their VTA neurones. Morphine analgesic tolerance developed faster and to a greater extent in MOP+/- mice than in WT mice. DOP-/- mice exhibited morphine analgesia with less tolerance, as did BAR2-/- mice. BAR2-/- mice also exhibited reduced morphine locomotion and an increased sensitivity to morphine reinforcement. Morphine tolerance was absent from BAR2-/-//DOP-/- mice. The inhibition of sIPSC frequency by morphine was reduced in BAR2+/- and BAR2-/- VTA neurones. Dasatinib and PP2 (c-Src tyrosine kinase inhibitors) prevented the development of morphine tolerance in WT and MOP+/- mice and dasatinib caused its reversal in the latter. The drugs had no significant analgesic effect alone. Dasatinib did not affect morphine preference or locomotor activation. PP2 reduced morphine's inhibition of sIPSC frequency.

As c-Src inhibition does not appear to alter the psychomotor effects produced by morphine and it acts to reduce morphine analgesic tolerance. We believe that c-Src is an attractive target to prevent the development of morphine analgesic tolerance without affecting hedonic homeostasis.

Chapter 1: Introduction

1.1 Opium

Opioid drugs have been exploited for centuries for both their analgesic and hedonistic effects. The poppy plant was cultivated in the ancient civilisations of Persia, Egypt, Greece and Mesopotamia. Opium is an extract from the poppy plant *Papavera Somniferum*, which is also known as the white poppy. Although there are many varieties of poppy there are very few from which active alkaloid compounds can be harvested. There have been findings of fossilized opium poppy seeds dating from 30,000 years ago suggesting that these plants were used by Neanderthal man (Dormandy, 2012).

The first known written reference to the opium poppy appears in a Sumerian text dating from approximately 4,000 BC, the Sumerians called the flower *hul gil*, 'the plant of joy' (Brownstein, 1993). The first written record of its use does not occur until much later when in the second century Galen, a prominent Greek physician described some of what he considered to be its medical indications (Waldhoer *et al*., 2004). He wrote that opium: "*...resists poison and venomous bites, cures chronic headache, vertigo, deafness, epilepsy, apoplexy, dimness of sight, loss of voice, asthma, coughs of all kinds, spitting of blood, tightness of breath, colic, the lilac poison, jaundice, hardness of the spleen stone, urinary complaints, fever, dropsies, leprosies, the trouble to which women are subject, melancholy and all pestilences*" (Braithwaite, 2007).

In the western world use of opium was not widespread until the 17th century. The drug became much more available as trade increased with Asian countries and the so called opium wars. The discovery that the potency of the drug was increased when it was extracted using alcohol instead of water by Paracelsus in the 16th century led to the development of laudanum (Jay, 2012). Thomas Sydenham a medical practitioner in 17th century England further adjusted and standardised this formula but kept the name laudanum (Van Ree *et al*., 1999). He wrote that; *"Among the remedies which it has pleased Almighty God to give to man to relieve his sufferings, none is so universal and so efficacious as opium"* (Braithwaite, 2007).

By the nineteenth century laudanum and other opium containing products were freely available from many outlets in England and the rest of Europe (Obladen, 2015). Opium and laudanum were viewed by the majority as medicine and not as drugs of abuse; there is very little documentation of harm associated with their use at this time (Dormandy, 2012). There were many well-known people that were consumers of the drug both within the general population and also the medical and scientific circles of the time. Several famous writers are known to have consumed the drug to varying degrees, including Samuel Coleridge, Sir Walter Scott and John Keats (Ober, 1968). One of the most well-known accounts of the effects of opium was published by Thomas De Quincey in 1821/2. He had previously been secretary to Samuel Coleridge and the publication was titled 'Confessions of an English opium eater' (Jay, 2012). There were areas of the

country where use of the drug in varying forms was particularly high, these included Lincolnshire, Cambridgeshire and Norfolk (Berridge, 1979).

Papavera Somniferum grows wild in areas ranging from Southern Europe and the Far East to the USA. The largest quantities come from three areas of the world: the 'Golden Triangle' (Laos, Burma, Thailand), the 'Golden Crescent' (Afghanistan, Pakistan, Iran), and Mexico (Dormandy, 2012). Opium is collected from the capsules that remain following the flowering of the plant. To do this shallow incisions are made into the wall of the capsule after which a milky substance called latex oozes out and it is this that is collected as it contains the active opiate alkaloid compounds (Schiff, 2002).

Opium resin, formed when the latex dries, contains a very complex chemical cocktail with inactive compounds and numerous active alkaloids, most notably morphine (8% - 17%), codeine (0.7% - 5%), noscapine (1% - 10%), papaverine (0.5% - 1.5%), and thebaine (0.1% - 2.5%) (Schiff, 2002). All of the latter, apart from thebaine, are or have been used for a long time medicinally as analgesics and to treat diarrhoea. Thebaine is now used as a precursor in the synthesis of several drugs including oxycodone, naloxone, naltrexone and buprenorphine (Pasternak, 2014a). In 1806 the German chemist Sertürner was the first to isolate the opium alkaloids, one of which he called morphine after Morpheus the Greek god of dreams (Schiff, 2002).

At this time the long-term consequences of opium use were becoming more noted as a public health issue due to the rapidly escalating use. Between 1825 and 1850 imports to Britain grew from 23,300 to 138,000 kilos per year (Dormandy, 2012). This was the start of the search that is still ongoing today for a non-addictive alternative to opium and morphine that still provides analgesia for severe pain. In 1874, an English pharmacist called C.R. Alder Wright produced diacetylmorphine; at the time that this finding was published its importance was not recognised. However the German pharmaceutical company, Bayer, went on to successfully repeat this experiment in the late 1800's and began to administer the compound first to animals and then people. They named the drug heroin (diamorphine) and it was marketed as a non-addictive and more powerful painkiller than morphine (Koob *et al*., 2014). The drug was very well received and sales swiftly increased. However, we now know that diamorphine is a prodrug that requires deacetylation to morphine to produce any significant effect (Way *et al*., 1960). It became increasingly apparent that while diamorphine was indeed a very potent analgesic, it was not free from the addictive side effects of morphine. Increasing public awareness of the problems associated with the use of these drugs and the associated morbidity and mortality led to the controls placed on their sale, distribution and use in the early twentieth century.

Despite these controls there are ongoing issues with illicit drug use and escalating legal prescriptions for opioid containing drugs. In parallel to the increasing use of opioid containing drugs is the rise in drug related deaths. In 2014 there were 613 deaths in Scotland related directly to drug use, this

represents a 16% rise on 2013 and a 72% rise since 2004 (Scotland, 2015). While these are figures for Scotland a similar pattern has been observed both in England and the USA (Kuo *et al*., 2015, Weisberg *et al*., 2014).

Opioid receptor agonists can be grouped into three categories, the first of these is the opiates, this group encompasses the opium alkaloids for example morphine, codeine and thebaine, the second is the opioids. This group includes the semi-synthetic opioids (for example diamorphine, oxycodone and etorphine) and the synthetic opioids methadone and fentanyl. The third group is that of the endogenous opioid peptides, these are peptides that are naturally occurring within the brain and the rest of the nervous system (Waldhoer *et al*., 2004). These and the rest of the opioid receptor agonists display differing degrees of selectivity for each of the opioid receptors (Table 1.1).

MOP receptor agonists remain the drugs of choice for treating both acute and chronic severe pain, (Stein *et al*., 2000, Trescot *et al*., 2008). As these drugs remain our most effective agents for the treatment of severe pain there is a significant ongoing problem with the side effects that are produced by their use. Their clinical utility continues to be limited by the compromise between efficacy and side effects. Some of the commonly described side effects include constipation, urinary retention, bronchospasm, nausea, sedation, respiratory depression, hypotension, miosis and cough suppression (Rang et al., 2000).

As well as the effects described previously opioid drugs also produce analgesic tolerance. Tolerance is defined as the requirement for an increasing dose of drug to gain the same analgesic effect over time (Way *et al*., 1969, Ossipov *et al*., 2004). They also cause physical dependence and can be addictive. Dependence is a physiological response to the chronic administration of opioid, it is observed in all subjects who are maintained on a chronic opioid regimen. In contrast addiction is defined in ICD-10 as "A cluster of behavioural, cognitive, and physiological phenomena that develops after repeated substance use" (WHO, 2015). Within this definition is included the description of an overwhelming compulsion to take the drug involved, an inability to control the use of the drug despite this being detrimental to their life and relationships, a description of drug use that is placed to be more important than any other events or obligations within life and a tolerance to the pharmacological effects of the drug.

It is now known that the opioid receptors have many different roles encompassing nociception, reward, respiration, cardiovascular function, bowel transit, consumption of food, learning and memory, locomotor activity, thermoregulation, hormone secretion and immune function (Rang *et al*., 2000). The fact that they are involved in so many varied pathways and behaviours suggests that this is a complex system with multiple different outcomes depending on the combination of signalling engaged. I will focus primarily on nociception, reward/ reinforcement and locomotor activity as the study of the effects of opioid drugs on these outcomes will provide useful information about

the signalling pathways involved in the development of the side effects of the drugs.

		MOP	DOP	KOP
Endogenous agonists	Beta-Endorphin	+++	+++	+++
	Met-Enkephalin	++	+++	-
	Leu-Enkephalin	+	+++	-
	Endomorphin	+++	-	-
	Dynorphin	++	+	+++
Exogenous agonists	Morphine	+++	+	+
	DAMGO	+++	-	-
	Fentanyl	+++	+	-
	Methadone	+++	-	-
	DPDPE	-	++	-
	SNC-80	-	++	-
	Deltorphin	-	++	-
	Salvinorin A	-	-	+++

Table 1.1: The differing selectivity's of the endogenous and exogenous opioid receptor agonists.

1.2 Opioid receptors

Opioid receptors belong to a large superfamily of seven transmembrane spanning (7TM) G protein coupled receptors (GPCRs). This family of receptors is of fundamental physiological importance due to its role in mediating the actions of the majority of known neurotransmitters and hormones (Waldhoer *et al.*, 2004). They can be activated by both exogenously administered agents and endogenously produced compounds. There are three opioid receptors, the mu opioid (MOP) receptor, the delta (DOP) opioid receptor and the kappa (KOP) opioid receptor (nomenclature as per IUPHAR guidelines; (Cox *et al.*, 2015). A

fourth receptor has also been identified that shares sequence homology with the other opioid receptors, it was previously called the opioid receptor like (ORL-1) receptor but this has now been altered and it is called the nociceptin/orphanin (NOP) receptor (Mollereau *et al*., 1994, Cox *et al*., 2015). The endogenous ligand for this receptor was identified soon after its discovery (Meunier *et al*., 1995). Neither the endogenous or exogenous ligands at the MOP, DOP or KOP receptors (Table 1.1) show significant binding activity through this receptor and I am therefore not going to discuss it further (Waldhoer *et al*., 2004).

Opioid receptors belong to the class A (rhodopsin) family of Gi/Go protein coupled receptors. Classically upon activation of the receptor by agonist binding, a conformational change leads to coupling to these heterotrimeric pertussis toxin sensitive proteins (Law *et al*., 2000). Following receptor activation and recruitment of the G protein complex GDP associated with the Gα subunit is replaced for GTP and the G protein complex dissociates into Gα and Gβγ subunits. Both G protein components interact with multiple cellular effector systems including inhibition of adenylyl cyclase and voltage activated Ca^{2+} channels (VACCs), and activation of inwardly rectifying K^+ channels (GIRKs) and phospholipase Cβ (Williams *et al*., 2001). The overall result is a decrease in neuronal cell excitability and a reduction in neurotransmitter release (Figure 1.1). There is a high degree of G protein heterogeneity, with 16 gene products for Gα, 5 for Gβ and 11 for Gγ (Raehal and Bohn, 2011). This allows for multiple different subunit compositions and differing signalling outcomes. There is

evidence that opioid receptors can recruit alternative G proteins other than Gi/Go under certain conditions.

Opioid receptor activation can also lead to the recruitment of beta-arrestin2 (BAR2), a scaffolding protein that interacts with various signal transducers including c-Src, Akt, and MAP kinases (Pierce and Lefkowitz, 2001, Yano *et al*., 2008) (Figure 1.1). BAR2 is recruited to the MOP receptor following phosphorylation by G protein receptor kinases (GRKs). Receptor phosphorylation can also occur through a number of different pathways including second messenger dependent protein kinases such as PKA and PKC (Ferguson, 2001). The BAR2 mediated pathway provides an alternative signalling mechanism that is thought to play a role in receptor desensitisation and endocytosis, in addition to the activation of alternative intracellular signalling pathways as mentioned previously.

The development in the understanding of this alternative signalling pathway that occurs following agonist binding at the opioid receptor and at other 7TMR's has led to the concept of biased agonism or functional selectivity. This concept describes the ability of a ligand to selectively recruit one signalling pathway over another to produce differing intracellular effects (Urban *et al*., 2007, Kelly *et al*., 2008, Kenakin and Miller, 2010).

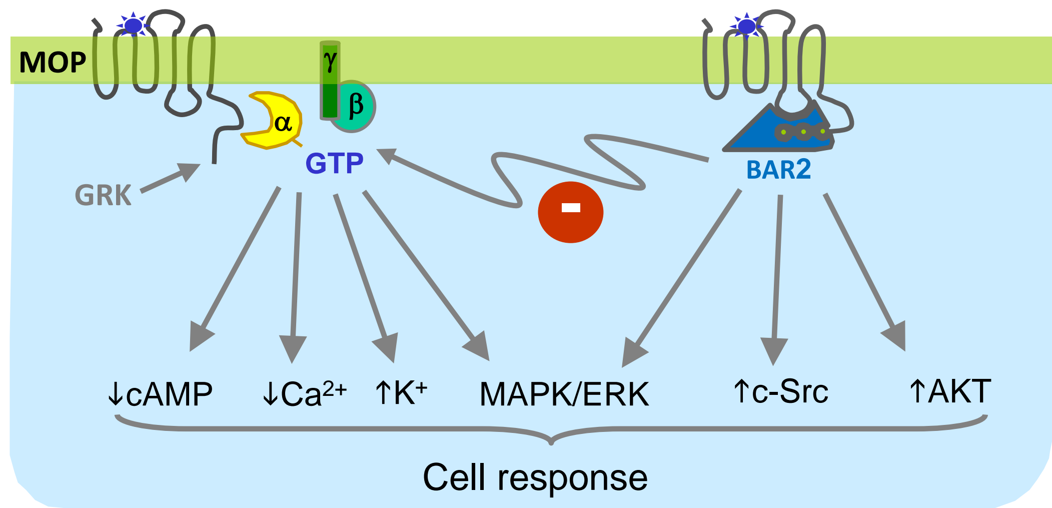


Figure 1.1: Opioid receptor signalling. The classical GPCR signalling pathway activates intracellular G proteins and leads to the inhibition of adenylyl cyclase, inhibition of VACCs, activation of GIRKs and an increase in inwardly rectifying K^+ channels and activation of the MAPK/ ERK signalling pathway. However the MOP receptors can also recruit BAR2 following the binding of agonist leading to a number of different intracellular events.

1.3 Constitutive activity

Costa and Herz (1989) were the first to demonstrate agonist independent constitutive receptor activity in their pioneering study of DOP receptors in the NG108-15 cell line. This led to a change in the way that activation of GPCRs was conceptualised. The existence of spontaneously active receptors gave rise to the idea that agonists stabilise the active state by conformational selection rather than inducing activation as a consequence of binding. A number of investigators subsequently demonstrated spontaneous activity of MOP receptors in various cellular models. The level of constitutive activity of MOP receptors under normal circumstances is thought to be low but it can be demonstrated when the receptors are overexpressed (Burford *et al.*, 2000) or altered by specific mutations (Brillet *et al.*, 2003). The concept of constitutive receptor activity led

to the designation of several ligands, previously considered antagonists, as inverse agonists. These are ligands that preferentially bind to, and stabilise, the inactive receptor conformation.

Since the original report by Costa and Herz (1989), DOP receptor constitutive activity has also been demonstrated in a variety of different cell based models using inverse agonists (Chiu *et al.*, 1996, Merkouris *et al*., 1997, Szekeres and Traynor, 1997). Inverse agonists are useful tools to investigate constitutive activity as when these drugs are added alone to the system they can produce a decrease in basal activity if there is constitutive activity and this action can be competitively inhibited by neutral competitive antagonists (Strange, 2002, Costa and Cotecchia, 2005). The drugs naloxone and naltrexone, long thought to be antagonists are now known to be MOP receptor inverse agonists (Bilsky *et al*., 2010, Liu and Prather, 2001, Liu *et al*., 2001), while 6α-naloxol, 6β-naloxol and 6β-naltrexol (hydroxyl derivatives of naloxone and naltrexone respectively) are neutral competitive antagonists (Wang *et al*., 2001, Wang *et al*., 2004, Raehal *et al*., 2005, Sadee *et al*., 2005).

Long term exposure to opioid drugs such as morphine can produce an increase in constitutive activity in both cell based systems (Wang *et al*., 2001, Burford *et al*., 2000), and in the striatum of morphine dependent mice (Wang *et al*., 2004). Mice that have been chronically treated with morphine also exhibit an increase in naloxone mediated conditioned place aversion and withdrawal behaviour, not

seen with administration of the neutral MOP receptor antagonists (Shoblock and Maidment, 2006). In DRG neurones from BAR2-/- mice it has been demonstrated that there is increased MOP receptor constitutive activity (Walwyn *et al.*, 2007). These animals also demonstrate basal analgesia, this phenomenon appears to be the result of MOP receptor constitutive activity within the pain pathway because it is inhibited by inverse agonists but not neutral antagonists (Lam *et al.*, 2011). This suggests that constitutive activity may be involved in opioid dependence and withdrawal. BAR2 appears to regulate constitutive activity of MOP receptors as its removal from the system results in basal analgesia and increased MOP receptor activity. Constitutive MOP receptor activity is also upregulated in the pain pathway during persistent hyperalgesia induced by injection of complete Freund's adjuvant into the mouse paw (Corder *et al.*, 2013). Mechanical hyperalgesia declines within 7 days and can be reinstated by administration of inverse agonists but not neutral MOP receptor antagonists.

1.4 The endogenous opioid system

Opioid binding sites were first proposed in the early 1950's (Beckett and Casy, 1954), and they were discovered in mammalian tissue in 1973 by Pert and Snyder, at which time they identified binding sites in the brain and intestine for the opioid antagonist naloxone (Pert and Snyder, 1973). In 1975 Hans Kosterlitz at the University of Aberdeen extracted the endogenous opioid met-enkephalin from pig brain. This compound inhibited contractions in guinea pig ileum (Hughes *et al.*, 1975), an effect that was later shown to be blocked by naloxone.

The endogenous opioid system is now known to be involved in the regulation of a number of different processes. It is thought to be responsible for setting pain threshold (nociception) and controlling nociceptive processing. It is also thought to participate in the modulation of gastrointestinal, endocrine and autonomic function and cognitive function (McDonald and Lambert, 2005). There are three genes encoding the endogenous opioid peptides that have been identified, they are pro-opiomelanocorntin (POMC), proenkephalin (Penk) and prodynorphin (Pdyn) (Trigo *et al.*, 2010). Most of these peptides have some activity at each of the three opioid receptors (Table 1.1). The endogenous peptides that exhibit increased selectivity for DOP receptors are met-enkephalin and leu-enkephalin, these peptides are encoded by proenkephalin and were the first to be identified (Hughes *et al.*, 1975). Prodynorphin encodes the endogenous opioid peptides with an increased selectivity for KOP receptors, dynorphin A and B. Pro-opiomelanocortin encodes beta endorphin, this peptide does not appear to be selective and exhibits agonist activity at all three of the opioid receptors. Endomorphin 1 and 2 have been identified as endogenous peptides at MOP receptors however, the precursor protein and the gene that encodes them remains unknown (Zadina *et al.*, 1997, Pasternak and Pan, 2013).

Genetic manipulation of the production of these peptides has allowed the study of their role in hedonic homeostasis. Following the generation of mice lacking beta-endorphin (Bend-/-) by manipulation of exon 3 of the POMC gene (Rubinstein *et al.*, 1996), Penk-/- mice (Konig *et al.*, 1996, Ragnauth *et al.*, 2001) and Pdyn-/- mice (Sharifi *et al.*, 2001, Zimmer *et al.*, 2001) numerous studies

have been performed. These have investigated the effects of these manipulations on natural reward and pharmacological agents that produce reward. Stress induced analgesia produced by the forced swim test is not present in the Bend-/- mice but is still present in the Penk-/- mice (Rubinstein *et al*., 1996, Konig *et al*., 1996). Naloxone administration can reduce the consumption of food and decrease the response rate to a normally reinforcing food reward suggesting a role for the opioid system; further supporting this is the fact that this behaviour is altered in both the Bend-/- and Penk-/- mice (Hayward *et al*., 2002, Hayward *et al*., 2004). When the aversive properties of naloxone are studied, Bend-/- mice find naloxone aversive but Penk mice do not suggesting that naloxone is aversive to WT mice due to the presence of endogenous enkephalin (Skoubis *et al.*, 2005, Shoblock and Maidment, 2007). Of interest is the observation that morphine conditioned place preference (CPP) is unchanged in both the Bend-/- and mice that have a double knockout for Bend-/-//Penk-/- expression when compared to WT mice, so opioid mediated reinforcement is not altered in the absence of these peptides (Skoubis *et al*., 2005). It appears that endogenous enkephalin may be involved in the normal hedonic state but that beta-endorphin and the dynorphins are important in stressful situations (Le Merrer *et al*., 2009).

1.5 The genetic manipulation of the opioid receptor system

In the early 1990's genes encoding MOP, DOP and KOP receptors were identified (termed *Oprm1*, *Oprd1* and *Oprk1,* respectively). The first of the receptor genes to be cloned was for DOP receptors (Evans *et al*., 1992, Kieffer *et al*., 1992), clones of genes encoding MOP receptors (Chen *et al*., 1993, Thompson *et al*., 1993, Wang *et al*., 1993), and the genes encoding KOP receptors (Meng *et al*., 1993, Minami *et al*., 1993, Kong *et al*., 1994), followed shortly thereafter.

There is a high degree of structural similarity across all three of the opioid receptors. They have three intracellular and three extracellular loops and all contain an intracellular C-terminus and an extracellular N-terminus (Allouche *et al*., 2014). The three opioid receptors (MOP, DOP and KOP) show a 60 - 70% sequence identity (Pasternak and Pan, 2013). The structural similarity of the receptors is highest in the intracellular loops which are involved in the interactions with intracellular G proteins and lowest in the extracellular loops that participate in ligand binding. The N-terminal region contains potential glycosylation sites that are thought to be important in DOP receptor maturation and trafficking to the cell membrane (Petäjä-Repo *et al*., 2000). In contrast glycosylation of this region of MOP receptors does not appear to affect function (Befort *et al*., 2001).

There are three known opioid receptor genes but alternative splicing is thought to explain the observed variety of pharmacological receptor phenotypes

(Pasternak, 2014b). Shortly after the cloning of these genes they were deleted from the genome of mice using the technique of homologous recombination. This has enabled the development of mouse lines that have had individual or combinations of the opioid receptors knocked out.

1.6 MOP knockout mice

There have been several MOP receptor knockout mice created. These models have targeted different exons of the gene. Targeted deletion of Exon 1 (Sora *et al*., 1997, Tian *et al*., 1997, Schuller *et al*., 1999), of the *Oprm1* gene produces a mouse that exhibits no signs of morphine analgesia compared to WT but in which the efficacy of diamorphine and morphine-6-glucuronide analgesia remains intact (Schuller *et al*., 1999). These mice also exhibit impaired sexual function and alterations in haematopoiesis (Tian *et al*., 1997). In contrast mice that have either or both Exon 2 and 3 targeted are healthy with no apparent detrimental effects on sexual health or immunity (Matthes *et al*., 1996, Loh *et al*., 1998). These mice gain no analgesia or reward from morphine (Matthes *et al*., 1996) and also demonstrate that MOP receptors are required for the immunosuppressive effects of morphine (Gavériaux-Ruff *et al*., 1998). These mice also exhibit no reward or locomotor activation following the administration of diamorphine (Contarino *et al*., 2002). There have been conflicting accounts of alterations in basal sensitivity to noxious heat in MOP-/- mice. An increased sensitivity to noxious heat was noted in the mice with a deletion of Exon 1 (Sora

et al., 1997) but no significant difference was reported in the Exon 2 deletion mice (Matthes *et al*., 1996).

Morphine CPP and self-administration are abolished in MOP receptor knockout mice (Becker *et al*., 2000). It is interesting to note that in these mice ethanol self-administration is abolished (Roberts *et al*., 2000, Becker *et al.*, 2002), and preference for nicotine (Berrendero *et al.*, 2002) and delta9-tetrahydrocannabinol (THC) (Ghozland *et al.*, 2002) are also abolished. These data suggest a role for the MOP receptors in the rewarding and reinforcing effects of drugs other than opioids. Behaviourally these mice also exhibit a number of other traits including decreased impulsivity (Olmstead *et al*., 2009), a decreased motivation to eat (Papaleo *et al*., 2007), decreased food anticipatory behaviour (Kas *et al.*, 2004) and decreased maternal attachment (Moles *et al*., 2004).

No compensatory changes to DOP or KOP opioid receptor expression have been identified in MOP-/- mice (Kitchen *et al*., 1997) and while a partial reduction in DOP receptor response to selective agonists was identified there was no significant alteration in KOP receptor response to selective agonist in MOP-/- mouse model (Matthes *et al*., 1998). Supporting this finding is the report that the analgesic efficacy of DOP receptor agonists may be decreased in these mice (Fuchs *et al*., 1999).

1.7 DOP receptor knockout mice

Mice lacking DOP receptors were produced by disruption of either Exon 2 of the *Oprd1* gene (Zhu *et al*., 1999) or Exon 1 (Filliol *et al*., 2000). There have been no detectable effects on the health or reproduction of these mice by removing DOP receptors. In radioligand binding studies the binding of [^{3}H]DPDPE and [^{3}H]deltorphin were completely abolished from brain tissue (Zhu *et al*., 1999). There have been no identifiable compensatory changes in the receptor expression levels of either MOP or KOP receptors in these mice (Zhu *et al*., 1999).

Behaviourally DOP knockout mice exhibit increased anxiety and depressive like behaviour (Filliol *et al*., 2000) and increased ethanol self-administration (Roberts *et al*., 2001). The increased ethanol consumption that these mice display appears to alleviate anxiety caused by DOP receptor gene deletion. There have been differing reports of morphine reinforcement in these mice, with it being reported as decreased (Chefer and Shippenberg, 2009) and unchanged (Le Merrer *et al.*, 2012). However, morphine self-administration, which is a more direct measure of the rewarding properties of a drug, is unchanged (Lutz and Kieffer, 2013). Therefore the role of DOP receptors in morphine preference and reward remains unclear.

1.8 KOP receptor knockout mice

One genetic knockout mouse model that targets KOP receptors has been developed. This KOP-/- mouse was produced by target deletion of exon 1 of the KOP receptor gene *Oprk1* (Simonin *et al*., 1998). These mice exhibit no alterations in the analgesia that they receive from morphine when tested using either the tail withdrawal assay or the hot plate test suggesting that the KOP receptor is not involved in this response. The mice also display no significant difference in morphine preference (tested using a CPP paradigm), locomotor alteration or anxiety when compared to WT mice (Simonin *et al*., 1998).

1.9 Structural models of the opioid receptors

Structural models of MOP, DOP and KOP receptors were published in 2012 (Manglik et al., 2012, Granier et al., 2012, Wu et al., 2012), respectively. Further structural models for DOP receptors (Fenalti *et al*., 2014) and MOP receptors (Huang *et al*., 2015) in association with agonists have recently been published.

These structural models have provided evidence that opioid receptors can form dimers particularly in the case of MOP receptors (Manglik *et al.*, 2012). This could be an artefact of crystallisation, but there is other experimental evidence supporting receptor oligomerisation. Cvejic and Devi (1997) first demonstrated opioid receptor dimerization in early work on recombinant epitope-tagged DOP receptors using cross linking and immunoprecipitation. Shortly thereafter, using similar approaches, Jordan and Devi showed that DOP receptors can also

heterodimerise with KOP receptors (Jordan and Devi, 1999). George and colleagues were the first to demonstrate dimerization of recombinant MOP and DOP receptors (George *et al*., 2000). Since then there have been numerous reports of MOP receptors combining with a variety of other GPCRs (Fujita *et al*., 2014). There is limited evidence for functional properties of MOP/DOP dimers distinct from those of MOP or DOP expressed alone. Their combined expression in GH_3 cells led to an elevation of intracellular Ca^{2+} when the MOP selective agonist DAMGO was applied (Charles *et al*., 2003, Charles and Hales, 2004). Signalling by putative MOP/DOP dimers was also resistant to pertussis toxin, which inhibits Gi/o mediated processes, implicating an alternative pathway to that of MOP and DOP receptors when expressed alone (George *et al*., 2000). However, all of this work was done with recombinant receptors expressed in cell lines. A role for heterodimers or, perhaps, larger hetero-oligomeric complexes *in vivo* is less well established. In DRG and brainstem neurones that have been chronically exposed to morphine there is a demonstrable increase in MOP/DOP heterodimers revealed by antibody staining (Gupta *et al*., 2010). An antibody selective for the MOP/DOP receptor interface was used in this study. This reveals the importance of MOP/ DOP heterodimers in nociceptive signalling both at the spinal level and within brainstem centres involved in regulating descending inhibitory pathways. A peptide that mimics the first transmembrane domain of the MOP receptor, when coupled to the TAT peptide, can cross cell membranes and disrupts MOP/DOP association (He *et al*., 2011). This approach was used to explore the requirement for MOP/DOP interactions in morphine analgesia. The peptide reduced morphine analgesic tolerance suggesting that MOP/DOP

receptors exist in the pain pathway where they mediated analgesic responses to morphine. A requirement for DOP receptor expression in DRG neurones for full function on MOP receptor agonists, including morphine also implies the existence of MOP/DOP receptors in the pain pathway (Walwyn *et al*., 2009).

There is also evidence for a role of MOP/DOP dimers in BAR2 recruitment. Studies have demonstrated that both MOP and DOP receptors need to be present to co-localise with BAR2 within the cell membrane in the absence of agonist application (Rozenfeld and Devi, 2007).

1.10 The arrestin system

As mentioned above, activation of many GPCRs including the opioid receptors leads to the recruitment of BAR1 and/or 2, this process has been implicated in signalling, receptor desensitisation and trafficking (Reiter and Lefkowitz, 2006). Agonist binding at the receptor also initiates GRK mediated phosphorylation and this leads to inhibition of G protein receptor coupling (Shenoy and Lefkowitz, 2003). The arrestin family consists of four members. Arrestin 1 and 4 are found to be located in the retinal rods and cones and arrestin 2 and 3 (also called β-arrestin1 and 2) which are ubiquitously expressed. The two BARs share a high sequence homology with each other and also with visual arrestin (arrestin-1). In vitro they act to desensitise GPCR's by physically preventing the interaction of the phosphorylated receptor and the heterotrimeric G-protein (Pierce and

Lefkowitz, 2001, DeWire *et al.*, 2007). As well as this role in the silencing of GPCRs they are also implicated in receptor trafficking and intracellular signalling.

The GRK family consists of seven different genes, GRK 1 and 7 have a restricted distribution being found only in the retinal rods and cones respectively, where they form the GRK1 subfamily. GRK4 also exhibits a restricted distribution pattern, it has been found in the testis, cerebellum and kidney and comprises the GRK2 subfamily. The remaining GRKs (2, 3, 5 and 6) are all ubiquitously expressed and comprise the GRK4 subfamily (Watari *et al*., 2014, Reiter and Lefkowitz, 2006).

Various studies have investigated the roles of GRK2, 3, 5 and 6 both *in vitro* and *in vivo*. The GRK2 knock out is embryonically lethal due to the ubiquitous nature of this protein, however cellular studies and *in vitro* studies utilising targeted siRNA knockdown have suggested that both GRK2 and GRK3 are involved in opioid receptor phosphorylation, BAR recruitment and functional uncoupling (Whistler and von Zastrow, 1998, Bohn *et al*., 2004). They have also been implicated in receptor endocytosis (Kim *et al*., 2008). GRK3 knock out mice exhibit a reduction in tolerance in response to fentanyl administration compared to WT mice but morphine induced tolerance is not altered (Terman *et al*., 2004, Kuhar *et al*., 2015). GRK6 is highly expressed in brain and gastrointestinal cells, *in vitro* it regulates MOP receptors by enhancing the recruitment of BAR2 and receptor internalisation. *In vivo* no difference in morphine induced tolerance

could be identified in GRK6 knockout mice but locomotor activation and sensitisation was altered and morphine was less constipating in these mice (Raehal *et al.*, 2009). GRK5 knockout mice exhibit reduced analgesia from morphine but not fentanyl with no differences identified in acute morphine tolerance when compared to WT mice. However these mice do exhibit significantly reduced preference for morphine tested using a conditioned place preference (CPP) paradigm (Gluck *et al.*, 2014). All of these results reveal the importance of opioid receptor phosphorylation in the subsequent cellular response to agonist.

Studies of GPCR phosphorylation by GRKs have led to the development of the barcode hypothesis (Butcher *et al.*, 2011). This suggests that the specific phosphorylation pattern created by an agonist following binding to the receptor influences the downstream effects that are produced (Reiter *et al.*, 2012). In addition to the role of BARs in receptor desensitisation and trafficking these proteins can also participate in signalling by recruiting various kinases to GPCRs. These include non-receptor tyrosine kinases such as the proto-oncogene Src (c-Src), other members of the c-Src family (Hck, Fgr, and Yes), c-Jun amino terminal kinase (JNK), ERK1 and 2, mitogen activated protein kinase (MAPK), Raf, MEK and Akt (Reiter and Lefkowitz, 2006).

1.11 Beta-arrestin2 knockout mice

Mice lacking β-arrestin1 (BAR1-/-) and BAR2-/- have been generated and characterised, (Schmid and Bohn, 2009). The double knock out of both BAR1 and 2 is embryonically lethal (Pierce and Lefkowitz, 2001). BAR2-/- mice appear phenotypically normal but develop negligible tolerance to opioid analgesia (Bohn *et al*., 1999, Bohn *et al*., 2000, Bohn *et al*., 2002). In addition the mouse's endogenous opioid pain killing mechanism becomes persistently active without the requirement for drug administration. This occurs with no evidence of the hedonic effects typically associated with opioid drug taking (Lam *et al*., 2011). The persistent analgesia is inhibited by naloxone and naltrexone (both inverse agonists), but not by neutral competitive antagonists (Hayward *et al*., 2004). It is also associated with constitutive MOP receptor coupling to VACCs in primary afferent neurones from BAR2-/- mice, a phenomenon that can be reproduced in WT neurones by directly inhibiting c-Src activity implicating tyrosine kinase-mediated phosphorylation in this process (Walwyn *et al*., 2007).

The BAR2-/- mice still exhibit physical dependence following morphine administration and this has been reported as both not significantly different to that observed in WT mice (Bohn *et al*., 2000) and decreased when compared to WT mice (Raehal and Bohn, 2011). However it has also been reported that these mice exhibit an increased preference for morphine when tested using a CPP paradigm (Bohn *et al*., 2003). Consistent with this observation is the report that morphine stimulated striatal extracellular dopamine levels are also increased in the BAR2-/- mice when compared to WT mice without any differences in the

baseline levels (Bohn *et al.*, 2003). As well as the reduction in morphine tolerance that has been recorded in these mice there is also a reduction in several other morphine induced side effects including locomotor activation, respiratory depression and constipation (Raehal *et al*., 2005) implicating BAR2 in these effects.

1.12 Pain and nociception

The International Association for the Study of Pain (IASP) defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”(IASP, 2014). The emotional aspects of pain, which may be affected by opioid administration, are challenging to study in animal models. Despite this limitation there are a number of well validated tests of nociception in rodents that are available. These encompass a number of different nociceptive mechanisms including mechanical, thermal, chemical and inflammatory pain models. Models of thermal nociception include the tail withdrawal assay using either radiant heat or hot water and the hot plate test. The latency for tail withdrawal is straightforward to study and provides a quantification of pain perception (nociceptive pain).

Both the hot plate test (Woolfe and MacDonald, 1944) and the tail withdrawal assay (D'Amour and Smith, 1941) involve exposing the rodent to a noxious thermal stimulus with a maximum exposure time to avoid tissue damage occurring. There are several different methods that have been described for the

tail withdrawal assay, some utilise radiant heat targeted to a specific area of the tail and others immersion of the tail in hot water. To investigate nociception the method that we have chosen to utilise is tail withdrawal from hot water following immersion, this is a modification of D'Amour and Smith's method (1941). The tail withdrawal assay is a simple and reproducible test that is highly responsive to opioid drugs (Wilson and Mogil, 2001, Barrot, 2012), but it does require restraint of the mouse which can cause a stress response. This stress response can manifest itself as measurable analgesia in the absence of exogenous drug (Butler and Finn, 2009). However, the advantage of this version of the test is that due to the significant surface area of the tail that is exposed to the hot water there is a rapid increase in the temperature of the tail and a reproducible spinal reflex response (Le Bars *et al*., 2001).

The tail flick reflex is a spinal reflex that is generally regarded to respond to spinal analgesia (Kieffer, 1999) although it is influenced by some descending supraspinal inputs (Irwin *et al*., 1951, Barton *et al*., 1980). This is in contrast to the hotplate test that is subject to a variety of supraspinal influences and requires the integration of a number of varying system inputs (Pasternak and Pan, 2013).

To assess nociception from mechanical stimuli Von Frey filaments can be used (Gregory *et al*., 2013). These filaments come in different sizes to produce a graded increase in pressure. The filaments are particularly useful for studying

mechanical allodynia. This is when a normally non-noxious stimulus results in pain. In rodents this results in paw withdrawal at a level of pressure that would not have previously produced this effect (Barrot, 2012). To study the response to electrical stimulation a technique called electrical threshold testing can be performed, however this is an unnatural non-specific stimulus and is therefore generally only used for baseline testing of animals involved in studies that use footshock techniques as part of the study protocol (Mogil, 2009). The response to noxious chemical stimuli can be investigated using a variety of different agents. These include the formalin paw injection, this process can also be used to model inflammatory pain. Other chemical agents that are commonly used to model inflammatory pain include the carrageenans and complete Freund's adjuvant (CFA). These agents are used more commonly as they produce a longer lasting inflammatory response when compared to formalin, this longer lasting response is due to their ability to produce immune activation (Barrot, 2012). Other animal models to study pain include models of neuropathic pain such as complete nerve transection which results in self-mutilation behaviour and chronic nerve constriction. Both of these models require the animal to undergo surgery, the most common nerve that is targeted for chronic nerve constriction is the sciatic nerve (Mogil, 2009, Barrot, 2012). Many of the tests that are currently used in pain studies measure spinal withdrawal reflexes, spino-bulbo spinal reflexes (such as jumping behaviour) or general behaviour such as vocalisation, licking or guarding.

Opioid analgesia is mediated within both the central and peripheral nervous system (Van Ree *et al*., 1999). Nociceptive stimuli are detected by a collection of peripheral nerve fibres termed nociceptors. Their cell bodies are located within the dorsal root ganglia (DRG) of the spinal cord. The inhibition of presynaptic VACCs within the DRGs is thought to give rise to the opioid-mediated reduction of excitatory neurotransmission in the pain pathway (Heinke *et al*., 2011). There may also be a role for neuronal hyperpolarisation through activation of the inwardly rectifying K^+ channel (Luscher and Slesinger, 2010). Yaksh and Rudy (1976) were the first investigators to demonstrate that morphine has direct analgesic effects on the spinal cord, by administering the drug intrathecally and subsequently testing nociceptive responses.

We now know that MOP, DOP and KOP receptors are all located in a high density within the superficial layers (lamina I and II) of the spinal cord with lower levels found in the deeper lamina. In the dorsal horn of the spinal cord MOP receptors account for 70% of the opioid receptors present with a small amount of DOP receptors (24%) and an even smaller proportion of KOP receptors (6%) (Davis and Pasternak, 2009). The opioid receptors have been identified at both pre and post synaptic sites within the dorsal horn of the spinal cord (Besse *et al*., 1990). As well as directly inhibiting receptors within the DRG neurones, morphine also activates descending inhibitory pathways that run from the brainstem to the DRG neurones and act to inhibit the activity of the nociceptive pathway (Basbaum and Fields, 1984).

A study by Corder (Corder *et al*., 2013) that used CFA administration to the hind paws of mice demonstrated that constitutively active MOP receptor activity limits the duration of inflammatory hyperalgesia. In this study they could reinstate hyperalgesia by the administration of inverse agonists such as naltrexone but not in the presence of neutral receptor antagonists suggesting the presence of constitutive activity of the MOP receptor.

1.13 Genetic influences on rodent nociceptive responses

It has been noted that there are significant strain differences in response to nociceptive testing between different commonly used laboratory mouse strains suggesting that genetic variation can significantly affect pain behaviour (Mogil *et al*., 1999, Leo *et al*., 2008). Gender differences in the response of rodents to opioid drugs have also been reported. In male rats an increased analgesic effect of morphine has been noted using both the hot plate and the tail withdrawal assays, suggesting that this gender difference affects both spinal and supraspinal mechanisms (Cicero *et al*., 1996). In contrast no gender differences were noted in the baseline sensitivity of mice to morphine but female mice exhibited a greater degree of tolerance to the analgesic effects of morphine following chronic administration and testing using the tail withdrawal assay (Kest *et al*., 2000). These differences may reflect a combination of species and genetic differences as studies investigating the analgesic effects of morphine over a wide range of laboratory mice with different genetic backgrounds have identified that

the observed sex differences are genotype dependent (Chesler *et al*., 2002, Kest *et al*., 1999, Kest *et al*., 2002).

There are also conflicting results regarding gender differences in tail withdrawal time from hot water, with a reported increase in baseline tail withdrawal time in male C57Bl/6 mice compared to females (Kest *et al*., 1999) but a subsequent study was not able to identify a gender difference using the same strain of mice and the identical nociceptive test (Kieffer *et al*., 1992). While there may be gender differences present it is apparent that they are not easy to identify and may well be multifactorial. The majority of animal studies that have been published to date have only included male rodents which also limit the conclusions that can be drawn at the present time.

1.14 Tolerance

Tolerance to opioid drugs requires escalating doses to gain the equivalent analgesic effect. This is a significant clinical problem limiting the use of analgesic opioids in clinical practice (Nestler, 1992, Koob *et al*., 1998, Williams *et al*., 2013). Tolerance develops to the opioid drug side effects at differing rates, meaning that analgesia may not be adequate due to a reduction in analgesic potency but at the time the drug may be producing respiratory depression that limits the dose that can be safely administered, it is also often specific to the administered opioid allowing improved analgesia with a switch to an alternative compound (Pasternak and Pan, 2013). Caution is required in this approach due

to the possibility of overdose. Although conversion tables for opioid switching exist and are useful, they provide a direct dose equivalent without allowing for the increased potency of the new drug. The new opioid prescription therefore requires to be of a significantly lower dose than the calculated equipotent amount of the existing drug (Mercadante and Bruera, 2006). The degree to which this occurs is not clear at the present time.

Tolerance to opioid analgesics develops on several different levels. It can be measured at a cellular, synaptic or network/whole animal level. It is currently unclear how changes at the cellular and synaptic level contribute to whole system tolerance but understanding the changes that occur at this level will allow the integration of knowledge of events over the larger pathways involved. With regards to the cellular mechanisms that have been linked to opioid analgesic tolerance, receptor desensitisation, receptor endocytosis, up regulation of adenylyl cyclase and CREB (cAMP response element binding protein) activation have all been investigated (Sim-Selley *et al*., 2000, Williams *et al*., 2001, Connor *et al*., 2004, Christie, 2008, Williams *et al.*, 2013).

Receptor desensitisation is thought to involve a reduction coupling of the MOP receptor to the intracellular G protein mediated signalling pathways (Williams *et al*., 2013). The extent to which this uncoupling occurs appears to vary across different neuronal populations within the CNS of rats treated with chronic opioid when binding studies are performed (Sim-Selley *et al*., 2000). The binding of

BAR2 to MOP receptors following receptor phosphorylation by GRK is thought to be involved in receptor desensitisation. This process is also implicated in the beginning of the receptor internalisation pathway as MOP receptor endocytosis is thought to occur through a BAR2/ dynamin dependent mechanism (Connor *et al*., 2004). However MOP receptors can still desensitise in the absence of BAR2 (Walwyn *et al*., 2007, Dang *et al*., 2011) suggesting that this is not the sole mechanism by which this occurs.

Following internalisation the receptors can be targeted for lysosomal degradation or recycling back to the cell membrane. Internalisation of the receptors is not required for desensitisation (Williams *et al*., 2001). Morphine produces less receptor internalisation compared to other MOP receptor agonists suggesting that it may be receptor desensitisation that has a larger role in the development of drug tolerance (Williams *et al*., 2001, Christie, 2008, Williams *et al*., 2013).

While one of the accepted end-points of classical GPCR signalling is a reduction in cAMP through inhibition of adenylyl cyclase, it is well documented that following continuous exposure of MOP receptors to agonist there is a point at which a switch to increased cAMP levels occurs (Christie, 2008). This compensatory increase in adenylyl cyclase activity has been suggested to be the underlying mechanism that results in tolerance and dependence (Koob and Bloom, 1988). It is thought that the phosphorylation of adenylyl cyclase and GRK

2/3 by protein kinases may be involved in the observed increase in adenylyl cyclase activity (Zhang *et al*., 2013).

CREB is a cellular transcription factor that can up or down regulate gene transcription. Activation of the MAPK/ERK pathway following agonist binding to MOP receptors can result in the phosphorylation of CREB within the cellular nucleus and subsequent alteration in gene transcription (Williams *et al*., 2001). It does not appear to be MOP receptors that are affected directly by this process as MOP receptor mRNA levels have not been found to be significantly different following treatment with chronic opioid (Christie, 2008). However it can also affect the transcription of multiple other receptor systems, adenylyl cyclase and a number of other signalling proteins (Carlezon *et al*., 2005).

1.15 The RAVE hypothesis

One theory as to why some opioid drugs produce much greater tolerance than others is the RAVE (Relative Activity Versus Endocytosis) theory (Whistler *et al*., 1999). The ability of MOP receptor agonists to promote endocytosis does not correlate with their potency or efficacy as activators of the receptor. For example morphine, which effectively triggers G protein signalling, is poor at initiating receptor endocytosis (Keith *et al*., 1996, Keith *et al*., 1998, Whistler and von Zastrow, 1998). This is in contrast to the selective MOP receptor agonist DAMGO (an encephalin analogue) that produces marked receptor endocytosis with a similar ability to trigger G protein mediated signalling. Morphine is

therefore said to have a high RAVE value when compared to DAMGO (Whistler *et al.*, 1999). Following exposure to morphine MOP receptors remain within the cell membrane where they undergo phosphorylation and desensitisation, in contrast exposure to DAMGO produces rapid endocytosis which is thought to allow resensitisation of the receptor and its return to the cell membrane (Mollereau *et al.*, 1994). It is the prolonged receptor activation that has been suggested to be responsible for analgesic tolerance with cAMP super-activation being a cellular marker of this phenomenon (Finn and Whistler, 2001, Williams *et al.*, 2001).

There are several studies that have co-administered a second opioid drug (that has a lower RAVE value) alongside morphine at a sub-analgesic dose. When DAMGO was administered alongside morphine to rats a reduction in analgesic tolerance was observed compared to the measured effects of morphine alone (He *et al.*, 2002). Also when methadone was co-administered to rats at a sub-analgesic dose then the development of acute tolerance was reduced in these animals compared to those treated with morphine alone (He and Whistler, 2005). These observations are of particular interest as they suggest that the presence of a second drug at MOP receptors can enable recovery from morphine induced tolerance. This implies that endocytosis is required for recovery of MOP receptor function.

1.16 Opioid receptor desensitisation in tolerance

An alternative theory as to the mechanism of drug tolerance involves receptor desensitisation rather than internalisation. Receptor desensitisation can occur through several different mechanisms, two of these have been described. The first is GRK mediated receptor phosphorylation that leads to the recruitment of the BARs and the second involves phosphorylation of the receptor by second messenger dependent protein kinases such as PKA, PKC and calcium/ calmodulin dependent kinase II (Ferguson, 2001, Bailey and Connor, 2005). There have been at least 20 phosphorylation sites identified on MOP receptors (Chavkin *et al*., 2001), these generally involve serine, tyrosine and threonine sites within the COOH tail of the receptor (Zhang *et al*., 2009).

Although early work suggested that morphine did not produce receptor desensitisation (Alvarez *et al*., 2002), several investigators have now demonstrated that morphine can produce desensitisation although to a lesser extent than other agonists at the receptor (Bailey *et al*., 2003, Bailey, 2004, Dang and Williams, 2005, Johnson *et al*., 2006, Bailey *et al*., 2009a). Its ability to do so appears to vary among tissue/ cell types. The mechanism by which MOP receptor desensitisation occurs appears to be dependent on the agonist that binds to the receptor possibly due to phosphorylation of the receptor at different binding sites. The binding sites that are available may depend on the conformational changes in receptor structure produced by the binding of different agonists. It has been suggested that desensitisation occurs via a PKC dependent mechanism following morphine binding (Bailey, 2004, Bailey *et al*.,

2009b, Johnson *et al*., 2006), and a GRK dependent mechanism following exposure to DAMGO (Johnson *et al*., 2006, Bailey *et al*., 2009b). This is important as PKC inhibition has been demonstrated to reduce morphine tolerance (Gabra *et al*., 2007, Hull *et al*., 2010), and BAR2-/- mice develop less tolerance to morphine (Bohn *et al*., 1999, Bohn *et al*., 2000, Bohn *et al*., 2002, Raehal *et al*., 2005). GRK inhibition alters tolerance to DAMGO and fentanyl but does not affect morphine tolerance to the same extent (Terman *et al*., 2004, Hull *et al*., 2010). Receptor internalisation does not correlate with desensitisation suggesting that these events are independent of each other and therefore that the RAVE hypothesis does not fully explain the mechanism of tolerance (Bailey and Connor, 2005).

1.17 The role of DOP receptors and BAR2 in tolerance

There is evidence to suggest that DOP receptors and BAR2 play important roles in both analgesia to morphine and in the development of tolerance to analgesic effects of the drug. When either MOP or DOP receptors are expressed alone in the cell membrane they are not co-localised with BAR2 but when they are expressed together they do. This suggests that both MOP and DOP receptors are required for the recruitment of BAR2 at the cell membrane and the subsequent activation of BAR2 mediated intracellular signalling (Rozenfeld and Devi, 2007). It is thought that DOP receptors are not required for morphine-mediated analgesia, but their absence leads to blunted morphine analgesic tolerance (Zhu *et al*., 1999). As previously mentioned it has been reported that the BAR2-/- mice

exhibit basal analgesia and also significantly reduced morphine tolerance compared to WT (Bohn *et al*., 1999, Bohn *et al*., 2000, Bohn *et al*., 2002, Raehal *et al*., 2005). These data implicate both BAR2 and DOP receptors in the development of morphine tolerance.

1.18 Pharmacokinetics of morphine in rodents

Morphine metabolism in mice differs considerably from the human metabolism. In humans morphine is broken down into morphine 6 glucuronide (M6G) and morphine 3 glucuronide (M3G) (Wittwer and Kern, 2006). These metabolites are themselves pharmacologically active. Mice produce only a small amount of M6G from morphine and M3G is therefore the main metabolite. The half-life of morphine in C57Bl/6J mice is 28 minutes following IV administration (Handal *et al*., 2002). Morphine administered subcutaneously (SC) to mice has a similar half-life to that administered via the IV route and it has a significantly higher bioavailability than an identical dose administered via the intraperitoneal route (IP) due to the first pass metabolism that occurs with this route of administration (Handal *et al*., 2002).

1.19 Mesocorticolimbic reward pathway

Opioid receptors are highly expressed throughout the central nervous system. They are particularly concentrated within the cortex, limbic system and brainstem. The expression of each of the receptor types varies between

structures with MOP receptors showing its highest expression levels in the amygdala, thalamus, mesencephalon and certain brainstem nuclei. The highest expression of DOP receptors occurs in the olfactory tract, cortices, regions of the amygdala and the striatum. While the highest expression of KOP receptors occurs in the olfactory tubercule, striatum (including the nucleus accumbens), preoptic area, hypothalamus and pituitary gland (Le Merrer *et al.*, 2009).

Although dopaminergic cells only make up <1% of the total brain neuronal population (Arias-Carrion *et al*., 2010), three areas in the midbrain (retrorubal field, substantia nigra pars compacta and the VTA) contain 70-75% of them (Grillner and Mercuri, 2002). Within the VTA differing cell populations have been reported. (Ungless and Grace, 2012) reported that the composition is 70% dopaminergic cells, 30% GABAergic and 2-3% glutamatergic, which agrees with the work of Chieng *et al* (2011). However, Margolis *et al* (2012) have reported that 22% VTA neurons are neither GABAergic nor dopaminergic when assessed using immunostaining. These differences may be due to differences between rat and mouse or to the precise location assessed within the VTA as this is a heterogeneous area. This heterogeneity has been increasingly appreciated as the role of this brain area and influence on behaviour has been investigated (Lammel *et al*., 2011, Lammel *et al*., 2012, Lammel *et al*., 2014). Yamaguchi *et al* (2015) reported that there were neuronal populations that stained for either VGlut2 alone, TH alone or co-stained for both. This is interesting as signalling involving both the dopaminergic and glutamatergic systems has been identified as important in motivated behaviours (Stuber *et al.*, 2010). Dopaminergic neurones

from the VTA project mainly to the nucleus accumbens (NA) within the ventral striatum as well as the pallidum, prefrontal cortex, amygdala and hippocampus (Figure 1.2). Together these projections form the mesocorticolimbic dopamine system (Korotkova *et al*., 2004).

There are a number of behaviours that are associated with survival of the species such as eating, drinking water and reproduction. These behaviours are naturally rewarding (Agmo *et al*., 1993) and have been demonstrated to involve the opioid receptors (Glass *et al*., 1999). Antagonists of MOP and DOP receptors suppress the reinforcing properties of natural rewards and those of the opioid and non-opioid drugs (Shippenberg and Elmer, 1998). The VTA in the midbrain has been identified to be important for a number of different behaviours including initiating reward and goal directed behaviour (McBride *et al*., 1999, Ungless and Grace, 2012). The rewarding effects of opioids are thought to occur through both dopamine dependent and dopamine independent mechanisms (Meye *et al*., 2012). MOP receptor agonists reduce the frequency of GABAergic inhibitory postsynaptic currents (IPSCs) in the VTA disinhibiting dopaminergic neurones, increasing dopamine release and stimulating reward (Johnson and North, 1992b, Matsui and Williams, 2011)(Figure 1.3). Both the GABAergic and the dopaminergic neurones of the VTA receive inputs from several different areas of the brain. These inputs can produce excitatory, modulatory or inhibitory effects. It is becoming increasingly apparent that neuronal subpopulations exist within the VTA and these subpopulations belong to different circuits that produce differing behavioural responses (Beier *et al*., 2015). A number of

different GABAergic inputs synapse onto VTA neurones, these originate from either local interneurones, NA or RMTg (Barrot *et al*., 2012, Cui *et al*., 2014, Matsui *et al*., 2014). Matsui *et al* (2014) demonstrated in rat brain slices that opioid inhibition of GABAergic IPSCs within the VTA was projection specific, with a significant input from terminals arising in the RMTg. Differences in cell type distribution and function have also been identified across the anterior/ posterior sections of the VTA (Sanchez-Catalan *et al*., 2014). It is increasingly recognised that this brain area is complex and further investigation is required to fully understand the role of the differing neuronal subtypes on function.

Pain is comprised of both sensory and affective components. The VTA is being increasingly recognised as important in the emotional/affective components of pain (Russo and Nestler, 2013). Sciatic nerve ligation in rodents (a model of chronic pain) leads to a decrease in the rewarding effects of MOP receptor agonists through a reduction in MOP receptor signalling within the VTA (Niikura *et al*., 2010). This is consistent with the report that the level of dopamine release observed in the nucleus accumbens following morphine treatment is significantly reduced in rodents that have had a sciatic nerve ligation performed compared to that of unaffected animals (Ozaki *et al*., 2003).

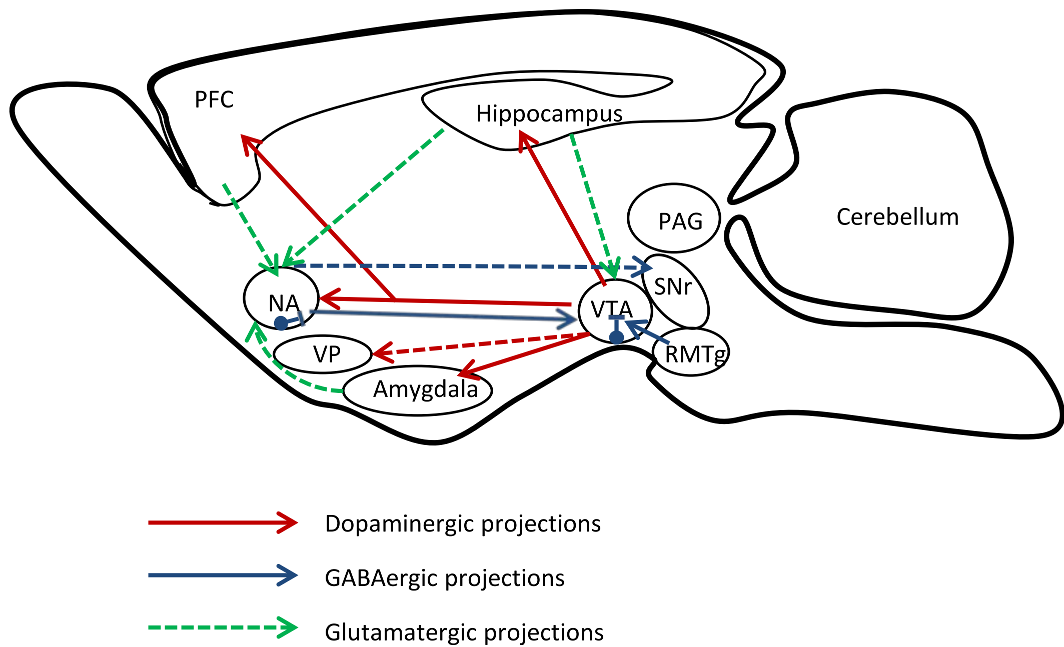


Figure 1.2: The dopaminergic component of the mesocorticolimbic reward pathway. Dopaminergic projections travel from the VTA to the nucleus accumbens and prefrontal cortex. There are also GABAergic and Glutamatergic projections to the VTA and other important brain regions involved in rewarding processes. PFC – prefrontal cortex, NA – nucleus accumbens, VP – ventral pallidum, VTA – ventral tegmental area, SNr – substantia nigra, PAG – periaqueductal gray, RMTg – rostromedial tegmental nucleus.

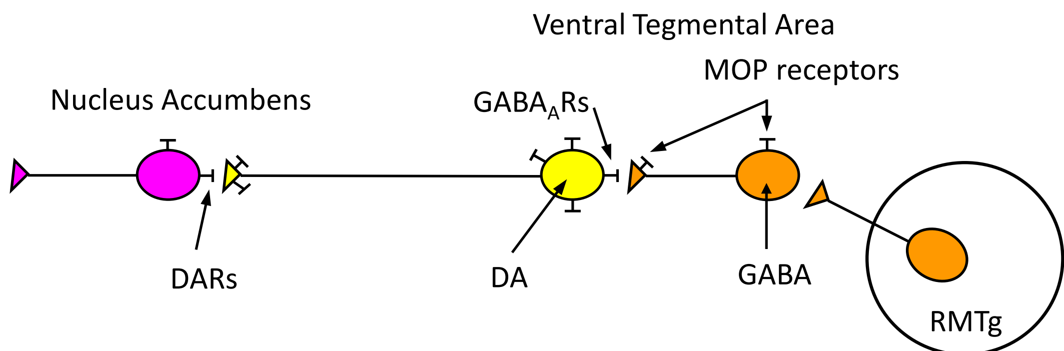


Figure 1.3: Signalling within the mesocorticolimbic reward pathway. GABA interneurones provide a continuous inhibitory tone onto the dopaminergic neurones of the VTA. Following agonist binding to presynaptic MOP receptors disinhibition of the dopaminergic signalling pathway occurs and dopamine release is stimulated in the nucleus accumbens.

1.20 Psychomotor effects of morphine

1.20.1 Locomotor activation

The administration of morphine in common with many other drugs of abuse results in increased locomotor activity in mice, the extent to which morphine stimulates locomotor activity is dependent on the genetic background of the mouse (Brase *et al*., 1977). C57Bl/6 mice exhibit a robust dose dependent increase in locomotor activity in response to morphine while in contrast DBA/2J mice do not display significant locomotor activation (Brase *et al*., 1977). The 129Sv mice from which embryonic stem cells are used in the development of MOP, DOP and BAR2 knockout mice also display a significant locomotor activation following the administration of morphine although the C57Bl/6 mice display a greater locomotor response (Murphy *et al*., 2001). This is important as alterations in psychomotor behaviour following genetic manipulation need to be understood as compared to the appropriate WT controls.

The other easily observed effect of the administration of morphine to mice is Straub tail, whereby mice hold their tails up over their back. This was first described by Straub in 1911 and it is produced by the effects of the drugs on the action of the sacro-coccygeus dorsalis muscle but also requires an intact lumbo-sacral cord outflow and blood supply (Bilbey *et al*., 1960). This process involves the activation of dopamine receptors within the CNS (Hasegawa *et al*., 1990).

The locomotor activation produced by opioids is important in the study of the rewarding effects of opioid drugs because increases in mesolimbic dopamine release have been implicated in both locomotor activation and drug seeking and reward related behaviours (Wise and Bozarth, 1987). This locomotor activation is thought to be mediated via a dopamine D1 receptor mechanism as D1-/- mice exhibit a reduced locomotor response to morphine (Urs *et al*., 2011). BAR2-/- mice also exhibit a reduced locomotor activation response compared to WT mice following the administration of morphine (Bohn *et al*., 2003). This implicates BAR2 in the locomotor response observed following the administration of morphine to WT mice. BAR1 is not involved in this process and does not compensate for the absence of BAR2 as BAR1-/- mice do not exhibit any significant difference in locomotor response to morphine when compared to WT mice (Urs *et al*., 2011).

Activation of the D1 receptor pathway involved in locomotor activity recruits the BAR2/MAPK complex. Five separate groups of MAPK have been identified at the present time, these include ERK 1+2, JNK1, 2+3, p38 isoforms, ERK 3+4, and ERK 5 (Sacks, 2006). The MAPK pathway consists of a three kinase cascade. MEKK (MAPK kinase kinase) activates MEK (MAPK/ERK kinase) following phosphorylation. This event in turn leads to a phosphorylation dependent increase in the activity of MAPK (Sacks, 2006). Systemic administration of the MEK inhibitor, SL327, to WT mice inhibits morphine induced locomotor activation in a dose dependent manner (Urs *et al*., 2011). SL327 has been shown to inhibit phosphorylation of ERK in the striatal brain regions of the mouse

(Beaulieu *et al.*, 2005). This suggests that the MEK-ERK pathway may be important in the locomotor activation produced by morphine.

1.20.2 Reinforcement

The reinforcing effects of addictive drugs in rodents can be established using the technique of conditioned place preference (CPP). This technique is dependent on Pavlovian conditioning, whereby the mice associate the drug effect with a particular environment that has been paired with it and display a preference for that environment (Shippenberg *et al.*, 1992). When WT mice are conditioned with naloxone this produces a conditioned place aversion which does not occur in MOP-/- mice. Suggesting that MOP receptors are involved in hedonic homeostasis (Skoubis *et al.*, 2001). This basal hedonic state is mediated by endogenous enkephalins but does not affect the acquisition of morphine preference (Skoubis *et al.*, 2005). WT mice will self-administer morphine directly into the VTA and the amount that they administer can be significantly reduced by the administration of systemic naloxone (David *et al.*, 2008). The ability of rodents to self-administer opioids and other rewarding drugs via cannulas implanted directly into the VTA and injection of these drugs into this area can be studied to investigate the effects of these drugs on CPP (see (McBride *et al.*, 1999) for review). Mice will also self-administer DAMGO and DPDPE directly into the VTA and this administration can produce CPP (Devine and Wise, 1994). Since DPDPE produces this effect through the VTA this implicates DOP receptors in the process of reward and reinforcement as this is thought to be an important part

of the reward pathway. It suggests that DOP receptors may be present and functional within the VTA although it does not exclude the possibility that the observed effect may be occurring via MOP receptors. There is some evidence that GABAergic neurones of the VTA express MOP receptors but not DOP opioid receptors (Matsui and Williams, 2011), however this is conflicting with other receptor localisation studies suggesting that DOP receptors are present within the VTA (Erbs *et al.*, 2015).

1.21 c-Src and the opioid receptors

Src family non-receptor tyrosine kinases (SFKs) are a family with several members. These kinases are expressed at variable levels in different tissue types. There are 11 tyrosine kinases that are currently recognised to be part of this SFK family, of which c-Src, Fyn, Yes, Lck and Lyn are expressed at a high level in brain tissue (Keenan *et al*., 2015). Src family kinases were initially described in processes relating to cell proliferation and differentiation but they are widely expressed throughout the central nervous system in varying levels and involved in many different cellular processes (Salter and Kalia, 2004). Neurones in particular express two different splice variants of c-Src that are known as N-Src (N1 and N2) as they have only been identified in neuronal cells (Keenan *et al*., 2015). Their role in intracellular signalling appears to be complex. They can be directly activated by GPCRs, this is thought to occur via either the Gα or Gβγ subunits with differing receptor systems favouring different subunit linkages (Luttrell and Luttrell, 2004). They are also involved in the activation of

intracellular signalling processes and pathways through the formation of signalling complexes with BAR2 and GRK2. It has also been suggested they are involved in opioid receptor phosphorylation (Zhang *et al*., 2009). c-Src has been identified in proximity to synaptic vesicles and it has been demonstrated that it will bind neuronal vesicular proteins including dynamin, α-adaptin and synapsin but does not directly phosphorylate these proteins suggesting that c-Src is involved in membrane trafficking in neuronal cells (Foster-Barber and Bishop, 1998). BAR2 not only mediates receptor desensitisation and internalisation but also the recruitment of c-Src following agonist binding. When BAR2 is bound to a GPCR it can provide a binding site for c-Src with the result that c-Src is part of a GPCR signalling complex with BAR2 (Luttrell and Luttrell, 2004).

There are a number of nonreceptor tyrosine kinase inhibitors that are in clinical use for the treatment of malignancy. Dasatinib is a c-Src inhibitor that is used clinically to treat leukaemia, it has the ability to cross the blood brain barrier without modification (Lagas *et al*., 2009, Porkka *et al*., 2008). It is a potent c-Src inhibitor that is normally administered orally to patients despite a low oral bioavailability. It has a K_d for c-Src of 0.21 nM but it has also been suggested to be able to target PDGFRβ with a K_d of 0.63 nM (Karaman *et al*., 2008).

The tyrosine kinase inhibitor, 3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (PP2), which is a specific inhibitor of c-Src ((Bain *et al*., 2007, Uitdehaag *et al*., 2012), has been demonstrated in WT DRG

neurones to replicate the enhanced constitutive MOP receptor inhibitory coupling to VACCs that occurs in BAR2-/- DRG neurones (Walwyn *et al*., 2007). This suggests that tyrosine kinase mediated phosphorylation may attenuate opioid analgesia and be a target to ameliorate opioid induced analgesic tolerance.

A study in rats suggested that the tyrosine kinase inhibitor, imatinib, abolished morphine analgesic tolerance (Wang *et al*., 2012). In this study imatinib had been modified to allow it to cross the BBB as it is not able to do this when administered in its standard formulation. The tyrosine kinase target of imitanib is thought to be PDGFRβ for which it has a dissociation constant (K_d) of 14 nM (Karaman *et al*., 2008). Imitanib can bind to c-Src but with a much lower affinity (K_d >10 μM) compared to its other kinase targets (Seeliger *et al*., 2007). However c-Src substrates have also been implicated in PDGFR signalling (Amanchy *et al*., 2009). There have been a number of different mechanisms for this suggested, with Src implicated in signalling upstream of the PDGFRβ (Tanimoto *et al*., 2002) and also as an intermediate in downstream receptor signalling (Barone and Courtneidge, 1995). Together this suggests that the reduction in tolerance observed in the Wang *et al* (2012) study may be mediated through c-Src inhibition of PDGFRβ signalling rather than a direct receptor effect.

1.22 Aims and Hypotheses

The studies reviewed above implicate BAR2, DOP receptors and c-Src in the failure of persistent MOP receptor mediated analgesia. Disruption of these components of the opioid signalling system may provide an approach for inhibiting tolerance to opioid analgesics such as morphine. It will be important if such an approach is to be clinically viable to establish whether there are associated changes in the actions of opioids in the reward pathway.

Our working hypothesis is that opioid receptors in the pain and reward pathways differ in their BAR mediated signalling mechanisms. We will test the hypothesis that the BAR2/c-Src system is differentially involved in opioid signalling in nociceptive pain and reward. To do this we will utilise mice that lack MOP receptors, DOP receptors and BAR2. We will also study the effects of morphine administration on the behaviour of a mouse that lacks both BAR2 and DOP receptors (BAR2-/-//DOP-/-). For all of these mouse models we aim to investigate the effects of these genetic manipulations on basal analgesia, analgesic tolerance and the development of morphine preference (Figure 1.4). We are also interested in the effects of c-Src on these parameters. The aims of the project are to test the following hypotheses:

1) The VTA lacks MOP/DOP receptor oligomers and consequently coupling through BAR2/c-Src does not participate in opioid reward. I will determine the involvement of DOP receptors and BAR2 in opioid-mediated reward and in opioid-induced disinhibition of dopaminergic neurones in the VTA.

2) MOP/DOP receptor oligomers in the pain pathway couple to the BAR2/c-Src signalling pathway. We hypothesise that this results in an absence of constitutive inhibitory coupling to VACCs and allows opioid analgesic tolerance. I will test the role of DOP receptors and BAR2 in opioid analgesia in vivo and opioid receptor signalling in primary afferent neurones in vitro.

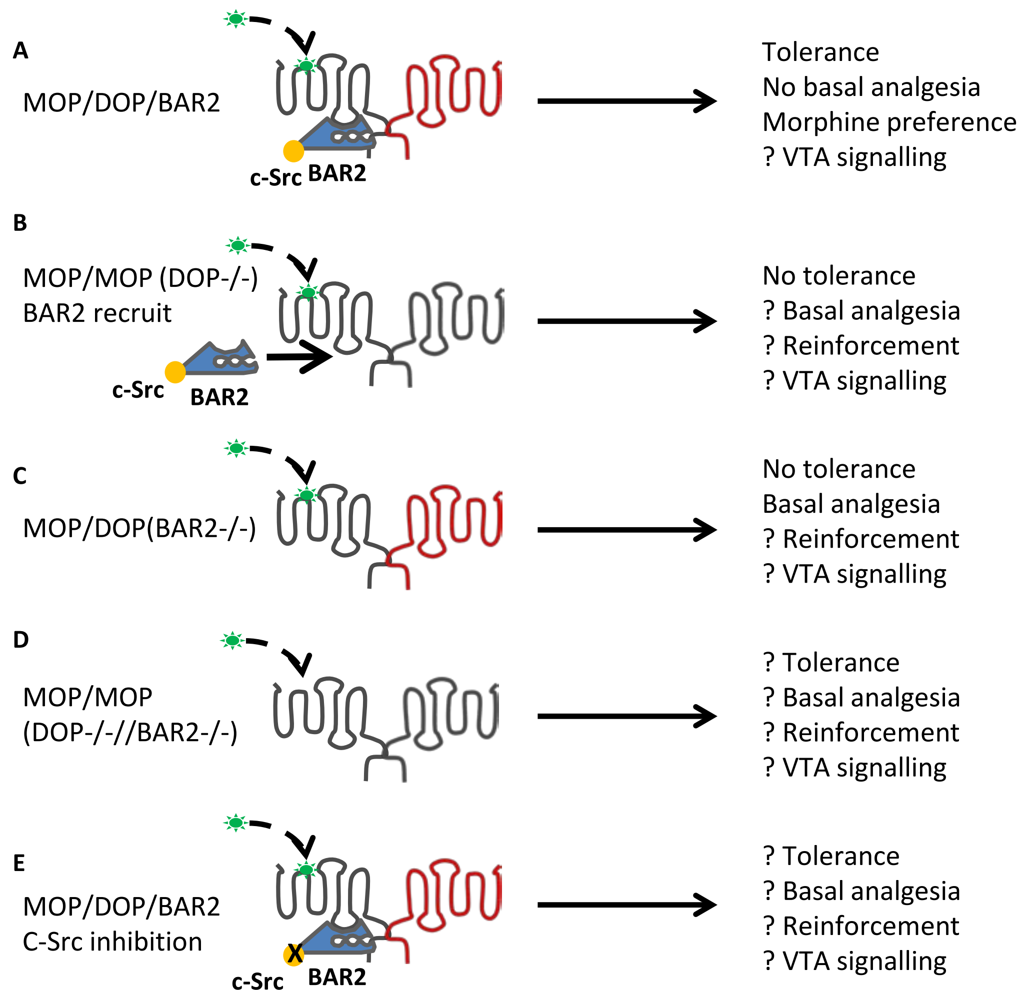


Figure 1.4: The involvement of BAR2, c-Src and MOP and DOP receptors in analgesia, tolerance and reinforcement. In the diagrams MOP and DOP receptors are depicted as physically interacting in the form of dimers. This is based on reports of dimer formation and the recent crystal structure of MOP receptors which supports this theory (Manglik *et al.*, 2012). (A) When expressed recombinantly MOP receptors, DOP receptors and BAR2 colocalise (Rozenfeld and Devi, 2007). This is depicted as the hypothetical scenario in WT neurones. The absence of DOP receptors (B), BAR2 (C), both DOP receptors and BAR2 (D), or the inhibition of c-Src (E) have the consequences summarised in each case. In each case the scenario is represented schematically.

Chapter 2: Methods

2.1 Breeding Colony Maintenance and Housing

MOP-/- mice were first generated by Dr Brigitte Kieffer's lab in 1996 (Matthes *et al.*, 1996), they disrupted the *Oprm1* gene utilising a technique called homologous recombination. This involved the insertion of a neomycin resistant cassette into Exon 2 of the gene and was performed on embryonic stem cells from the 129/Sv mouse line. Continued breeding was performed with C57BL/6J mice and the mice are now available fully backcrossed onto C57BL/6J background from Jackson Labs (stock number 007559). Exon 1 of the gene has also been targeted but this produced mice with impaired sexual function and breeding and also altered haematopoiesis (Sora *et al.*, 1997, Tian *et al.*, 1997).

DOP-/- mice were first generated by Dr John Pintar's lab in 1999 (Zhu *et al.*, 1999). The DOP-/- mice transferred to our lab from Dr Wendy Walwyn were first generated by Dr Brigitte Kieffer's lab. They inactivated the *Oprd1* gene by targeting Exon 1 for deletion with a neomycin cassette. This was inserted into embryonic stem cells from the 129/Sv mouse line and further breeding was performed with C57BL/6J mice (Filliol *et al.*, 2000). The mice are now available fully backcrossed onto C57BL/6J background from Jackson Labs (stock number 007557).

The BAR2-/- mice were initially developed by Dr Robert Lefkowitz's lab in 1999. These mice were created by utilising a homologous recombination technique targeting Exon 2 of the β-arrestin 2 gene on Chromosome 11. These mice were again created using embryonic stem cells from the mouse line 129/Sv and

further breeding and backcrossing performed on the C57BL/6J background (Bohn *et al*., 1999). These mice are also available from Jackson Labs stock number 011130.

MOP-/-, DOP-/-, BAR2-/- and BAR2-/-//DOP-/- mice were kindly transferred from Dr Wendy Walwyn at UCLA, these mice were maintained on a C57BL/6J background. BAR2-/-//DOP-/- mice were obtained by mating BAR2-/- and DOP-/- mice and subsequent offspring until the required double knockout was obtained. Since these mice have arrived with us they have been further backcrossed onto the C57BL/6J background. BAR2-/-//DOP-/- mice have been subsequently outbred using JAX C57BL/6J mice and appropriately re-crossed to obtain the double knockout model. All mice were maintained in the Medical Resource Unit in accordance with Home Office regulations. They had free access to food and water with 12 hour cycles of light and dark corresponding to day/ night externally. All of our experiments were performed in the light phase. When required mice were culled utilising a schedule 1 method.

2.2 Genotyping

Genotyping was initially performed in house using the following protocols but subsequently contracted to Transnetyx (USA). All test samples utilised tissue from ear clipping, tissue was collected from the mice once they were greater than six weeks of age and they were ear-tagged. For mice that were used to

generate brain slices or DRG preparations ear tissue was collected post mortem and analysed to confirm genotype.

Genomic DNA was extracted from ear clippings using the following protocol. Extraction solution (Sigma) 50 µL was added to the ear clip sample along with 12.5 µL of tissue preparation solution (Sigma); these were allowed to incubate at room temperature for 10 minutes then at 95°C on the heat block for a further 3 minutes. Neutralisation solution (50 µL, Sigma) was then added to stop the reaction. The resulting genomic DNA was either used immediately for PCR or stored at -20°C until required.

A master-mix was created for each PCR reaction as required (Table 2.1) and the samples set up on the thermo-cycler using the appropriate programme (Table 2.3). The primers used for each reaction consisted of a forward, reverse and middle primer, the sequences of which can be viewed in Table 2.2, they were obtained from Sigma. Following completion of the PCR reactions the samples were kept at 4°C until an electrophoresis gel could be run.

We used 1% agarose TAE gel with ethidium bromide (10 µL / 100 mls, Sigma) to run the MOP and BAR2 reactions and a 2% agarose TAE gel with ethidium bromide for the DOP reaction as this allowed better separation of the bands for this reaction. We added 10 µL of loading dye to each 50 µL PCR reaction and loaded 30 µL of each reaction to the gel. A 1 kb DNA ladder (Fisher) was used for the MOP and DOP reactions and a 100 bp ladder (Invitrogen) for the BAR2

reaction. Electrophoresis gels were run at 100 V for 60 – 80 minutes until adequate band separation had occurred and imaged using a UV light source. For the MOP results we expect to see a WT band visible at 700 bp and a KO band visible at 400 bp. For the DOP reaction we expect to observe a band at 1000 bp and a KO band at 600 bp. The BAR2 reaction produced a WT band at 188 bp and a KO band at 400 bp. For all of the reactions one band was present to represent either WT or KO and heterozygote animals were identified by the presence of both bands.

Reagent	MOP Reagents (µL)	DOP Reagents (µL)	BAR2 Reagents (µL)
Forward primer 10 µM	0.4	0.3	0.75
Middle primer 10 µM	0.6	0.3	0.5
Reverse primer 10 µM	0.8	0.3	0.75
Deoxynucleotide triphosphate mix (dNTP) 10 mM	2	2	1
10x Dream Taq Buffer	5	5	5
Dimethyl sulfoxide (DMSO) 100%	2.5	3.5	0
50 mM MgCl2	2.5	1.5	0
Dream Taq 5U/ µL	0.4	0.5	0.2
Template DNA	4	2	2
Sterile ddH20	31.8	34.6	39.8

Table 2.1: The PCR master-mix recipe for each of the mouse genotypes. This was varied between the genotypes as detailed above. The primer sequences for each reaction can be viewed in a separate figure. Reagents were supplied by Sigma and Fisher.

	MOP	DOP	BAR2
Forward	5' GAG TTA GGA GAA TCA GGA GTT CAA G 3'	5' TCC ATC AGA GAA CAC GCA GCA CAA 3'	5' TCT TCC CTG CCC CGA TTT C 3'
Reverse	5' TGC CAT GAA CAT TAC GGG CAG AC 3'	5' CGC CTC CGG ACC ACG TGG 3'	5' AGG TGA GAG CCC CAA GAT G 3'
Middle	5' ACC GCT TCC TCG TGC TTT ACG GTA 3'	5' ACC GCT TCC TCG TGC TTT ACG GTA 3'	5' ATG TGG AAT GTG TGC GAG GCC AGA G 3'

Table 2.2: The forward, reverse and middle primer sequences required for genotyping each genetically modified mouse line.

A

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	5 minutes	1
Denaturation	94	60 seconds	30
Annealing	62	60 seconds	
Extension	72	60 seconds	
Final extension	72	5 minutes	1
Hold	4	As required	1

B

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	15 minutes	1
Denaturation	94	60 seconds	40
Annealing	64	120 seconds	
Extension	72	120 seconds	
Final extension	72	10 minutes	1
Hold	4	As required	1

C

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	2 minutes	1
Denaturation	94	45 seconds	40
Annealing	60	45 seconds	
Extension	68	90 seconds	
Final extension	72	5 minutes	1
Hold	4	As required	1

Table 2.3: The PCR thermo-cycler conditions for (A) the MOP PCR reaction (B) the DOP PCR reaction and (C) the BAR2 PCR reaction.

2.3 Behavioural Studies

For three days prior to each experiment mice were handled and habituated to the room where the tests were to take place. The room temperature was maintained between 19 and 21°C. All experiments took place during daytime hours. All drug doses were calculated using individual body weight, maximum volume administered in a single injection was 200 µL. Groups of mice were made up of equal numbers of males and females where possible and balanced numbers when not. Mice were aged from 7 weeks to 24 weeks of age at the time of their participation in the tasks. At the end of each experimental protocol the mice were killed using a schedule one method.

2.3.1 Assessing morphine analgesia

The hot water tail withdrawal assay was used to assess morphine analgesia. We used an electronic thermostatic circulating water bath (Thermostatic circulator bath Optima general purpose digital +5°C to 100°C, 12L stainless steel tank Fisher Scientific) that maintained the temperature within ± 0.1 °C of that set. Prior to the start of each experiment we performed a baseline tail withdrawal assay using 48°C water with a maximum cut-off exposure time of 15 seconds. If a mouse left its tail in the water for the maximum time at any point throughout the experiment we removed it to prevent damage. The mice were restrained gently in a plastic tube from their home cage to allow the immersion of the distal 3 cm of their tail in the hot water. They were habituated to this restraint during

the three day handling period prior to the start of the experiment. Before and after each test the mice were maintained in their home cages.

To investigate analgesic dose response mice were treated with cumulative doses of morphine sulphate (Sigma) of 0.1, 0.3, 1, 3, 10 and 30 mg/Kg; this was prepared in an aseptic environment and filtered using a 0.2 µm syringe filter prior to use. Morphine was diluted in 0.9% NaCl at varying concentrations to allow correct dose/volume of the drug to be administered. Injections of morphine were performed subcutaneously (SC) into the scruff of the neck. Thirty minutes after each morphine dose the tail withdrawal assay was performed. Results were calculated as a percentage of maximal possible effect (MPE: % MPE = 100*((drug latency – basal latency) / (15 s – basal latency))). Once a mouse had reached the 15 second maximum it received no further doses of drug.

2.3.2 Morphine tolerance

To investigate the development of analgesic tolerance to morphine we treated mice with 10 mg/Kg morphine sulphate via a subcutaneous injection once daily for 10 days. The injections were performed at the same time each day and all experiments took place during the light phase. On each experimental day we performed a baseline tail withdrawal assay using the circulating hot water bath (bath settings 48°C, 15 s maximum exposure time). The mice then received a 1 mg/Kg injection of morphine sulphate administered subcutaneously and a repeat tail withdrawal assay was performed thirty minutes later. Following this they

received an injection of 10 mg/Kg morphine sulphate administered subcutaneously, with a repeat tail withdrawal assay again performed thirty minutes after the morphine dose.

2.3.3 Psychomotor testing: Locomotor activation and CPP

We used a two compartment model of conditioned place preference to investigate morphine reinforcement in mice. One chamber had a wallcovering consisting of black and white horizontal stripes and the other black and white vertical stripes. The floor material was the same in each of the chambers; it consisted of 1 cm square wire grid flooring material. The grid direction of this material differs depending on its orientation. We utilised this property to provide a difference in the floor between each chamber. The direction of the grid matched the wall stripe direction in each chamber. Each test arena measured 28 x 28 cm and was 19 cm high. Two test arenas, each consisting of a two compartment apparatus, are contained within an operant box (Figure 2.1). The test apparatus was matched to mice of specific genders and only mice of the same gender were placed in the same operant box set-up. The majority of the mice placed in the same operant box for testing were cage mates, but this could not always be ensured for the male mice. These boxes are soundproofed and allow the light levels to be controlled at approximately 70 lumens, the temperature of the room was maintained between 21 and 23°C. The boxes also contain fans but we did not use them during the course of these experiments as due to their location on one side of the operant box we were concerned that they would affect the conditioning phase of the experiment for the test arena

closest to the fan. For each experimental protocol we used eight mice, four male and four female. All of the mice used for the behavioural experiments were handled for three days prior to the start of the study. This allowed habituation to handling, the experimental room and other mice to be used in the experiment. On day one of the study the mice were habituated to the testing environment and allowed free access to both chambers for 15 minutes. Time spent in each chamber and the distance that they travelled was recorded using a CCTV camera connected to a PC and analysed using AnyMaze software with a point fixed on the mid-point of each mouse's body.

After habituation equal numbers of mice were assigned to either the horizontal or vertical chamber to receive a subcutaneous injection of 0.9% saline (volume matched to that of the morphine injection). Four hours later the mice received a subcutaneous injection of morphine sulphate in the opposite chamber from where they received their first injection. After each injection they were confined to the corresponding chamber for thirty minutes and the distance that they travelled was tracked using AnyMaze software. In between conditioning sessions the mice were returned to their home cages. We performed the injections in this order due to the duration of action of morphine. If we counterbalanced the injections there may still be a morphine effect at the time of the second conditioning session. All mice were killed using a schedule 1 method when they completed their involvement in the study.

WT, MOP-/-, DOP-/-, BAR2-/- and BAR2-/-//DOP-/- mice were conditioned at both 3 mg/Kg and 10 mg/Kg of morphine sulphate. MOP+/- mice were conditioned at both 10 mg/Kg and 30 mg/Kg. We used groups of 8 mice from each genotype for each dose of morphine that was tested. On the fifth day of the CPP protocol the mice were allowed free access to both of the chambers for fifteen minutes, this was the test period. We recorded the time that they spent in each chamber and then compared the two. Preference scores for morphine paired chambers were calculated by subtracting the time spent in the saline paired chamber from the time spent in the morphine paired chamber.

For the experiments involving c-Src inhibition, dasatinib (Bristol Squibb Myer) was dissolved in DMSO (Sigma) to give a 50 mg/ml stock. The final concentration of the solution for injection was 1 mg/ml dasatinib to allow administration of 5 mg/Kg. This consisted of a 2% DMSO and 2% Kolliphor EL (Sigma) in a 0.9% saline solution.

The matched vehicle injection contained the same constituents but without the active drug. PP2 (Tocris) was also dissolved in DMSO at its solubility limit of 25 mM and diluted in a 0.9% saline solution to give a final concentration of 1 mg/ml (16% DMSO and 16% Kolliphor EL). PP3 (1-Phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine; Tocris) has a higher solubility limit and so was made up in DMSO at 100 mM and diluted in a 0.9% saline solution to give a final concentration of 1 mg/ml (5% DMSO and 5% Kolliphor EL). These drugs were all administered via the intraperitoneal (IP) route.

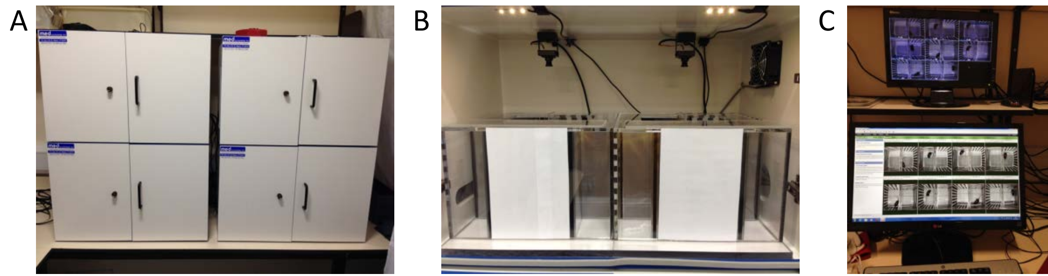


Figure 2.1: CPP apparatus. (A) We have four operant boxes each containing two sets of CPP apparatus. (B) Each box has a camera positioned directly above it to monitor mouse movement and location, the light level has been set at 70 lumens using LED lighting strips. (C) The camera system feeds into both a recording system and the computer AnyMaze software.

2.4 Dorsal Root Ganglion neurone culture preparation

Mice were killed using cervical dislocation, following which dorsal root ganglia (DRG) were harvested from C57BL/6J mice at postnatal day (PN) 17 to 21. When possible, matched mice were utilised for brain slicing and DRG harvest. Cells were collected in ice cold Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS, Invitrogen) and then dissociated both enzymatically (collagenase type 1 (Sigma)/ dispase (Sigma, 4 mg/ml) and papain (Sigma, 40 units/ml)) and physically by trituration using a fire polished glass pipette. The neurones were plated onto poly-D-lysine (Sigma) and laminin (Sigma) coated 13 mm diameter coverslips housed within 35 mm cell culture dishes (VWR) (Walwyn *et al.*, 2007). Cells were cultured in Dulbecco's modified Eagle medium (DMEM)/ F-12 nutrient mixture (Invitrogen), supplemented with heat inactivated fetal bovine serum (FBS, 10%, Invitrogen) nerve growth factor (NGF, 50 ng/ml, Life technologies) and penicillin (100 µg/ml) and streptomycin (100 units/ml, Invitrogen) at 37°C and 5% CO_2.

Neurones were used for electrophysiological recordings on days 1 to 3 in culture. After this, if dishes remained cells were re-suspended and plated on newly coated coverslips to allow recording for up to 3 more days at appropriate cell density to allow single neurone recording. This procedure did not affect the properties of Ca^{2+} currents with regard to the effects of morphine.

Whole cell patch recordings were obtained from the DRG neurones, Ca^{2+} currents mediated by VACCs were activated by depolarising small diameter (<25 µm) DRG neurones from -80 mV to +10 mV (as described previously by (Walwyn *et al.*, 2007, Walwyn *et al.*, 2009). The experiments were performed using an extracellular solution containing 130 mM TEA, 10 mM $CaCl_2$, 5 mM HEPES, 25 mM glucose and 500nM tetrodotoxin (TTX, Tocris) at a pH of 7.2 (obtained by adjusting with CsOH).

Borosilicate glass pipettes of 2-3 MΩ (World Precision Instruments, 1.5/1.12 OD/ID (mm)) were manufactured for recording and filled with intracellular solution. The intracellular solution contained 105 mM CsCl 40 mM HEPES, 2.5 mM $MgCl_2$, 10 mM EGTA, 5 mM glucose, 2 mM ATP and 0.5 mM GTP, this solution was also pH adjusted to 7.2 using CsOH. Morphine sulphate was diluted into the extracellular solution from frozen stocks as required on the day of the experiment and subsequently spritzed onto the cell of interest at 15 psi.

Data was collected and recorded using an Axopatch 200A amplifier (Molecular devices, CA, USA). Data were low pass filtered at 2kHz, digitised at 8 kHz using a

digidata 1322A interface, acquired and analysed using pClamp 10.2 software (from molecular devices). A linear regression was performed for each recording to compensate for rundown and inhibition of VACC by morphine calculated.

2.5 Immunohistochemistry

Brain slices were fixed overnight in a 1% PFA solution containing picric acid (TAAB Labs). To make 200 ml of the fixative solution 1% paraformaldehyde (2 g) was heated in ddH_2O in a fume cupboard until hot at which point 400 µl of 4 M NaOH was added to dissolve the PFA. To this solution 30 ml of saturated picric acid and 100 ml of 0.2 M phosphate buffer were added and the whole solution was diluted with ddH_2O to reach a total volume of 200 ml, the final pH was between 7.2 and 7.4.

The 0.2 M phosphate buffer solution contains a 4:1 ratio of (A) 0.2 M dibasic sodium phosphate ($Na_2HPO_4*7H_2O$) and (B) 0.2 M monobasic sodium phosphate ($NaH_2PO_4*2H_2O$). After fixation the slices were washed three times in 0.1 M phosphate buffer (0.2 M phosphate buffer diluted 1: 1 with ddH_2O) containing 0.05% sodium azide and stored at 4°C until required.

The first step of the immunohistochemistry staining protocol was to wash the brain slices three times, (each wash was for 15 minutes), in 0.1 M phosphate buffer containing 0.3% Triton (PBT) to permeabilise the cells. We then blocked in 10% bovine serum in PBT for 30 minutes at room temperature. The primary

antibody we used to identify the dopaminergic neurones was rabbit anti-tyrosine hydroxylase which we used at a 1:1000 dilution (Millipore AB152) in 10% bovine serum PBT. This was incubated with the slices for 2 nights at 4°C gently rocking. The slices were then washed three times, again for 15 minutes each wash, in PBT. To identify the neurones that we had recorded from we added biocytin 1% to our intracellular recording solution and allowed this to diffuse into the cell.

For the second blocking step we used 0.2% BSA in 0.1 M PBT for 1 hour at room temperature. Incubation in our secondary antibody mix then took place in the dark in 0.2 % BSA in PBT overnight at 4°C gently rocking. The secondary antibody mix comprised anti-rabbit IgG conjugated AlexaFluor 594 at a dilution of 1:1000 (Invitrogen A-21207) to identify the TH labelled cells and Streptavidin conjugated AlexaFlour 488 at a dilution of 1: 200 (Invitrogen S-11223) to identify the biocytin containing cells. The slices were then washed in PB (three times 15 minute washes) in the dark and mounted on slides with FluorSave reagent (Millipore). The slides were then left to cure at 4°C in the dark and subsequently imaged using confocal microscopy.

The slices were examined under a Leica TCS SP-5 confocal microscope (Leica) and images were obtained at 10x and 40x magnification. The AlexaFluor 594 bound to the primary anti-tyrosine hydroxylase antibody was excited at 594 nm and the emitted fluorescence was collected between 600 nm and 650 nm. The Streptavidin conjugated AlexaFluor 488 was excited at 488 nm and the emitted fluorescence was collected between 500 nm and 550 nm.

2.6 Slice Electrophysiology

Mouse brain slices were prepared from C57BL/6J (Wild type) mice aged postnatal day (PN) 17 – 21. Mice were killed by cervical dislocation. Immediately following decapitation the brain was removed and placed in ice cold solution bubbled with 95% O_2/5% CO_2. This solution used for slicing contained a high concentration of sucrose instead of NaCl as this is thought to improve the VTA cell viability. The solution comprised: 234 mM sucrose, 26 mM $NaHCO_3$, 10 mM glucose, 10 mM $MgSO_4$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, and 0.5 mM $CaCl_2$. Horizontal slices of 250 μm thickness containing the VTA were cut in ice cold sucrose solution using a Leica vibratome, set at a speed of 0.14 mm/s and amplitude of 1 mm. These horizontal slices corresponded to Bregma -4.12 to Bregma -4.44 (Paxinos and Franklin, 2013). Slices were then incubated in extracellular solution bubbled with 95% O_2/5% CO_2 for $\geq$ 1 hr at room temperature, this solution consisted of 126 mM NaCl, 26 mM $NaHCO_3$, 10 mM Glucose, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1.3 mM NaH_2PO_4. We added 2 mM kynurenic acid sodium salt (Abcam) to this solution as required for recording this was done to inhibit glutamatergic events. When mIPSC recording was required tetrodotoxin (TTX) was added to this solution at a final concentration of 500 nM, this was omitted when recording of sIPSC events was required.

Borosilicate glass pipettes of 2-5 MΩ (World Precision Instruments, 1.5/1.12 OD/ID (mm)) were manufactured for recording and filled with intracellular solution containing: 138 mM CsCl, 10 mM HEPES, 10 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Mg-ATP, 5 mM QX-314 (Tocris) (pH 7.4 with CsOH). Slices were

held in position with a small grid fashioned from platinum wire and nylon tights. Recordings were made from slices superfused with a continuously oxygenated (95% O_2/5% CO_2) saline based extracellular solution and maintained at 36°C with an in-line temperature controller (HPT-2 heated perfusion tube, ALA Science). An infrared differential interference contrast microscope with a water immersion objective (x40) was used to obtain images displayed on a video monitor.

Recordings were also obtained in current clamp mode. This allowed the study of the effects of morphine and naloxone on action potential (AP) frequency. For these recordings the intracellular solution consisted of 123 mM KCl, 15 mM NaCl, 10 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM ATP and 1 mM $CaCl_2$. This solution was adjusted to pH 7.2 – 7.3 using CsOH. For these recordings kynurenic acid was omitted from the extracellular solution.

Whole cell voltage clamp recordings were obtained at -60mV and current clamp recordings at 0 mA using an Axopatch 200B, the data collected were filtered at 5 kHz, digitised at 10 kHz (National Instruments NADAQ-MX) and acquired using Strathclyde Electrophysiology Software. All chemicals were supplied by Sigma unless otherwise stated.

Spontaneous inhibitory postsynaptic current (sIPSC) parameters were investigated in the absence and presence of a variety of drugs (morphine (Sigma), DAMGO (Sigma), DPDPE (Abcam), ondansetron (Sigma), PP2 (Tocris),

PP3 (Tocris), SL327 (Tocris), naloxone (Tocris) and bicuculline (Sigma)). All drug solutions were prepared as required on the day of use from frozen stock solutions and all were diluted in the extracellular solution containing 2 mM kynurenic acid (Abcam).

All recordings were obtained and analysed offline using Strathclyde Electrophysiology Software, WinEDR and WinWCP. Each recording segment (i.e. control or drug) consisted of at least 450 s, this was then divided into three 120 s segments and the event frequency of this segment analysed and averaged. To allow for the kinetic analysis the IPSCs were threshold detected (amplitude -4 pA, duration 3 ms) and visually inspected, a minimum of 50 events per recorded segment were used for analysis. This analysis included peak amplitude, rise time and decay time.

The decay phase of the averaged IPSCs (from each control/ drug treated segment) were best fitted (98–5% of the peak amplitude) with a double exponential function ($y^{(t)} = A_{fast}.e^{(-t / \tau\ fast)} + A_{slow}.e^{(-t / \tau\ slow)}$), where t was time, A was the amplitude, and τ was the decay time constant. A weighted decay time constant (T_{ω}) was also calculated for the averaged events from the calculation: $T_{\omega} = T_1P_1 + T_2P_2$, where T_1 and T_2 are the decay time constants and P_1 and P_2 are the proportions of the decay relative to each of the component parts (Maguire *et al.*, 2014).

2.7 SW620 cell culture

SW620 human colon cancer cells (Catalog No. CCL-227) were obtained directly from American Type Culture Collection (ATCC). These cells have high levels of c-Src activity and were used here to confirm the inhibitory effects of dasatinib and PP2. Phosphorylated c-Src (pc-Src) is the activated form of c-Src within the cell, comparison of the ratio of c-Src to pc-Src can provide information about activity (Roskoski Jr, 2015). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO_2. Cells were passaged twice weekly, plated on 60 mm culture dishes and incubated with dasatinib, PP2 and PP3 all at a 10 µM concentration for 24 hours prior to collection.

2.8 Western Blotting

To obtain protein for western blotting the SW620 cells were collected and lysed using RIPA buffer (Fisher). Alternatively tissue was harvested post mortem from mice treated with the drugs of interest during behavioural studies. Brain and DRG tissue were homogenised using RIPA buffer and the Tissue-Tearor (Biospec products). The protein levels obtained from both methods were measured using the Pierce BCA protein assay kit (Fisher).

For each sample between 5 and 50 µg of protein was added to an SDS loading buffer (containing 125 mM Tris-HCl, 40 % glycerol, 20 % β-mercaptoethanol, 8 % SDS and 0.008 % bromophenol blue) and H_2O to give a final sample volume of 20 µl for each well required. This mix was heated at 95°C for 5 minutes and then loaded onto a NuPAGE Bis-Tris 4 -12 % 10 well mini gel (Invitrogen). The gel was run with a MOPS-SDS running buffer (Invitrogen) for 90 minutes at 120 V. We used actin (Abcam) as a loading control for the SW620 experiment, GAPDH (Abcam) for the experiments utilising brain and DRG tissue and the ladder used for both was SeeBlue Plus 2 prestained standard (Invitrogen).

For protein transfer chromatography paper (6 pieces per gel)(Sigma) and nitrocellulose transfer membrane (GE healthcare)(1 piece per gel) were soaked in transfer buffer containing Tris (2.42 g/ L) and glycine (14.4 g/ L) in ddH2O and 20 % methanol and compiled. The transfer was performed in transfer buffer at 4°C at 200 mA for 90 minutes. After completion the nitrocellulose membrane was removed and soaked in Ponceau solution (1 mg/ml) for 2 minutes to reveal the protein bands. The membrane was then washed with ddH_2O and cut to size. The first one hour blocking step was performed at room temperature in 5% BSA for the membrane containing Src and p-Src and 5% milk protein for the membrane containing the loading controls. The primary antibodies were then diluted in the relevant blocking buffer to which 0.02% sodium azide was also added. The Src antibody (rabbit)(Cell signalling #2108) was diluted to 1:2000 as was the p-Src antibody (rabbit)(Cell signalling #2101). The actin antibody (mouse)(Abcam) was diluted to 1:1000 and the GAPDH (rabbit)(Abcam) was

diluted to 1:1000. They were incubated with the membranes at 4°C overnight gently rocking and then washed 4 times with TBS-T, this contained Tris (2.4 g/L), NaCl (8.8 g/L) and Tween-20 (0.1%). The secondary antibodies were goat anti-rabbit HRP (Abcam) at a 1:5000 in 5 % BSA and anti-mouse HRP (Abcam) at 1:1000 in 5 % milk protein. These were incubated with the membranes at room temperature for an hour. The membranes were then washed for ten minutes 4 times and prepared for the enhanced chemiluminescence reaction. This reaction was performed using ECL prime western blotting detection media (GE Healthcare) and the blots developed in the dark room with the required exposure time.

2.9 Statistical Analysis

Data are represented as mean ± standard error of the mean (SEM). The graph plots and statistical analysis were performed using GraphPad Prism 5 software. Statistical significance ($p < 0.05$) was determined either by Student's t test (paired or unpaired as appropriate) or by analysis of variance (ANOVA), (one or two way as appropriate), with the relevant *post hoc* tests.

Chapter 3: Analgesia and Tolerance

3.1 Introduction

Morphine analgesia is mediated by MOP receptors. Morphine administration to MOP-/- mice does not cause analgesia (Matthes *et al*., 1996, Sora *et al*., 1997). There are a number of simple behavioural assays for assessing nociception and analgesia in mice (Wilson and Mogil, 2001, Le Bars *et al*., 2001, Barrot, 2012). These include nociceptive responses to heat, cold, mechanical stimuli and chemical stimuli. With regards to morphine analgesia there are several tests that are accepted as useful and reproducible. These include the tail flick test with radiant heat or immersion, paw withdrawal, the hot plate test and response to cold stimuli.

The method that we have chosen here is tail withdrawal from hot water. This is a modification of D'Amour and Smith's method (1941). The tail flick reflex is a spinal reflex that is generally regarded to reflect spinal analgesia (Kieffer, 1999). This is in contrast to the hotplate test that is subject to supraspinal influences and requires integration of a number of varying system inputs. The tail withdrawal assay is a simple and reproducible test that is highly responsive to opioid drugs (Wilson and Mogil, 2001, Barrot, 2012), but it does require restraint of the mouse which we perform using a plastic tube that is normally present in their cage. To minimise the stress associated with this the mice are habituated to the test room and handling for three days prior to the start of each test. The tail flick response can also be affected by the ambient temperature of the room (Tjølsen and Hole, 1993); we therefore maintain the test room at a temperature between 19 and 21°C. An advantage of this test is that due to the significant

surface area of the tail exposure there is a rapid increase in temperature of the tail and a spinal reflex response (Le Bars *et al*., 2001).

It has been noted that there are significant strain differences in pain behaviour between different commonly used laboratory mouse strains (Brase *et al*., 1977, Mogil *et al*., 1999, Leo *et al*., 2008). All of our mice are back crossed over many generations to the C57Bl/6J genetic background to allow comparison between groups and to a control population of C57Bl/6J mice. There are conflicting results reported with regard to gender differences in tail withdrawal time from hot water. Kest *et al* (1999) found an increase in baseline tail withdrawal time in male C57Bl/6J mice compared to females but a subsequent study was not able to identify a gender difference using the same strain of mice and the identical nociceptive test (Kieffer *et al*., 1992). We have used balanced groups of male and female mice across all of behavioural experiments to avoid introducing bias by gender.

Nociceptive stimuli are detected by a collection of peripheral nerve fibres termed nociceptors. Their cell bodies are located within the dorsal root ganglia (DRG). Binding of an opioid to MOP receptors leads to activation of inhibitory G proteins leading to several intracellular effects (Williams *et al*., 2001). This includes inhibition of Ca^{2+} entry through voltage-activated Ca^{2+} channels (VACCs) in primary afferent nociceptor DRG neurones. The inhibition of presynaptic

VACCs gives rise to the opioid-mediated reduction of excitatory neurotransmission in the pain pathway (Heinke *et al*., 2011).

As previously discussed opioid agonists do not produce analgesia in MOP-/- mice. However analgesia and a number of other opioid induced side effects including tolerance, locomotor activation (in rodents), constipation and respiratory depression are not solely MOP receptor mediated (Schmid and Bohn, 2009). We know from previous studies that DOP receptors are not required for morphine-mediated analgesia, but their absence leads to blunted morphine analgesic tolerance (Zhu *et al*., 1999). In DRG neurones DOP receptors are required for the full functional expression of MOP receptors and inhibition of VACCs by DAMGO (Walwyn *et al*., 2009).

MOP receptors in primary afferent neurones constitutively internalise, a process that is inhibited by either the absence of BAR2 or inhibition of c-Src (Walwyn *et al*., 2007). This implies that there is constitutive recruitment by MOP receptors of BAR2/c-Src in these neurones. BAR2 is a scaffolding protein that has been implicated in analgesia and tolerance. BAR2-/- mice appear phenotypically normal but develop negligible tolerance to opioid analgesia (Bohn *et al*., 1999, Bohn *et al*., 2000, Bohn *et al*., 2002). In addition the mouse's endogenous opioid pain killing mechanism becomes persistently active without the requirement for drug administration. This occurs with no evidence of the hedonic effects typically associated with opioid drug taking (Lam *et al*., 2011). When expressed alone in

the absence of agonist Rozenfeld and Devi detected no co-localization between MOP receptors and BAR2 using confocal microscopy (Rozenfeld and Devi, 2007). However, co-expression of MOP and DOP receptors led to co-localization of all three proteins at the cell membrane; consistent with the idea that MOP/DOP receptor heterodimerisation is required for constitutive recruitment of BAR2.

We confirmed a role for DOP receptors in the constitutive recruitment of BAR2 using a protein complementation assay in which inactive fragments of β-galactosidase are attached to MOP receptors and of BAR2 (Baptista-Hon *et al*., 2013). The introduction of recombinant DOP receptors into cells expressing tagged MOP receptors and BAR2 led to enhanced agonist-independent β-galactosidase activity.

The basal analgesia observed in BAR2-/- mice is associated with constitutive MOP receptor coupling to VACCs in primary afferent neurones from these mice, a phenomenon that can be recapitulated in wild type neurones by directly inhibiting c-Src activity implicating tyrosine kinase-mediated phosphorylation (Walwyn *et al*., 2007).

Tolerance to opioids is a major problem particularly in the management of chronic pain including cancer pain, where to control symptoms escalating drug doses are often required (Rang *et al*., 2000). This then exposes patients to many other worsening drug side effects. Understanding the mechanisms of tolerance is therefore very important to allow alternative methods of drug design and administration.

3.2 Analgesia

3.2.1 The role of MOP receptors

Female WT mice have a significantly shorter baseline tail withdrawal latency suggesting that they have a higher sensitivity to noxious stimuli compared to the male mice (Figure 3.1A). Morphine administration to wild type C57Bl/6J (WT) mice produces dose-dependent analgesia (Figure 3.1B). The latency for tail withdrawal from hot (48°C) water was established with a maximum exposure time of 15 s to prevent tail damage. The percentage of maximum possible effect (MPE) was then calculated using the formula; % MPE = 100*((drug latency – basal latency) / (15 s – basal latency)). ED_{50} values were established from logistic fits to the morphine dose-response relationships. For WT mice ED_{50} = 1.2 ± 0.1 mg/Kg morphine, n = 29 (Table 3.1). Despite the apparent gender difference in pain sensitivity, when the morphine ED_{50} values for male and female WT mice were compared there was no significant sex difference (ED_{50} male WT 1.1 ± 0.2 mg/Kg, n = 14 and ED_{50} female WT 1.3 ± 0.1 mg/Kg morphine, n = 15, unpaired t test $p = 0.2$).

MOP+/- mice, in which one copy of the MOP receptor gene has been deleted, have a 50% reduction in MOP receptor number compared to WT mice (Sora *et al*., 2001). In these mice we observe a rightward shift in the dose-response curve indicating a reduction of morphine potency (ED_{50} MOP+/- = 6.2 ± 0.8 mg/Kg morphine, n = 15), without altered efficacy (Figure 3.1B and Table 3.1). It is not possible to compare sex differences across the genotypes at this time due to the small n numbers in the MOP+/- and MOP-/- groups. As future experiments are completed utilising these mice this will be addressed.

MOP-/- mice, which lack MOP receptors, were unaffected by morphine (10 mg/Kg) confirming the requirement for MOP receptors in opioid analgesia (Figure 3.1B). There was no significant difference in the basal tail withdrawal latencies between WT, MOP+/- and MOP-/- mice, one way ANOVA p = 0.1 (Figure 3.1C). This implies that there is no critical role for MOP receptors in the basal sensitivity of mouse tails to noxious heat.

The inhibition of VACCs in primary afferent nociceptive DRG neurones plays an important role in morphine analgesia (Schroeder and McCleskey, 1993, Rusin and Moises, 1995, Heinke *et al*., 2011). DRG neurones were cultured from WT, MOP+/- and MOP-/- mice as described in the methods section. Whole-cell currents mediated by VACCs were activated by depolarising small diameter (<25 µm) DRG neurones from -80 mV to +10 mV. Morphine caused an inhibition of Ca^{2+} currents recorded from WT DRG neurones (Figure 3.1D). In MOP+/- mice

there was a reduction in the ability of morphine to inhibit the VACC and in MOP-/- it was absent, one way ANOVA $p < 0.009$, WT $n = 4$, MOP+/- $n = 7$, MOP-/- $n = 5$. This confirms the importance of MOP receptors in morphine-evoked inhibition of VACC activity.

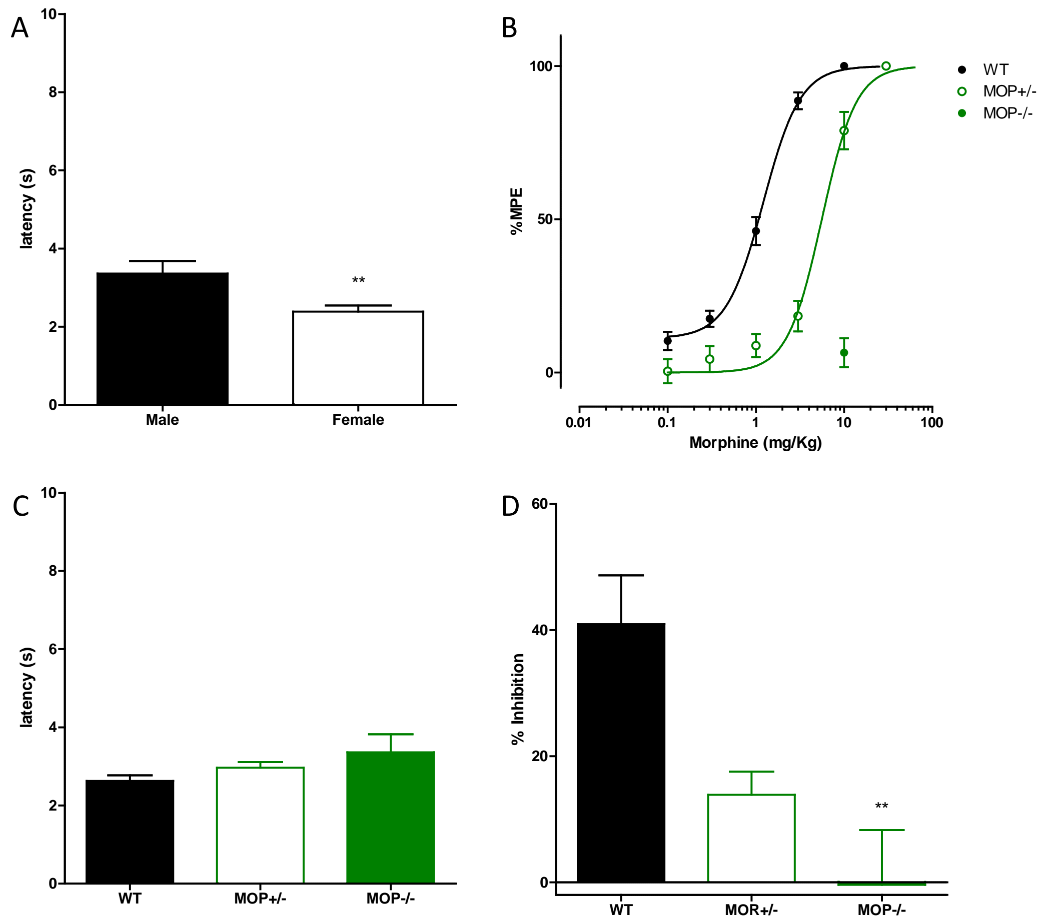


Figure 3.1: MOP receptors are required for morphine analgesia and inhibition of VACCs in DRG neurones. (A) Female WT mice have a significantly faster baseline tail withdrawal latency compared to the male mice. Male mice 3.4 ± 0.3 s, n = 39, female mice 2.4 ± 0.2 s, n = 46. Unpaired t test p = 0.006. (B) Dose response relationship for morphine analgesia in WT, MOP+/- and MOP-/- mice of mixed gender. ED_{50} WT = 1.2 ± 0.1 mg/Kg morphine, n = 29, ED_{50} MOP+/- = 6.2 ± 0.8 mg/Kg morphine, n = 15. MOP-/- n = 10, no significant analgesia received from morphine sulphate. (C) Baseline tail withdrawal latencies for WT, MOP+/- and MOP-/- mice do not differ significantly (one way ANOVA). (D) Mean VACC inhibition in DRG neurones by morphine is reduced in a gene dependent manner compared to the WT neurones, one way ANOVA p < 0.001 *post hoc* Tukey results are shown on the graph, n = 4 - 7. For all figures the vertical lines represent ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.

3.2.2 The Role of DOP receptors

We established dose-response relationships for morphine in WT, DOP+/- and DOP-/- mice (Figure 3.2A and B). The ED_{50} for morphine analgesia differs between WT and DOP-/- mice (Figure 3.2C). There is also a reduction in the slope for morphine analgesia in DOP-/- mice compared to WT mice (Figure 3.2D and Table 3.1). Basal tail withdrawal times did not differ between WT, DOP+/- and DOP-/- mice (one way ANOVA) (Figure 3.2E). These data suggest that, while not necessary for morphine analgesia, DOP receptors are involved. This is consistent with previous work in DRG neurones demonstrating that the inhibition of VACCs is reduced in the absence of DOP receptors (Walwyn *et al.*, 2009). The altered slope for morphine analgesia is evidence of an interaction between MOP and DOP receptors in the pain pathway.

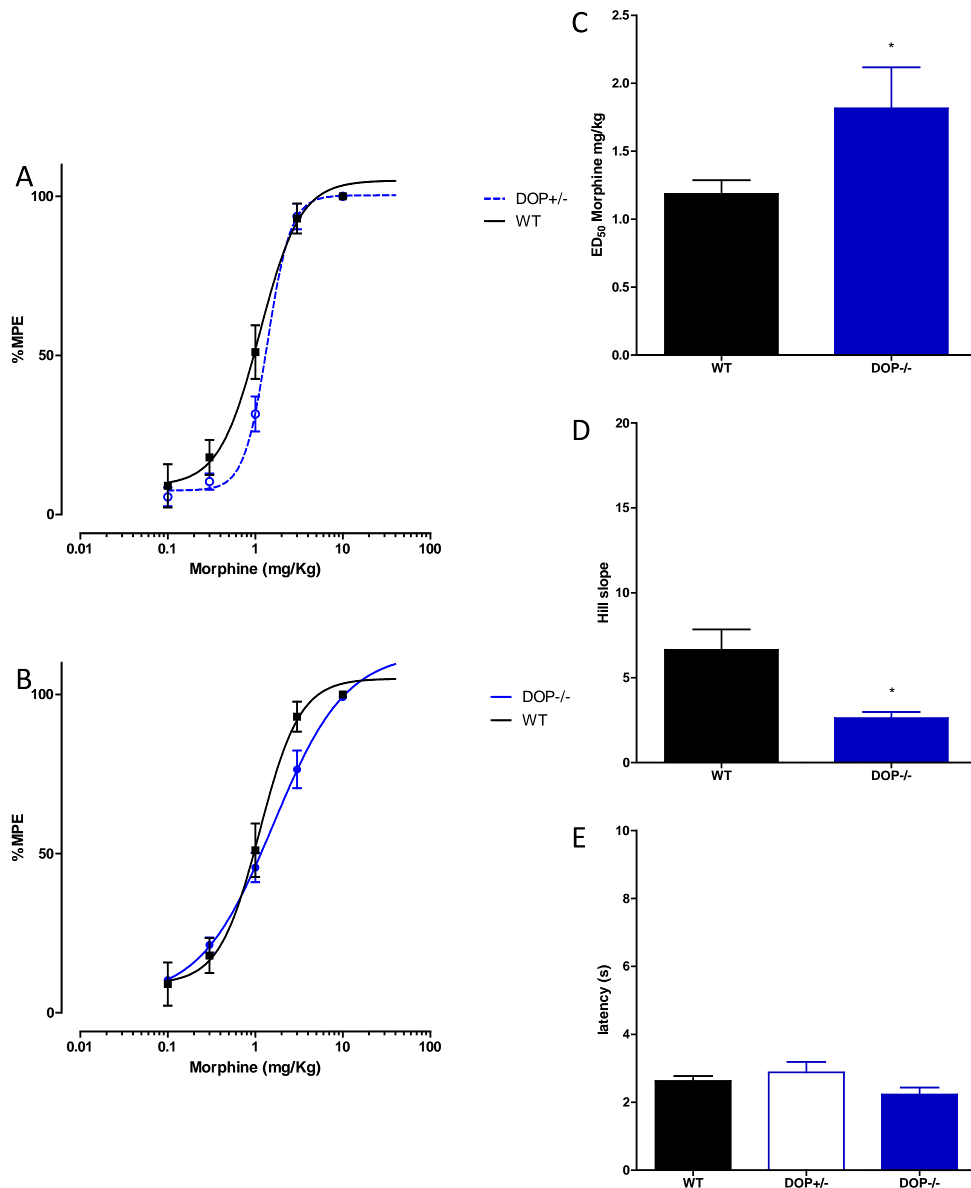


Figure 3.2: Morphine is less potent as an analgesic in DOP-/- mice. (A) Morphine dose response relationship for WT and DOP+/- mice. The ED_{50} for DOP+/- mice is 1.4 ± 0.2 mg/Kg, n = 14. This compares to the WT ED_{50} of 1.2 ± 0.1 mg/Kg, n = 29. The slope of the curve is not significantly different from that of the WT dose response curve, slope DOP+/- 6.8 ± 1.0 and slope WT 6.6 ± 1.2, unpaired t test p = 0.95. (B) Morphine dose response curve for WT and DOP-/- mice. The DOP-/- ED_{50} is 1.8 ± 0.3 mg/Kg, n = 14. (C) The DOP-/- mice show a significantly increased ED_{50} for morphine compared to the WT mice. Unpaired t test $p < 0.02$. (D) The morphine analgesia dose response relationship for DOP-/- mice also exhibited a significantly reduced slope (2.6 ± 0.4) compared to that for WT mice, unpaired t test p = 0.03. (E) DOP-/- mice do not show basal analgesia. Baseline tail withdrawal times are shown for WT, DOP+/- and DOP-/- mice, which do not differ significantly (one way ANOVA). Vertical lines represent ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2.3 The role of β-arrestin2

We investigated morphine analgesia in BAR2+/- and BAR2-/- mice and compared this to WT mice. The BAR2-/- mice do not have a significantly different ED_{50} for morphine analgesia tested using the tail withdrawal assay (Figure 3.3A and Table 3.1). As previously reported BAR2-/- mice exhibit a significantly longer basal tail withdrawal latency in a genotype dependent manner (one way ANOVA) (Figure 3.3B).

As described previously for WT, MOP+/- and MOP-/-, we recorded VACCs from DRG neurones to investigate the effects of morphine in the presence of these modifications. Morphine (3 µM) caused a 41 ± 8% inhibition of VACC recorded from WT DRG neurones, in the BAR2-/- neurones this inhibition was significantly reduced to 5.1 ± 2.0% (Figure 3.3C). This reduction in the ability of morphine to inhibit the VACC in BAR2-/- DRGs has been previously reported (Walwyn *et al.*, 2007). It is thought to be due to an increase in constitutive activity of MOP receptors in the absence of BAR2. This renders the receptor unavailable to the exogenous agonist and so the overall effect of the drug is reduced.

In light of the evidence for an interaction between MOP and DOP receptors in the pain pathway we hypothesised that basal analgesia in the absence of BAR2 is caused by constitutively active MOP/DOP oligomers (Figure 1.4 Chapter 1).

We tested this hypothesis by breeding mice that lack genes for both BAR2 and DOP receptors (BAR2-/-//DOP-/-). The rationale being that if MOP/DOP

oligomers are required for constitutive analgesia these double knockout mice should lack the phenomenon. The ED_{50} for the BAR2-/-//DOP-/- mice is not significantly different to that for the WT mice (Figure 3.4 and Table 3.1). These mice differ from WT mice by exhibiting basal analgesia, (unpaired t test $p < 0.0001$, WT n = 110, BAR2-/-//DOP-/- n = 48), (Figure 3.4B). This suggests that DOP receptors are not required for the phenomenon of basal analgesia and constitutive opioid receptor signalling observed in mice lacking BAR2.

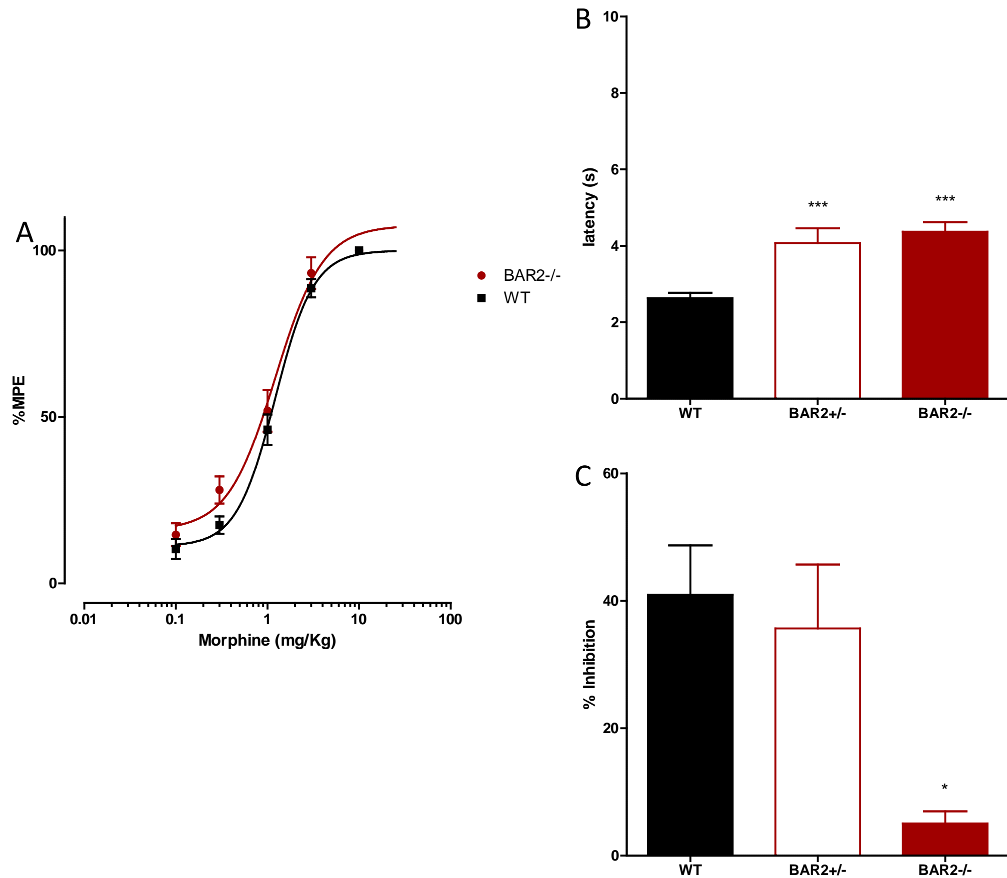


Figure 3.3: A lack of BAR2 has no significant effect on the dose-response relationship for morphine analgesia but causes basal analgesia. (A) Dose response relationships for morphine analgesia in BAR2-/- and WT mice. There is no difference in ED_{50} value for morphine analgesia between WT and BAR2-/- mice. ED_{50} WT = 1.2 ± 0.1 mg/Kg, ED_{50} BAR2-/- = 1.5 ± 0.4 mg/Kg morphine, WT n = 29, BAR2-/- n = 16. (B) BAR2-/- mice show basal analgesia. Their baseline tail withdrawal latencies are significantly prolonged compared to WT mice, (one way ANOVA p < 0.0001, *post hoc* Dunnett's shown on graph, WT n= 110, BAR2+/- n= 30 and BAR2-/- n = 75). (C) Mean VACC inhibition by morphine is reduced in BAR2-/- neurones compared to the WT neurones, one way ANOVA with post hoc Dunnett's, p < 0.01. Data points are averages of between 4 and 6 recordings. Vertical lines represent ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.

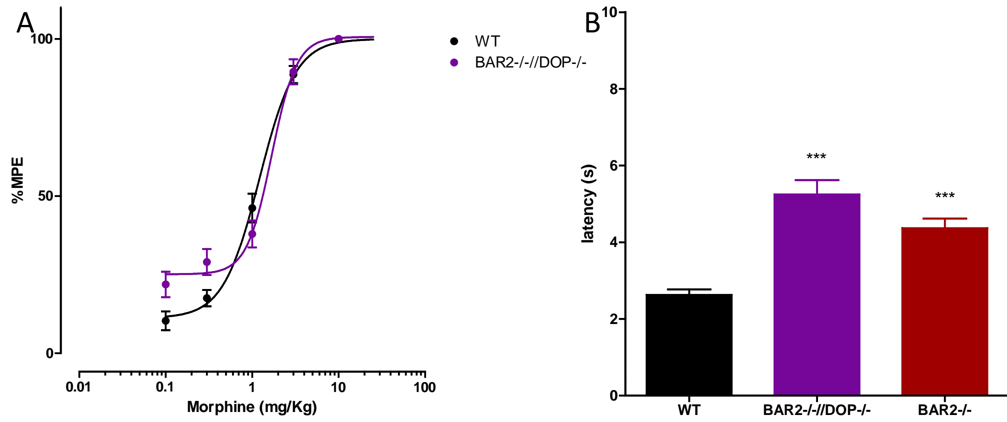


Figure 3.4: Mice that lack DOP receptors and BAR2 exhibit basal analgesia. (A) Dose response relationships for morphine analgesia in WT and BAR2-/-//DOP-/- mice. WT ED_{50} = 1.2 ± 0.1 mg/Kg, n = 29 and BAR2-/-//DOP-/- ED_{50} = 1.4 ± 0.2 mg/Kg morphine, n = 16. (B) Like BAR2-/- mice, BAR2-/-//DOP-/- mice exhibit basal analgesia. One way ANOVA p < 0.0001 *post hoc* Dunnett's shown on graph. WT n = 110, BAR2-/-//DOP-/- n = 48, BAR2-/- n = 75. Vertical lines represent ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.

3.3 Tolerance

3.3.1 The role of MOP receptors

Analgesic tolerance in WT mice leads to a reduction in opioid potency, observed as a rightward shift in the dose response relationship. The subcutaneous administration of 10 mg/Kg morphine once daily for 10 days, caused tolerance in WT mice as evidenced by a significant reduction in the tail withdrawal latency following morphine administration on day 10, one way ANOVA $p < 0.0001$, $n = 16$ (Figure 3.5A).

Compared to the WT mice, morphine analgesic tolerance developed significantly faster in MOP+/- mice. When these mice were given 10 mg/Kg morphine once daily for ten days there was a dramatic reduction in the analgesic effect of morphine, one way ANOVA $p < 0.0001$, $n = 15$ (Figure 3.5B). The mice show significant tolerance from day 4 onwards, despite starting with an analgesic effect of morphine that is not significantly different to WT mice. When compared directly to the WT mice (Figure 3.5C), there is a significant decrease in morphine analgesia in MOP+/- mice from day 4 onwards, (two way ANOVA, time $p < 0.0001$, genotype $p < 0.0001$, $n = 15$ for MOP+/- mice and $n = 16$ for WT mice).

As previously discussed, the morphine dose response relationship was established for naïve MOP+/- mice (Figure 3.1B), this revealed an ED_{50} of 6.3 ± 0.8 mg/Kg morphine. We examined the dose-response relationship in MOP+/- mice that had received five days of 10 mg/Kg morphine. At this time point

MOP+/- mice exhibit tolerance to morphine analgesia (Figure 3.5B). The dose-response relationship in these tolerant mice reveals both a further reduction in potency (rightward shift of the dose response curve) without an apparent reduction in the efficacy of morphine. The ED_{50} for morphine analgesia shifted from 6.3 ± 0.8 mg/Kg in naïve MOP+/- mice, to 43 ± 15 mg/Kg in tolerant MOP+/- mice, n = 7 (Figure 3.5D).

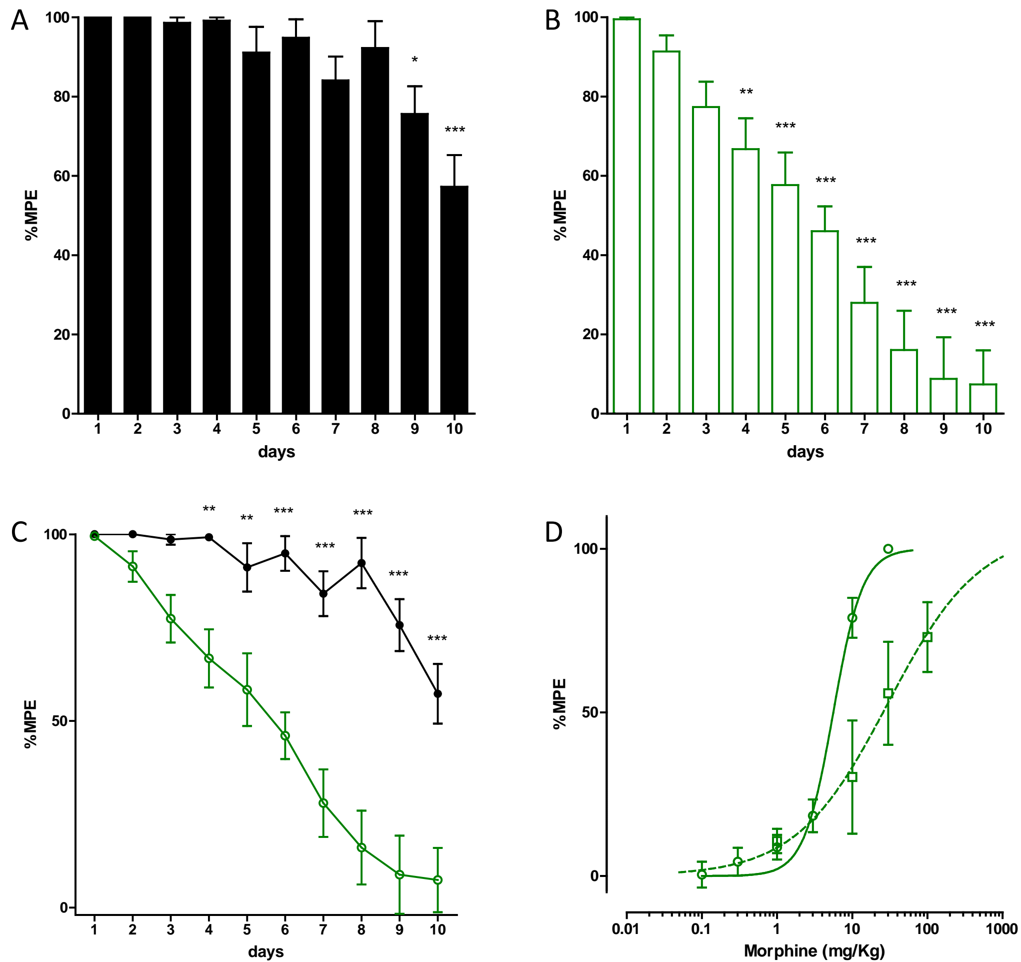


Figure 3.5: Development of morphine tolerance after repeated daily dosing in mice. For all figures analgesic effect of morphine has been measured using the tail immersion assay and expressed as %MPE, described in Chapter 2 (%MPE = 100*((drug latency – basal latency) / (15 s – basal latency))). (A) WT mice exhibit significant tolerance to morphine after 10 days of once daily subcutaneous administration. One way repeated measures ANOVA $p < 0.0001$, *post hoc* Tukey results vs day 1 shown on graph (n = 16). (B) Induction of tolerance in MOP+/- mice. One way repeated measures ANOVA $p < 0.0001$, *post hoc* Tukey vs day 1 shown on graph (n = 15). (C) Morphine tolerance develops significantly earlier and to a greater extent in MOP+/- mice compared to WT mice. Two way ANOVA, time $p < 0.0001$, genotype $p < 0.0001$, *post hoc* Bonferroni results are shown on graph. n = 15 for MOP+/- mice and n = 16 for WT mice. For all figures the black symbols represent WT mice and the open green symbols represent MOP+/- mice. (D) Effects of chronic morphine treatment on dose response for morphine analgesia in MOP+/- mice. ED_{50} analgesia for morphine naïve mice is 6.3 ± 0.8 mg/Kg morphine. The ED_{50} following 5 days of morphine treatment in the MOP+/- mice is 43 ± 15 mg/Kg morphine, n = 7. The solid line represents opiate naïve MOP+/- mice and the dotted line tolerant MOP+/- mice. Vertical lines represent ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3.2 A role for DOP receptors in morphine tolerance

DOP+/- and DOP-/- mice also exhibit differences compared to WT mice in the development of tolerance to morphine. Significant morphine tolerance occurs by day 10 in DOP+/- and DOP-/- mice (DOP+/- mice one way ANOVA $p < 0.0001$, $n = 15$. DOP-/- one way ANOVA DOP-/- $p = 0.01$, $n = 16$) (Figure 3.6A and B). While significant morphine tolerance does develop in the DOP+/- and DOP-/- mice, this is significantly reduced from that seen in the WT mice. On days 9 and 10 a significantly higher level of morphine analgesia remains in DOP-/- mice compared to the WT mice, two way ANOVA shows a significant difference in genotype compared to WT ($p = 0.03$), WT $n = 16$ and DOP-/- $n = 16$ (Figure 3.6C). The DOP receptor is important in the development of tolerance to morphine, removing the DOP receptor results in significantly reduced tolerance to morphine.

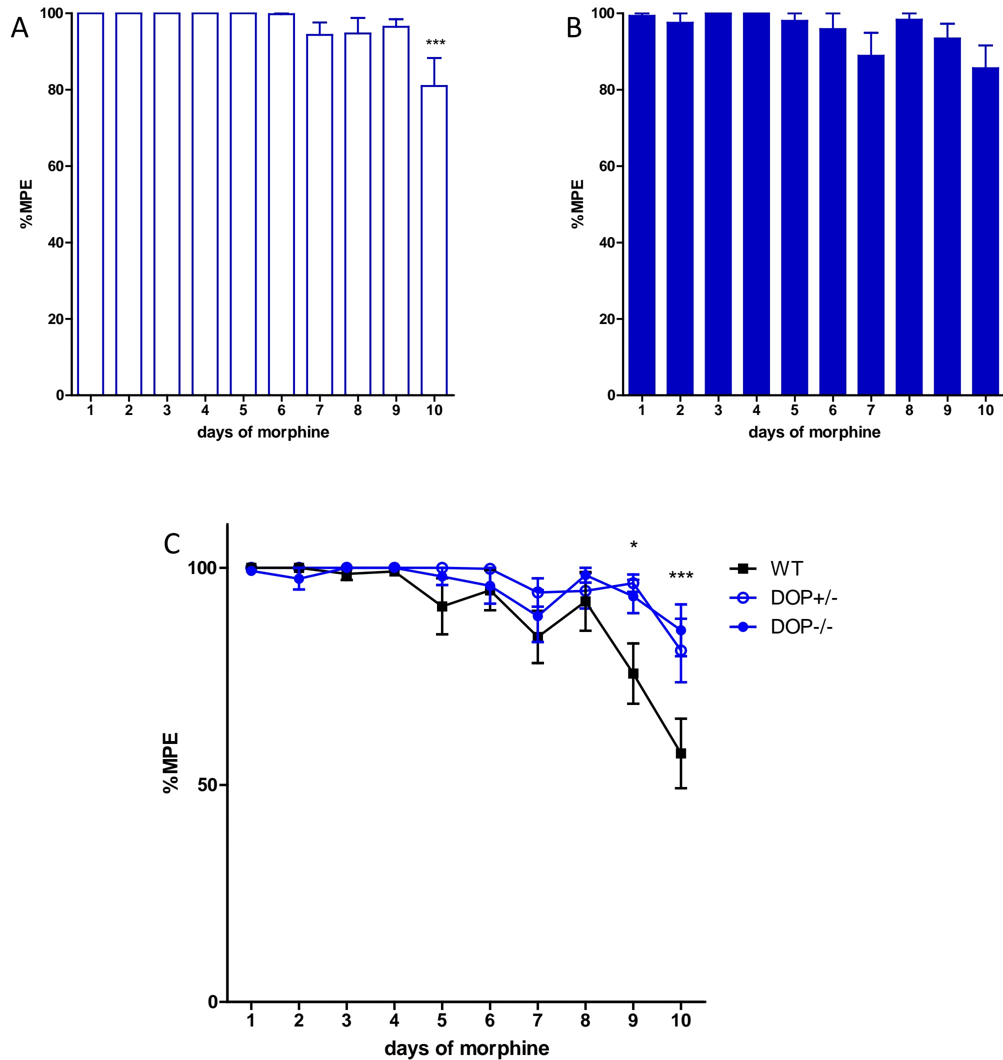


Figure 3.6: Mice lacking DOP receptors exhibit reduced morphine analgesic tolerance. (A) DOP+/- show significant morphine tolerance following 10 days of once daily morphine 10 mg/Kg. One way repeated measures ANOVA $p < 0.0001$, n = 15, *post hoc* Tukey results shown on the graph. (B) DOP-/- mice show reduced morphine tolerance over 10 days of once daily morphine administration. One way repeated measures ANOVA DOP-/- $p = 0.01$, n = 16. No *post hoc* significant differences were identified. (C) Morphine tolerance is significantly reduced in DOP+/- compared to WT mice. Two way ANOVA reveals a significant reduction in tolerance in the DOP+/- mice compared to WT mice ($p < 0.01$). Two way ANOVA comparing the WT and DOP-/- mice reveals a significant difference in genotype ($p = 0.03$), *post hoc* Bonferroni results shown on graph, WT n = 16 and DOP-/- n = 16. Vertical lines represent ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3.3 A role BAR2 in morphine tolerance

BAR2 is also important in the development of tolerance to morphine analgesia. When the BAR2-/- mice were given morphine daily for the 10 day period they exhibited a blunted, but significant tolerance, one way ANOVA p = 0.02, n = 15 (Figure 3.7B). Both the BAR2+/- and BAR2-/- mice exhibited a significant reduction in morphine tolerance during the 10 day protocol when compared to the WT mice, two way ANOVA, genotype p= 0.0007, WT n = 16, BAR2+/- n = 8, BAR2-/- n = 15, (Figure 3.7C).

When the tolerance paradigm was performed in BAR2-/-//DOP-/- mice morphine caused no tolerance even after 10 days of exposure (Figure 3.8A). This was significantly different to the WT mice, two way ANOVA, time p = 0.0005, genotype p = 0.007, WT n = 16, BAR2-/-//DOP-/- n = 7, and also appears different to BAR2-/- and DOP-/- mice (Figure 3.8B and C). These data suggest that both BAR2 and DOP receptors are involved in the development of tolerance to the analgesic effects of morphine.

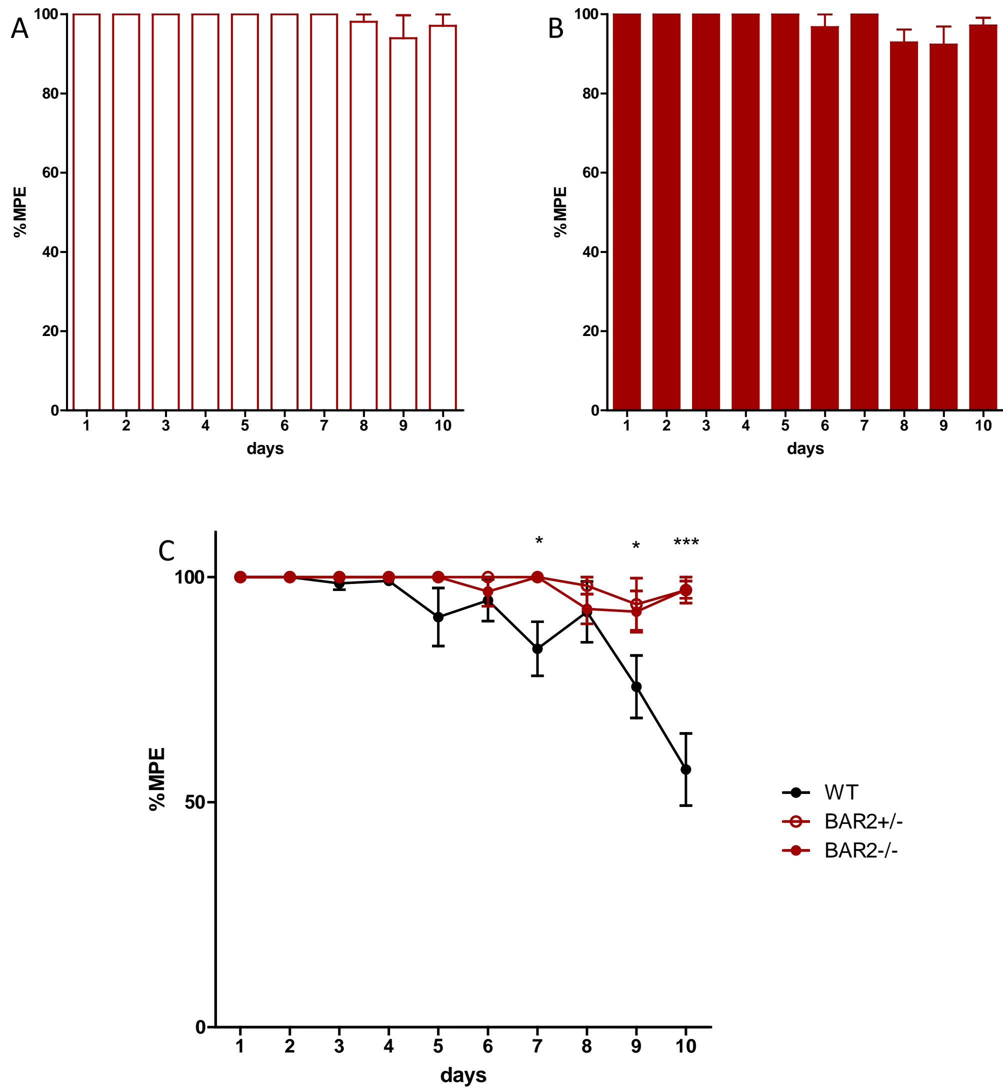


Figure 3.7: BAR2+/- and BAR2-/- mice exhibit reduced tolerance to morphine analgesia. (A) BAR+/- mice do not exhibit significant morphine analgesic tolerance following 10 days of once daily dosing, one way ANOVA ns. (B) Likewise, morphine tolerance in BAR2-/- mice is minimal at day 10, one way ANOVA $p = 0.02$ no significant differences were identified on *post hoc* testing, $n = 15$. (C) Both BAR2+/- and BAR2-/- mice show significantly less tolerance to morphine than WT mice, two way ANOVA, time $p < 0.0001$, genotype $p = 0.0007$, *post hoc* Bonferroni test results for BAR2-/- mice compared to day 1 are shown on the graph. BAR2+/- mice are significantly different from WT mice on days 9 and 10 (not shown). WT $n = 16$, BAR2+/- $n = 8$, BAR2-/- $n = 15$. Vertical lines represent ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

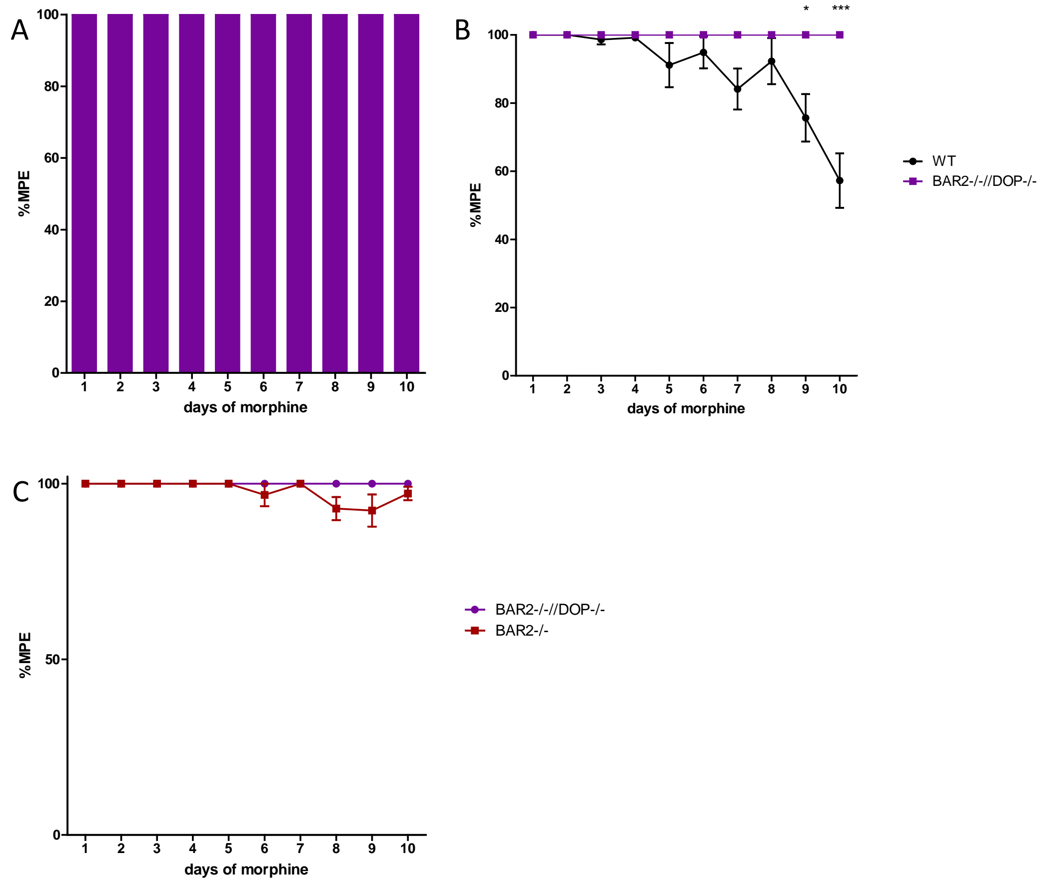


Figure 3.8: (A) BAR2-/-//DOP-/- mice do not become tolerant to morphine. Following 10 days of administration the level of morphine (10 mg/Kg) analgesia had not diminished. (B) BAR2-/-//DOP-/- mice exhibit significantly reduced tolerance compared to WT mice, two way ANOVA time p = 0.0005, genotype p = 0.007. Differences between WT and BAR2-/-//DOP-/- in *post hoc* Bonferroni tests are shown on the graph. WT n = 16, BAR2-/-//DOP-/- n = 7. Vertical lines represent ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001. (C) BAR2-/-//DOP-/- mice show less tolerance to morphine than the BAR2-/- mice.

3.4 Summary

We have confirmed in MOP-/- mice that morphine (10 mg/Kg) does not cause analgesia in the absence of MOP receptors. Morphine also does not inhibit VACC's in DRG neurones from MOP-/- mice. These observations are in keeping with previous findings (Matthes *et al*., 1996, Sora *et al*., 1997, Walwyn *et al*., 2005). In MOP+/- mice we observe a reduced analgesic potency of morphine with a rightward shift in the dose response curve, there does not appear to be a change in efficacy of the drug in opioid naïve mice. This reduction in potency is significant, while the slope of the morphine dose response relationship remained unchanged (Table 3.1).

While we identified a significant difference in the baseline tail withdrawal latency between male and female mice indicating a higher sensitivity of female mice to noxious heat, there were no significant differences between male and female mice in ED_{50} or development of morphine tolerance. However, the numbers in our groups are relatively small (a total of 29 mice in the analgesia experiment and a total of 16 mice in the tolerance experiment) and so it is likely that we would not be able to detect a subtle difference in morphine's actions between genders. A gender difference in baseline sensitivity to noxious heat of C57Bl/6 mice has been reported previously (Kest *et al*., 1999) and other investigators using much larger cohorts of mice have demonstrated gender differences in morphine analgesia with male rodents receiving greater analgesia from morphine (Cicero *et al*., 1996) and the development of tolerance with increased tolerance to the morphine recorded in female mice (Kest *et al*., 2000,

Mogil and Chanda, 2005). To account for this within our results and attempt to avoid introducing bias by gender we kept the genders of all experimental groups balanced.

MOP+/- mice exhibited a very rapid onset of tolerance following daily treatments with 10 mg/Kg morphine. They have significantly less analgesia at day 4 of the study protocol compared to day 1. This contrasts with WT mice in which there was no significant difference in morphine analgesia until day 9 of the protocol. MOP+/- mice also developed a significantly greater degree of tolerance to the analgesic effects of morphine by day 10 than that observed for WT mice. This suggests that MOP receptor number is important in the development of tolerance to morphine's analgesic effects. MOP+/- mice have 50% fewer MOP receptors than WT mice due to the deletion of one copy of the *oprm1* gene (Sora *et al.*, 1997). In WT mice the development of morphine tolerance appears to be limited by the existence of an excess of MOP receptors. The fact that morphine has a similar analgesic efficacy in naïve MOP+/- and WT mice suggests that the loss of 50% of the MOP receptors does not affect this and there are therefore spare receptors. The existence of spare receptors in WT mice presumably prevents a reduction in efficacy during morphine tolerance despite receptor down regulation.

The potency of morphine is affected by an absence of DOP receptors, the ED_{50} is significantly increased in the DOP-/- mice compared to the WT mice (Table 3.1).

The slope of the morphine analgesia dose response relationship is also significantly reduced in the DOP-/- mice compared to the WT mice. This could be explained by a difference in the way that morphine interacts with MOP/DOP receptor oligomers compared to homomeric MOP receptors on nociceptive neurones. It is possible that binding of morphine to MOP receptors is influenced by occupancy of adjacent DOP receptors leading to cooperativity.

While DOP-/- mice do develop tolerance to morphine this is significantly reduced compared to WT mice after 10 days of once daily dosing. This finding is consistent with work that has been previously published demonstrating the importance of DOP receptors in the development of tolerance (Zhu *et al*., 1999).

BAR2-/- mice exhibited no significant alteration in ED_{50} value for morphine analgesia but did show basal analgesia as previously discussed (Bohn *et al.*, 1999, Bohn *et al*., 2000, Bohn *et al*., 2002, Lam *et al*., 2011). Removal of BAR2 does not appear to affect binding of morphine as the ED_{50} and slope of the analgesic dose response curve to morphine are unaltered in the BAR2-/- mice.

BAR2-/- mice did develop tolerance to morphine over the course of our 10 day protocol, but this was significantly reduced when compared to the WT mice. We then investigated the double knockout BAR2-/-//DOP-/- mice. These mice did not show any morphine tolerance during our 10 day test protocol. They have the same measurable analgesic effect of morphine on day 1 of the protocol as on

day 10. Removing both BAR2 and DOP receptors appears to have completely abolished tolerance to morphine.

BAR2-/- mice show basal analgesia, their baseline tail withdrawal times, in the absence of exogenously administered drug, are significantly prolonged when compared to the WT mice. These data are consistent with previously reported studies that have also observed this phenomenon (Bohn *et al*., 1999, Bohn *et al*., 2000, Bohn *et al*., 2002, Lam *et al*., 2011). We also observed a prolonged baseline tail withdrawal time in the BAR2-/-//DOP-/- mice. These mice show basal analgesia that is not significantly different to that observed in the BAR2-/- mice. DOP-/- mice baseline tail withdrawal times are not significantly prolonged when compared to those of the WT mice. It appears that the removal of BAR2 allows constitutive signalling of MOP receptors to occur resulting in basal analgesia. There does not appear to be an involvement of DOP receptors in this process. However both BAR2 and DOP receptors are implicated in the development of tolerance to morphine.

These results reveal the importance of MOP receptor number, DOP receptors and BAR2 in the development of tolerance to morphine. When there is a normal intact system, as in the WT mice, MOP and DOP receptors and BAR2 are all functioning, then there is an absence of basal analgesia and tolerance develops following repeated morphine administration. When DOP receptors are removed from this system (MOP receptors and BAR2 remain) morphine tolerance is

significantly reduced and there is no basal analgesia. When BAR2 is removed leaving MOP and DOP receptors alone this causes basal analgesia and a reduction in the development of morphine tolerance. When both BAR2 and DOP receptors are removed, leaving MOP receptors alone, basal analgesia occurs with no demonstrable tolerance to morphine (Figure 3.9). The significance of these findings will be discussed in the context of the entire project in Chapter 7.

	ED_{50} (mg/Kg morphine)	slope	n number
WT	1.2 ± 0.1	6.6 ± 1.2	29
MOP+/-	6.2 ± 0.8*	5.7 ± 1.1	15
DOP+/-	1.4 ± 0.2	6.8 ± 1.0	13
DOP-/-	1.8 ± 0.3*	2.6 ± 0.4*	14
BAR2-/-	1.5 ± 0.4	8.2 ± 2.7	16
BAR2-/-//DOP-/-	1.4 ± 0.2	3.3 ± 1.0	16

Table 3.1: Summary of morphine ED_{50} (mg/Kg) and slope values for morphine analgesia in WT, MOP+/-, DOP+/-, DOP-/-, BAR2-/- and BAR2-/-//DOP-/- mice. * $P < 0.05$ on Student's t test compared to WT.

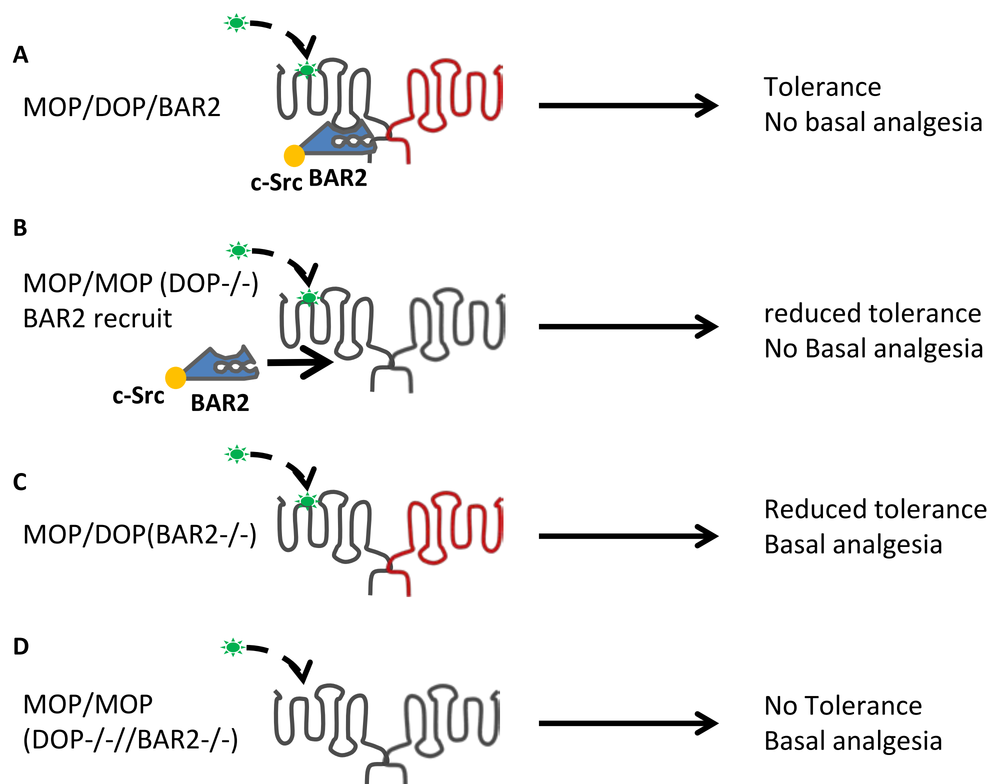


Figure 3.9: The roles of MOP and DOP receptors and BAR2 in basal analgesia and the development of morphine tolerance. (A) WT mice do not exhibit basal analgesia and develop tolerance to the analgesic effects of morphine. (B) DOP-/- mice also do not exhibit basal analgesia, however they do develop significantly reduced morphine tolerance when compared to the WT mice. (C) BAR2-/- mice exhibit the development of significantly reduced tolerance to morphine analgesia, but they also have prolonged basal tail withdrawal times indicating basal analgesia. (D) In mice that lack both BAR2 and the DOP receptor we observe basal analgesia and they do not develop any significant tolerance to the analgesic effects of morphine over the 10 day test period.

Chapter 4: Psychomotor effects of morphine

4.1 Introduction

Opioid receptors expressed throughout the reward pathway are involved in several natural (Agmo *et al*., 1993) and pharmacological rewards (Hall *et al*., 2001). MOP receptors are required for the rewarding effects of opioid drugs. MOP-/- mice, which have the MOP receptor gene deleted, neither self-administer opioids or exhibit opioid induced conditioned place preference (Matthes *et al*., 1996, Sora *et al*., 2001).

We used conditioned place preference (CPP) to examine the reinforcing effects of morphine. Conditioned place preference is a Pavlovian behavioural paradigm that has been developed to allow the study of the reinforcing properties of drugs (Bardo and Bevins, 2000). In order for this test to function the animal must be able to associate the context that they are placed in with the drug effect. The associative reward learning required for the mice to perform CPP needs dopaminergic neurones located within the VTA. Rodents will learn to self-administer opioid drugs directly into the VTA (Bozarth and Wise, 1984). These neurones are thought to encode the relationship between predictive cues and future events (Day *et al*., 2013). However, although the dopaminergic neurones of the VTA have been heavily implicated in rewarding processes, they can also be activated by aversive stimuli (Lammel *et al*., 2011). It is thought that they are crucial to decision making and so can respond to both rewarding or aversive stimuli and aid in the processing of an overall response. Dopamine D2 receptor knock-out mice do not self-administer opioid drugs (Elmer *et al*., 2002) and when they are conditioned with morphine in a CPP paradigm they do not exhibit

preference for the morphine paired chamber (Maldonado *et al*., 1997). However the D2 receptor knockout mice do retain the ability to demonstrate a preference for natural rewards suggesting that the reward processes for natural and drug induced effects may be instigated by separate mechanisms (Maldonado *et al*., 1997). In dopamine deficient (Th-/-) mice it was still possible to demonstrate a preference for morphine in the CPP paradigm in all but the lowest doses of morphine that were tested (2.5 mg/Kg), suggesting that although dopaminergic systems are important in reward seeking it may not be the only system involved in this process (Hnasko *et al*., 2005). Further work investigating the dopamine D1 receptor involvement in reward and locomotion following opioid administration has revealed that dopamine D1 receptors are not required for the mice to demonstrate a preference for morphine in the CPP paradigm but they are involved in the locomotor activation observed in rodents (Urs *et al*., 2011).

Many drugs of abuse, including opioids, increase locomotor activation in mice following administration to drug naïve animals. The extent to which morphine stimulates locomotor activity is dependent on the genetic background of the mouse. C57Bl/6J mice, which are the genetic background of all knockout mice used in this study, exhibit a robust dose dependent increase in locomotor activity in response to morphine (Brase *et al*., 1977). This locomotor activation is important in the study of the rewarding effects of opioid drugs because increases in mesolimbic dopamine release have been implicated in both locomotor activation and drug seeking and reward related behaviours (Robinson and Berridge, 2000). The psychomotor stimulation theory of addiction proposed

by Wise and Bozarth (1987), suggests that forward locomotion is a necessary manifestation of the goal directed behaviours required for rewarding processes.

Locomotor activity can be stimulated in rodents by direct administration of an opioid into the VTA (Kelley *et al*., 1980), implicating the VTA not only in reward related behaviour but also the observed locomotor activation. However, studies have failed to reliably correlate increased mesolimbic dopamine levels with locomotor activity following opioid administration (Murphy *et al.*, 2001), suggesting that there may be both dopamine dependent and dopamine independent mechanisms that underlie the locomotor activation observed with opioid drugs. Studies in dopamine deficient mice reveal that Th-/- mice do not display a significant locomotor response to morphine compared to matched controls except at very high doses of drug (Hnasko *et al*., 2005). The remaining locomotor activation observed in these mice is only 5% of that observed in the control mice. So, although dopamine independent mechanisms exist, they appear to only play a small part in this process in rodents.

The dopamine dependent component of locomotor activation is thought to be mediated via a dopamine D1 receptor mechanism. Activation of this pathway appears to recruit BAR2 and mitogen-activated protein kinase (MAPK). Phosphorylation and activation of MAPK requires the activity of mitogen-activated protein kinase kinase (MEK). Systemic administration of the MEK inhibitor, SL327, to WT mice inhibits morphine induced locomotion in a dose

dependent manner (Urs *et al*., 2011). SL327 has been shown to inhibit the activity of ERK in the brain (Beaulieu *et al*., 2006). This suggests that this pathway is important in the locomotor activation produced by morphine.

Repeated administration of MOP receptor agonists causes an increased locomotor response. This increase in response when drugs are repeatedly administered is known as sensitisation. It is postulated that this behaviour may be indicative of a switch from voluntary to compulsive intake of the drug and therefore indicative of vulnerability to addiction (Charbogne *et al*., 2014).

We measured locomotor activity during the conditioning phases of the CPP paradigm. This was done using a camera to track the position of the mouse within the apparatus based on a point coinciding with the position of the mid-body. Immediately following injection of drug or vehicle mice were placed within the corresponding box and monitored for 30 minutes. Our test apparatus consisted of two compartments. One chamber had a wallcovering consisting of black and white horizontal stripes and the other black and white vertical stripes. The floors in each chamber both consisted of 1 cm square wire grid flooring material, however the direction of the bars was different in each chamber to match the stripe direction. Each test chamber measured 28 x 28 cm and was 19 cm high. Two test arenas, each consisting of a two compartment apparatus, were contained within an operant box (Figure 2.1). These boxes are soundproofed and allow the light levels to be controlled at approximately 70

lumens; the temperature of the room was maintained between 21 and 23°C. The boxes also contain fans but we did not use them during the course of these experiments.

4.2 Locomotor effects of morphine

4.2.1 The role of MOP receptors

WT mice do not exhibit locomotor activation following the administration of morphine (3 mg/Kg). There are no significant changes in either the distance travelled or their average speed of travel following morphine or saline injection (Figure 4.1A – 4.1D). On day 1 the distance travelled in the 30 minutes following saline injection was 20.8 ± 1.1 m and following morphine 3 mg/Kg injection the distance travelled was 23.8 ± 2.0 m. The speed of travel of the mice on day 1 following saline injection was 0.012 ± 0.0007 m/s and following morphine 3 mg/Kg injection their speed of travel was 0.013 ± 0.001 m/s. The speed of travel was not significantly increased following three days of morphine 3 mg/Kg injection (0.014 ± 0.002 m/s).

However the WT mice did exhibit locomotor activation following administration of morphine (10 mg/Kg). Both their speed and the total distance travelled was significantly increased (Figure 4.1E – 4.1H). In this group of mice the distance travelled on day 1 in the 30 minutes following saline injection was 18.5 ± 1.4 m and following morphine 10 mg/Kg injection the distance travelled was 81.1 ± 7.0

m. Their average speed of travel for the 30 minutes was 0.01 ± 0.0008 m/s after saline injection and 0.045 ± 0.004 m/s after morphine 10 mg/Kg injection.

The WT mice also developed sensitisation to the locomotor effects of morphine, on day 3 of conditioning they travelled significantly further than they had on day 1 (118.6 ± 13.1 m) and their speed was similarly increased (0.07 ± 0.007 m/s) (Figure 4.1F and 4.1H). The distance travelled and the average speed following saline injection was unchanged on day 3 of the study (15.0 ± 2.0 m and 0.008 ± 0.001 m/s respectively).

MOP receptors have been implicated in the locomotor response to morphine (Matthes *et al.*, 1996, Sora *et al.*, 2001). Consistent with this, there was no significant locomotor activation following morphine administration to MOP-/- mice (Figure 4.2). Their distance travelled in the 30 minutes following saline injection on day 1 was 19.0 ± 1.6 m and their average speed of travel was 0.011 ± 0.0009 m/s. After an injection of morphine 10 mg/Kg the total distance travelled over the 30 minutes observation time was 16.9 ± 2.2 m and their average speed of travel was 0.009 ± 0.001 m/s.

There was locomotor activation in MOP+/- mice treated with 10 mg/kg morphine, but not sensitisation (Figure 4.3). The total distance travelled by the MOP+/- mice after saline injection was 14.3 ± 2.0 m and their speed of travel was 0.008 ± 0.001 m/s. Following administration of morphine 10 mg/Kg the total

distance travelled was 26.2 ± 1.3 m and their speed of travel was 0.015 ± 0.0007 m/s.

By day 3 of conditioning the distance travelled was 33.5 ± 3.8 m after morphine 10 mg/Kg injection; this was not a significant increase from day 1, $p = 0.12$. Furthermore, the locomotor response to morphine of the MOP+/- mice was significantly reduced compared to that of the WT mice. We investigated whether the reduced locomotor activation was due to a reduction in morphine potency in MOP+/- compared to WT mice. Consistent with this, 30 mg/Kg morphine caused a significant locomotor activation, which was increased compared to that observed after administration of 10 mg/Kg morphine (Figure 4.3). However, even at this increased dose of morphine there was no significant sensitisation of mice to the locomotor effects of morphine (30 mg/Kg). The locomotor activation that we see for both the 10 and the 30 mg/Kg morphine, although significant, is reduced compared to WT mice (Figure 4.4).

Consistent with previous observations these data demonstrate that morphine-evoked locomotor activity is mediated through MOP receptors. As observed for morphine analgesia, the number of MOP receptors is important; MOP+/- mice exhibited reduced locomotor activation and also the absence of significant sensitisation to morphine.

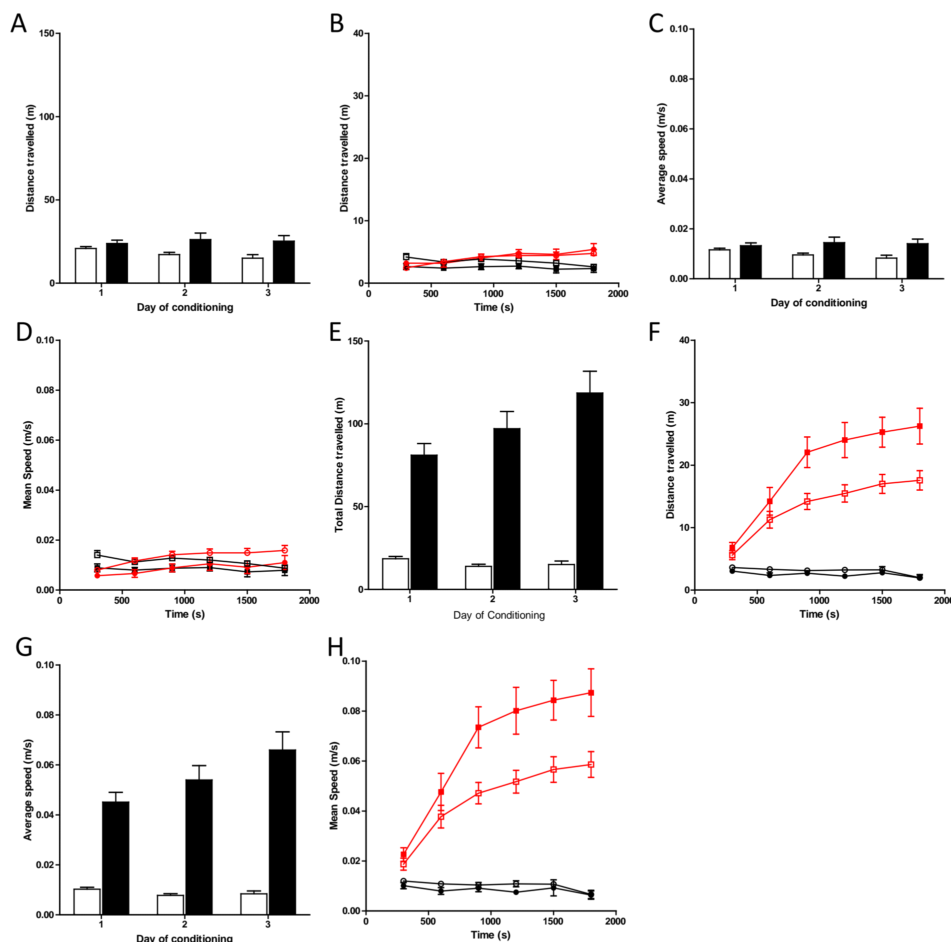


Figure 4.1: Morphine causes a dose dependent locomotor activation in WT mice. WT mice do not show significant locomotor activation after 3 mg/kg morphine. (A) There is no significant alteration in distance travelled or sensitisation following injection of morphine 3 mg/Kg over the 3 days of conditioning, two way ANOVA (ns). (B) There is no significant alteration in distance travelled following 3 mg/kg morphine administration and no sensitisation. (C) There are no significant changes in speed following injection of morphine 3 mg/Kg, two way ANOVA (ns). (D) There is no significant alteration in speed of travel over the 3 day protocol following morphine 3 mg/Kg administration. (E) WT mice exhibit locomotor activation following morphine 10 mg/Kg administration and sensitisation with repeated exposure. Distance travelled following morphine 10 mg/Kg is significantly increased compared to saline treatment, two way repeated measures ANOVA, time $p = 0.0002$ and morphine treatment $p < 0.0001$, $n = 8$. (F) WT mice show sensitisation to the locomotor effects of morphine. They travel a significantly increased distance on day 3 of conditioning compared to day 1. The saline injection does not affect locomotion and there is no significant change in the distance travelled in the saline compartment between day 1 and day 3. (G) Speed of travel is significantly increased following morphine 10 mg/Kg, two way repeated measures ANOVA, time $p = 0.0002$ and morphine treatment $p < 0.0001$, $n = 8$. (H) WT mice exhibited a significant increase in the speed of travel following morphine treatment. They also exhibit sensitisation to this effect with a significant increase in speed on day 3 compared to day 1. Bar graphs – white represents saline and black represents morphine. Line graphs – red square symbols represent morphine and black circle symbols represent saline. Open symbols are day 1 and closed symbols are day 3 of the study.

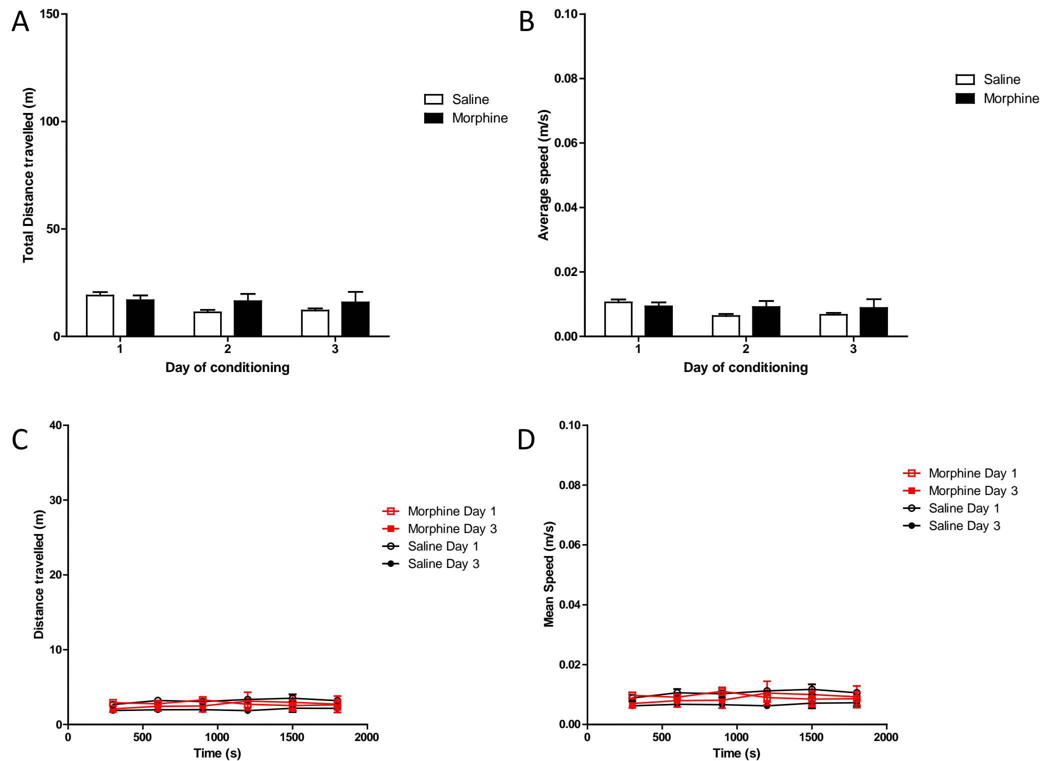


Figure 4.2: MOP-/- mice do not exhibit locomotor activation or sensitisation to morphine. (A) Distance travelled is not significantly different following morphine 10 mg/kg administration or saline injection, two way repeated measures ANOVA (ns), n = 8. (B) MOP-/- mice show no changes in speed of travel following conditioning with 10 mg/Kg morphine, two way repeated measures ANOVA (ns), n = 8. (C) MOP-/- mice did not exhibit sensitisation to the effects of morphine 10 mg/Kg over the conditioning period. The distance travelled was not significantly different to that of saline treated mice. (D) They did not exhibit a significant change in speed following administration of 10 mg/Kg morphine compared to the saline treated mice.

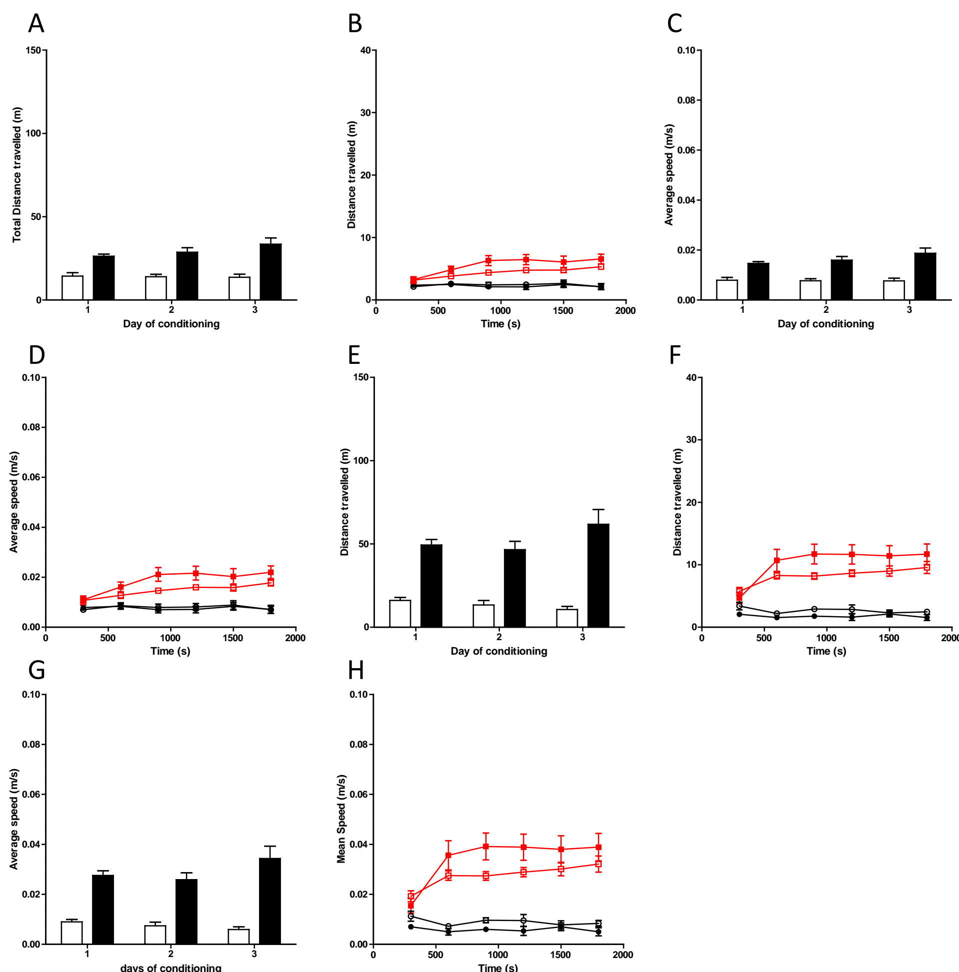


Figure 4.3: MOP+/- mice show a significant dose-dependent locomotor activation by morphine without sensitisation. (A) MOP+/- mice administered 10 mg/Kg morphine travelled significantly further than saline treated mice, two way repeated measures ANOVA, time (ns) and morphine treatment $p < 0.0001$, $n = 8$. (B) MOP+/- mice exhibited an increase in speed following conditioning with 10 mg/Kg morphine but no sensitisation to this effect over the three day conditioning period. Two way repeated measures ANOVA, time (ns), morphine treatment $p < 0.0001$, $n = 8$. (C) MOP+/- mice treated with 10mg/kg morphine do not show sensitisation over the three day conditioning period. There are no significant differences in their distance travelled between day 1 and day 3. (D) There are also no significant differences in the speed of travel between days 1 and 3 of morphine 10 mg/Kg treatment. MOP+/- treated with 30mg/kg morphine show locomotor activation to morphine but do not sensitise. (E) There is significant locomotor activation following administration of 30 mg/kg morphine to MOP+/- mice, two way repeated measures ANOVA, time (ns), morphine treatment $p < 0.0001$, $n = 8$. (F) There is a corresponding significant increase in the speed of travel following administration of morphine 10 mg/Kg, two way repeated measures ANOVA time (ns), morphine treatment $p < 0.0001$, $n = 8$. (G) MOP+/- mice treated with 30mg/kg morphine do not show sensitisation to locomotor activation. There is no significant increase in the distance travelled between day 1 and day 3. (H) No significant increase in speed is seen on day 3 compared to day 1, confirming that there is no sensitisation over this time period. Bar graphs – white represents saline and black represents morphine. Line graphs – red square symbols represent morphine and black circle symbols represent saline. Open symbols are day 1 and closed symbols are day 3 of the study. Vertical lines represent ± SEM.

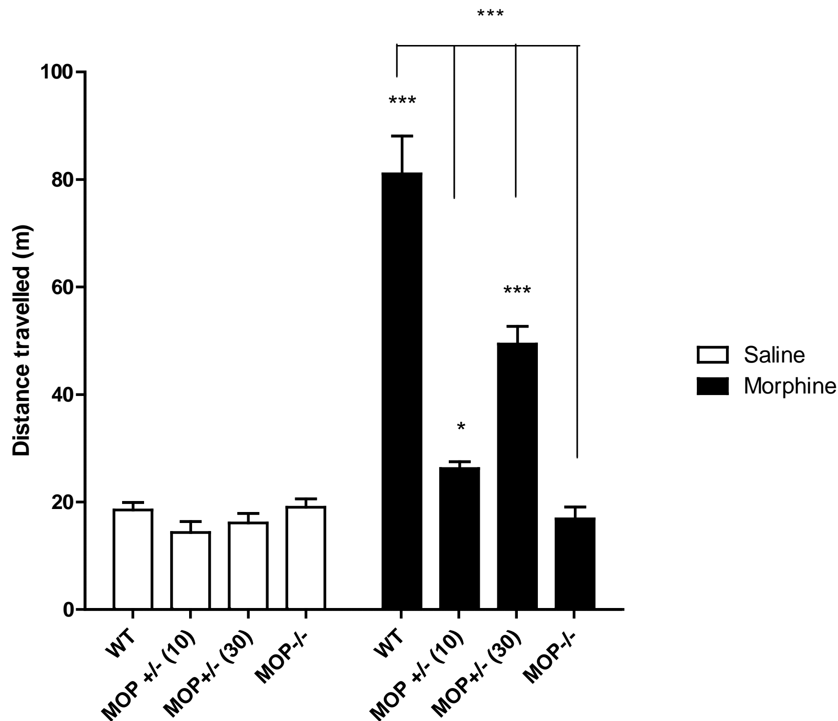


Figure 4.4: Locomotor activation by morphine is dependent on the MOP receptors. One way ANOVA distance travelled following saline injection (ns, $p = 0.21$). When distance travelled in the saline conditioning session is compared to the morphine 10 mg/Kg session for each genotype using a paired t test we observe locomotor activation in all groups except the MOP-/-, WT $p < 0.0001$, MOP+/- (10) $p = 0.0004$, MOP+/- (30) $p < 0.0001$, MOP-/- $p = 0.47$ (ns), all groups $n = 8$. One way ANOVA of the morphine treated group is significantly different, $p < 0.0001$. All are significantly reduced compared to WT (*post hoc* Tukey results are shown on the graph). There are also significant differences between MOP+/- (10) and MOP+/- (30) (**). MOP+/- (30) and MOP-/- (***). There is no significant difference between MOP+/- (10) and MOP-/-. Two way repeated measures ANOVA interaction, genotype and drug are all significant ($p < 0.0001$). *Post hoc* Bonferroni results compared to saline are shown on graph (immediately above bars). Vertical lines represent ± SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

4.2.2 The role of DOP receptors

The DOP receptor influences morphine analgesia and tolerance (Chapter 4). As described previously, WT mice do not show significant locomotor activation following morphine 3 mg/Kg administration (Figure 4.1A-D). By contrast, administration of 3 mg/Kg morphine to DOP-/- mice caused significant locomotor activation (Figure 4.5). The distance that DOP-/- mice travelled during the 30 minutes following saline injection was 21.3 ± 2.6 m and after morphine 3 mg/Kg this distance was 28.7 ± 3.1 m. Their average speed of travel following saline injection was 0.012 ± 0.001 m/s and after morphine 3 mg/Kg was 0.016 ± 0.002 m/s. They did not exhibit the development of significant sensitisation to morphine's locomotor effects following three days once daily administration of 3 mg/kg morphine. The total distance travelled on day 3 of morphine 3 mg/Kg was 29.0 ± 4.3 and their average speed was 0.016 ± 0.002 m/s. These data suggest that DOP-/- mice show increased sensitivity to the locomotor activation produced by morphine.

When the mice receive 10 mg/Kg morphine the DOP+/- and the DOP-/- mice show a significant locomotor activation which was similar to that observed in WT mice (Figure 4.5 and 4.6 compared to Figure 4.1). For the DOP+/- mice the distance travelled in the 30 minutes after saline injection was 25.2 ± 3.8 m and their average speed was 0.014 ± 0.002 m/s. When morphine 10 mg/Kg was administered the distance travelled significantly increased to 63.8 ± 4.5 m (Figure 4.6) and their speed of travel was also significantly increased at 0.035 ± 0.003 m/s (data not shown). The DOP+/- mice also sensitised to the locomotor

effects of morphine as on day 3 of the conditioning phase their total distance travelled following morphine 10 mg/Kg administration was 95.9 ± 3.3 m and their average speed was 0.053 ± 0.002 m/s.

The DOP-/- mice also exhibited significant locomotor activation and sensitisation following the administration of morphine 10 mg/Kg. Their distance travelled in the 30 minutes following saline injection was 16.3 ± 1.5 m and their speed of travel was 0.009 ± 0.0008 m/s. After morphine 10 mg/Kg injection their total distance travelled in the 30 minutes was 74.1 ± 3.8 m and their average speed was 0.041 ± 0.002 m/s. They also sensitised to the locomotor effects of morphine following the administration of the 10 mg/Kg dose, their total distance travelled on day 3 of conditioning was 107.5 ± 7.4 m and their average speed was 0.06 ± 0.004 m/s (Figure 4.5 and 4.6).

Overall these results suggest that DOP receptors are not required for locomotor activation but influence the potency of morphine in this respect.

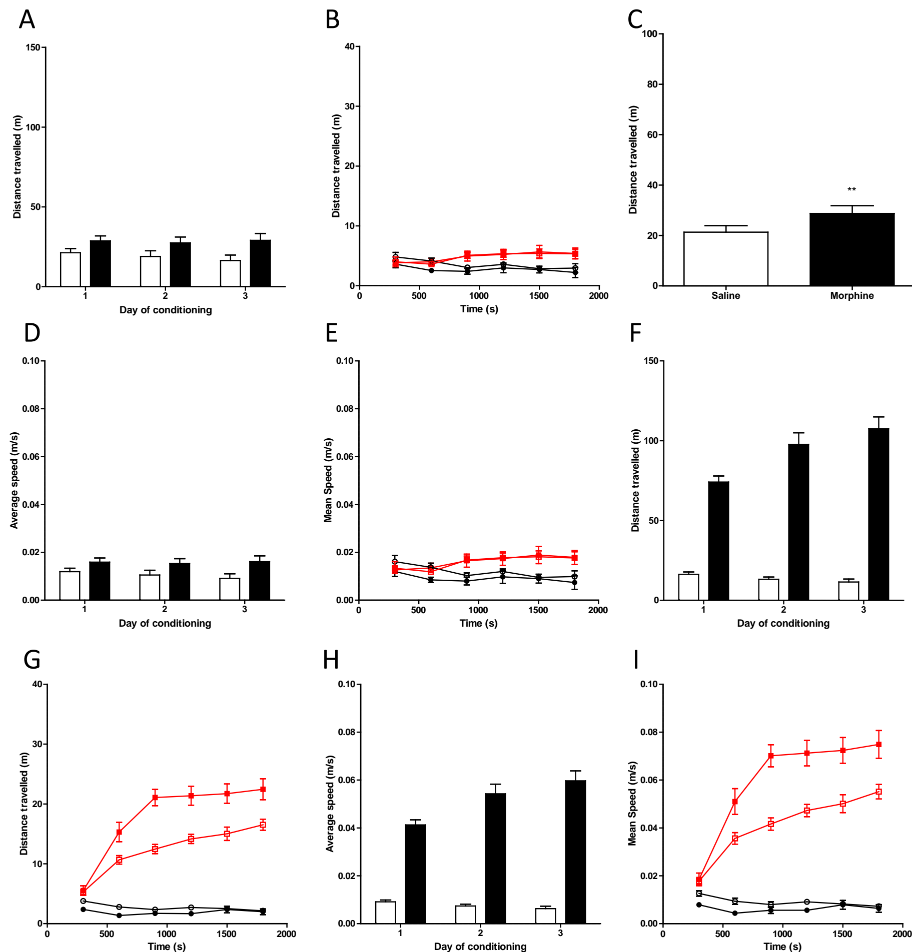


Figure 4.5: DOP-/- mice show significant locomotor activation following both 3 mg/kg and 10 mg/Kg morphine. (A) DOP-/- mice administered 3 mg/Kg morphine travelled significantly further than saline treated mice, two way repeated measures ANOVA, time (ns), morphine treatment $p < 0.01$. (B) The DOP-/- mice do not display sensitisation to the effects of 3 mg/kg morphine over the three days of conditioning, the distance that they travel on day 3 is not significantly different to that travelled on day 1. (C) DOP-/- mice displayed significant locomotor activation following the administration of 3 mg/kg morphine, paired t test $p = 0.006$. (D) The average speed of travel of DOP-/- mice is increased following administration of 3 mg/Kg morphine. (E) The DOP-/- mice do not sensitise to the effects of morphine 3 mg/Kg over the three days of conditioning, the speed they travel on day 3 is not significantly different from that on day 1. DOP-/- mice do not exhibit significantly altered locomotor activation following morphine 10 mg/Kg administration when compared to WT mice. (F) DOP-/- mice show locomotor activation following 10 mg/kg morphine injection, two way repeated measures ANOVA, time $p < 0.0001$, drug $p < 0.0001$, $n = 8$. (G) DOP-/- mice show sensitisation in speed of travel over the three day conditioning period. They travel significantly faster following treatment with 10 mg/ kg morphine on day 3 compared to day 1, two way repeated measures ANOVA, time $p < 0.0001$, drug $p < 0.0001$, $n = 8$. (H) DOP-/- mice travel significantly further following three days of morphine 10 mg/kg compared to day 1, $n = 8$. (I) DOP-/- mice travel significantly faster on day 3 of conditioning compared to day 1, $n = 8$. Bar graphs – white represents saline and black represents morphine. Line graphs – red square symbols represent morphine and black circle symbols represent saline. Open symbols are day 1 and closed symbols are day 3 of the study. Vertical lines represent ± SEM.

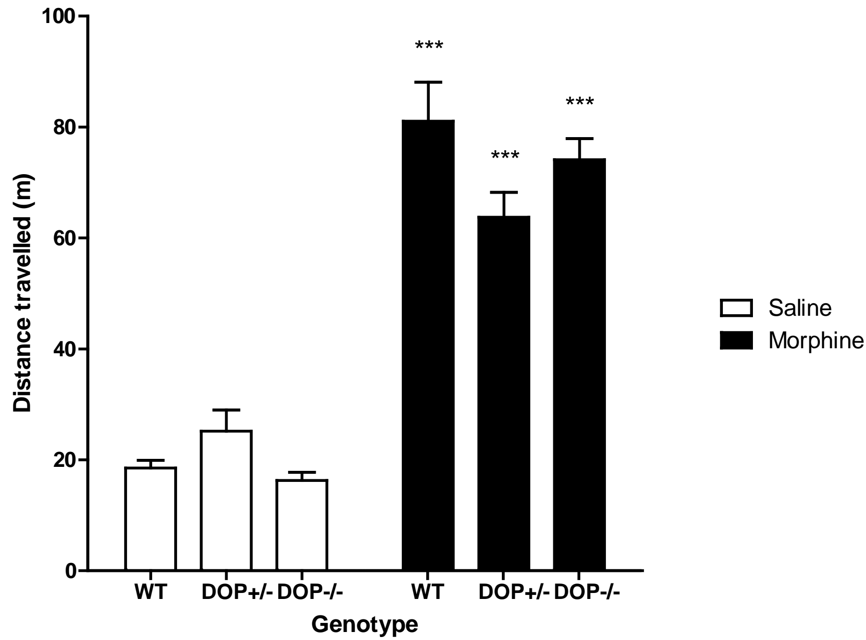


Figure 4.6: The absence of DOP receptors does not affect locomotor activation following morphine 10 mg/Kg. All mice (WT, DOP+/- and DOP-/-) show significant locomotor activation with morphine on day 1 of conditioning as shown on graph. WT $p < 0.0001$, DOP+/- $p = 0.0002$, DOP-/- $p < 0.0001$ (paired t test). There are no significant differences in baseline locomotion with saline (one way ANOVA). There were also no significant differences in locomotor activation observed following morphine treatment, one way ANOVA $p = 0.1$. Vertical lines represent ± SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

4.2.3 The role of β-arrestin2

A previous study has implicated BAR2 in the morphine-evoked locomotor response (Bohn *et al*., 1999). In keeping with WT mice (Figure 4.1) BAR2-/- mice in the present study did not exhibit locomotor activation following the administration of morphine at 3 mg/Kg (Figure 4.7A-4.7D). The distance travelled in the 30 minutes following saline administration on day 1 of conditioning was 11.4 ± 0.9 m and after morphine 3 mg/Kg the distance travelled was 15.3 ± 2.1 m. The average speed of travel after saline was 0.006 ± 0.0005 m/s and after morphine 3 mg/Kg was 0.008 ± 0.001 m/s. In addition, BAR2-/- mice did not sensitise to the locomotor effects of morphine during the three days of conditioning, on day 3 the distance travelled after morphine 3 mg/Kg was 15.6 ± 2.9 m and their average speed of travel was 0.009 ± 0.002 m/s.

However, BAR2-/- mice exhibited locomotor activation and sensitisation to morphine following administration of 10 mg/Kg (Figure 4.7E-H). The distance travelled in the 30 minutes following the saline injection on day 1 was 15.6 ± 1.7 m and the distance travelled following the morphine 10 mg/Kg injection was 50.8 ± 6.6 m. Their average speed of travel was 0.009 ± 0.0009 m/s after the saline injection and 0.028 ± 0.004 m/s after the morphine 10 mg/Kg injection.

By day 3 of the conditioning period the distance travelled after the morphine 10 mg/Kg was significantly increased (75.6 ± 12.0 m) and their average speed of travel had also increased (0.042 ± 0.007 m/s).

BAR2-/-//DOP-/- mice also did not exhibit locomotor activation or sensitisation after administration of morphine 3 mg/Kg. Their distance travelled in the 30 minutes following injection of saline on day 1 was 24.6 ± 0.5 m and their average speed of travel was 0.014 ± 0.0002 m/s. After morphine 3 mg/Kg injection their total distance travelled over the 30 minutes was 23.5 ± 3.3 m and their average speed was 0.013 ± 0.002 m/s. On day 3 of the conditioning period there were no significant differences compared to day 1 in either total distance travelled after morphine 3 mg/Kg (27.2 ± 6.7 m) or their average speed of travel (0.015 ± 0.004 m/s) (Figure 4.8A-D).

After morphine 10 mg/Kg administration BAR2-/-//DOP-/- mice also exhibited significant locomotor activation and sensitisation to morphine (Figure 4.8E-H). The distance travelled by the BAR2-/-//DOP-/- mice in the 30 minutes following saline injection was 17.1 ± 1.6 m and after morphine 10 mg/Kg injection the distance travelled was 42.5 ± 2.7 m. Their average speed after the saline injection was 0.009 ± 0.0009 m/s and after the morphine 10 mg/Kg injection was 0.024 ± 0.002 m/s. On day 3 of the conditioning period the total distance travelled following morphine 10 mg/Kg had significantly increased to 73.1 ± 5.0 m and their average speed of travel was 0.041 ± 0.003 m/s.

Consistent with the previous report (Bohn *et al*., 1999), locomotor activation by morphine (10 mg/Kg) in BAR2-/- mice was significantly reduced compared to WT mice (Figure 4.9). As discussed previously morphine (10 mg/Kg) produced

locomotor activation in the BAR2-/-//DOP-/- mice but this was also significantly reduced when compared to the WT mice (Figure 4.9). There was no significant difference in locomotor activation by morphine when the BAR2-/- and the BAR2-/-//DOP-/- mice were compared.

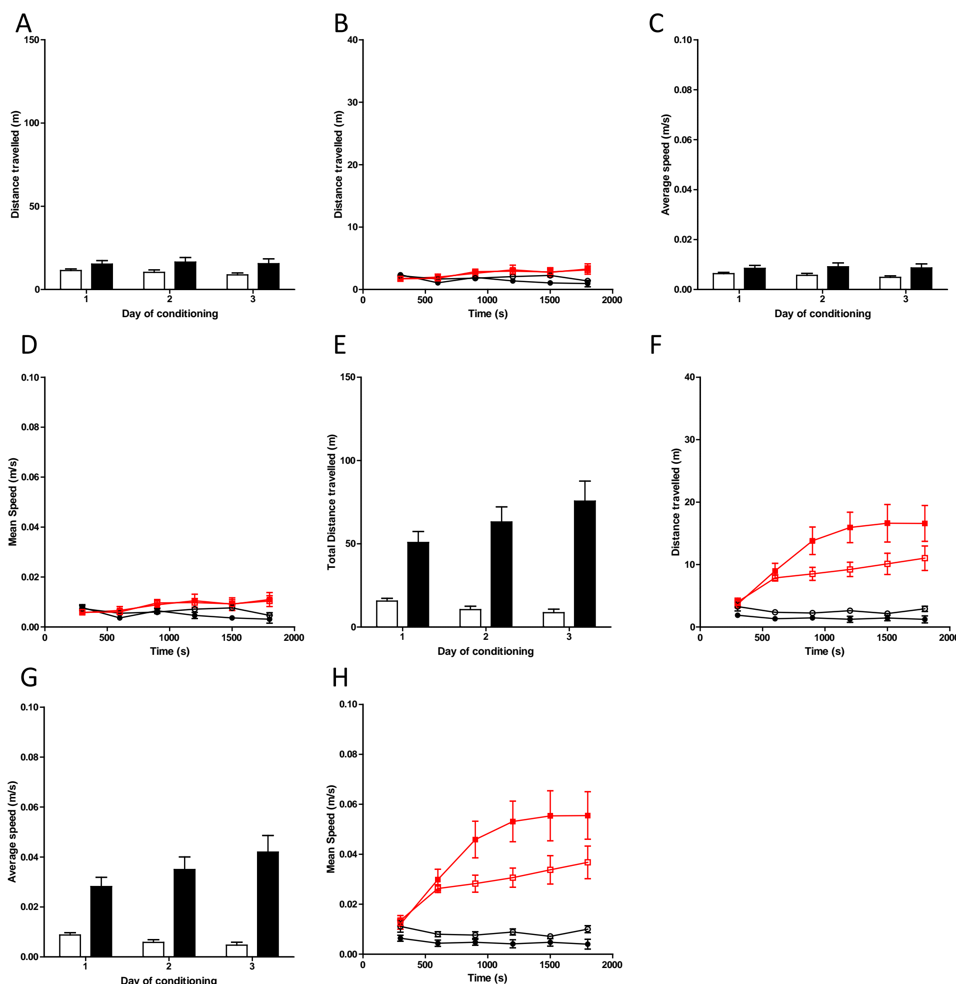


Figure 4.7: BAR2-/- mice exhibit locomotor activation by morphine. (A) BAR2-/- mice exhibit no significant alteration in distance travelled following the administration of 3 mg/Kg morphine. (B) BAR2-/- mice do not exhibit locomotor sensitisation following morphine 3 mg/KG, there are no significant differences in the distance travelled between day 1 and day 3. (C) BAR2-/- mice exhibit no significant differences in average speed of travel following administration of morphine 3 mg/Kg. (D) BAR2-/- mice exhibit no significant alterations in speed of travel following morphine 3 mg/Kg administration. Morphine 10 mg/Kg produces significant locomotor activation and sensitisation in BAR2-/- mice. (E) The distance travelled was significantly increased following morphine administration, two way repeated measures ANOVA, time $p = 0.004$, morphine $p < 0.0001$. (F) The mice exhibit locomotor activation to morphine and sensitise to its locomotor activating effects over the conditioning period. The distance travelled on day 3 was significantly greater than that on day 1. (G) The speed of travel is also significantly increased, two way repeated measures ANOVA, time $p = 0.005$, morphine $p < 0.0001$. (H) The speed travelled on day 3 was significantly greater than on day 1. Bar graphs – white represents saline and black represents morphine. Line graphs – red square symbols represent morphine and black circle symbols represent saline. Open symbols are day 1 and closed symbols are day 3 of the study. Vertical lines represent ± SEM.

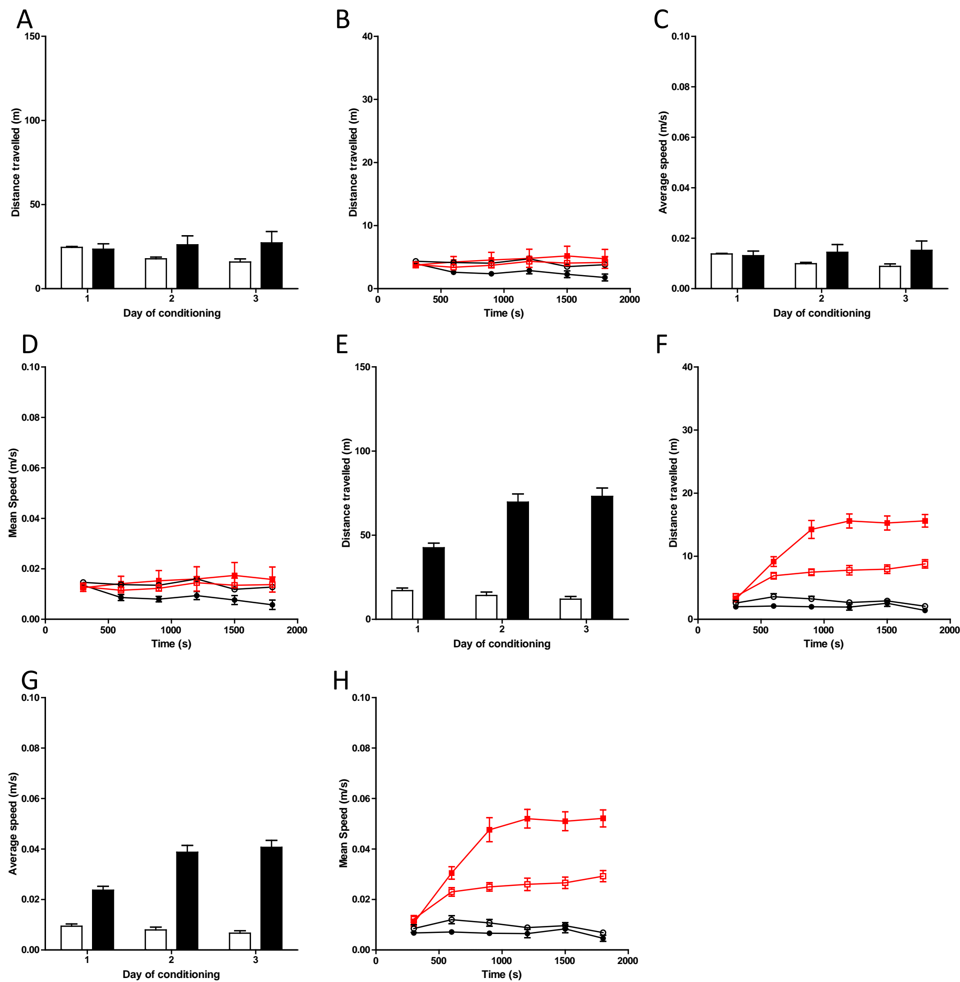


Figure 4.8: BAR2-/-//DOP-/- mice exhibit locomotor activation following morphine administration. (A) There are no significant differences in the distance travelled following saline or morphine 3 mg/Kg injection in the BAR2-/-//DOP-/- mice. (B) BAR2-/-//DOP-/- mice also show no sensitisation to the locomotor effects of morphine 3mg/Kg. The distance travelled is not significantly different between day 1 and day 3. (C) The average speed of travel is not significantly different between the saline and morphine 3 mg/Kg injections. (D) Again the BAR2-/-//DOP-/- mice do not exhibit sensitisation, their speed of travel is not significantly different from day 1 to day 3. BAR2-/-//DOP-/- mice do show significant locomotor activation and sensitisation to morphine 10 mg/Kg. (E) Locomotor activation over the conditioning sessions is intact, two way ANOVA time $p < 0.0001$, morphine $p < 0.0001$. (F) The mice show locomotor activation to morphine and sensitise to its locomotor activating effects over the conditioning period. (G) The BAR2-/-//DOP-/- mice exhibit a significant increase in speed following morphine 10 mg/Kg administration, two way ANOVA time $p < 0.0001$, morphine $p < 0.0001$. (H) There is also significant sensitisation to morphine between day 1 and day 3. The mice travel significantly faster by day 3. Line graphs – red square symbols represent morphine and black circle symbols represent saline. Open symbols are day 1 and closed symbols are day 3 of the study. Vertical lines represent ± SEM.

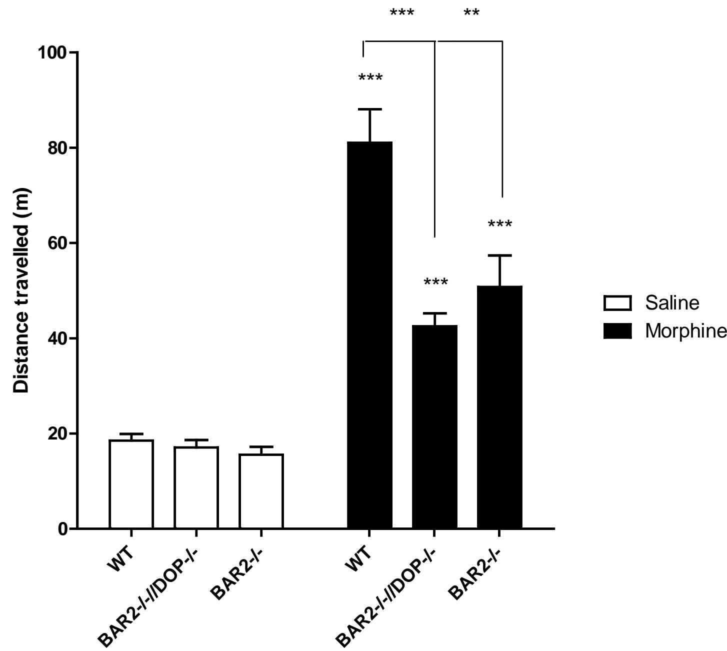


Figure 4.9: Removing β-arrestin2 significantly reduced locomotor activation following morphine 10 mg/Kg administration. This is true for the BAR2-/- mice and the BAR2-/-//DOP-/- mice. There are no significant differences in locomotor activity between these mice (unpaired t test p = 0.3), suggesting that the DOP receptor is not involved in this aspect of behaviour. Two way ANOVA reveals significant locomotor activation by morphine 10 mg/Kg in WT, BAR2-/- and BAR2-/-//DOP-/- mice, $p < 0.001$, *post hoc* Bonferroni results are shown on the graph. Vertical lines represent ± SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

4.3 Conditioned place preference

4.3.1 The role of MOP receptors in reinforcement

The experiments described above for establishing the locomotor effects of morphine were conducted during the conditioning phase of the CPP experiments described here. Prior to the conditioning phase mice showed no preference for either of the compartments which were distinguishable on the basis of differences in the wallcoverings and floor orientation. As previously described one chamber had a wallcovering consisting of black and white horizontal stripes and the other black and white vertical stripes. As discussed previously, Chapter 2, the floors in each chamber both consisted of 1 cm square wire grid flooring material. The grid direction of this material differs depending on its orientation. We utilised this property to provide a difference in the floor between each chamber. The direction of the grid matched the wall stripe direction in each chamber. The mice investigated both chambers equally as shown by the fact that there was no significant difference in time spent in each chamber during the habituation phase of the experiment, time spent in saline paired chamber was 455.7 ± 21.8 s and the time spent in the morphine paired chamber was 444.3 ± 21.8 s (Figure 4.10A). Morphine administered at 3 mg/Kg to WT mice did not cause CPP and the time spent in the saline and morphine chambers was consistent throughout the test period (Figure 4.10B and C). The time spent in saline paired chamber was 412.9 ± 31.6 s and the time spent in the morphine 3 mg/Kg paired chamber was 487.1 ± 31.6 s (Figure 4.10B). By contrast, WT mice showed a significant preference for the 10 mg/Kg morphine paired chamber following three days of conditioning. Time spent in the saline paired chamber

was 353.3 ± 11.5 s and the time spent in the morphine 10 mg/Kg paired chamber was 546.8 ± 11.5 s (Figure 4.10D). Mice spent a significantly longer time in the morphine-paired chamber, $p<0.0001$. Furthermore, the time spent in the morphine paired chamber compared to the saline paired chamber was consistent throughout the test period suggesting that there was no extinction during this observation period (Figure 4.10E).

The development of morphine preference in MOP+/- and MOP-/- mice was investigated using the same conditioning protocol. MOP-/- mice did not show a preference for morphine. The time spent in saline paired chamber was 441.4 ± 20.2 s and the time spent in morphine 10 mg/Kg chamber was 458 ± 20.2 s (Figure 4.10F), confirming the importance of MOP receptors in reinforcement. Importantly, there was no initial chamber bias during habituation, the time spent in the saline paired chamber was 445.1 ± 23.0 s and the time spent in the morphine paired chamber was 454.9 ± 23.0 s (data not shown). There was also no significant difference in the time spent in the saline and morphine paired chambers throughout the test period (Figure 4.10G).

The MOP+/- mice did not show a significant preference for the morphine paired chamber following conditioning with 10 mg/Kg morphine. The time spent in the saline paired chamber was 419.8 ± 25.0 s and the time spent in the morphine 10 mg/Kg paired chamber was 480.2 ± 25.0 s (Figure 4.11A). Closer examination of the data reveals that MOP+/- mice initially exhibited a preference for the

morphine paired chamber but this preference was extinguished during the test period (Figure 4.11B and C). The MOP+/- mice show a significant reduction in preference score for 10 mg/Kg morphine compared to WT mice, 60.3 ± 49.9 compared to 193.5 ± 23.0 for the WT mice (Figure 4.11D).

The reduced preference of MOP+/- mice for morphine in this paradigm appears to be caused by reduced drug potency. When the conditioning dose of morphine was increased to 30 mg/Kg the MOP+/- mice exhibited a preference for the morphine paired chamber over the saline paired chamber. The time spent in the saline paired chamber was 371.0 ± 25.8 s and the time spent in the morphine paired chamber was 529.0 ± 25.8 s. The preference score for the morphine paired chamber was 157.9 ± 51.6 (Figure 4.11E). The MOP+/- mice no longer exhibited extinction of the morphine preference during the testing period when conditioned with morphine 30 mg/Kg (Figure 4.11F). There was no initial chamber bias for the MOP+/- mice during habituation, time spent in the saline paired chamber was 453.9 ± 28.1 s and the time spent in the morphine paired chamber was 446.1 ± 28.1 s (data not shown).

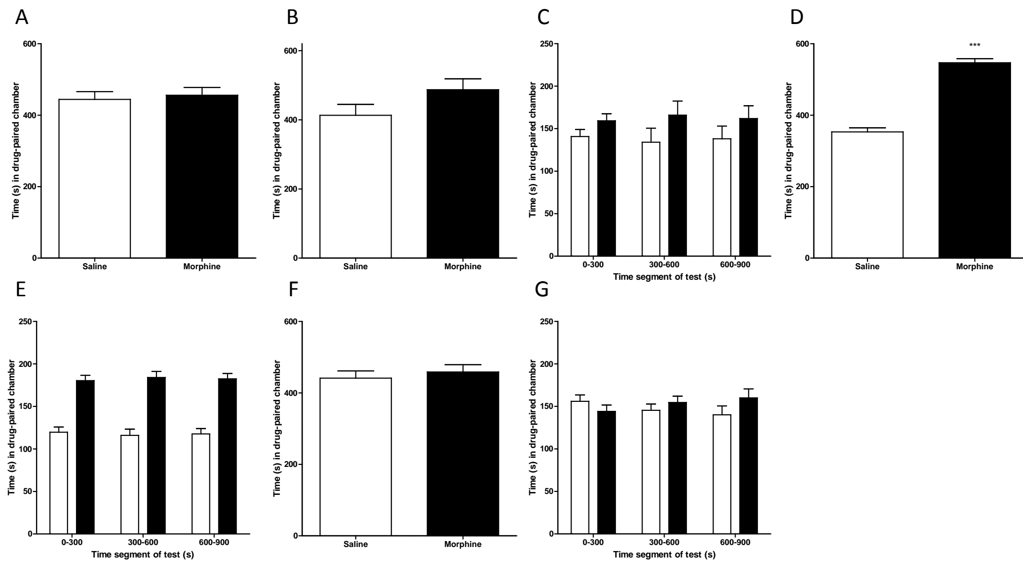


Figure 4.10: MOP receptors are required for the reinforcing effect of morphine. (A) WT mice show no chamber preference during the habituation phase of conditioned place preference (CPP). Student's t test p = 0.8, n = 8. (B) WT mice exhibit no preference for the morphine paired chamber following conditioning with morphine 3 mg/Kg, paired t test p = 0.3, n = 8. (C) Time spent by the WT mice in the saline and morphine paired chambers by segment of test following conditioning with morphine 3 mg/Kg. Two way ANOVA ns, n = 8. (D) WT mice do show significant preference for the morphine paired chamber after three days of conditioning with morphine 10 mg/Kg, paired t test p < 0.0001, n = 8. (E) Time spent in each chamber on test day. WT mice demonstrated a continued preference for the morphine paired chamber throughout the test period, with no sign of extinction within the test period of 900 s. (F) MOP-/- mice exhibit no preference for morphine following conditioning with morphine 10 mg/ Kg for three days, paired t test (ns) p = 0.7, n = 7. (G) The time that the MOP-/- mice spent in the saline and morphine paired chambers is not significantly different throughout the test period. Two way ANOVA ns, n = 7. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.

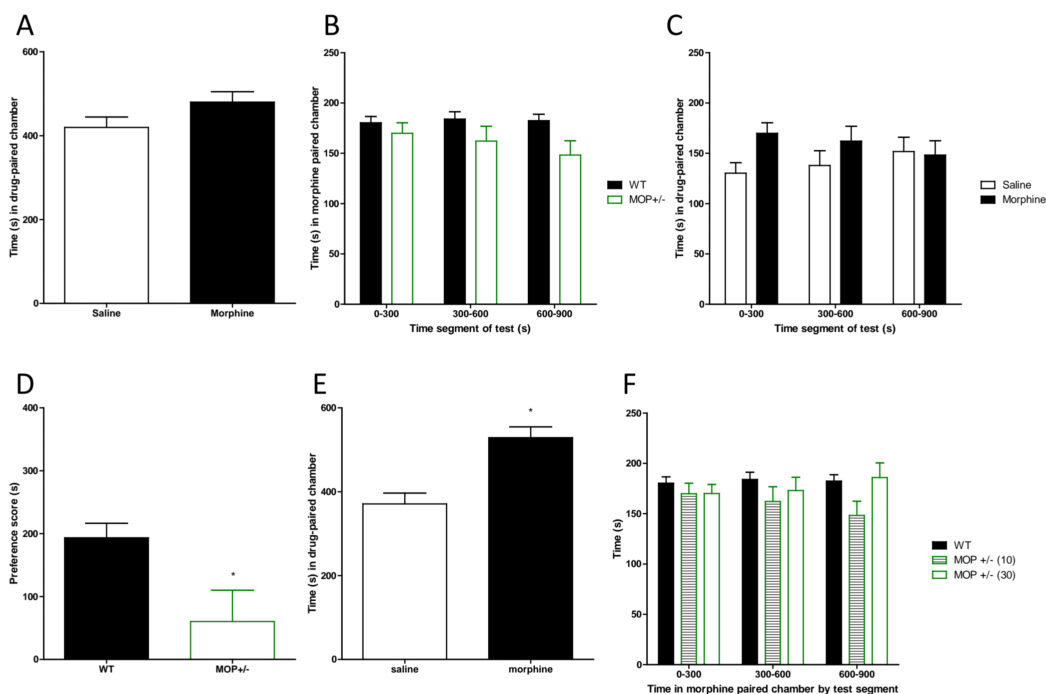


Figure 4.11: Morphine has a lower potency for reinforcement in MOP+/- mice. (A) MOP+/- mice do not show a significant preference for the morphine paired chamber following conditioning with 10 mg/Kg morphine, n = 8 t test p = 0.27. (B) MOP+/- mice show a reduction in morphine preference over time during the test period compared with the WT mice. (C) MOP+/- mice show extinction of morphine preference during the test period following conditioning with 10 mg/kg morphine. During the first 300 s of the test period there are no significant differences between the MOP+/- and WT mice, suggesting that the MOP+/- mice are at this point showing a preference, unpaired t test p = 0.4, n = 8. During the last 300s of the test period (Segment 600-900 s) there is now a significant difference in the time spent in the drug paired chamber between the WT and MOP+/- mice. This suggests extinction of preference in the MOP+/- mice, unpaired t test p = 0.04. (D) The MOP+/- mice show a significant reduction in preference score for morphine 10 mg/Kg compared to WT mice, unpaired t test p = 0.03. (E) MOP+/- mice conditioned with 30 mg/Kg morphine exhibit significant morphine preference, paired t test p = 0.02, n = 8. (F) Morphine has reduced reinforcement potency in MOP+/- mice compared to WT mice. Time in morphine paired chamber shown for WT mice, MOP+/- mice (10 mg/kg) and MOP+/- mice (30 mg/kg). This is restored following conditioning with 30 mg/Kg. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.

4.3.2 The role of DOP receptors in reinforcement

The DOP+/- and DOP-/- mice did not display any initial chamber preference on the habituation day. The DOP+/- mice spent 452.3 ± 15.1 s in the saline paired chamber and 447.7 ± 15.1 s in the morphine paired chamber. The DOP-/- mice spent 451.3 ± 13.7 s in the saline paired chamber and 448.7 ± 13.7 s in the morphine paired chamber (data not shown). DOP-/- mice did not exhibit a significant preference for the morphine paired chamber following conditioning with morphine 3 mg/Kg. The time spent in the saline paired chamber was 433.3 ± 12.2 s and the time spent in the morphine 3 mg/Kg paired chamber was 466.7 ± 12.1 s resulting in a preference score of 33.3 ± 24.3 (Figure 4.12A).

During CPP both strains exhibited a significant preference for the morphine 10 mg/Kg paired chambers on test day. DOP+/- mice spent 380.5 ± 16.8 s in the saline paired chamber and 519.5 ± 16.8 s in the morphine paired chamber (preference score 138.9 ± 33.7) (data not shown) and the DOP-/- mice spent 370.8 ± 21.7 s in the saline paired chamber and 529.3 ± 21.7 s in the morphine 10 mg/Kg paired chamber (preference score 158.5 ± 43.5) (Figure 4.12B). There was no significant difference in the preference scores between these genotypes (Figure 4.12E).

Conditioning with 10 mg/Kg morphine is significantly more reinforcing in the DOP-/- mice compared to conditioning with morphine 3 mg/Kg (Figure 4.12C). There was no significant difference in the time that the DOP-/- mice spent in the morphine and saline chambers over the duration of the test (Figure 4.12D). The

DOP-/- mice do not exhibit extinction of morphine preference during the test period.

These data suggest that the removal of DOP receptors does not significantly affect morphine reinforcement in our model. Using this paradigm and dose of morphine there were no significant differences between WT, DOP+/- and DOP-/- mice (Figure 4.12E).

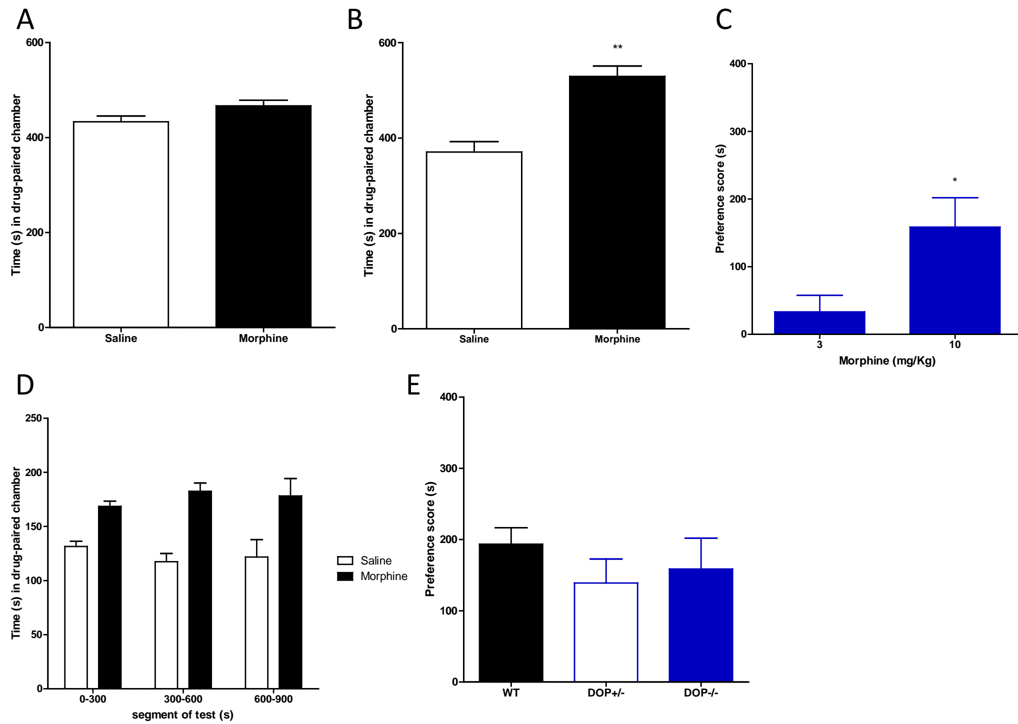


Figure 4.12: DOP receptors are not required for morphine reinforcement. (A) DOP-/- mice do not exhibit a morphine preference following conditioning with morphine 3 mg/Kg, paired t test p = 0.2 n = 8. (B) DOP+/- and DOP-/- show a significant preference for morphine 10 mg/Kg. DOP+/- mice exhibited a significant preference for morphine following conditioning with 10 mg/Kg, paired t test p = 0.004, n = 8 (data not shown). DOP-/- mice showed a significant preference for morphine following conditioning with morphine 10 mg/Kg, paired t test p = 0.008, n = 8. (C) There is significantly more preference for the morphine paired chamber when the DOP-/- mice are conditioned with 10 mg/Kg morphine, p = 0.02 n = 8. (D) Time spent in the saline and morphine paired chambers during the test period by the DOP-/- mice reveals a lack of extinction of morphine preference during the test period. (E) DOP-/- and DOP+/- mice show no difference in morphine preference for 10 mg/Kg morphine compared to WT mice, one way ANOVA ns, n = 8. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.

4.3.3 The role of β-arrestin2 in reinforcement

It has previously been demonstrated that BAR2-/- mice show an increased preference for morphine compared to WT mice in the CPP paradigm (Bohn *et al*., 2003). Therefore mice were tested with 3 mg/Kg morphine, a dose that was without effect in WT mice (Figure 4.10B). By contrast to WT mice, BAR2-/- mice exhibited a significant preference for morphine following three days of conditioning to 3 mg/Kg. The time spent in the saline paired chamber was 330.7 ± 19.2 s and the time spent in the morphine 3 mg/Kg paired chamber was 569.3 ± 19.2 s (the preference score for 3 mg/Kg morphine was 238.6 ± 38.4) (Figure 4.13A).

When the BAR2-/- mice were conditioned and tested with morphine 10 mg/Kg there was also significant morphine CPP. The time spent in the saline paired chamber was 326.8 ± 41.4 s and the time spent in the chamber paired with morphine 10 mg/Kg was 573.2 ± 41.4 s (Figure 4.13B). The preference of BAR2-/- and WT mice for 10 mg/Kg morphine was not significantly different. WT preference score for 10 mg/Kg morphine was 193.5 ± 23.0 and the BAR2-/- preference score was 246.5 ± 82.7 (Figure 4.13C). As the preference was maintained throughout the test period (Figure 4.13D), BAR2-/- mice did not demonstrate significant extinction.

There was no initial preference for either chamber. On habituation day the BAR2-/- mice spent 450.0 ± 22.4 s in the saline paired chamber and 450.0 ± 22.4 s in the morphine paired chamber (data not shown). There was no significant

difference between the preference scores for 3 and 10 mg/Kg morphine in BAR2-/- mice suggesting that these doses lie at the top of the morphine dose-response relationship for reinforcement. These data suggest that there is a considerable increase in the potency of morphine reinforcement in BAR2-/- mice.

The BAR2-/-//DOP-/- mice did not show a significantly increased preference for the morphine paired chamber following conditioning with 3 mg/Kg morphine, although there was a trend towards a preference ($p = 0.07$). The time that they spent in the saline paired chamber on the test day was 391.2 ± 27.2 s and the time that they spent in the chamber paired with morphine 3 mg/Kg was 508.8 ± 27.2 s, this gave a preference score of 117.6 ± 54.6 for the morphine 3 mg/Kg paired chamber (Figure 4.14A).

Like WT and BAR2-/- mice, BAR2-/-//DOP-/- mice also showed a significant preference for morphine following conditioning with 10 mg/Kg. The time spent in the saline paired chamber was 380.1 ± 18.0 s and the time spent in the morphine 10 mg/Kg paired chamber was 520.0 ± 18.0 s, resulting in a preference score of 139.9 ± 36.0 for the morphine (10 mg/Kg) paired chamber (Figure 4.14B). Again these mice did not show any extinction of this preference during the test period (Figure 4.14D). Their preference scores for the morphine paired chambers were not significantly different from either that of the WT mice or the BAR2-/- mice (Figure 4.14C). There was no significant initial chamber bias during

habituation, time spent in the saline paired chamber was 469.0 ± 19.1 s and time spent in the morphine paired chamber was 431.0 ± 19.1 s (data not shown).

When the preference scores are compared across the genotypes we observed a trend for a dose response relationship in the WT and DOP-/- mice. However in the BAR2-/- and BAR2-/-//DOP-/- mice both 3 mg/Kg and 10 mg/Kg morphine appear to be at the top of the dose response relationship. This suggests that the BAR2-/- and BAR2-/-//DOP-/- mice are more sensitive to the conditioned reinforcing properties of morphine.

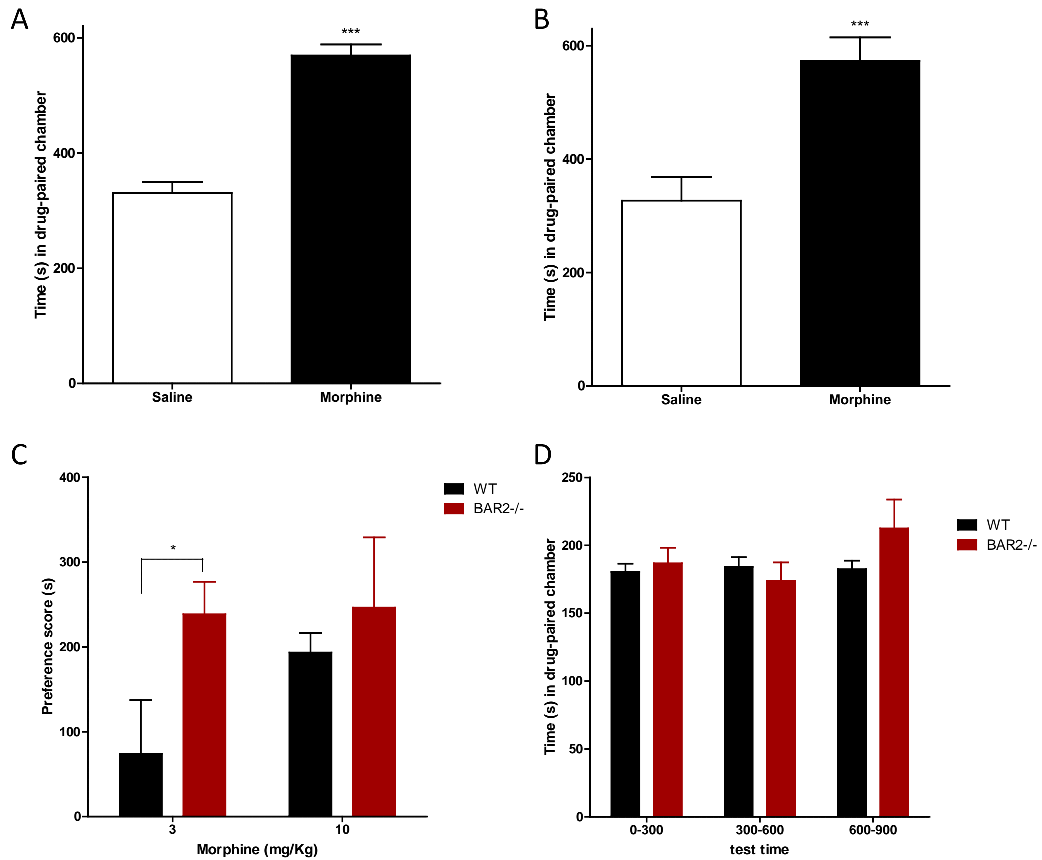


Figure 4.13: BAR2-/- mice exhibit increased sensitivity to morphine reinforcement. (A) 3 mg/Kg morphine is reinforcing in BAR2-/- mice. BAR2-/- mice show a preference for morphine following conditioning with 3mg/Kg morphine, paired t test p = 0.0004 n = 8. (B) BAR2-/- mice show morphine preference following 3 days of conditioning to 10 mg/Kg morphine, t test p = 0.0009, n = 8. (C) BAR2-/- show a significantly increased preference for morphine compared to WT mice following conditioning with 3 mg/Kg morphine, t test p = 0.04 n = 8. There is no difference in preference score between 3 mg/Kg and 10 mg/Kg for BAR2-/- mice, t test ns, p = 0.9 n = 8. At 10 mg/Kg there is no significant difference in morphine preference between the WT and BAR2-/- mice, t test p = 0.55, n = 8. (D) BAR2-/- mice show continued preference for the morphine 10 mg/Kg paired chamber during the entire test period, similar to the WT mice they do not show extinction of the preference, two way ANOVA ns, n = 8. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.

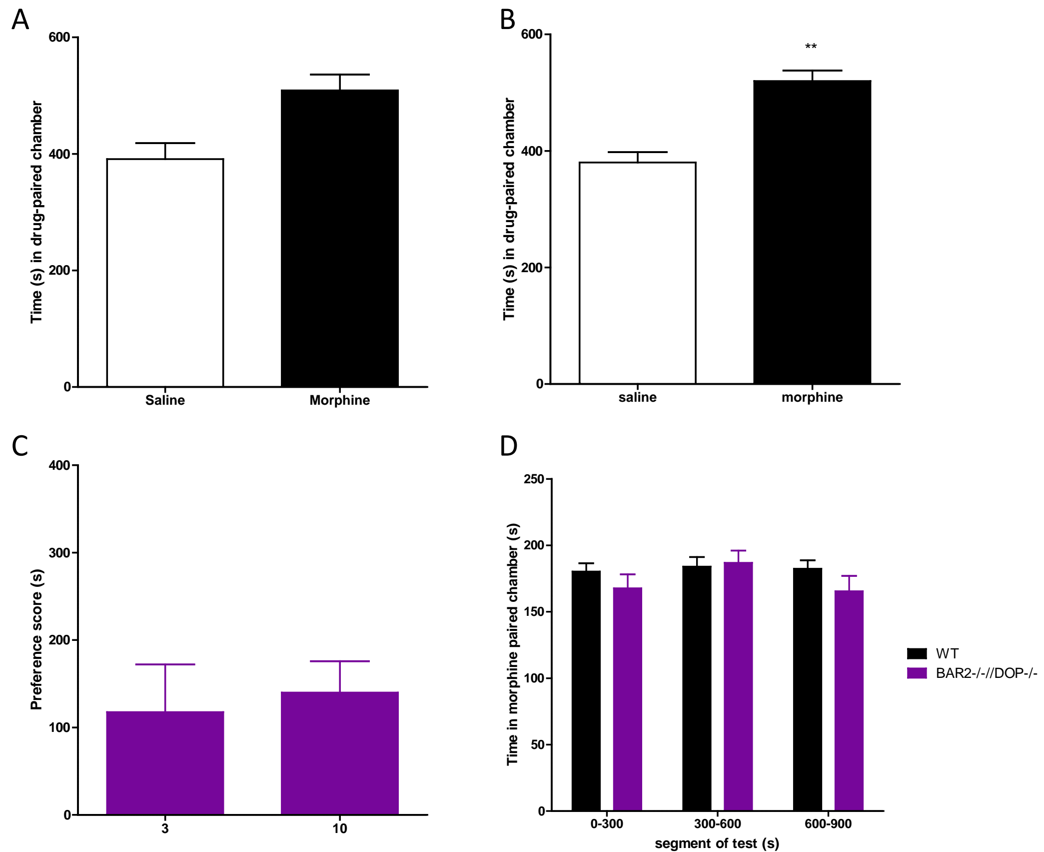


Figure 4.14: BAR2-/-//DOP-/- mice exhibit morphine reinforcement. (A) BAR2-/-//DOP-/- mice appear to spend more time in the morphine 3 mg/Kg paired chamber on test day but this is not significant, paired t test p = 0.07 n = 8. (B) BAR2-/-//DOP-/- mice do exhibit a significant preference for the 10 mg/Kg morphine chamber on test day, paired t test p = 0.006, n = 8. (C) There is no significant difference in the preference score of these mice for morphine when we compare the measured preference at 3 mg/Kg and 10 mg/Kg morphine, p = 0.7 n = 8. (D) Time spent in morphine 10 mg/Kg chamber on test day is not significantly different from WT mice, two way ANOVA ns. The BAR2-/-//DOP-/- mice do not show extinction of preference over the test period. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.

4.4 Summary

WT mice exhibited a robust dose dependent locomotor activation by morphine and they sensitised to these effects, this is consistent with work previously published (Brase *et al*., 1977). They also exhibited a dose dependent relationship in the preference that they express for morphine in the CPP paradigm. None of the mice tested exhibited a significant preference for either chamber on the habituation day. The distance travelled in the 30 minutes after saline injection on day 1 was not significantly different across the genotypes tested.

Locomotor activation following morphine administration requires MOP receptors, it does not occur in the MOP-/- mice. MOP receptors are also required for the reinforcing effects of morphine. The MOP-/- mice expressed no preference for the morphine paired chamber during CPP. These results are consistent with previously published work (Matthes *et al*., 1996, Sora *et al*., 2001).

The MOP+/- mice reveal that, as demonstrated for analgesic response (Chapter 4), there is a reduction in the potency of morphine for both locomotor activation and reinforcement. Locomotor activation in these mice is significantly reduced compared to WT mice. Even when the locomotor activation produced by the highest dose tested in the MOP+/- mice (morphine 30 mg/Kg) is compared to that produced by morphine (10 mg/Kg) in the WT mice we still observe a significant reduction in the locomotor activation produced by morphine. MOP+/-

mice do not show any sensitisation to the locomotor activating effects of morphine even at a dose of 30 mg/Kg. This suggests that MOP receptor number is critical for the process of sensitisation.

In the MOP+/- we observed a dose dependency in the development of morphine preference, with no significant preference at 10 mg/Kg but a restored preference when the mice are conditioned with 30 mg/Kg morphine. Interestingly, the MOP+/- mice displayed a preference for the 10 mg/Kg morphine paired chamber during the first 300 s of the test period but this was extinguished by the last 300 s (segment 600 – 900 s). This suggests a reduction in potency of morphine in these mice as we have observed for the analgesic and locomotor effects previously. This pattern of extinction observed in the MOP+/- mice conditioned with morphine (10 mg/Kg) did not occur in the other groups of mice tested. WT mice do not display a preference for the morphine paired chamber following conditioning with morphine 3 mg/Kg at any of the time points. There are no significant differences in the time spent in each chamber across the test period. This is also the case for the MOP-/- mice conditioned with morphine (10 mg/Kg). These data suggest that MOP receptors alone are responsible for this effect and that receptor number may have a role in this process.

The DOP-/- mice exhibited no significant alteration in locomotor activation at 10 mg/Kg morphine compared to the WT mice but interestingly did demonstrate increased sensitivity to its locomotor activating effects. They exhibited significant

locomotor activation at the 3 mg/Kg dose which the WT mice do not. This implies that the DOP receptor may be involved in limiting the sensitivity of the locomotor system to activation by morphine. Utilizing a different morphine dose and test protocol it has been previously reported that DOP-/- mice exhibit a greater degree of locomotor activation to morphine when compared to WT mice and also sensitise to this effect at a faster rate (Chefer and Shippenberg, 2009). We have only tested our mice over the three day conditioning period for CPP and so we are not able to determine if this occurs in our study.

The DOP-/- mice exhibited no significant alteration in morphine preference compared to the WT mice at either the 3 mg/Kg or 10 mg/Kg morphine doses, suggesting that DOP receptors are not involved in morphine reinforcement. The results obtained here for morphine CPP differ in some respects from findings previously published. One study demonstrated that the DOP-/- mice developed a morphine preference only when paired with a previously non-preferred chamber and not when paired with a preferred chamber (Chefer and Shippenberg, 2009). This is hard to interpret when compared to our results as we commenced the conditioning phase with no initial chamber bias. They also maintained the CPP apparatus in darkness whereas we use controlled low light levels. The study does not state whether the mice were tested in their normal light phase as ours were. The only difference between their chambers was floor texture whereas we use both a pattern and floor difference to provide cues to the mice that the chambers are different. Other groups have suggested that the acquisition of morphine CPP in the DOP-/- mice is state dependent, testing with no drug

resulted in no preference and testing with drug present revealed a preference for the previously paired chamber (Le Merrer *et al*., 2011). Further work has suggested that it is the drug-context association that is important in the acquisition of morphine CPP in these mice, giving a time, sound or drug cue restored morphine CPP (Le Merrer *et al*., 2012).

Our experimental protocol was the same for all genotypes, it involved an AM saline injection and a PM morphine injection separated by a 4 hour interval, testing took place at a time point midway between the administration points. It is conceivable that this protocol constituted enough of a circadian cue to allow our DOP-/- mice to develop morphine preference.

BAR2-/- mice exhibited a significant reduction in locomotor activation following the administration of 10 mg/Kg morphine as has previously been reported (Bohn *et al*., 2003). We could observe no significant locomotor activation following the administration of morphine 3 mg/Kg in either the BAR2-/- or WT mice. The BAR2-/- mice also did not demonstrate any sensitisation to the locomotor activating effects of morphine at the 10 mg/Kg dose, this is significantly different from the behaviour of the WT mice. The BAR2-/- mice exhibited a marked preference for the morphine paired chamber at both 3 and 10 mg/Kg morphine. While the WT and DOP-/- mice demonstrate a dose response relationship in the development of morphine preference, this is lost in the BAR2-/- mice. The preference score for morphine at 3 and 10 mg/Kg are not significantly different.

This is likely because these doses lie at the top of the dose response relationship for morphine preference in these mice and reveal a significant increase in the potency of morphine to produce reinforcement when BAR2 is absent.

BAR2-/-//DOP-/- mice also exhibited significantly reduced locomotor activation following administration of morphine 10 mg/Kg compared to WT mice. This is not significantly different to that observed in the BAR2-/- mice. These mice do not exhibit a significant preference for morphine following conditioning with morphine 3 mg/Kg, although there is a trend towards significance. They do demonstrate a preference for the morphine paired chamber following conditioning with morphine 10 mg/Kg. They exhibited no extinction of preference during the test period at either dose and their preference scores for morphine are not significantly different from those of the BAR2-/- mice.

MOP-/- mice do not exhibit morphine preference confirming that the MOP receptor is required for the development of preference for opioid. By contrast, there were no significant differences between WT mice and DOP-/- mice in the development of morphine preference. This conflicts to some extent with previously published work but may be due to differences in our experimental protocol. Removing β-arrestin2 increases the sensitivity of the mice to the reinforcing effects of morphine and suggests that the potency of morphine to produce a preference is increased in this circumstance.

The role of opioid receptors and BAR2 in the psychomotor effects of morphine will be discussed further in the context of analgesia in the final discussion (Chapter 7).

Chapter 5: Opioid receptor signalling within the VTA

5.1 Introduction

The VTA in the midbrain is important for a number of behaviours but particularly in the development of reward and goal directed behaviour (Ungless and Grace, 2012). Although dopaminergic (DA) cells only make up <1% of the total brain neuronal population (Arias-Carrion *et al.*, 2010), three areas in the midbrain (retrorubal field, substantia nigra pars compacta (SNc) and the VTA) contain 70-75% of them (Grillner and Mercuri, 2002).

Within the VTA differing cell populations have been reported. Ungless *et al* (2012) suggest that the composition is 70% DA cells, 30% GABAergic and 2-3% glutamatergic, which is similar to that suggested by Chieng *et al* (2011). However, Margolis *et al* (2012) have reported that 22% VTA neurons are neither GABAergic nor dopaminergic when assessed using immunostaining. These differences may be due to species differences between rat and mouse or to the precise location assessed within the VTA as it appears that this is a heterogeneous area.

Johnson and North (Johnson and North, 1992b, Johnson and North, 1992a) suggested that cells could be distinguished into two groups in the rat VTA, principal cells and secondary cells as in the SNc reported by Lacey *et al* (1989). They suggested that tyrosine hydroxylase (TH) positive, spontaneously active (1.7 +/- 0.1 Hz) principal cells were dopaminergic with a long duration AP (half peak amplitude 0.92 +/- 0.02 ms) and hyperpolarised to dopamine but not met-

enkephalin. In contrast, TH negative secondary cells were usually quiescent with short duration APs (0.50 +/- 0.03 ms), were hyperpolarised by met-enkephalin, but not dopamine (Johnson and North, 1992b).

Margolis *et al* (2006) looked at a variety of parameters within rat brain slices to attempt to distinguish cell type. It was noted that there were differences in cell morphology between TH(+) and GAD67-GFP(+) cells. Most elliptical neurones were TH(+) and most multipolar neurones were GAD67-GFP(+) but no morphology was exclusive to a single cell type. No differences were observed in the cell cross sectional area. Furthermore no differences were found in whole cell action potential duration between the cell types, (I_h(+) TH(+) cells 2.4 +/- 0.1 ms, I_h(-) 2.3 +/- 0.3 ms and I_h(+) TH(-) 2.1 +/- 0.2 ms). They found overall that none of the following: I_h magnitude, R_{input}, or dopamine D2 receptor agonist inhibition could be used to differentiate DA neurones from non-DA neurones in the VTA (Margolis *et al*., 2006).

Chieng *et al* (2011) utilised GAD67-GFP mice to investigate differences between cell types, they studied both cell attached and whole cell recording configurations. In cell attached mode the GAD67-GFP(+) cells had shorter AP durations (0.43 +/- 0.02 ms) and higher spontaneous AP frequencies (3.6 +/- 0.8 Hz) than did negative cells (AP duration: 1.49 +/- 0.04 ms and firing frequency: 1.6 +/- 0.2 Hz). There was no overlap between these cell groups and so they could be distinguished using these parameters. This was not the case in whole

cell recording, where there was considerable overlap between the cell types. The AP duration was generally less in GAD67-GFP positive cells but could not be used to reliably distinguish cell type. They also looked at cell size, GFP(+) cells were generally smaller than other cells, but there was again considerable overlap between groups.

In mouse VTA, Hnasko *et al* (2012) found I_h to be an unreliable marker of cell phenotype. They also found that AP durations between DA and glutamatergic cells were not significantly different. Despite these inconsistencies, a variety of people have used I_h as a marker of DA neurones (Madhavan *et al.*, 2010, Zhang *et al.*, 2010). Zhang *et al* (2010) found that the magnitude of I_h varies according to the projection target of the individual neurone and its anatomical location within the VTA. This heterogeneity has been increasingly appreciated as the role of this brain area and influence on behaviour has been investigated (Lammel *et al.*, 2011, Lammel *et al.*, 2012, Lammel *et al.*, 2014). Both the GABAergic and the dopaminergic neurones of the VTA receive inputs from several different areas of the brain. These inputs can produce excitatory, modulatory or inhibitory effects. It is becoming increasingly apparent that neuronal subpopulations exist within the VTA and these subpopulations belong to different circuits that produce differing behavioural responses (Beier *et al.*, 2015). A number of different GABAergic inputs synapse onto VTA neurones, these originate from either local interneurones, NA or RMTg (Barrot et al., 2012, Cui et al., 2014, Matsui et al., 2014). Matsui *et al* (2014) demonstrated in rat brain slices that opioid inhibition of GABAergic IPSCs within the VTA was projection specific, with a significant

input from terminals arising in the RMTg. Differences in cell type distribution and function have also been identified across the anterior/ posterior sections of the VTA (Sanchez-Catalan *et al*., 2014).

DA neurones from the VTA project mainly to the nucleus accumbens (NA) within the ventral striatum as well as the pallidum, prefrontal cortex, amygdala and hippocampus. Together these projections form the mesocorticolimbic dopamine system (Korotkova *et al*., 2004). Recent optogenetic studies support a role for DA neurones within the VTA in reinforcement. Rats and mice will learn a conditioned response in order to receive a photostimulation that selectively excites the DA neurones within the VTA (Tsai *et al*., 2009). Photostimulation of the VTA DA neurones using this method produces CPP in a manner similar to that of natural rewards (Kim *et al*., 2012).

MOP receptor agonists produce analgesia in part by reducing Ca^{2+} entry through VACCs on primary afferent neurones, reducing the frequency of excitatory postsynaptic currents (EPSCs) in dorsal horn (DH) neurones of the spinal cord (Heinke *et al*., 2011). By contrast, MOP receptor agonists reduce the frequency of GABAergic inhibitory postsynaptic currents (IPSCs) in the VTA disinhibiting dopaminergic neurones, increasing dopamine release and stimulating reward (Johnson and North, 1992b, Matsui and Williams, 2011, Fields and Margolis, 2015, Xi and Stein, 2002). The mechanism by which opioid drugs mediate the observed inhibition of IPSC frequency in the VTA remains unknown (Williams *et*

al., 2013). There appear to be significant differences in the behaviour of MOP receptors following agonist binding depending on their location within the neurone (Lowe and Bailey, 2015).

There is conflicting evidence regarding the expression of DOP receptors within the VTA. It has been proposed that GABAergic neurones of the VTA express MOP receptors but not DOP receptors (Matsui and Williams, 2011). This would suggest that in these neurones there may be an absence of MOP/DOP receptor heterodimers and consequently a lack of basal recruitment of BAR2 (Rozenfeld and Devi, 2007). However, further studies have identified neuronal expression of DOP receptors within the VTA (Erbs *et al*., 2015, Erbs *et al*., 2014) and It has been suggested that DOP receptors are involved in addiction behaviours (Gendron *et al*., 2015). Rats will learn to self-administer both MOP receptor agonists and DPDPE directly into the VTA (Devine and Wise, 1994) and both MOP and DOP agonists cause an increase in the striatal dopamine levels following VTA injection (Devine et al., 1993a). We have investigated the role of DOP receptors in CPP and locomotor activation produced by morphine (Chapter 4). No difference in morphine preference could be identified between the WT and DOP-/- mice.

We examined the involvement of MOP, DOP receptor and BAR2 in opioid-mediated signalling within the VTA. To do this brain slices were taken from WT mice and mice that lack one or both copies of the MOP, DOP or BAR2 genes.

5.2 VTA cell type identification

Horizontal brain slices of 250 µm thickness were used to study the electrophysiological consequences of activating opioid receptors within the VTA. Slices were labelled with a tyrosine hydroxylase antibody and a secondary antibody conjugated to Alexa Fluor 594 to reveal dopaminergic cells (Figure 5.1). During whole-cell recording biocytin 1% was used within the intracellular solution. This was allowed to dialyse into the cell. Slices were subsequently exposed to streptavidin-Alexa Flour 488 secondary antibody. Slices were then imaged using confocal microscopy (Figure 5.1C and D).

The protocol worked reliably for labelling with the TH antibody and dopaminergic cells of the VTA were routinely identified within brain slices used for recording. Labelling with intracellular biocytin during whole cell recording was considerably less reliable. There are many possible reasons for the unreliable nature of the approach. These include washout of the biocytin during long recordings performed particularly for the concentration response curves. Inability to locate the biocytin labelled cell within the slice due to rotation of the slice during fixation and staining and potential leakage of biocytin from the cell during removal of pipette, fixation and/or staining. An example of a successfully biocytin labelled TH positive cell is provided in Figure 5.1C and D.

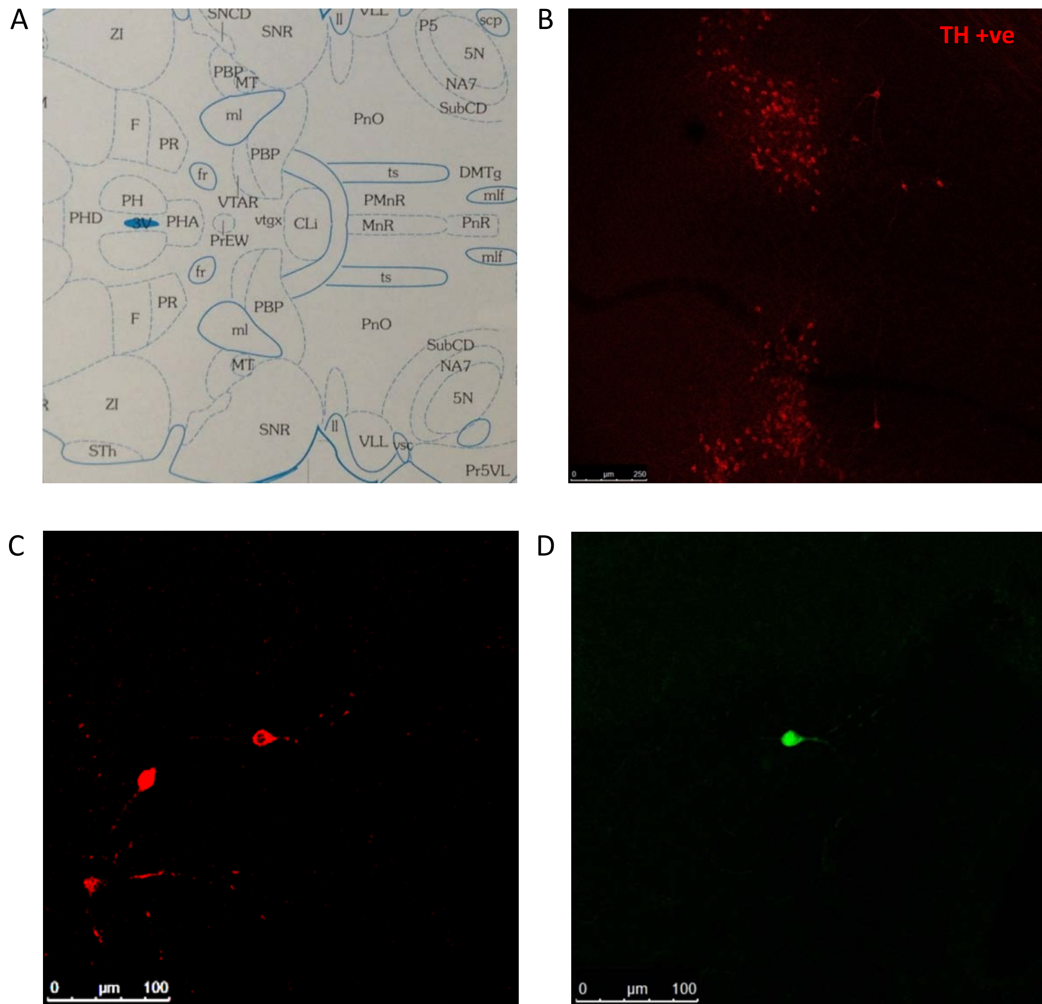


Figure 5.1: The identification of dopaminergic cells within the VTA. (A) Area of interest (i.e. the VTA) is labelled PBP (parabrachial pigmented nucleus) in the brain atlas (Paxinos and Franklin, 2013). (B) Confocal microscopy image at 10x magnification of a brain slice stained with antibody to tyrosine hydroxylase (TH) primary and secondary antibody conjugated to Alex Fluor 594. Revealing TH stained neurones within the VTA. (C) This is a 40x magnification image of a cell labelled with a tyrosine hydroxylase primary antibody and a secondary antibody conjugated to Alexa Fluor 594, this labelling process allows the identification of dopaminergic cells. (D) Biocytin labelled cell identified by a streptovidin secondary antibody conjugated to Alexa Fluor 488. As can be appreciated from panel C, the biocytin labelled cell is also dopaminergic.

The electrophysiological parameters of the neurones within the VTA were examined using whole cell current clamp recordings to study action potentials (APs) and their response to morphine. All of the recordings were performed on slices at 36°C with continuous perfusion of oxygenated saline (see Methods Chapter 2). After a control recording period morphine (10 µM) was bath applied. The drug effects observed allowed the neurones to be grouped into either responders to morphine (i.e. the spontaneous action potential frequency was inhibited by morphine) or non-responders (i.e. the action potential frequency was not inhibited by morphine) (Figure 5.2). On the basis of previous reports using MOP receptor agonists, these two groups likely represent recordings from presynaptic GABAergic neurones and postsynaptic DA neurones, respectively (Johnson and North, 1992a). On the basis of this we analysed the responders and non-responders for differences in baseline frequency of firing, action potential duration, threshold potential, peak amplitude or rise time. We could identify no significant differences in these parameters between these groups. There was a trend towards a reduction in both rise time, ($p = 0.36$ unpaired t test), and peak amplitude ($p = 0.56$ unpaired t test) and an increase in the baseline firing frequency, ($p = 0.25$ unpaired t test), in the cells which demonstrated inhibition of AP firing following morphine exposure (Table 5.1). We may not have been able to identify any significant differences in these parameters due to the small n numbers. Power analysis indicates that a minimum of 18 experiments would be required to identify a possible difference in the baseline frequency of firing, 112 to identify a difference in the peak amplitude and 47 to identify a difference in the event rise time.

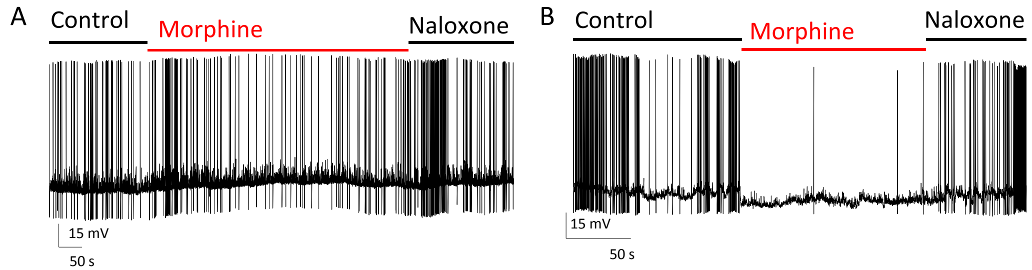


Figure 5.2: Current clamp recordings from WT VTA neurones in the presence of morphine (10 µM) and naloxone (10 µM). (A) An example trace from a WT VTA neurone in which morphine (10 µM) did not result in inhibition of spontaneous action potential frequency. (B) There were a second group of cells in whom morphine (10 µM) application resulted in a reduction in the frequency of spontaneous action potential firing.

	Non-Responders n = 4	**Responders n = 3**
Action potential duration (ms)	2.5 ± 0.3	2.3 ± 0.2
Threshold potential (mV)	-49.3 ± 5.8	-39.3 ± 2.4
Peak amplitude (mV)	58.7 ± 12.7	49.2 ± 2.7
Rise time (ms)	1.2 ± 0.4	0.72 ± 0.06
Baseline frequency	7.4 ± 4.5	15.9 ± 4.4
Frequency with morphine (10 µM)	12.1 ± 6.9	0.62 ± 0.5*

Table 5.1: Characteristics of action potentials recorded from spontaneously firing WT VTA neurones in current clamp mode. The neurones were grouped according to the response to the application of morphine (10 µM). The * represents $p < 0.05$ on Student's t test. The distinguishing feature between these groups is the response to morphine.

5.3 The influence of opioid drugs on IPSC events within the VTA

Recordings of spontaneous IPSCs from WT VTA neurones within horizontal brain slices in voltage clamp mode at -60 mV and 36°C reveal a baseline event frequency of 2.2 ± 0.2 Hz, n = 64. These events were inhibited by the $GABA_A$ receptor antagonist bicuculline (30 μM), confirming that they are $GABA_A$ receptor mediated IPSCs (Figure 5.3).

Morphine inhibited the frequency of sIPSC events in a concentration dependent manner (Figures 5.4 and 5.6). The application of TTX (500 nM) to the recording solution did not significantly affect the baseline IPSC event frequency. In the absence of TTX the baseline IPSC frequency was 2.2 ± 0.3 Hz and in the presence of TTX (500 nM) the frequency of IPSC events was 2.7 ± 0.7 Hz (Table 5.2). No significant difference in the degree of inhibition with morphine (10 μM) was identified, for the sIPSC recordings there was an inhibition of 58.2 ± 3.7% and for the mIPSC recordings the inhibition was of 49.6 ± 4.3% (Figure 5.5).

The kinetic parameters, which include peak amplitude, rise time, T70 and the decay time constant (T_{ω}) were not significantly different for the sIPSC events compared to the mIPSC events and the application of morphine (10 μM) did not significantly alter these parameters (Table 5.2). These observations suggest that events recorded in the absence of TTX are mono-synaptic in nature.

As previously discussed morphine inhibits the frequency of sIPSC events in WT neurones within the VTA in a concentration dependent manner. The MOP receptor is required for the effects of opioid drugs on sIPSC frequency within the VTA, there was no significant inhibition of sIPSC frequency in neurones from MOP-/- mice, paired t test $p = 0.08$ $n = 5$ (Figure 5.6A-C). Morphine (100 µM) caused a significant reduction in the ability of morphine to inhibit sIPSC frequency in WT and MOP+/- neurones but no significant inhibition of sIPSC frequency in MOP-/- neurones (Figure 5.6B).

Receptor number is also important in the ability of morphine to produce this effect as removing 50% of MOP receptors, as is the case in MOP+/- mouse (Sora *et al*., 1997, Sora *et al*., 2001), caused a reduction in the potency of morphine. This is indicated by the rightward shift in the concentration response relationship for morphine (Figure 5.6A). The ability of morphine (100 µM) to inhibit sIPSC frequency in MOP+/- neurones was significantly reduced compared to WT neurones (Figure 5.6B).

There was a trend towards baseline inhibition of sIPSC frequency even with low concentrations of morphine (25.4 ± 5.2% with 0.03 µM morphine) in WT neurones, although this was not significant (paired t test p 0.06, $n = 5$), but it is likely that this will reach significance with additional experiments (Figure 5.6A). Although not significant, as stated earlier, there was also a trend towards inhibition in MOP-/- neurones (14.4 ± 5.0%, $p < 0.08$, $n = 6$). Additional

experiments are needed to establish whether this is in fact a small but significant MOP receptor independent inhibitory effect of morphine. Interestingly there was no trend towards inhibition when recordings were performed with DAMGO (Figure 5.8).

It has previously been reported that morphine is an antagonist at the 5-HT_3 receptor (Baptista-Hon *et al*., 2012) and 5-HT receptors are present within the VTA (Cameron *et al*., 1997, Rodd *et al*., 2007). The 5HT_3 receptor antagonist ondansetron was used to test the possibility of an involvement of 5-HT_3. Consistent with a role for 5-HT_3 receptors in the release of GABA from presynaptic neurones in the VTA, ondansetron alone caused a significant inhibition of sIPSC frequency in WT neurones (Figure 5.7A) by 23.1 ± 6.2%. When applied with morphine the effects of ondansetron were additive. The inhibition of sIPSC frequency with morphine (10 µM) alone is 58.2 ± 3.6% and in the presence of ondansetron (100 nM) is 79.7 ± 10.2%. However, the level of sIPSC inhibition observed in the presence of ondansetron is not significantly different from when morphine was applied alone (Figure 5.7C). Taken together these data suggest that if morphine is inhibiting 5-HT_3 receptors within the VTA this is a minor effect compared to the inhibition mediated by MOP receptors.

DAMGO, a selective MOP receptor agonist, also inhibited sIPSC frequency in a concentration dependent manner in WT neurones (Figure 5.8). The EC_{50} of

DAMGO was 0.25 µM in the WT neurones. The potent inhibition of sIPSC frequency observed with morphine was not evident with DAMGO.

It appears that morphine causes a small high potency inhibition of sIPSC frequency that is not replicated by the selective agonist DAMGO. While negligible this inhibition by morphine appears to remain in slices from MOP-/- neurones. The observation that ondansetron and morphine are additive in their combined inhibition of sIPSC frequency suggests that the 5-HT_3 receptor is not the target of this action. Morphine can activate DOP receptors. DOP-/- mice were used to establish a possible role for DOP receptors in the actions of morphine.

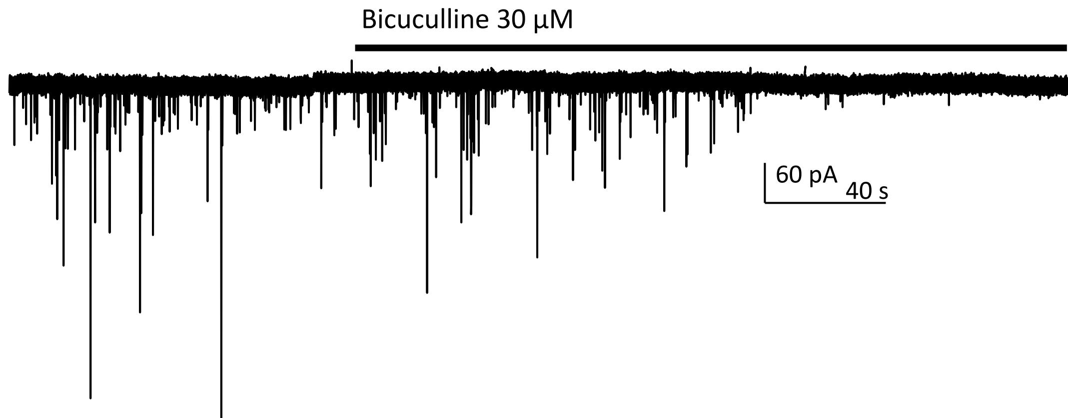


Figure 5.3: sIPSC events within the VTA are $GABA_A$ receptor mediated. The above example trace reveals inhibition of the sIPSC events following the addition of the $GABA_A$ receptor antagonist bicuculline (30 µM) to the recording solution.

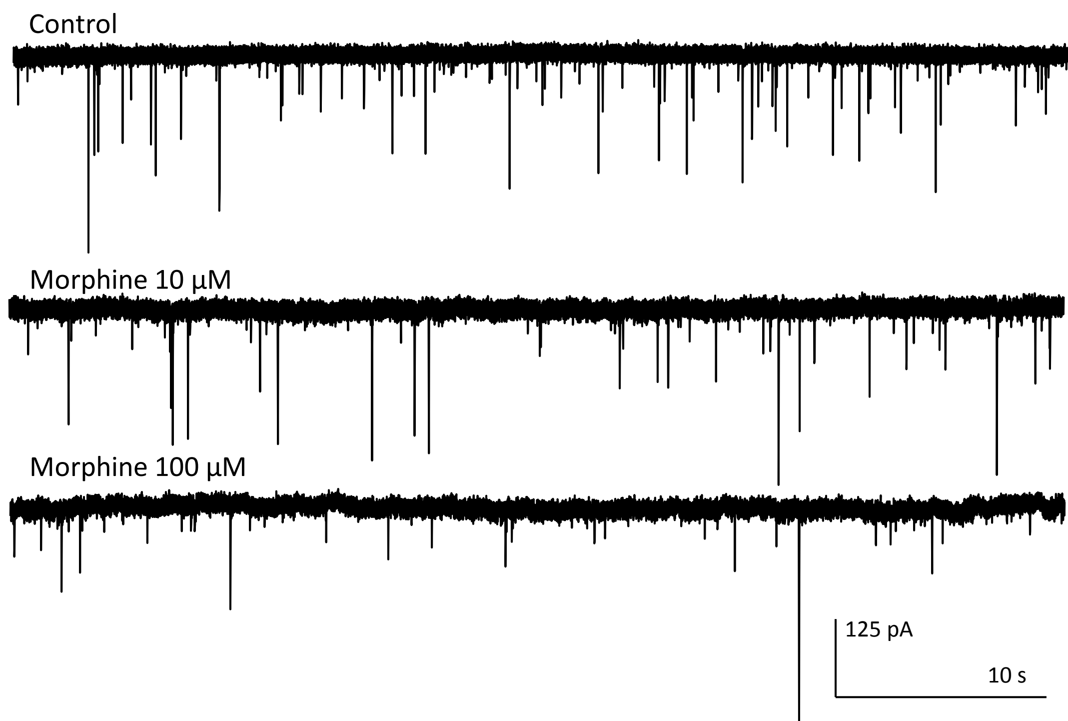


Figure 5.4: Morphine inhibits sIPSC activity in a concentration dependent manner in WT neurones. sIPSCs were recorded from voltage-clamped VTA neurones before and during the application of increasing concentrations of morphine.

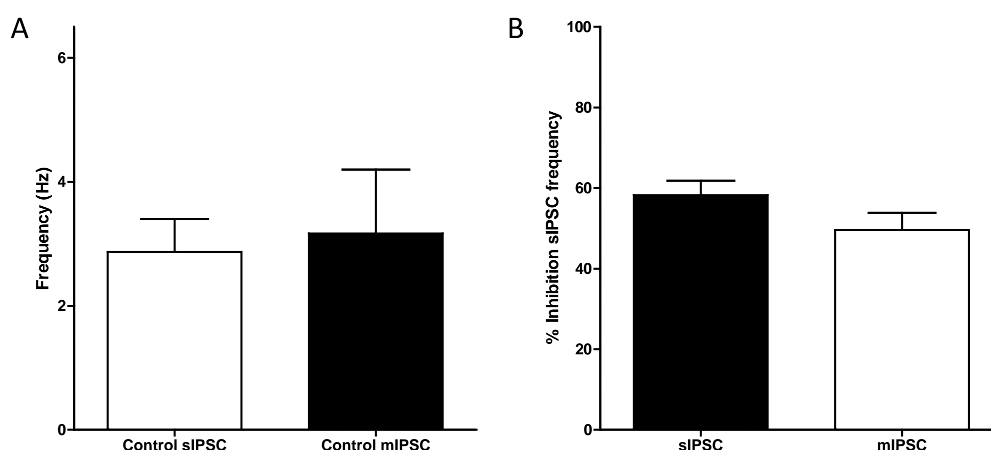


Figure 5.5: TTX does not affect IPSC frequency or inhibition by morphine. (A) There is no difference in IPSC frequency in the presence or absence of TTX (500 nM). Control frequency, sIPSC n = 10, mIPSC n= 4, unpaired t test p = 0.8. (B) TTX does not affect the degree of inhibition of IPSC frequency observed in the presence of morphine (10 µM), sIPSC n = 7, mIPSC n = 12, unpaired t test p = 0.2. Vertical lines represent ± SEM.

	sIPSC		mIPSC	
	Control	Morphine 10 µM	Control	Morphine 10 µM
Peak Amplitude (pA)	-83.7 ± 6.8	-94.8 ± 13.6	-81.1 ± 8.9	-86.6 ± 9.3
Rise time (ms)	0.64 ± 0.02	0.65 ± 0.04	0.64 ± 0.03	0.67 ± 0.03
T70 (ms)	7.5 ± 0.4	7.6 ± 0.6	7.4 ± 0.7	8.1 ± 0.8
Tau ω (ms)	6.5 ± 0.4	6.8 ± 0.7	7.0 ± 1.0	6.8 ± 0.7
Frequency (Hz)	2.2 ± 0.3	1.0 ± 0.3 *	2.7 ± 0.7	1.2 ± 0.3 *

Table 5.2: TTX does not significantly affect IPSC parameters within the VTA. Morphine inhibits IPSC frequency but does not alter the kinetics of the events. There are no significant changes in the kinetic parameters of the IPSC events in the presence of TTX (500 nM) or morphine (10 µM). The frequency of events is significantly decreased following morphine exposure; this is unchanged in the presence of TTX. For the sIPSC recordings n = 8 and for the mIPSC recordings n = 12, * represents $p < 0.05$ on Student's t test.

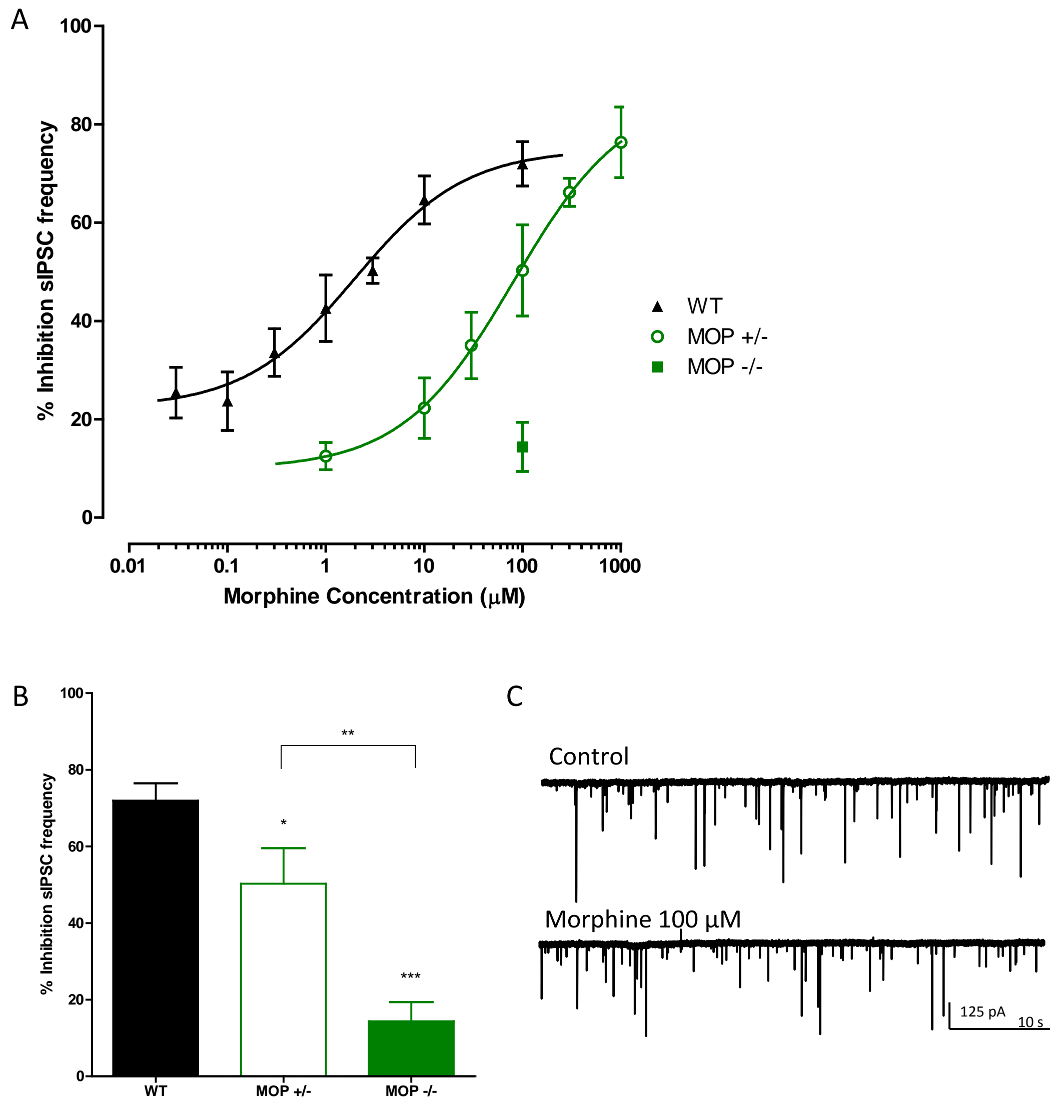


Figure 5.6: Morphine inhibits sIPSCs in a concentration dependent manner in WT and MOP+/- neurones; it does not cause significant inhibition in MOP-/- neurones. (A) Concentration response curve for morphine inhibition of sIPSC frequency within the VTA. WT neurones EC_{50} 2 μM n = 5 – 10, MOP+/- neurones EC_{50} 81.2 μM n = 4 – 6, and MOP-/- neurones n = 5. (B) Inhibition of sIPSC frequency by 100 μM morphine. There is a significant reduction in the ability of morphine to inhibit sIPSC frequency in the MOP+/- and MOP-/- neurones compared to the WT neurones. One way ANOVA $p < 0.001$, *post hoc* Tukey test results are shown on the graph. Vertical lines represent ± SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. (C) Morphine had a negligible effect on sIPSC frequency in MOP-/- neurones.

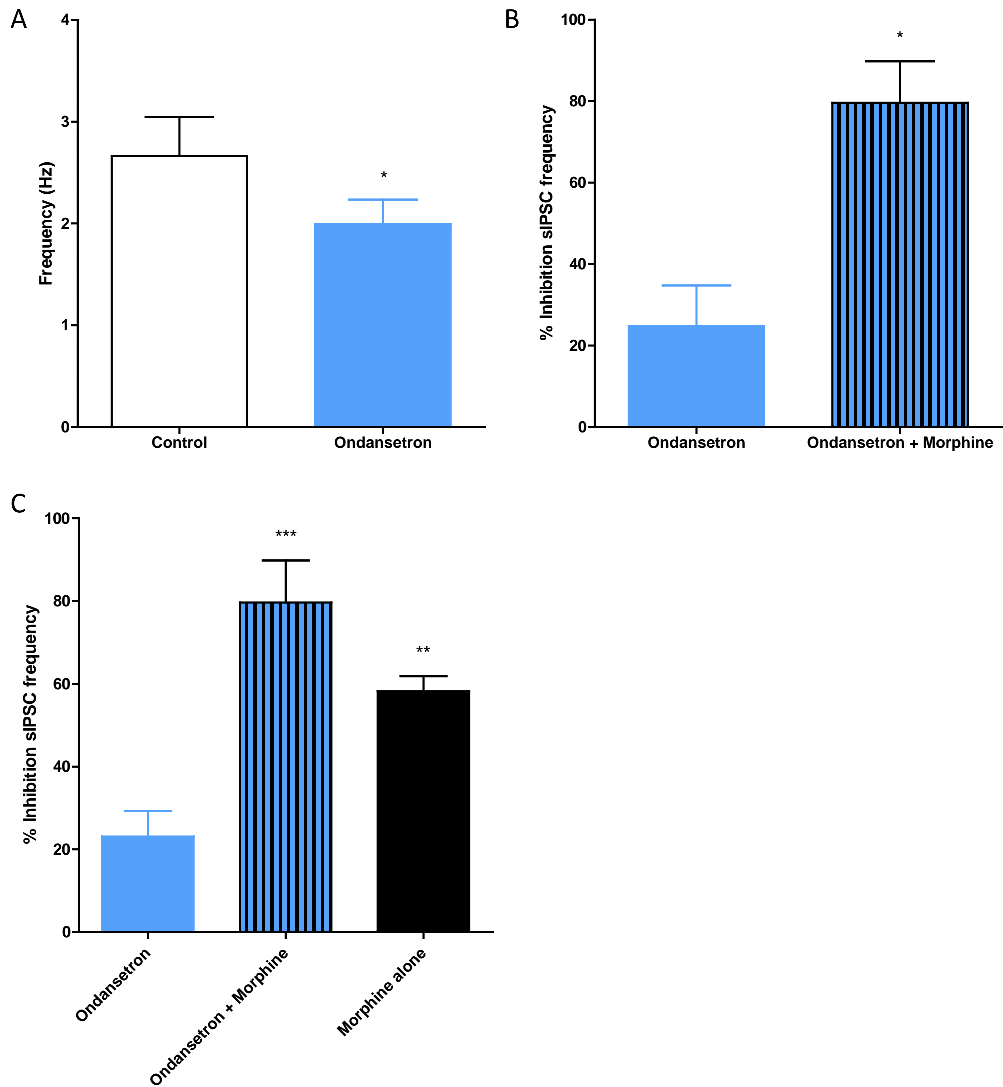


Figure 5.7: Addition of the 5-HT$_3$ receptor antagonist ondansetron results in inhibition of sIPSC frequency within the VTA. It does not affect the ability of morphine to inhibit sIPSC frequency. (A) Ondansetron (100 nM) significantly inhibits sIPSC frequency in WT VTA neurones, t test p = 0.04 n = 6. (B) Morphine (10 µM) significantly inhibits sIPSC frequency in WT neurones in the presence of ondansetron (100 nM), t test p = 0.01 n = 3. (C) The presence of ondansetron does not affect the ability of morphine to inhibit sIPSC frequency in WT neurones within the VTA, one way ANOVA p < 0.0001 *post hoc* Tukey results are shown on the graph, ondansetron alone n = 6, ondansetron plus morphine n = 3, morphine alone n = 7. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.

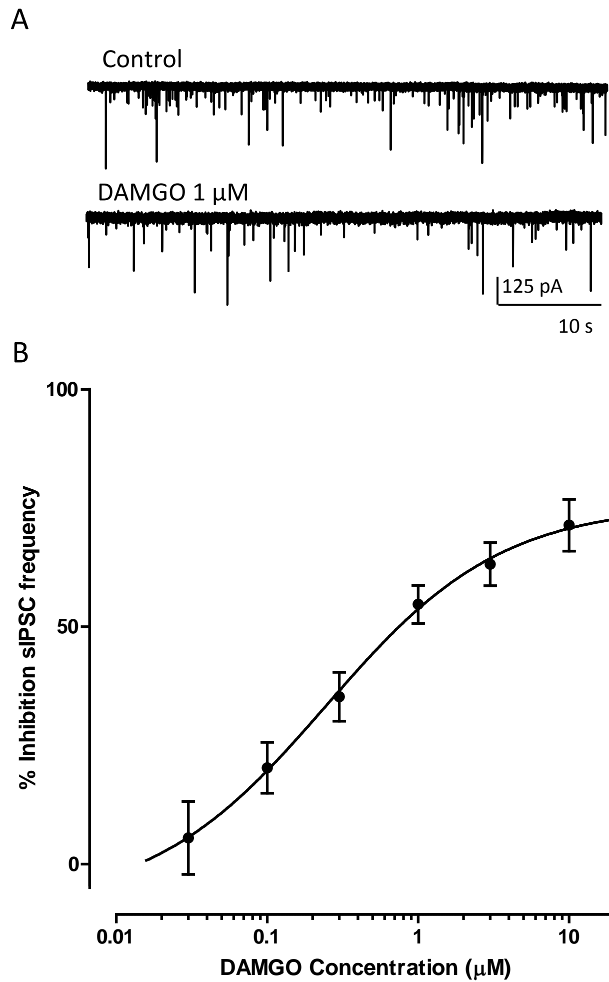


Figure 5.8: DAMGO inhibits sIPSC frequency in a concentration dependent manner in WT neurones within the VTA. (A) DAMGO (1 μM) inhibits sIPSC frequency in a WT neurone. (B) Concentration response curve for inhibition of sIPSC frequency by DAMGO in WT neurones, n = 3 – 7, EC_{50} DAMGO 0.25 μM. Vertical lines represent ± SEM.

5.4 DOP receptors within the VTA

DPDPE (1 µM) significantly inhibited sIPSC frequency in WT neurones (37.3 ± 7.4% n = 6; paired t test p = 0.001). The inhibition observed was not significantly different in MOP-/- neurones suggesting that the response is mediated by DOP receptors. In confirmation of this DPDPE (1 µM) has no significant effect on sIPSC frequency when applied to DOP-/- neurones (Figure 5.9). This confirms the presence of DOP receptors within the VTA and suggests that they can function independently of MOP receptors.

The inhibition by morphine (10 µM) of sIPSC frequency within the VTA was significantly reduced in the DOP+/- and DOP-/- neurones (Figure 5.10). While DOP receptors appear to be required for the full inhibitory effect of morphine on IPSCs the inhibition of IPSC frequency by DAMGO was not significantly different in the WT and DOP-/- VTA neurones (Figure 5.11).

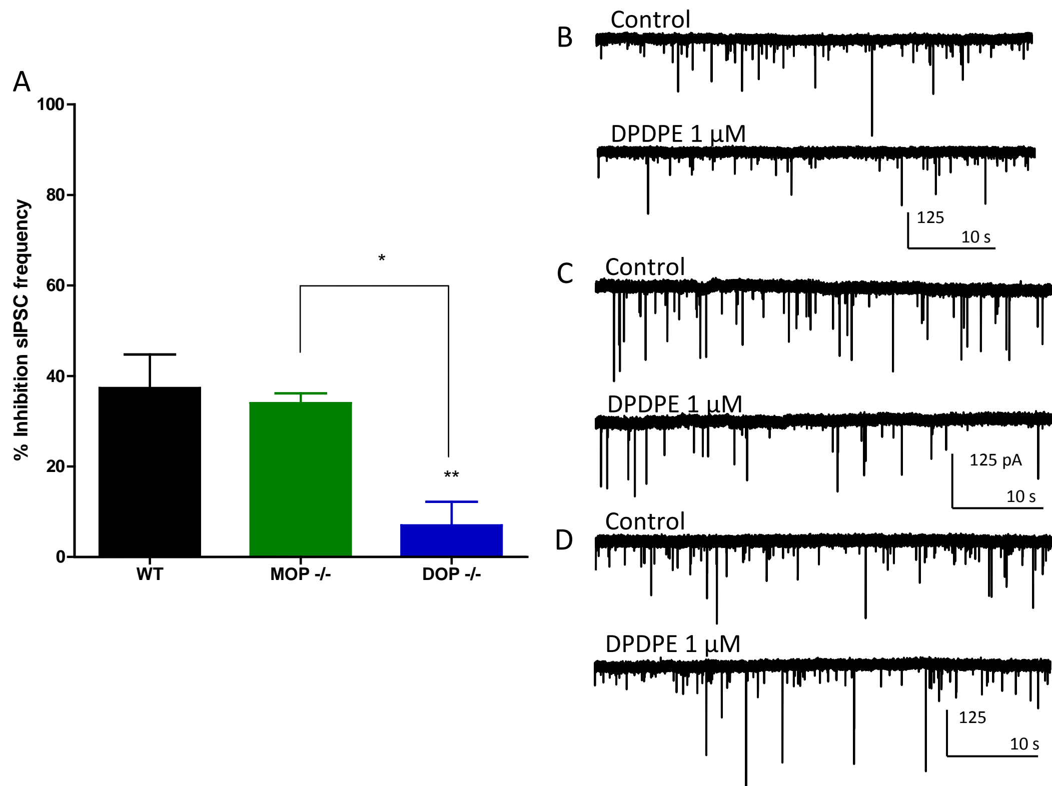


Figure 5.9: DOP receptors are functional and can signal independently of MOP receptors within the VTA. (A) In WT neurones we observe inhibition of sIPSC activity in the presence of DPDPE 1 μM, (37.3 ± 7.4%). In MOP-/- neurones there is no significant difference in the ability of DPDPE to inhibit sIPSC frequency (34.0 ± 2.2%), unpaired t test p = 0.7. DPDPE is selective for the DOP receptor, there are no significant changes in sIPSC frequency in response to DPDPE exposure in DOP-/- neurones (7.0 ± 5.2%), one way ANOVA p = 0.0032, *post hoc* Tukey results shown on graph. WT n = 6, MOP-/- n = 5, DOP-/- n = 6. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001. (B) DPDPE inhibits sIPSC frequency in WT neurones. (C) DPDPE inhibits sIPSC frequency in MOP-/- neurones. (D) DPDPE does not inhibit sIPSC frequency in DOP-/- neurones.

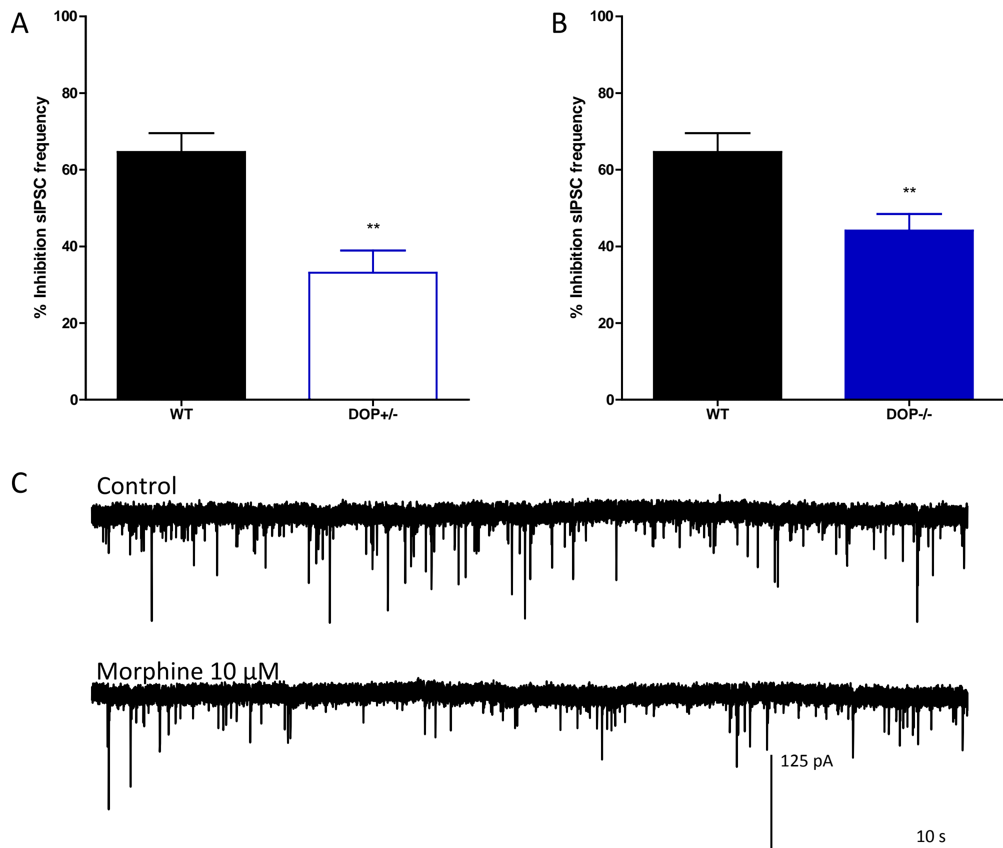


Figure 5.10: The absence of DOP receptors in the VTA significantly reduces the ability of morphine to inhibit sIPSC frequency. (A) The ability of morphine (10 µM) to inhibit sIPSC frequency is reduced in DOP+/- neurones (33.1 ± 5.8 %) compared to WT neurones (64.6 ± 4.9 %), unpaired t test p = 0.001, WT n = 10, DOP+/- n = 6. (B) The ability of morphine (10 µM) to inhibit sIPSC frequency is reduced in DOP-/- neurones (44.1 ± 4.3 %) compared to WT neurones, unpaired t test p = 0.009, WT n = 10, DOP-/- n = 7. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001. (C) Inhibition of sIPSC events by morphine in a DOP-/- neurone within the VTA.

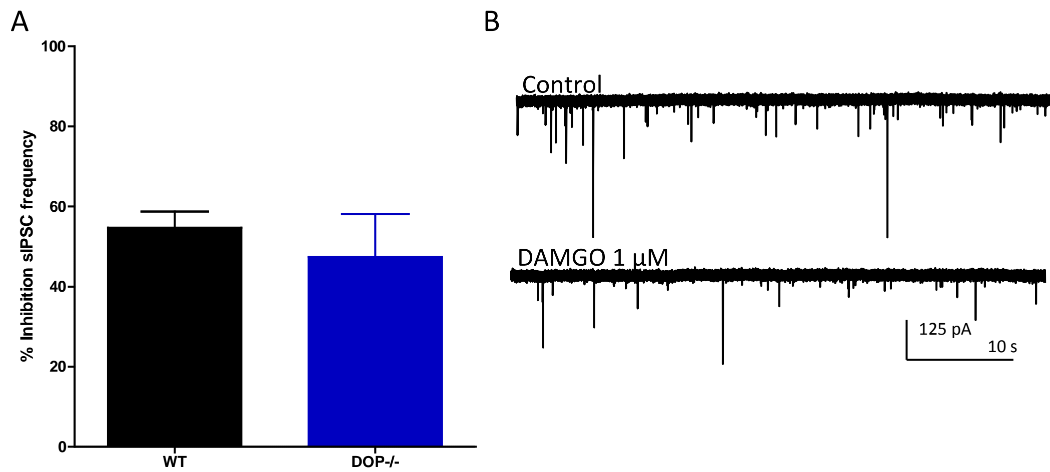


Figure 5.11: DOP receptors are not involved in signalling within the VTA in response to DAMGO. (A) The inhibtion by DAMGO (1 µM) of sIPSC frequency in DOP-/- neurones (47.4 ± 10.8 %) is not significantly different to that seen in WT neurones (54.7 ± 4.0 %), unpaired t test p = 0.54, WT n = 7, DOP-/- n = 7. Vertical lines represent ± SEM. (B) DAMGO (1 µM) exposure results in significant inhibition of sIPSC's in DOP-/- neurones. Vertical lines represent ± SEM.

5.5 The role of β-arrestin2 in signalling within the VTA

BAR2 is involved in the analgesic and psychomotor effects of morphine (see Chapter 4). However, the role of BAR2 in opioid receptor mediated inhibition of IPSCs in the VTA is unknown. Surprisingly, there was a significant decrease in the ability of morphine (10 μM) to inhibit sIPSC frequency in BAR2-/- VTA neurones compared to WT VTA neurones (Figure 5.12A). Increasing the concentration of morphine (from 10 to 100 μM) increased the inhibition suggesting that the absence of BAR2 leads to a reduction in morphine potency (Figure 5.12B). This effect appears not to be specific to morphine as the inhibition of sIPSC frequency by DAMGO in the BAR2-/- neurones was also less than in WT neurones (Figure 5.12C).

There was also a significant decrease in the inhibition of sIPSC frequency by morphine (37.0 ± 6.6 %) in BAR2-/-//DOP-/- neurones compared to WT neurones (Figure 5.13). Overall there is a decrease in the ability of morphine to inhibit sIPSC frequency in the DOP-/-, BAR2-/- and the BAR2-/-//DOP-/- neurones (Figure 5.13). There are no significant differences in the level of inhibition observed between these genotypes. This suggests that both BAR2 and DOP receptors are involved in signalling in response to morphine within the VTA.

While an absence of BAR2 led to reduced inhibition of sIPSC frequency by DAMGO (Figure 5.12C) the absence of DOP receptors had no effect (Figure 5.11). This suggests that DOP receptors are not involved in signalling in response to

DAMGO within the VTA but that BAR2 is. Together these data provide evidence that different opioid drugs may utilise differing combinations of opioid receptor subtypes to achieve inhibition of sIPSC frequency in the VTA. This may have important implications regarding their tendency to initiate tolerance and dependence.

While there were differences in the degree of inhibition of sIPSC frequency in response to agonist stimulation, there were no significant differences in baseline sIPSC frequency across the genotypes (Figure 5.14). WT VTA neurones have a baseline sIPSC frequency of 2.2 ± 0.2 Hz, MOP+/- neurones: 2.0 ± 0.5 Hz, MOP-/- neurones: 1.8 ± 0.3 Hz, DOP-/- neurones: 1.4 ± 0.3 Hz, BAR2-/- neurones: 1.6 ±0.3 Hz and BAR2-/-//DOP-/- neurones: 2.0 ± 0.3 Hz. This implies that there is little basal MOP or DOP mediated inhibition of sIPSCs, because the absence of these receptors (or their signalling pathway) would otherwise be expected to result in enhanced tonic sIPSC frequency.

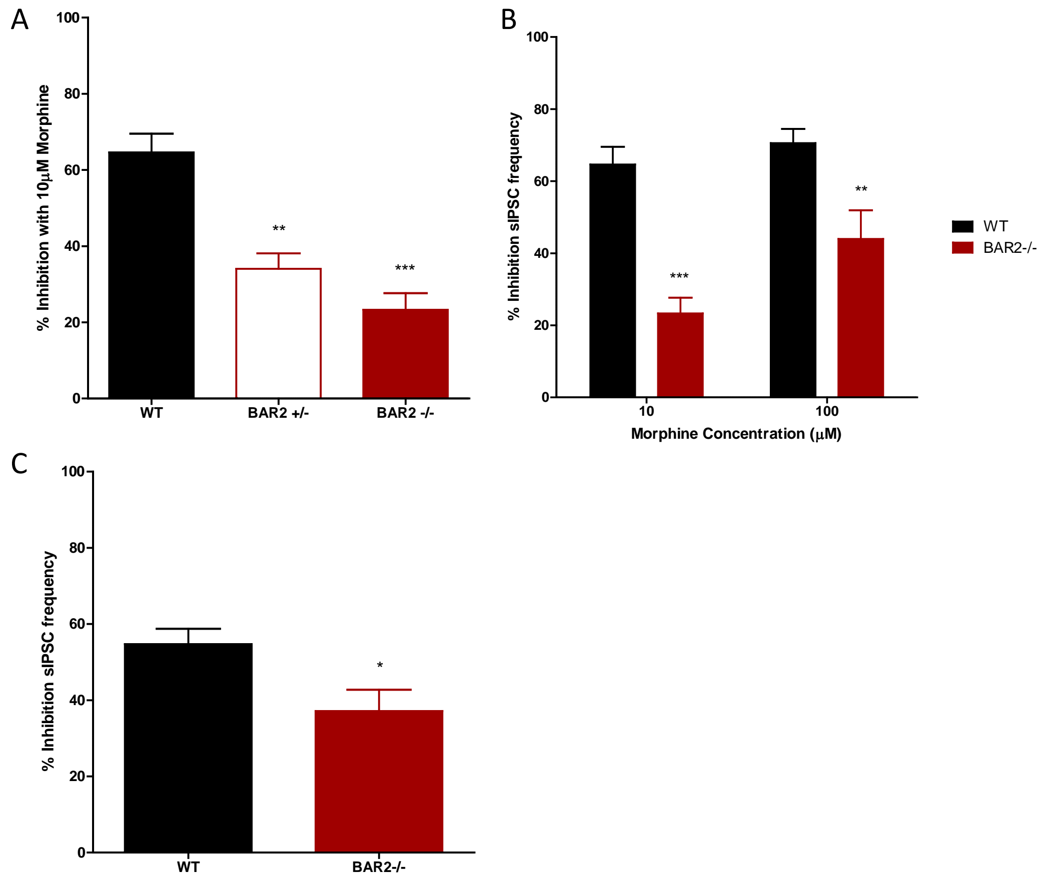


Figure 5.12: The absence of BAR2 reduces inhibition of the frequency of sIPSC events in the VTA by opioid drugs. (A) There is a genotype dependent reduction in the ability of morphine to inhibit the frequency of sIPSCs within the VTA WT 64.6 ± 4.9 % n = 10, BAR2+/- 34 ± 4 % n = 4, BAR2-/- 23.3 ± 4.4 % n = 5. (B) The inhibition of sIPSC in BAR2-/- neurones is concentration dependent. The difference in the ability of morphine to inhibit sIPSC frequency in the presence of 10 and 100 µM morphine in WT and BAR2-/- neurones, unpaired t test (100 µM morphine) $p = 0.0085$, WT n = 7, BAR2-/- n = 5. (C) The ability of DAMGO 1 µM to inhibit sIPSC frequency within the VTA was also significantly reduced in the BAR2-/- neurones, unpaired t test $p = 0.03$, WT 54.7 ± 4 % n = 7, BAR2-/- 37.1 ± 5.6 % n = 8. Vertical lines represent ± SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

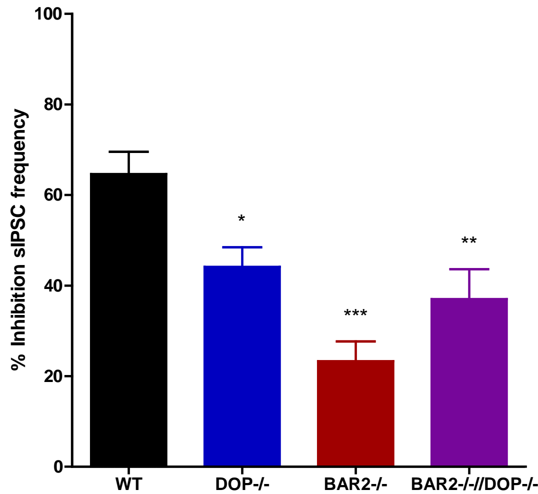


Figure 5.13: BAR2 and the DOP receptor are involved in the response of VTA neurones to morphine. The ability of morphine 10 µM to inhibit sIPSC frequency within the VTA is significantly reduced in DOP-/-, BAR2-/- and BAR2-/-//DOP-/- neurones compared to WT neurones, one way ANOVA $p < 0.0001$, *post hoc* Tukey results are shown on graph. WT n = 10, DOP-/- n = 7, BAR2-/- n = 5 and BAR2-/-//DOP-/- n = 4. Vertical lines represent ± SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

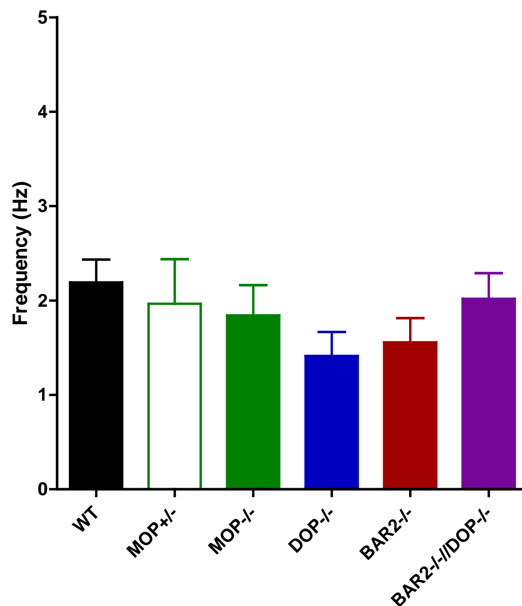


Figure 5.14: Deletion of MOP receptors, DOP receptors or BAR2 does not affect the sIPSC frequency in the absence of drug. One way ANOVA p = 0.4 *post hoc* Dunnett's tests were ns. WT n = 64, MOP+/- n = 19, MOP-/- n = 18, DOP-/- n = 27, BAR2-/- n = 18 and BAR2-/-//DOP-/- n = 12. Vertical lines represent ± SEM.

5.6 Effects of naloxone on sIPSC frequency within the VTA

Naloxone competitively antagonises the actions of agonists at MOP receptors and causes conditioned place aversion when administered to morphine dependent mice (Shoblock and Maidment, 2006). Systemic naloxone is also somewhat aversive in morphine naive mice. This effect is mediated through MOP receptors as it does not occur in MOP-/- mice (Skoubis et al., 2001). Surprisingly, MOP receptor antagonists administered into the VTA cause increased DA release in the NA (Devine et al., 1993b). We examined whether naloxone affects basal IPSC frequency recorded from VTA neurones. Naloxone (10 µM) consistently inhibited sIPSC frequency recorded from WT VTA neurones. The inhibition was independent of MOP receptors as it was not significantly different in recordings from WT and MOP+/- or MOP-/- neurones (Figure 5.15).

In DOP-/- neurones there was no significant difference between the sIPSC frequency under control conditions or during naloxone (10 µM) application. The sIPSC frequency with naloxone added was 96.2 ± 6.4% of control, paired t test (ns) $p = 0.5$, $n = 6$. Interestingly, naloxone caused a small enhancement of sIPSC frequency in BAR2-/- neurones in which sIPSC frequency in the presence of naloxone was 115.7 ± 12.7% of control frequency (paired t test $p = 0.3$, $n = 5$).

This facilitation was not observed in BAR2-/-//DOP-/- neurones, in which the sIPSC frequency in the presence of naloxone was 92.9 ± 11.6 % of control (paired t test was $p = 0.3$, $n = 6$) (Figure 5.16). These data suggest that the inhibition of

sIPSC frequency by naloxone involves both BAR2 and DOP receptors. The small facilitatory effect of naloxone in the absence of BAR2 appears to require DOP receptor expression (Figure 5.16).

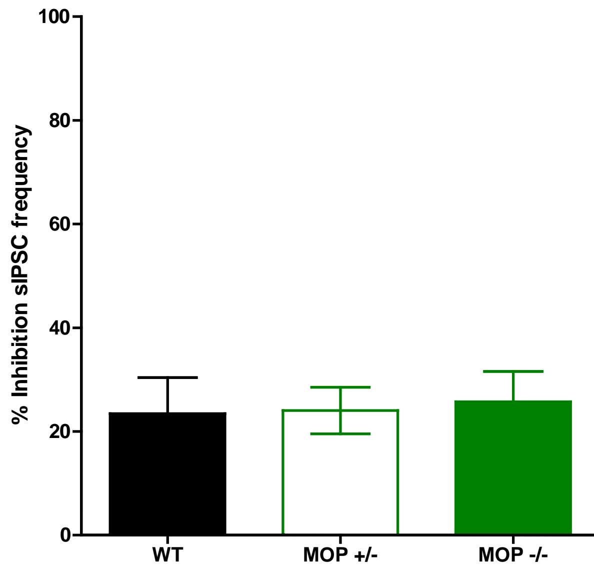


Figure 5.15: Naloxone causes an inhibition of IPSC frequency that is independent of MOP receptors. Inhibition of sIPSC frequency by naloxone in WT neurones is 23.4 ± 6.9%, n = 5 paired t test versus control p = 0.03. One way ANOVA of inhibition of sIPSc frequency by naloxone in WT, MOP+/- and MOP-/- is not significantly different, p = 0.96. MOP+/- inhibition of sIPSC events is 24.0 ± 4.5 % n = 5, MOP-/- inhibition is 25.7 ± 5.8 % n = 8. Vertical lines represent ± SEM.

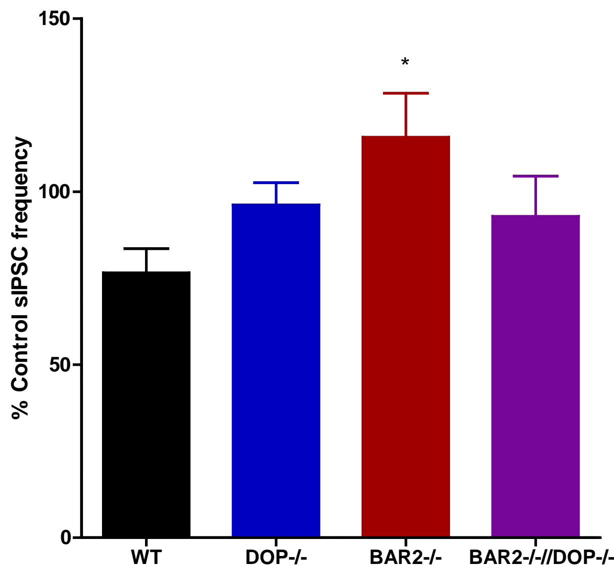


Figure 5.16: Naloxone does not cause significant inhibition of sIPSC events in DOP-/-, BAR2-/- or BAR2-/-//DOP-/- neurones. One way ANOVA p = 0.1, *post hoc* Dunnett's results shown on graph. Vertical lines represent ± SEM. *, p < 0.05. This suggests that the inhibition of sIPSC frequency by naloxone involves both BAR2 and DOP receptors. The small facilitatory effect of naloxone in the absence of BAR2 appears to require DOP receptor expression.

5.7 Summary

Identifying cell type within the VTA using electrophysiological techniques is currently controversial as previously discussed. The IHC protocol used to label brain slices revealed nuclei of TH positive cells corresponding to the VTA. The identification of biocytin labelled TH positive cells following whole-cell recording demonstrates that the postsynaptic DA neurones were effectively targeted in these cases, however, this approach was not routine and in future studies the reliability of cell identification will need to be improved. Nevertheless, the vast majority of cells targeted for whole-cell voltage-clamp recording exhibited a reduction in sIPSC frequency upon application of either DAMGO or morphine indicating their likely identification as DA neurones postsynaptic to GABAergic inhibitory neurones.

Other investigators have suggested that the response to MOP receptor agonists is a reliable marker of cell type (Johnson and North, 1992b, Johnson and North, 1992a, Cameron *et al*., 1997, Chieng *et al*., 2011, Ford *et al*., 2006). The cells that exhibit a reduced AP frequency with opioids are likely to be presynaptic GABAergic neurones, while those that do not are likely to be post-synaptic DA neurones. Therefore the neurones identified in current-clamp recordings as morphine responders are likely to be the GABAergic neurones and the non-responders are dopaminergic neurones. We could detect no significant differences between action potential (AP) duration, amplitude, threshold potential or rise time in current clamp recordings from VTA neurones. The AP duration in cells that exhibited inhibition of AP frequency upon morphine

application did not differ significantly from those published previously (Margolis *et al.*, 2006). There was a trend for an increase in the baseline frequency in the neurones that were responders to morphine (i.e. morphine inhibited AP frequency) but this was not significant.

This corresponds to previously published work that suggests that the firing frequency is increased in non-dopaminergic neurones when compared to dopaminergic neurones (Margolis *et al.*, 2006, Johnson and North, 1992a). The T_{70} of our sIPSC events in WT neurones was 7.5 ± 0.4 ms, this is similar to a previously reported T_{75} for dopaminergic cells within the VTA of 7.55 ms but significantly different from the T_{75} reported for GABAergic neurones within the VTA of 3.16 ms (Tan *et al.*, 2010). Together these findings suggest that DA neurones were effectively targeted for voltage-clamp recordings of IPSCs. However, further work will be required to further identify postsynaptic neurones and the source of their inhibitory input.

The addition of bicuculline to the recording solution abolished the sIPSC events in the VTA confirming that these are GABAergic events. Bicuclline did not produce any alteration in the baseline current suggesting that tonic GABAergic receptor activity is not present in this region under these recoding conditions.

We could identify no significant differences in baseline frequency, kinetics or effects of morphine between mIPSCs and sIPSCs. Recordings of sIPSC events

were used subsequently for the remainder of the study. In recordings performed in the VTA of guinea pig brain slices mIPSC frequency was 2.4 ± 1.3 Hz (Bonci and Williams, 1997) and in VTA containing mouse brain slices the mIPSC frequency was 1.5 Hz in a study published by Meye *et al* (2012). These results are similar to the event frequency observed here.

Previously published studies of MOP receptor activation in the VTA predominately used DAMGO and reported inhibition in responding cells at single concentration points for each drug. One study by Lecca *et al* (2012) bath applied morphine (1 µM) to rat brain slices while recording from DA cells in the VTA and reported an inhibition of sIPSC frequency of 42 ± 4%. This is similar to the inhibition that we observe with morphine (1 µM) in WT mouse VTA neurones (43 ± 7%). With DAMGO (1 µM) the inhibition of mIPSC frequency has been reported as 57.5 ± 9.9% (Bergevin *et al*., 2002) in cultured VTA neurones and 56.94 ± 9.53% and 75.35 ± 9.94% respectively in VTA neurones within mouse brain slices (Meye *et al*., 2012, Madhavan *et al*., 2010). I observed an inhibition of 54.7 ± 4.0% (n = 7) with DAMGO (1 µM) in WT VTA neurones, which is not dissimilar from these results. Unlike previous studies, the current study examined full concentration response relationships for morphine and DAMGO. The addition of increasing concentrations of DAMGO, a selective MOP receptor agonist, or morphine resulted in a concentration dependent inhibition of sIPSC events in the VTA.

In the MOP+/- neurones we observed a rightward shift in the concentration response relationship indicating a reduction in potency of morphine in these neurones. There was no apparent change in morphine's efficacy. These data are similar to results in MOP+/- mice for analgesia (Chapter 3) and psychomotor assays (Chapter 4) in which a higher dose of morphine was required for analgesia, for the development of a preference to morphine and also for significant locomotor activation. In the MOP-/- neurones there is no significant inhibition of sIPSC frequency following exposure to morphine. This is consistent with the previous demonstration that these mice do not show a preference for morphine (Chapter 4). However, there is a trend towards inhibition which may be caused by an action of morphine on DOP receptors.

Even very low concentrations of morphine inhibit sIPSC frequency in WT neurones, although this is not significant in sample of neurones tested (25.4 ± 5.2%, paired t test $p = 0.06$ $n = 5$). There was no such trend in the presence of DAMGO. Morphine is not entirely selective for MOP receptors and therefore may be having off target effects that could contribute to this high potency inhibition of sIPSCs. There are 5-HT_3 receptors within the VTA (Cameron *et al*., 1997, Rodd *et al*., 2007) and morphine potently inhibits this ligand-gated ion channel (Baptista-Hon *et al*., 2012). 5-HT_3 antagonists can also decrease morphine self-administration in rats (Hui *et al*., 1993), modulate tolerance and opioid induced hyperalgesia in mice (Hui *et al*., 1996, Liang *et al*., 2011) and prevent morphine CPP (Carboni *et al*., 1989). Ondansetron alone produced a significant inhibition of sIPSC frequency and, when applied together, the actions

of ondansetron and morphine appear to be additive. These data suggest that there are 5-HT_3 receptors within the VTA, but these are not responsible for the inhibitory effect of morphine.

The ability of morphine to inhibit sIPSC frequency was reduced in DOP-/-, BAR2-/- and BAR2-/-//DOP-/- neurones. These findings suggest that both DOP receptors and BAR2 are involved in signalling within the VTA in response to morphine. With regard to DOP receptors, we observed a significant reduction in the ability of morphine to inhibit sIPSC frequency even after the deletion of one copy of the oprd1 gene in the DOP+/- neurones. However in the DOP-/- neurones inhibition with DAMGO was not changed compared to the WT neurones. By contrast, BAR2-/- neurones exhibited a reduction in the inhibition of sIPSC frequency by both morphine and DAMGO. This suggests that differing opioid drugs may signal through differing opioid receptor combinations. The observation that the selective DOP receptor agonist DPDPE also inhibits sIPSC frequency in the WT VTA neurones, but not DOP-/- neurones suggests that the DOP receptors can function independently of MOP receptors.

In BAR2-/- neurones there is a gene dependent reduction in the ability of morphine to inhibit sIPSC frequency. In these neurones DAMGO is also less effective at producing inhibition of sIPSC frequency. These data support the role of BAR2 in signalling in response to different opioid drugs within the VTA.

The results of CPP in the BAR2-/- mice suggest that these mice are more sensitive to the reinforcing effects of morphine and are more likely to develop a preference at lower doses than the WT mice (Chapter 4). The fact that we observe a reduction in the ability of morphine to inhibit sIPSC frequency and therefore higher concentrations of drug are required to produce inhibition in BAR2-/- neurones suggests that this synapse may not be involved in the reinforcing effects of morphine.

The reduction in the ability of morphine to inhibit sIPSC events is also observed in the BAR2-/-//DOP-/- neurones. Overall our data suggest that both DOP receptors and BAR2 are important in signalling within the VTA their involvement is dependent on the agonist used.

Surprisingly exposure to naloxone caused a small but significant decrease in the frequency of sIPSC events in the WT neurones. It had been anticipated that there would either be no change in frequency or an increase in frequency if there was tonic inhibition of sIPSC frequency mediated by the opioid receptors. This effect persisted in the MOP+/- and MOP-/- neurones and is therefore independent of MOP receptors. The inhibition in sIPSC frequency was absent in both the DOP-/- and BAR2-/- neurones, revealing that this action is occurring through DOP receptors perhaps coupled to BAR2. Interestingly, naloxone caused a small facilitation of sIPSC frequency in BAR2-/- VTA neurones which was not seen in

BAR2-/-//DOP-/- neurones suggesting that this effect, unmasked by the absence of BAR2, is mediated through DOP receptors.

Overall these results confirm that MOP receptors are required for the actions of morphine and DAMGO within the VTA. There are functional DOP and 5-HT_3 receptors within the VTA. The former, but not the latter appear to be important for the full inhibition of sIPSC frequency by morphine. BAR2 is also involved in the response to DAMGO suggesting that it may have a wider role in VTA signalling in response to opioid drugs. This is the most surprising finding of this study implicating the BAR2 pathway in the control of vesicular release. The next chapter explores a possible role for c-Src in this and other consequences of MOP receptor activation mediated through the BAR2 signalling pathway.

Chapter 6: The effects of c-Src on opioid receptor signalling

6.1 Introduction

BAR2-/- mice exhibit decreased sensitivity to noxious heat and a marked reduction in analgesic tolerance to morphine (Chapter 3). These findings are in line with previous reports suggesting that the BAR2 signalling system impairs MOP receptor mediated analgesia (Bohn *et al*., 1999, Lam *et al*., 2011). The absence of BAR2 enhances MOP receptor constitutive activity leading to tonic inhibition of VACCs in primary afferent neurones (Walwyn *et al.*, 2007). Inhibition of c-Src activity in DRG neurones of WT mice also enhanced constitutive MOP receptor inhibitory coupling to VACCs suggesting that tyrosine kinase mediated phosphorylation may attenuate opioid analgesia. This study utilised PP2, a selective inhibitor of the Src family tyrosine kinases and compared its actions to those of an inactive chemical analogue called PP3, used as a negative control. This work raises the possibility that sustained MOP receptor mediated analgesia might be produced by inhibiting c-Src (Walwyn *et al*., 2007). Furthermore, the basal analgesia and reduced morphine tolerance observed in BAR2-/- mice might be caused by uncoupling of MOP receptors from c-Src. Therefore, a primary goal of this study was to examine whether inhibition of c-Src in mice causes sustained MOP receptor mediated analgesia.

A previous study in rats has suggested that a tyrosine kinase inhibitor used in the treatment of leukaemia (imatinib) can abolish morphine analgesic tolerance (Wang *et al*., 2012). For this study the drug had been modified to allow it cross the blood brain barrier as the normal clinically administered drug cannot do this. One of the targets of imatinib is the PDGFRβ receptor and this study focused on

this receptor as a target strategy to reduce morphine tolerance (Wang *et al*., 2012). There are a number of other anti-cancer drugs related to imatinib that inhibit tyrosine kinases. These include dasatinib, a drug also licensed for clinical use to treat leukaemia. It is thought that it may be a useful treatment for other cancers, including colorectal cancer as an adjunct to existing therapies (Sharma *et al*., 2012). Dasatinib has the capability to cross the blood brain barrier without any alterations to its structure/ pharmacology (Lagas *et al*., 2009, Porkka *et al*., 2008). Dasatinib is a potent c-Src inhibitor that is normally administered orally to patients. However, the oral bioavailability is low (14 – 34%), after oral dosing it reaches a peak plasma concentration between 30 minutes and 3 hours, it has a high volume of distribution (> 3 l/Kg) and high serum protein binding (> 90%) (Kamath *et al*., 2008).

We used this drug for its capacity to inhibit c-Src in mice. When oral and IP dosing has been compared in mice there are no significant differences in brain accumulation at six hours when either 10 mg/Kg is administered orally or 5 mg/Kg is administered via the IP route. The C_{brain} after oral dosing was 6.5 ± 2.1 ng/g and after IP dosing was 5.5 ± 0.4 ng/g. These corresponded to plasma levels (C_{max}) of 0.37 ± 0.08 mg/L for oral dosing and 0.94 ± 0.07 mg/L for IP dosing (Lagas *et al*., 2009). We chose to use a dose of 5 mg/Kg via the IP route for ease of administration at a defined time point.

It is important to establish whether approaches developed to modify opioid receptor signalling, in an attempt to reduce tolerance and enable persistent analgesia, affect reward. We have demonstrated that an absence of BAR2 causes persistent analgesia and an increase in the sensitivity of BAR2-/- mice to the reinforcing effects of morphine (Chapters 3 and 4). We know from the animal studies that dasatinib can cross the blood brain barrier (Lagas *et al*., 2009, Porkka *et al*., 2008). We examined whether c-Src affects the psychomotor effects of morphine.

6.2 PP2 and Dasatinib inhibit c-Src *in vitro* and *in vivo*

As previously discussed, in Chapter 1 (Section 1.21), PP2 is a tyrosine kinase inhibitor that is selective for c-Src (Bain *et al*., 2007, Uitdehaag *et al*., 2012). This contrasts with dasatinib that affects several different tyrosine kinases but which is a potent inhibitor of c-Src with a K_d of 0.21 nM (Karaman *et al*., 2008). PP2 has been widely used *in vitro* but it is rarely administered *in vivo*, however, dasatinib is a clinically used drug that has been widely tested *in vivo*. PP3 is an inactive chemical analogue of PP2 that provides a control for studies utilising PP2 (Tocris).

Both PP2 and dasatinib inhibit the phosphorylation of c-Src in SW620 cells following a 24 hour exposure to the drug at a concentration of 10 μM (Figure 6.1A). The presence of phosphorylated c-Src was confirmed in samples only exposed to DMSO or PP3. A modified protocol was performed on DRG neurones

obtained post mortem from mice treated with either vehicle control, PP2 (5 mg/Kg), PP3 (5 mg/Kg) or dasatinib (5 mg/Kg). The samples obtained from the mice treated with dasatinib (5 mg/Kg) exhibited a marked reduction in the phosphorylation of c-Src compared to the control vehicle treated sample. However, there was no detectable difference in the level of phosphorylated c-Src between the PP2 and the PP3 treated mice (Figure 6.1B).

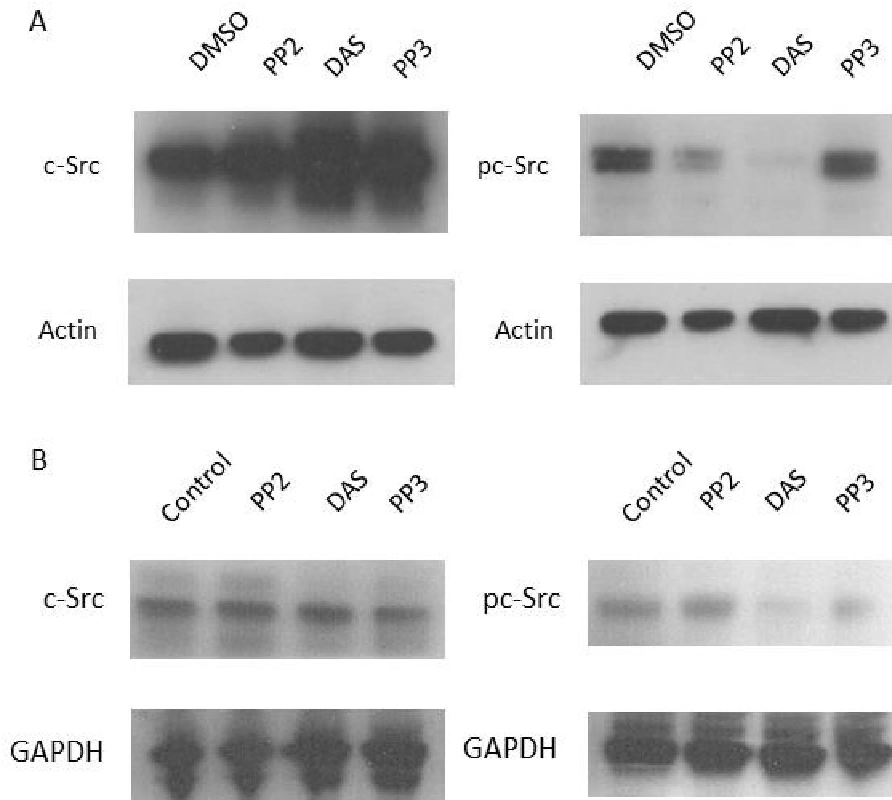


Figure 6.1: PP2 and dasatinib inhibit the phosphorylation of c-Src. (A) Western blot demonstrating that PP2 and dasatinib inhibit the phosphorylation of c-Src in SW620 cells. SW620 cells were incubated with PP2, dasatinib and PP3 (10 µM) and DMSO control for 24 hours prior to collection. Primary antibodies were used against actin (loading control) total c-Src and phosphorylated c-Src (pc-Src) Both PP2 and dasatinib reduced pc-Src with DMSO and PP3 having no effect. (B) c-Src inhibition in DRG neurones following systemic administration of PP2 and dasatinib. Dasatinib but not PP2 exhibits a reduction in pc-Src in DRG neurones collected post mortem from drug treated WT mice. GAPDH was used as a loading control for these experiments.

6.3 The role of c-Src in morphine tolerance

As previously discussed (Chapter 3), WT mice exhibit tolerance to morphine following repeated administration (Figure 3.5). Tolerance was markedly reduced in BAR2+/- and -/- mice (Figure 3.7). Previous work has implicated c-Src in BAR2-mediated signalling in the pain pathway (Walwyn *et al.*, 2007). Therefore, we hypothesised that c-Src may participate in tolerance. We investigated the effects of administering dasatinib (5 mg/Kg) or vehicle via the IP route, 30 minutes prior to the SC injection of morphine (10 mg/Kg) in WT mice for 10 days, n = 8 in each group. The baseline tail withdrawal latencies of the two groups were not significantly different, the vehicle treated mice had a baseline tail withdrawal latency of 2.3 ± 0.1 s and the dasatinib treated group had a baseline tail withdrawal latency of 2.3 ± 0.4 s on day 1 of the protocol.

Morphine was administered 30 minutes after the IP injection, immediately prior to this a repeat tail withdrawal assay was performed. On day 1 the vehicle treated mice had an average tail withdrawal time of 2.7 ± 0.4 s and the dasatinib treated mice had an average tail withdrawal time of 3.6 ± 0.3 at this point. This was not significantly different (t test $p = 0.08$) (data shown as %MPE, Figure 6.2A). The baseline tail withdrawal times expressed as MPE at this time point were also not significantly different between the groups. The vehicle treated group had an MPE of 3.0 ± 4.0% and the dasatinib treated group had an MPE of 10.1 ± 4.8% (Figure 6.2A). There were also no significant changes in tail withdrawal times or equivalent values expressed as %MPE for analgesia between the vehicle treated and dasatinib treated mice prior to morphine administration

over the ten days of the protocol two way repeated measures ANOVA time p = 0.2 and treatment p = 0.8 (Figure 6.2D). These data suggest that dasatinib administration did not produce consistent basal analgesia when administered to WT mice.

Baseline tail withdrawal times were also investigated in the MOP+/- mice, which exhibited latencies of 2.5 ± 0.3 s for the vehicle allocated group and 2.1 ± 0.2 s for the dasatinib allocated group, n = 8 in each group. After either vehicle or dasatinib injection a tail withdrawal assay was repeated. At this point the vehicle treated group had a tail withdrawal time of 3.1 ± 0.3 s (MPE of 3.3 ± 0.52%) and the dasatinib treated group a time of 4.2 ± 2.2 s (MPE of 9.4 ± 3.5%)(Figure 6.3A). These times were not significantly different between the groups and the mice did not exhibit any significant differences over the 5 days of testing (data not shown). These data demonstrate that dasatinib administration did not produce basal analgesia in the MOP+/- mice.

Mice (MOP+/-) treated with either PP3 (5mg/Kg) or PP2 (5mg/Kg) IP 30 minutes prior to morphine also did not exhibit any significant differences in baseline tail withdrawal times (Figure 6.3B). For these groups the average baseline tail withdrawal times for the PP3 allocated group were 3.3 ± 0.4 s and for the PP2 allocated group they were 3.4 ± 0.2 s.

Following PP3/ PP2 administration the tail withdrawal times were 4.0 ± 0.3 s (5.1 ± 1.6% MPE) and 3.8 ± 0.3 s (3.8 ± 2.2% MPE) respectively, n = 8 in each group. When these results were compared over the 5 days of the experiment there were no significant differences between the PP3 and the PP2 treated MOP+/- mice (data not shown). PP2 administration did not produce basal analgesia in the MOP+/- mice.

Having investigated the actions of c-Src inhibition on baseline sensitivity to noxious heat the effect of dasatinib and PP2 were examined against morphine analgesic tolerance. Interestingly, dasatinib treated WT mice did not develop significant tolerance to the analgesic effects of morphine (10 mg/Kg) over the ten day test period (Figure 6.2B). On day 10 vehicle treated mice exhibited a decline in morphine analgesia to 57.6 ± 11.4% of the MPE. By contrast, dasatinib treated mice maintained morphine (10 mg/Kg) analgesia at 99.3 ± 0.7% of MPE. The difference between dastinib and vehicle treated groups was significant on day 10, t test p = 0.003 (Figure 6.2C).

MOP+/- mice develop a more rapid and profound morphine analgesic tolerance than do WT mice (Chapter 3, Figure 3.5). This provides an additional model in which to investigate the capacity of c-Src inhibition to affect the development and maintenance of tolerance. Either vehicle or dasatinib (5 mg/Kg) were injected IP, 30 minutes prior to morphine (10 mg/Kg) administration, n = 8 for each group. On day 5 the tail withdrawal latency for control mice treated with

morphine was 32.0 ± 9.2% of MPE, while dasatinib treated mice exhibited a significantly increased analgesic effect of morphine with an MPE of 88.1 ± 7.9% (Figure 6.3C).

The effect of the c-Src inhibitor, PP2, on the development of morphine analgesic tolerance in MOP+/- mice was also investigated. For this experiment mice received either the inactive analogue, PP3 (10 mg/Kg), or PP2 (10 mg/Kg) injected IP, 30 minutes prior to SC morphine (10 mg/Kg), n = 12 in each group. Like dasatinib, PP2 administration also significantly reduced the development of analgesic tolerance in the MOP+/- mice. On day 5 the PP3 treated mice had a MPE of 17.0 ± 4.1% for morphine and the PP2 treated mice had a significantly larger analgesic response to morphine with an MPE of 87.4 ± 4.7% (Figure 6.3D).

Dasatinib was applied next using a paradigm to determine whether the drug affects morphine tolerance after the process had already developed. MOP+/- mice received morphine (10 mg/Kg) for three days, n = 8 in each group. Tail withdrawal latencies were established 30 minutes after morphine administration. As previously observed a significant reduction in the morphine analgesia developed in both groups of mice. The group that subsequently received IP vehicle treatment exhibited an MPE of 64.8 ± 12.7% following the administration of morphine (10 mg/Kg). The other group that subsequently received dasatinib exhibited a morphine analgesic MPE of 69.8 ± 12.7%. On days 4 and 5 one group received a vehicle injection IP, 30 minutes prior to the

administration of morphine (10 mg/Kg) and the other group received dasatinib (5 mg/Kg) IP, 30 minutes prior to morphine (10 mg/Kg) administration. Not only was there no further development of analgesic tolerance, in the dasatinib treated group, the previously existing analgesic tolerance appears to have reversed.

On day 5 the vehicle treated group had an MPE of 30.3 ± 17.3% following the administration of morphine, indicating a significant morphine analgesic tolerance. By contrast, the dasatinib treated group had an MPE of 88.9 ± 5.8% following the administration of morphine. In addition to being significantly different from the vehicle treated group, the analgesic effect of morphine in the mice treated with dasatinib on day 4 was significantly greater than that observed on the previous day (t test $p = 0.04$)(Figure 6.3E).

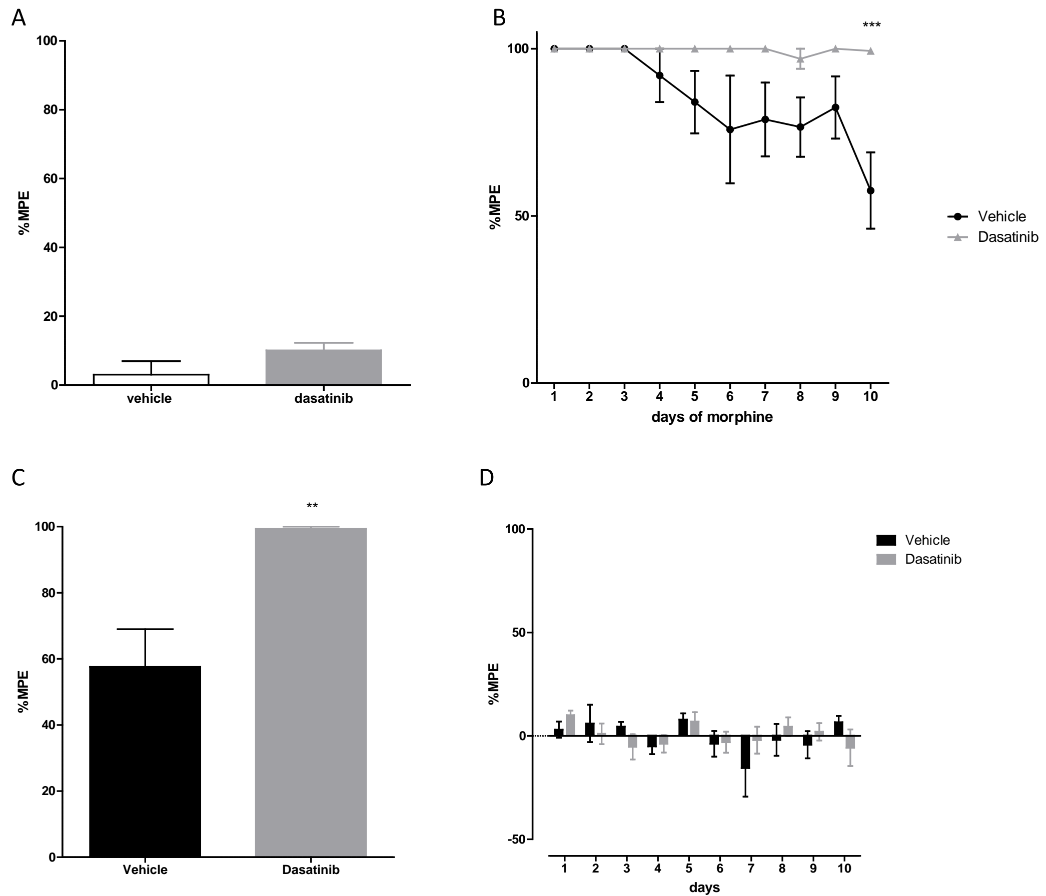


Figure 6.2: The c-Src inhibitor dasatinib inhibits the development of morphine tolerance in WT mice. (A) Dasatinib does not significantly alter baseline tail withdrawal when compared to vehicle injection on day 1, t test p = 0.14, n = 8 in each group. (B) Administration of dasatinib (5 mg/Kg) prior to the administration of morphine (10 mg/Kg) prevented the development of tolerance in WT mice, two way repeated measures ANOVA time p = 0.009, dasatinib administration p = 0.002, the *post hoc* Bonferroni results are shown on graph. (C) WT mice treated with dasatinib prior to morphine administration exhibited significantly more analgesia on day 10 compared to the vehicle treated mice, unpaired t test p = 0.003, n = 8 in each group. (D) Dasatinib administration did not produce persistent analgesia in WT mice over a 10 day administration period, two way repeated measures ANOVA, time p = 0.2, treatment p = 0.8. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.

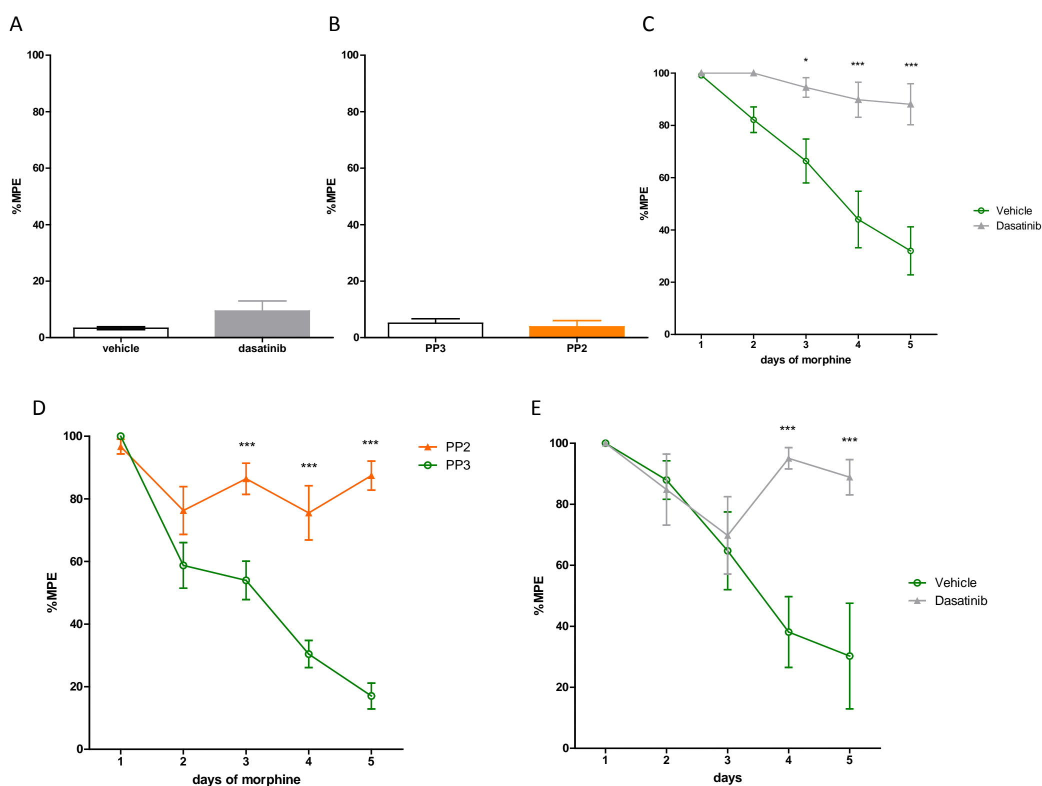


Figure 6.3: c-Src inhibition inhibits morphine tolerance in MOP+/- mice. (A) Dasatinib does not cause basal analgesia in MOP+/- mice. Tail withdrawal times (not shown) and MPE 30 minutes after either vehicle or dasatinib injection were not significantly different, t test p = 0.11 n = 8 in each group. (B)PP2 does not cause basal analgesia in MOP+/- mice. The tail withdrawal times (not shown) and MPE are not significantly different for the PP2 treated mice when compared to those that have received PP3, t test p = 0.64, n = 12 in each group. (C) Dasatinib (5 mg/kg) administered 30 minutes prior to morphine (10 mg/Kg) prevented the development of significant morphine tolerance in MOP+/- mice, two way repeated measures ANOVA, time p < 0.0001, dasatinib treatment p = 0.0005, *post hoc* Bonferroni results are shown on the graph, n = 8 in each group. (D) PP2 also inhibits the development of morphine tolerance. PP2 (5 mg/Kg) administered IP 30 minutes prior to subcutaneous morphine (10 mg/Kg) administration prevented the morphine tolerance observed in the PP3 (5 mg/Kg IP) treated mice, two way repeated measures ANOVA, time p < 0.0001, drug difference p < 0.0001, *post hoc* Bonferrroni results are shown on the graph. Each treatment group was n = 12, (5 female and 7 male). (E) Dasatinib reversed the development of tolerance in MOP+/- mice. 16 mice (5 male and 3 female in each group) were treated with morphine (10 mg/Kg) daily for three days, on days 4 and 5 one group received dasatinib (5 mg/Kg IP) 30 minutes prior to subcutaneous morphine administration and the other group received a vehicle injection at the equivalent time. The mice that received dasatinib exhibited a significantly greater morphine analgesia compared to the vehicle treated mice; two way repeated measures ANOVA revealed a significant difference between the two groups on days 4 and 5. The *post hoc* Bonferroni test results are shown on graph. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.

6.4 The effects of c-Src inhibition on locomotor activation by morphine

As described previously (Chapter 4, Figure 4.1), morphine (10 mg/Kg) administration causes locomotor activation and sensitisation in WT mice. Before investigating the effects of dasatinib on the locomotor activating effects of morphine we investigated whether administration of dasatinib alone produced any locomotor effects. The two chamber CPP apparatus, was used to establish locomotor responses (see Methods, Chapter 2), n = 8 for each experiment. Saline was administered in both compartments but one compartment was paired with a vehicle injection and the other dasatinib (5 mg/Kg) injection 30 minutes prior to the saline administration. There were no significant differences in distance travelled following vehicle or dasatinib administration (Figure 6.4A). There were also no significant differences in distance travelled or speed of travel over the three days of conditioning (Figure 6.4B). On day 1 of conditioning the saline alone treated mice travelled 16.2 ± 2.2 m with an average speed of 0.009 ± 0.001 m/s and the dasatinib and saline treated mice travelled 14.2 ± 1.9 m with an average speed of 0.008 ± 0.001 m/s during the 30 minute time period.

To determine whether administering an IP injection 30 minutes prior to conditioning with morphine affects the CPP protocol and to control for the other aspects of the IP injection a vehicle was administered 30 minutes prior to morphine (10 mg/Kg) administration.

On day 1 of conditioning these mice travelled 21.5 ± 1.2 m with an average speed of 0.012 ± 0.0007 m/s following saline administration and 57.1 ± 2.5 m with an average speed of 0.03 ± 0.001 m/s following vehicle and morphine (10 mg/Kg) administration (Figure 6.4E). WT mice administered a vehicle injection IP prior to morphine exhibited both locomotor activation following morphine 10 mg/Kg administration and also sensitisation to its affects (Figure 6.4F). However, the locomotor activation observed following morphine administration was significantly reduced compared to the WT mice that received morphine alone which travelled 81.1 ± 7.0 m following morphine administration (data not shown), suggesting that the administration of the vehicle injection affected the morphine-evoked locomotor activation.

Morphine (3 mg/Kg) administered 30 minutes after an IP injection of dasatinib (5 mg/Kg) to WT mice did not produce significant locomotor activation or sensitisation (Figures 6.4C and D). After saline injection the mice travelled 23.2 ± 2.2 m and after dasatinib and morphine injection they travelled 26.0 ± 1.5 m. This is not different to the effect observed when morphine (3 mg/Kg) is administered alone to WT mice (Chapter 4, Figure 4.1).

Dasatinib (5 mg/Kg) administered 30 minutes prior to morphine (10 mg/Kg) had the same effect as vehicle injection. On day 1 the distance travelled following saline injection was 18.5 ± 1.7 m with an average speed of 0.01 ± 0.0009 m/s and the distance travelled following dasatinib and morphine administration was 61.1

± 6.1 m with an average speed of 0.03 ± 0.003 m/s (Figure 6.4G). This was not significantly different to that observed in the vehicle and morphine treated mice. However, similar to vehicle treated mice the distance travelled was significantly reduced compared to the morphine alone group (data not shown).

By contrast to vehicle treated mice, which exhibited significant sensitisation to the locomotor effects of morphine (10 mg/ Kg; Figure 6.4F), mice that received dasatinib prior to the administration of morphine did not. The distance that they travelled on day 3 was not significantly increased compared to the distance travelled on day 1 of the conditioning phase (Figure 6.4H).

However, when day 3 conditioning distances are directly compared there were no significant differences in the distance travelled following administration of morphine 10 mg/Kg in the WT alone (118.6 ± 13.1 m), WT vehicle treated (88.5 ± 3.0 m) and WT dasatinib treated (87.9 ± 11.6 m), one way ANOVA p = 0.08 (data not shown). The difference observed between the vehicle treated and dasatinib treated mice may be due to the increased variability in the distance travelled on day 3 in the dasatinib treated mice compared to the vehicle treated mice. The range of distances travelled in the last 300 s of the morphine conditioning phase on day 3 were 18.1 to 24.7 m for the vehicle treated and 10.3 to 30.8 m for the dasatinib treated mice. Overall this suggests that the absence of significant sensitisation observed in the dasatinib treated mice is likely to increased variability rather than a systematic reduction caused by dasatinib.

On day 1 of conditioning WT mice treated with either vehicle or dasatinib alone do not exhibit locomotor activation (Figure 6.5). Furthermore, the mean distance travelled after morphine (10 mg/Kg) was similar in vehicle and dasatinib treated mice, suggesting that c-Src inhibition does not affect the locomotor activation produced by morphine (Figure 6.5).

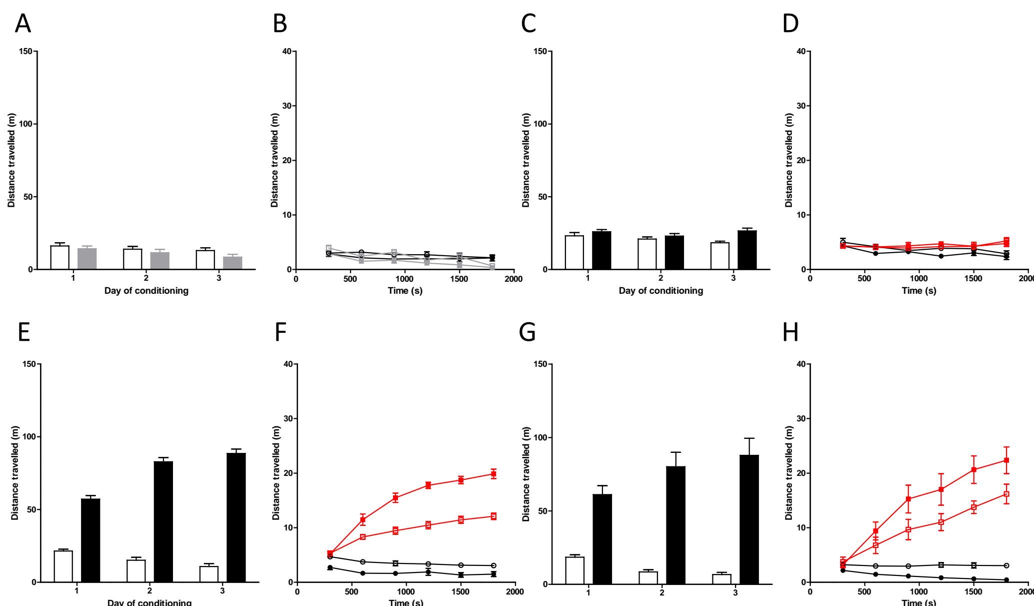


Figure 6.4: Dasatinib has no consistent effect on locomotor activation by morphine. (A) Dasatinib (5 mg/Kg IP) does not affect locomotion, two way repeated measures ANOVA ns, n = 8 in each group. White bars represent vehicle treatment, grey bars represent dasatinib treatment. (B) Dasatinib alone does not cause locomotor activation or sensitisation following repeated dosing. The distance travelled on day 3 is not greater than the distance travelled on day 1. The grey squares represent dasatinib and the black circles represent vehicle, open symbols are day 1 and closed symbols are day 3. (C) WT mice treated with dasatinib (5 mg/Kg) prior to conditioning with morphine (3 mg/Kg) do not exhibit significant locomotor activation, two way ANOVA ns, n = 8. (D) Mice treated with dasatinib prior to morphine (3 mg/Kg) do not exhibit locomotor sensitisation, two way ANOVA ns. (E) WT mice injected with vehicle 30 minutes prior to morphine (10 mg/Kg) conditioning exhibit locomotor activation, two way repeated measures ANOVA time $p < 0.0001$, n = 8 in each group. (F) WT mice sensitise to the locomotor effects of morphine when injected with vehicle prior to morphine conditioning, the distance travelled on day 3 following morphine injection was significantly further than on day 1. (G) Dasatinib pre-treatment did not affect locomotor activation with morphine. The WT mice exhibit significant locomotor activation, two way repeated measures ANOVA, morphine effect $p < 0.0001$. (H) WT mice treated with dasatinib prior to morphine do not exhibit significant sensitisation to morphine over the three days of conditioning, two way repeated measures ANOVA time $p = 0.2$ (ns). White bars represent saline administration and the black bars morphine administration, while on the line graphs red squares represent morphine administration and the black circles represent saline administration, open symbols are day 1 and the closed symbols are day 3.

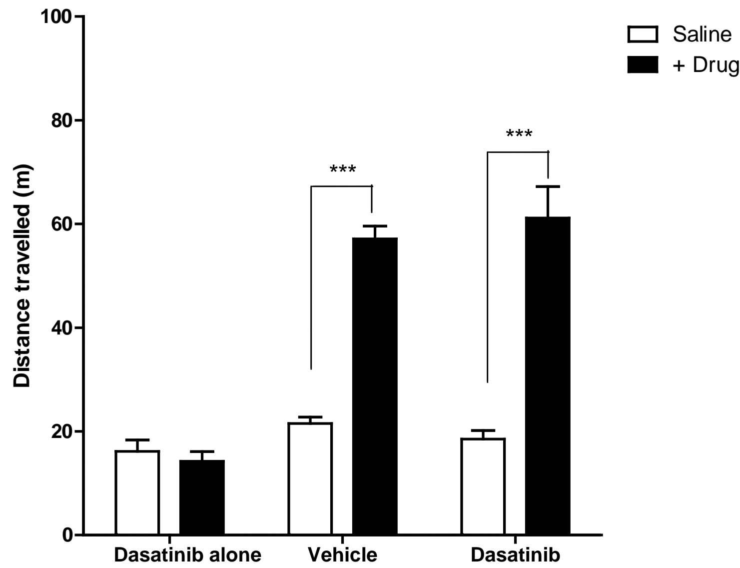


Figure 6.5: Dasatinib has no direct locomotor effect and does not differ from vehicle in its effect of locomotor stimulation by morphine. The distance travelled by the WT mice was not significantly different following saline treatment on day 1 of conditioning for the dasatinib alone or the mice treated with vehicle or dasatinib prior to morphine (10 mg/Kg), one way ANOVA ns ($p = 0.6$). Dasatinib administered alone did not produce significant locomotor activation. WT mice administered vehicle then morphine (10 mg/Kg) exhibit significant locomotor activation on day 1 of conditioning, WT mice treated dasatinib prior to morphine (10 mg/Kg) administration also exhibit significant locomotor activation, two way repeated measures ANOVA *post hoc* Bonferroni results are shown on the graph. Vertical lines represent ± SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

6.5 The effects of c-Src inhibition on conditioned place preference to morphine

Dasatinib (5 mg/Kg) administered alone is not rewarding or aversive to WT mice (Figure 6.6A). The mice spent 464.6 ± 302 s in the saline alone paired chamber and 435.4 ± 30.2 s in the dasatinib and saline paired chamber, t test p = 0.6. When WT mice were conditioned to saline or dasatinib (5 mg/Kg) and morphine (3 mg/Kg) they did not exhibit a significant preference for the morphine paired chamber (Figure 6.6B). The mice spent 411.6 ± 17.5 s in the saline paired chamber and 488.2 ± 17.6 s in the chamber paired with dasatinib and morphine (3 mg/Kg). there were no significant differences in the preference score for the morphine paired chamber between mice administered dasatinib prior to morphine and those that received morphine alone, t test p = 0.97. The preference score for the morphine (3 mg/Kg) paired chamber was 76.6 ± 35.1 s, this compares to 74.2 ± 63.1 s for WT mice administered morphine (3 mg/Kg) alone (Figure 6.6C).

The mice that received a vehicle injection prior to morphine (10 mg/Kg) during the conditioning phase exhibited a preference for the morphine paired chamber on test day (Figure 6.6D). The time spent in the saline paired chamber was 402.3 ± 20.4 s and the time spent in the vehicle and morphine paired chamber was 497.7 ± 20.4 s. There were no significant differences in chamber preference on habituation day (data not shown).

When WT mice received dasatinib (5 mg/Kg) prior to morphine (10 mg/Kg) during the conditioning phase of the experiment the mice demonstrated a preference for the morphine paired chamber on test day (Figure 6.6E). The time spent in the saline paired chamber was 375.2 ± 15.6 s and the time spent in the dasatinib and morphine paired chamber was 524.8 ± 15.6 s. There was no significant difference in preference score for the morphine (10 mg/Kg) between the vehicle treated mice and the dasatinib treated mice (Figure 6.6F). The vehicle treated mice had a preference score of 95.5 ± 40.8 s and the dasatinib treated mice had a preference score of 149.7 ± 31.2 s.

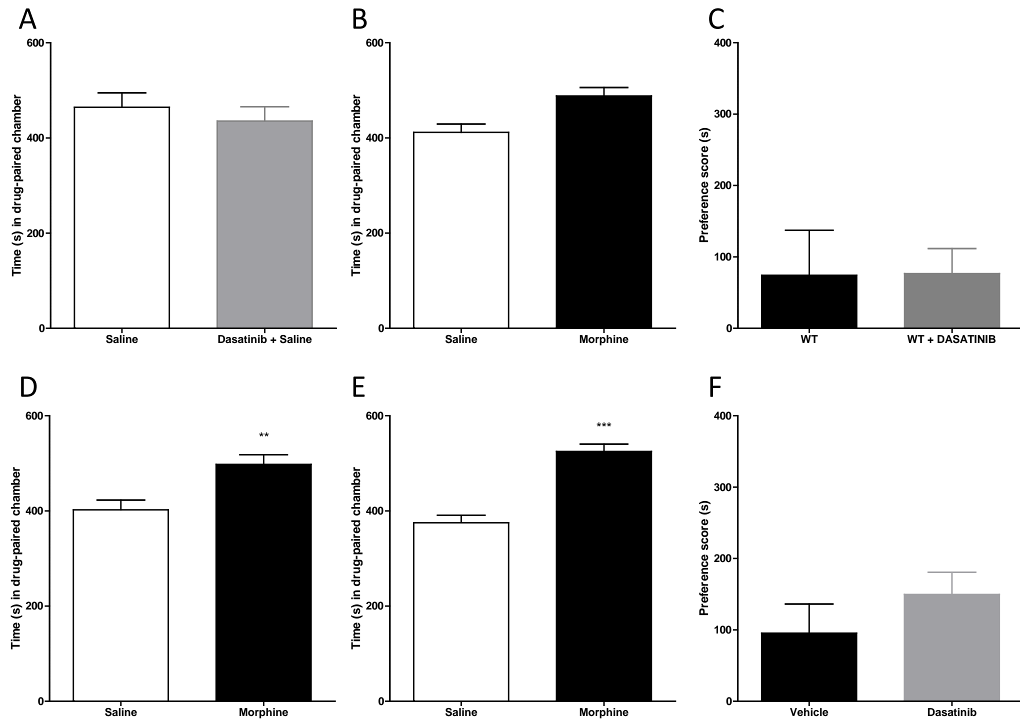


Figure 6.6: Dasatinib does not affect morphine conditioned place preference. (A) Saline both sessions, but 30 minutes prior to second session all mice received dasatinib (5 mg/kg IP). Mice did not show a preference for, or aversion to, dasatinib alone, t test ns p = 0.6, n = 8. (B) WT mice treated with dasatinib prior to morphine (3 mg/Kg) did not exhibit a significant preference for the morphine paired chamber on test day, t test p = 0.07 n = 8. (C) The preference score for morphine (3 mg/Kg) was not significantly different between WT mice treated with morphine alone and those that received dasatinib prior to morphine , t test p = 0.97 n = 8. (D) WT mice that received vehicle treatment 30 minutes prior to morphine (10 mg/Kg) conditioning exhibited a significant morphine preference, t test p = 0.005. (E) WT mice administered dasatinib (5 mg/Kg) 30 minutes prior to the morphine (10 mg/Kg) conditioning session exhibited a preference for the morphine paired chamber, t test p < 0.0001. (F) There was no significant difference in preference score for the morphine paired chamber between vehicle treated and dasatinib treated mice subsequently conditioned with morphine (10 mg/Kg), t test p = 0.3. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.

6.6 The effects of c-Src inhibition on sIPSC frequency in the VTA

PP2 mimics the effects of the absence of BAR2 in DRG neurones by increasing the constitutive inhibitory coupling of MOP receptors to voltage-activated Ca^{2+} channels (Walwyn *et al*., 2007). We investigated whether inhibition of c-Src affects neurones within the VTA. PP2 (10 µM) alone had no effect on the sIPSC frequency of WT neurones (Figure 6.7B), the control sIPSC frequency was 1.6 ± 0.3 Hz and the sIPSC frequency following PP2 (10 µM) exposure was 1.4 ± 0.2 Hz, n = 6. PP3, the inactive chemical analogue of PP2, also had no effect on sIPSC frequency of WT neurones within the VTA when applied alone (Figure 6.7C), control sIPSC frequency was 2.1 ± 1.3 Hz and the sIPSC frequency following PP3 (10 µM) exposure was 1.8 ± 1.0 Hz, n = 5. Exemplar recordings from WT neurones of the VTA exposed to both PP2 and morphine are provided in Figure 6.7A.

As described in Chapter 5, morphine inhibits sIPSC frequency in VTA neurones (Figure 5.6) and this effect was diminished in BAR2-/- mice (Figure 5.12). PP2 and PP3 were applied with morphine to determine whether c-Src plays a role in the inhibition of sIPSCs by morphine. There was a significant reduction in the inhibition of sIPSC frequency in VTA neurones that received morphine (10 µM) in the presence of PP2 (10 µM) (25.5 ± 3.1%) compared to those that received morphine in the presence of PP3 (10 µM) (52.2 ± 4.5%). In this respect neurones exposed to PP2 resembled BAR2-/- neurones, which exhibited a 23.3 ± 4.4% inhibition of sIPSC frequency by morphine (10 µM).

The inhibition of sIPSC frequency produced by exposure of the neurones to morphine was not significantly different in the presence or absence of PP3 (Figure 6.7D). These results, together with the effects of the BAR2 knockout, suggest that BAR2 and c-Src are important in the response of VTA neurones to morphine.

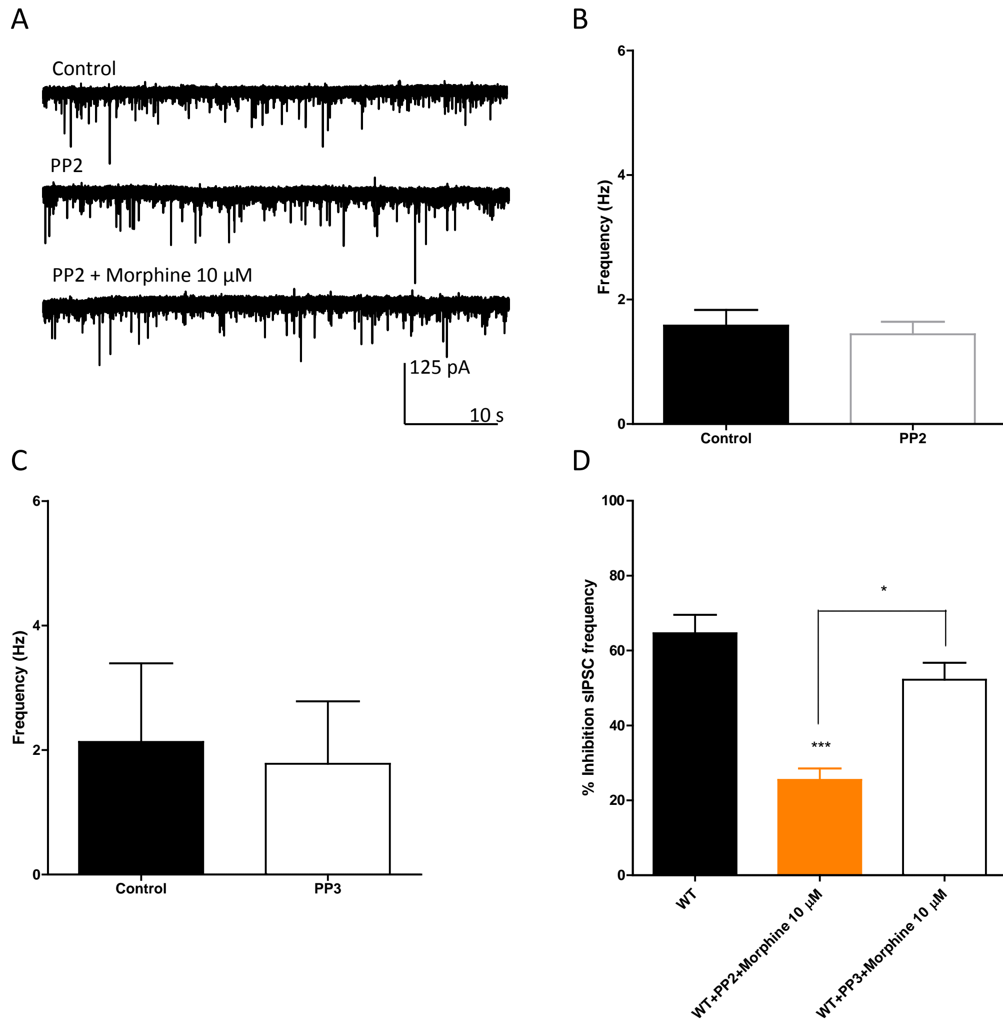


Figure 6.7: PP2 significantly reduces the inhibition of sIPSC frequency in VTA neurones by morphine. (A) This is an exemplar recording from a WT neurone in the presence of PP2 (10 µM) and PP2 plus morphine (10 µM). (B) PP2 (10 µM) alone does not significantly affect sIPSC frequency, t test ns $p = 0.2$, $n = 6$. (C) PP3 (10 µM) alone does not significantly alter sIPSC frequency within the VTA, t test ns $p = 0.3$, $n = 5$. (D)The ability of morphine 10 µM to inhibit sIPSC frequency is significantly reduced in the presence of PP2 10 µM and unchanged in the presence of PP3 10 µM. One way ANOVA $p < 0.0001$, the *post hoc* Tukey results are shown on the graph. WT n = 10, WT + PP2 + morphine n = 5, WT + PP3 + morphine n = 5. Vertical lines represent ± SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

6.7 The effects of MEK inhibition on sIPSC frequency within the VTA

BAR2-/- mice exhibit a reduced locomotor activation compared to WT mice following the administration of morphine (10 mg/Kg). As previously discussed the dopamine dependent component of locomotor activation is thought to be mediated via a dopamine D1 receptor mechanism. Activation of this pathway recruits the BAR2/MAPK complex. Phosphorylation and activation of MAPK requires the activity of MEK. Systemic administration of the MEK inhibitor, SL327, to WT mice inhibits locomotion in a dose dependent manner (Urs *et al*., 2011). The effect of SL327 on morphine induced locomotor activation in BAR2-/- mice has not yet been investigated. SL327 has been shown to inhibit phosphorylation of ERK in the striatal brain regions of the mouse (Beaulieu *et al*., 2006). This suggests that the BAR2-MEK-ERK pathway is important in the locomotor activation by morphine. It is possible that the MEK-ERK pathway is also involved in the inhibition of sIPSC frequency by morphine. To test this, WT neurones within the VTA were treated with the MEK inhibitor, SL327 (Figure 6.8). Control sIPSC frequency was 3.0 ± 1.6 Hz in the absence and 3.0 ± 1.7 Hz in the presence of SL327 (1 µM). Morphine (10 µM) significantly inhibited sIPSC frequency in the presence of SL327 (1 µM) (51.8 ± 7.4%) (Figure 6.8C). This was not significantly different to the inhibition of sIPSC frequency observed in the WT neurones exposed to morphine 10 µM alone (64.6 ± 4.9%). In contrast, the presence of PP2 significantly reduces the ability of morphine to inhibit sIPSC frequency.

The inhibition of sIPSC frequency observed in the presence of PP2 was not significantly different to that recorded in the BAR2-/- neurones. These data suggest that the MEK-ERK pathway does not contribute to the inhibitory effect of morphine on sIPSC frequency in VTA neurones but that c-Src is involved in this process.

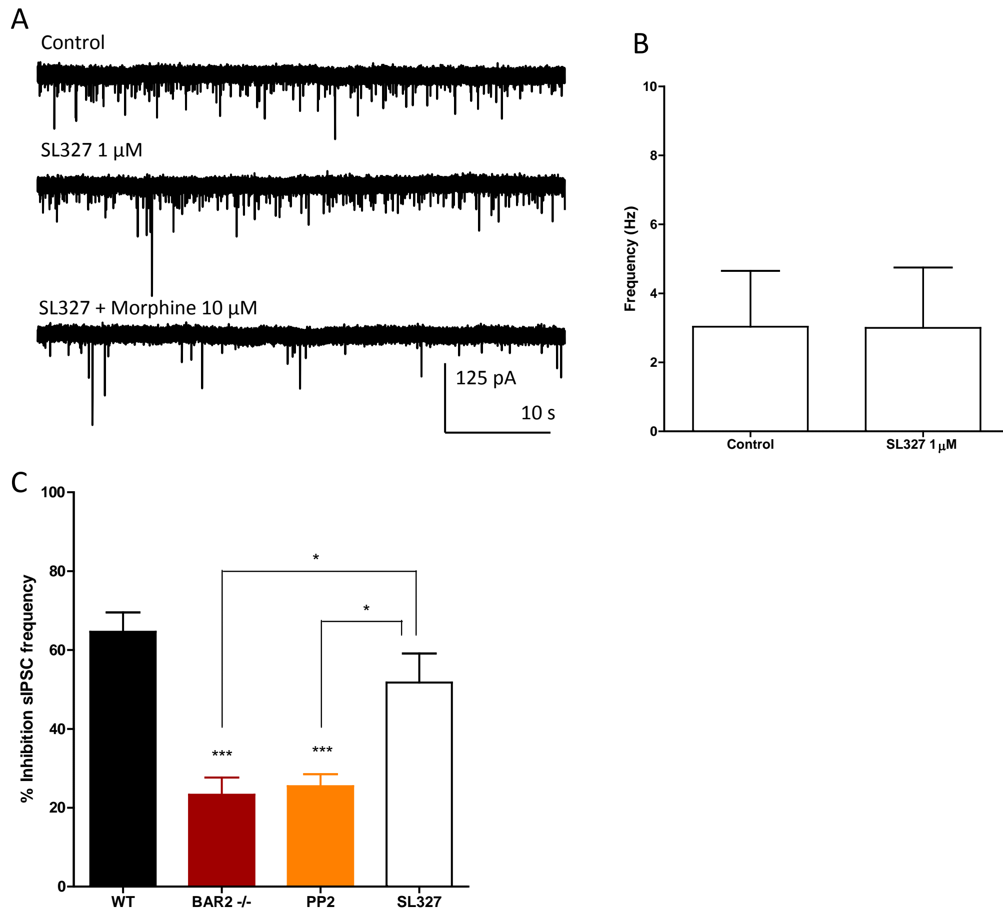


Figure 6.8: MEK inhibition by SL327 has no significant effect on morphine inhibition of sIPSC frequency within the VTA. (A) Example of a recording from a neurone exposed to SL327 (1µM) and morphine (10µM). (B) SL 327 (1 µM) alone had no significant effect on sIPSC frequency, t test, p = 0.8, n = 6. C) SL327 (1 µM) has no significant effect on the ability of morphine (10 µM) to inhibit sIPSC frequency in WT neurones within the VTA, t test p = 0.15, WT n = 10, WT + SL327 n = 6. This is significantly different to the reduction in sIPSC inhibition observed in response to morphine (10 µM) exposure in the BAR2-/- neurones and when PP2 is present, one way ANOVA p <0.0001 the *post hoc* Tukey results are shown on the graph. Vertical lines represent ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.

6.8 Summary

Dasatinib, administered prior to morphine, prevents the development of significant morphine tolerance in both WT and MOP+/- mice. It does not significantly alter basal tail withdrawal latency, this is interesting because BAR2-/- mice exhibit prolonged basal tail withdrawal latencies and this suggests that another signalling pathway may be responsible for this aspect of behaviour. PP2 also inhibits the development of morphine analgesic tolerance; this inhibition is not significantly different from that observed following the administration of dasatinib. Dasatinib also reversed the analgesic tolerance that had already developed to morphine in the MOP+/- mice. The observed reversal and return to almost full analgesia suggests that these effects can be rapidly reversed and implicated c-Src in these processes.

Dasatinib had no psychomotor effects when administered alone. Mice administered either vehicle or dasatinib injections prior to morphine exhibited a reduced locomotor response. This may be due to an effect of the components of the vehicle injection (DMSO / Kolliphor EL) on locomotor activity or a behavioural effect due to a further period of restraint and injection. Further experiments will be required to determine whether either of these play a role in locomotor activation following morphine administration. Our data suggested that dasatinib may inhibit sensitisation to the locomotor effects of morphine, however, on closer inspection the average distances travelled by WT alone, vehicle treated and dasatinib treated mice administered morphine are not significantly different. The reason that there appears to be a difference is due to

an increase in the variability of response in the dasatinib treated mice, which is not present in the vehicle treated mice or mice treated with morphine alone. To explore this effect further more experiments will be required to diminish the variance in the dasatinib data and to investigate whether dasatinib is affecting locomotor sensitisation.

Inhibition of c-Src does not produce reinforcement or aversion in the absence of morphine. Furthermore, when mice were treated with either vehicle or dasatinib prior to morphine during the conditioning phase of CPP there was no significant differences in the preference for the morphine paired chamber on test day. Dasatinib does not appear to affect morphine reinforcement. This contrasts with the differences that we have observed in the BAR2-/- mice, which exhibit an increased sensitivity to morphine reinforcement compared to the WT mice. BAR2-/- mice exhibited a significant preference for morphine following conditioning with morphine at both 3 and 10 mg/Kg. This was not the case for WT mice or for WT mice administered dasatinib prior to morphine. In these mice reinforcement was only exhibited following conditioning with morphine (10 mg/Kg) not the lower dose. This suggests that c-Src is not implicated in this process of sensitisation to the reinforcing effects of morphine.

The c-Src inhibitor, PP2, reduced the ability of morphine to inhibit sIPSC frequency in VTA neurones. The diminished sIPSC inhibition by morphine in the presence of PP2 was similar to that observed in recordings from BAR2-/-

neurones. By contrast, PP3, an inactive chemical analogue of PP2, did not significantly alter the ability of morphine to inhibit sIPSC frequency compared to the WT neurones exposed to morphine alone. This suggests that BAR2 and c-Src are important components of the signalling pathway within the VTA in response to morphine.

Systemic administration of the MEK inhibitor SL 327 has been demonstrated to inhibit locomotion in a dose dependent manner that involves D1 dopamine receptors in WT mice, the effect in BAR2-/- mice has not been investigated. By contrast, MEK inhibition was without effect on morphine evoked CPP (Urs *et al*., 2011). We investigated whether inhibition of the MEK/ERK pathway using SL327 would affect the ability of morphine to inhibit sIPSC frequency within the VTA. There were no significant differences in the ability of morphine to inhibit sIPSC frequency in WT VTA neurones treated with morphine alone or WT VTA neurones treated with SL327 and morphine. This suggests that the inhibition by morphine of vesicular release of GABA from presynaptic GABAergic neurones in the VTA does not involve the BAR2-MEK-ERK signalling pathway.

Chapter 7: Discussion and Conclusions

7.1 Discussion

This study in mice confirms that morphine causes analgesia, analgesic tolerance, and has psychomotor effects leading to enhanced locomotion and conditioned reinforcement. In VTA neurones morphine and the selective MOP receptor agonist DAMGO caused concentration-dependent inhibition of the frequency of IPSCs. All these actions of morphine were absent from MOP-/- mice that lack MOP receptors. The absence of 50% of MOP receptors (in MOP+/- mice) reduced the potency of morphine in all of these actions, with no evidence of reduced efficacy, and greatly accelerated the development of analgesic tolerance. Even after the development of profound tolerance the dose-response relationship in MOP+/- mice for analgesia suggests that morphine maintains its efficacy. Taken together these observations suggest that there is a remarkable surplus of MOP receptors.

A lack of DOP receptors resulted in a significant decrease in morphine analgesia and in the development of analgesic tolerance compared to the WT mice. However, these mice still displayed significant tolerance to the analgesic effects of morphine. DOP receptors are not required for the locomotor activation produced by morphine, but they do contribute to this effect as demonstrated by the increased locomotor activation produced in DOP-/- mice by a low dose of morphine when compared to WT mice. They are not involved in the development of sensitisation to the locomotor activating effects of morphine and are not required for the development of the reinforcing effect of morphine as the absence of DOP receptors did not alter morphine CPP. Interestingly, DOP

receptors are required for the full inhibitory effect of morphine on sIPSC frequency within the VTA. This is not the case for DAMGO, as its inhibition of sIPSC frequency in the DOP-/- VTA neurones was not different to that observed in the WT neurones. There was no difference in the effects on morphine function of a lack of a single or both copies of the *Oprd1* gene (i.e. DOP+/- and DOP-/- mice, respectively). This suggests that in contrast to MOP receptors there is not a large receptor reserve as the loss of 50% of the receptors in the DOP+/- mouse creates the full phenotype seen in the KO animals.

A lack of BAR2 does not significantly affect morphine analgesia, but these mice display basal analgesia. They also exhibit significantly reduced tolerance to morphine compared to the WT mice, although the analgesia that they receive from morphine is itself significantly reduced by day 10 of the tolerance protocol. This demonstrates that the absence of BAR2 alone does not completely abolish morphine analgesic tolerance and that other signalling mechanisms may be involved. BAR2 is also involved in the locomotor activating effects of morphine as BAR2-/- mice exhibit significantly reduced locomotor activation after the administration of morphine when compared to WT mice. They do still sensitise to this effect over the conditioning period suggesting that BAR2 is not involved in the process of sensitisation. The BAR2-/- mice exhibit an increased sensitivity to the rewarding aspects of morphine as they have an increased preference for the morphine paired chamber at lower doses of drug when compared to WT mice. However, unexpectedly we found that the ability of morphine to inhibit sIPSC frequency within the VTA of BAR2-/- mice was significantly decreased. This was

also the case when we used DAMGO, suggesting that BAR2 is involved in the response to both morphine and DAMGO within the VTA.

In most cases the combined lack of DOP receptors and BAR2 had no additional phenotypic effects. The double KO mice replicated the effects seen in mice lacking either DOP receptors or BAR2. The exception to this was for analgesic tolerance. Mice lacking both genes exhibited a complete absence of tolerance, which while negligible, was still detectable in DOP-/- and BAR2-/- mice.

Similar to the effect of the absence of DOP receptors and/or BAR2, inhibition of c-Src activity by dasatinib or PP2 also reduced morphine analgesic tolerance. Furthermore, similar to VTA neurones from BAR2-/- mice, WT VTA neurones treated with PP2 exhibited reduced morphine inhibition of sIPSC frequency. Dasatinib alone was neither rewarding nor aversive to WT mice and IP administration did not significantly alter the psychomotor effects (reinforcement or locomotion) of morphine.

In contrast to our original hypothesis that DOP receptors are absent from the VTA leading to a lack of MOP/DOP oligomers, the evidence suggests that DOP receptors are present and functional in this brain region. They appear to be involved in the locomotor activating effects of morphine and their absence results in an increased sensitivity to this effect. They do not appear to be involved in the development of morphine preference although their presence is

required for the full inhibitory effect of morphine on sIPSC frequency within the VTA. BAR2 also appears to be involved in the rewarding effects of morphine. Its absence results in an increase in morphine preference and a decrease in the ability of morphine to produce locomotor activation. It, like DOP receptors, is required for the full inhibitory effect of morphine on sIPSC frequency within the VTA. Inhibition of c-Src resulted in a reproduction of this effect implicating both BAR2 and c-Src in VTA signalling in response to morphine. This raises a number of questions about the role of BAR2 and c-Src in the VTA. It would be very interesting to investigate whether c-Src inhibition has an effect on sIPSC frequency and the ability of morphine to inhibit these events in BAR2-/- slices. It will also be important to determine whether inhibitors of c-Src administered via the IP route inhibit the activity of c-Src within the VTA. The lack of psychomotor effects of systemically administered dasatinib might simply reflect a failure to reach a concentration in the VTA sufficient to inhibit c-Src activation.

In the pain pathway it appears that both DOP receptors and BAR2 are involved in the development of morphine analgesic tolerance. The absence of either component reduced tolerance but when both were removed in the BAR2-/-//DOP-/- mice then tolerance to the analgesic effect of morphine was abolished. Inhibition of c-Src also significantly reduced the development of tolerance but did not produce basal analgesia. This suggests that a reduction in morphine tolerance can be produced without altering basal MOP receptor signalling. These findings are summarised in Figure 7.1.

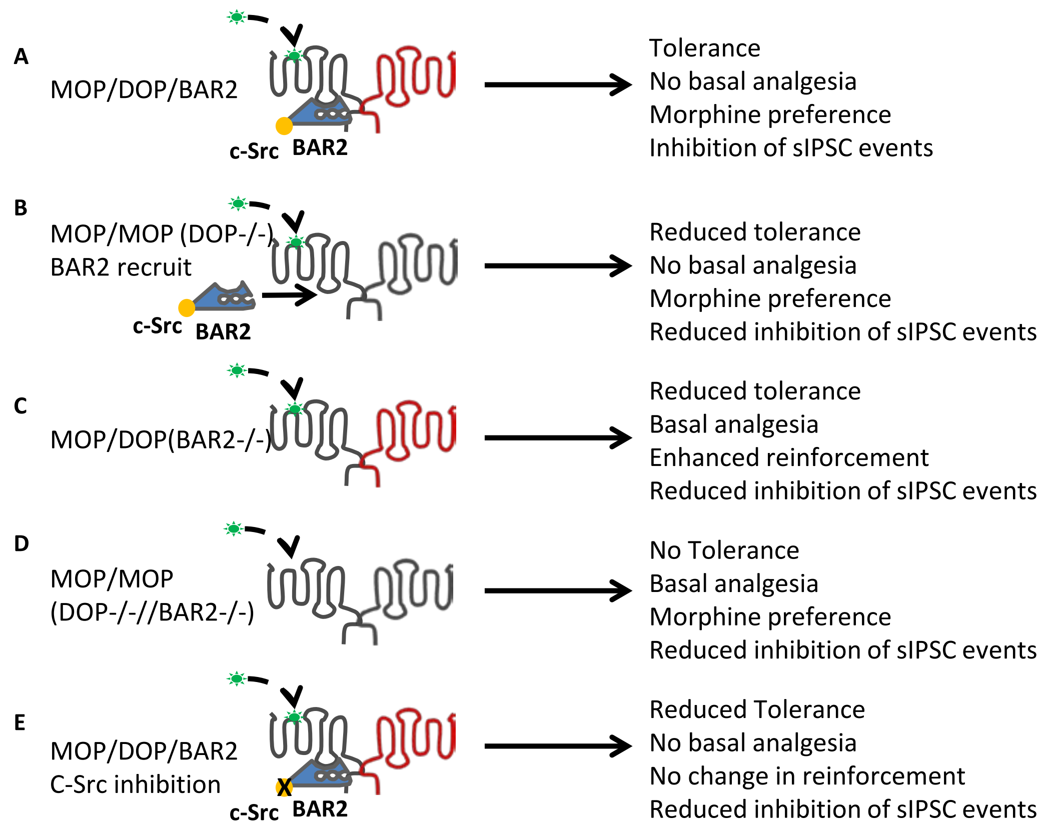


Figure 7.1: The role of MOP and DOP receptors, BAR2 and c-Src in opioid receptor signalling in the pain and reward pathway. In the diagrams MOP and DOP receptors are depicted as physically interacting in the form of dimers. This is based on reports of heterodimerisation and the recent crystal structure of MOP receptors which reveals the existence of dimers (Manglik *et al.*, 2012). (A) When expressed recombinantly MOP receptors, DOP receptors and BAR2 colocalise (Rozenfeld and Devi, 2007). This is depicted as the hypothetical scenario in WT neurones. The absence of DOP receptors (B), BAR2 (C), both DOP receptors and BAR2 (D), or the inhibition of c-Src (E) have the consequences summarised in each case. In each case the scenario is represented schematically.

7.2 Analgesia and Tolerance

7.2.1 MOP receptors in analgesia and tolerance

Work with MOP-/- mice confirms previously published data that MOP receptors are required for the analgesic effect of morphine (Matthes *et al*., 1996, Sora *et al*., 1997, Tian *et al*., 1997, Schuller *et al*., 1999, Becker *et al*., 2000). MOP-/- neurones in slices of VTA and from DRGs harvested from these mice do not respond to drugs that act as selective agonists at MOP receptors. Small DRG neurones are largely primary afferent nociceptors and a loss of opioid inhibition of VACCs here is likely to contribute to the absence of morphine analgesia. The VTA has also been implicated in the analgesic activity of opioids (Niikura *et al*., 2010, Schifirnet *et al*., 2014) and a lack of opioid inhibition of sIPSCs recorded from dopaminergic neurones is also likely to contribute to the analgesic deficit. Dissection of the contribution of these two neuronal populations to the analgesic actions of morphine will require conditional MOP knockouts and/or neurone specific reintroduction of MOP receptors into MOP-/- mice.

We could not identify any alteration in baseline tail withdrawal times in MOP-/- mice implying that basal sensitivity to noxious heat is not affected by tonic MOP receptor mediated activity in the mouse. This is consistent with previously published work (Matthes *et al*., 1996). We did however identify differences in the baseline tail withdrawal latency between male and female WT mice, indicating a higher sensitivity of female mice to noxious heat. A gender difference in baseline sensitivity to noxious heat of C57Bl/6J mice has been reported previously (Kest *et al*., 1999) and other investigators using much larger

cohorts of mice have demonstrated gender differences in morphine analgesia with male rodents receiving greater analgesia from morphine (Cicero *et al*., 1996) and the development of tolerance with increased tolerance to the morphine recorded in female mice (Kest *et al*., 2000, Mogil and Chanda, 2005). Additional experiments using MOP-/- mice will be required to determine whether this difference relates to a sex difference in the contribution of MOP receptors to thermal nociception.

Mice lacking one copy of the *Oprm1* gene, MOP+/- mice, have 50% less MOP receptors than WT mice (Sora *et al*., 2001). The analgesic potency of morphine was reduced in these mice as observed by a rightward shift in the dose response curve. This occurred without any significant change in the efficacy of morphine. They also exhibit a very rapid onset of tolerance to the analgesic effects of morphine. This is significantly different from the development of tolerance in WT mice. Interestingly, there appears to be no loss of analgesic efficacy in MOP+/- mice, even after 5 days of morphine daily exposure, a time point at which these mice exhibit profound morphine analgesic tolerance. This suggests that MOP receptor number in WT animals massively exceeds that required for maximum analgesia. This has been described previously as spare receptors and receptor reserve (Chavkin and Goldstein, 1984). A number of investigators have examined MOP receptor reserve and attempted to identify changes following opioid exposure. In cell and tissue models there are demonstrable effects of decreasing MOP receptor reserve (Chavkin and Goldstein, 1984, Adams *et al*., 1990). A previous study using MOP+/- mice revealed a decreased locomotor activation,

decreased self-administration and an increased lethal dose of morphine in these mice (Sora *et al*., 2001). MOP+/- mice in this study also developed morphine analgesic tolerance following repeated dosing, which appears to be greater than in WT mice but was not directly compared (Sora *et al*., 2001). They did not comment on the pattern of development of tolerance in the differing genotypes. The mice that were used for this study were also a different genetic background as they were maintained on a C57/129 base. The rapid development of morphine analgesic tolerance observed in the MOP+/- mice reveals the importance of MOP receptor number in this process. A recently published study using a genetically modified mouse that expresses humanised MOP receptors with the A118G mutation, which decreases receptor expression, demonstrates the importance of receptor reserve in the response to morphine (Robinson *et al*., 2015).

Morphine is a low efficacy partial agonist at MOP receptors but causes tolerance whereas fentanyl is a high efficacy full agonist at the receptor that appears to cause little tolerance in comparison to morphine (Duttaroy and Yoburn, 1995). This has led to the concept that receptor internalisation/ desensitisation and endocytosis are important in this process (Alvarez *et al*., 2002, Koch *et al*., 2005, Johnson *et al*., 2006). Consistent with this is the demonstration that morphine is poor at initiating receptor internalisation compared to other MOP agonists such as DAMGO and methadone (Keith *et al*., 1996, Keith *et al*., 1998, Koch *et al*., 2005, Walwyn *et al*., 2006). Initial studies suggested that the degree of internalisation produced by agonists at MOP receptors correlated with their

ability to cause endocytosis (Alvarez *et al*., 2002). However subsequent work suggests that receptor internalisation is inversely correlated with desensitisation leading to the RAVE hypothesis (Whistler *et al*., 1999, Finn and Whistler, 2001). In this hypothesis the ratio of receptor activation versus endocytosis is considered to be the key determinant of desensitisation/tolerance. Endocytosis is considered to be a requirement for receptor resensitisation. This is further complicated by the fact that morphine will internalise MOP receptors in striatal and DRG neurones (Haberstock-Debic *et al*., 2005, Walwyn *et al*., 2006). This suggests that the internalisation of receptors produced by morphine differs across cell types and locations. It would be very interesting to look at DRG neurones from these mice stained for MOP receptors and investigate the effects of exposure to morphine and the differences from WT cells. It would also be informative to establish the level of receptor endocytosis/recycling in these neurones.

7.2.2 DOP receptors in analgesia and tolerance

DOP-/- mice exhibit slightly reduced analgesic potency of morphine, with an increased ED_{50}. This corresponds with previously published data (Zhu *et al*., 1999) and suggests that both MOP and DOP receptors are involved in the antinociceptive effects of morphine. While MOP receptors must be present for any analgesic effect it appears that DOP receptors also play a role. This is possibly in the binding of morphine to the receptor as the slope of the analgesia dose response curve is altered in DOP-/- mice and suggests a reduced

cooperativity of agonist binding. DOP-/- mice also exhibit reduced morphine tolerance confirming previous reports of this effect (Zhu *et al*., 1999). These mice do not exhibit any alteration in baseline tail withdrawal time compared to the WT mice.

There is evidence for opioid receptor dimerisation, this was first evident for DOP receptors in recombinant models (Cvejic and Devi, 1997, Jordan and Devi, 1999) and subsequently for MOP receptors with DOP receptors (George *et al*., 2000) and also with other GPCRs (Fujita *et al*., 2014). The potential for the opioid receptors to dimerise has been supported by the publication of the structural models for the receptors, in particular that of MOP receptors (Manglik *et al*., 2012). From further work it is known that DOP receptors are required for the complete inhibitory response on VACCs in DRGs by morphine (Walwyn *et al*., 2009). Disruption of the MOP/DOP association results in decreased analgesic tolerance (He *et al*., 2011) and the exposure of DRGs and brainstem neurones to chronic morphine results in an increase in MOP/DOP receptor antibody staining (Gupta *et al*., 2010, Costantino *et al*., 2012). Interestingly the presence of DOP receptors appears to increase the ability of morphine to induce internalisation (Whistler *et al*., 1999, Haberstock-Debic *et al*., 2003, Walwyn *et al*., 2006). This suggests that morphine would produce a greater degree of tolerance when only MOP receptor homomers are present when compared to a mixed population including MOP/DOP heteromers. This occurs as these heteromers exhibit an increased degree of internalisation with morphine (Walwyn *et al*., 2006, Finn and Whistler, 2001). Although we observe decreased morphine tolerance in DOP-/-

mice and the presence of MOP/DOP heteromers in the cell membrane is increased after chronic morphine treatment (Gupta *et al*., 2010, Costantino *et al*., 2012), this is thought to reduce the analgesic effects of opioid drugs (Milan-Lobo *et al*., 2013). This is contradictory and requires further investigation, it is also interesting that the compensatory increases in several second messenger systems associated with the detrimental long term adaptations that occur with chronic opioid exposure, for example increases in cAMP and MAPK kinase signalling, occur to a greater extent with agents that produce more internalisation (Koch *et al*., 2005, Walwyn *et al*., 2006). Dual labelling for DOP and MOP receptors would allow the study of whether decreased receptor number, (as in the MOP+/- mice), affects the ability of these two opioid receptors to associate and interact at the cell membrane. MOP/DOP heterodimers are also involved in the recruitment of BAR2 to the cell membrane (Rozenfeld and Devi, 2007).

7.2.3 The role of BAR2 in analgesia and tolerance

Morphine causes dose-dependent analgesia in BAR2-/- mice that is not significantly different to that observed in WT mice. BAR2-/- mice exhibit basal analgesia and decreased morphine tolerance after 10 days of once daily morphine administration. These effects had been previously reported (Bohn *et al*., 1999, Bohn *et al*., 2000, Bohn *et al*., 2002, Lam *et al*., 2011). DOP receptors exhibit constitutive activity (Costa and Herz, 1989). This raises the possibility that DOP receptors in the putative MOP/DOP heterodimers of the pain pathway may

drive constitutive activity when BAR2 is absent. This possibility was tested using the double KO mouse model (BAR2-/-//DOP-/-). These mice exhibit basal analgesia that is not significantly different to that observed in the single BAR2-/- mice. So DOP receptors are not required for constitutive signalling seen in the absence of BAR2. It would be interesting to investigate the effects of combining the BAR2-/- mouse model with the MOP+/- mouse line to create a BAR2-/-//MOP+/- mouse model. As we know that MOP receptor number is reduced in the MOP+/- mice this would assist in the elucidation of the importance of receptor number and spare receptors in the activation of constitutive signalling involving MOP receptors. It would also be interesting to see if a lack of BAR2 is able to inhibit the profound analgesic tolerance observed in MOP+/- mice.

7.2.4 The effects of c-Src inhibition within the pain pathway

We used two tyrosine kinase inhibitors to investigate the role of c-Src in opioid receptor signalling and the development of the side effects associated with the use of opioid drugs. PP2 is a selective inhibitor of c-Src that has been used predominately *in vitro* (Bain *et al*., 2007, Uitdehaag *et al*., 2012). It is unclear whether PP2 administered systemically crosses the BBB, however the benefit of using this drug is that it has an inactive analogue, PP3, that can be used as a matched control. The second drug that we have used is dasatinib, this a clinically licensed drug that is used for the treatment of leukaemia. It has been extensively tested *in vivo* and does cross the blood brain barrier (BBB) (Porkka *et al*., 2008, Lagas *et al*., 2009). However, dasatinib is not a selective inhibitor of c-Src and

also affects a number of other tyrosine kinases and their receptors including PDGFRβ. This is important as PDGFRβ has been implicated in the development of morphine tolerance (Wang *et al.*, 2012).

Dasatinib prevented the development of tolerance in WT mice. Furthermore, dasatinib, PP2 (but not PP3) inhibited the development of morphine tolerance in MOP+/- mice, which are a good model to study tolerance development as they are significantly tolerant to the analgesic effects of morphine after 5 days of treatment. Importantly, dasatinib also reversed morphine tolerance in MOP+/- mice. This is very exciting as it suggests that tolerance is not an irreversible process only recoverable on stopping morphine, but instead tyrosine kinase related signalling is involved in its maintenance. In the study from Wang *et al* (2012) they were unable to reverse the development of existing tolerance though the administration of imatinib although if this drug was administered prior to morphine it prevented the development of tolerance. Suggesting that there may be different processes involved and that dasatinib may be more effective as an adjunct approach to mitigating tolerance. It would be very interesting to know if PP2 is also able to reverse tolerance as this would confirm the involvement of c-Src in this process. An important question that remains is whether there are differences in the level of phosphorylated c-Src between BAR2-/- neurones and WT neurones. It is currently unclear whether the activation of c-Src by morphine requires MOP receptors, DOP receptors and/or BAR2 as implied by the hypotheses depicted in Figure 7.1. This can now be

addressed by assaying phospho-Src (the activated form of c-Src) in WT, MOP-/-, DOP-/- and BAR2-/- mice following morphine treatment.

7.2.5 Summary analgesia and tolerance

DOP receptors and BAR2 are required for the normal development of tolerance to the analgesic effects of morphine. This is in spite of the fact that selective DOP receptor agonists have only limited analgesic efficacy in acute pain models *in vivo* (Gavériaux-Ruff *et al*., 2008, Gaveriaux-Ruff and Kieffer, 2011, Gendron *et al*., 2015, Peppin and Raffa, 2015). Some of the selective DOP agonists that have been developed, for example SNC-80, have demonstrated proconvulsant effects limiting their use (Chu Sin Chung and Kieffer, 2013). Upregulation of DOP receptors within the cell membrane of DRG neurones can significantly increase the inhibitory effect of selective DOP receptor agonists on VACCs (Walwyn *et al*., 2005). In models of chronic pain including neuropathic, inflammatory and bone cancer pain models, selective agonists at DOP receptors produce antinociceptive effects (Contet *et al*., 2006, Nadal *et al*., 2006, Gavériaux-Ruff *et al*., 2008). Knockout of DOP receptors produce a significant increase in neuropathic and inflammatory pain and abolishes the antinociceptive effect of DOP selective agonists, demonstrating their importance in chronic pain (Martin *et al*., 2003, Nadal *et al*., 2006, Gavériaux-Ruff *et al*., 2008). In inflammatory pain models DOP receptor function is upregulated through improved coupling to VACC signalling (Pradhan *et al*., 2013). Recombinant MOP and DOP receptors form heterodimers (Gomes *et al*., 2004) and the presence of both MOP and DOP receptors is

required for recruitment of BAR2 in the absence of agonist (Rozenfeld and Devi, 2007, Baptista-Hon *et al*., 2013). This implicates DOP receptors in BAR2 signalling produced by agonist activation of MOP receptors. Following chronic morphine treatment it appears that MOP/DOP receptor heteromers are upregulated (Gupta *et al*., 2010, Costantino *et al*., 2012) and that there is an anti-analgesic effect of MOP/DOP heteromers in the pain pathway (Milan-Lobo *et al*., 2013). Methadone, a MOP receptor agonist, administered alone produces rapid endocytosis but when administered in combination with a low dose of a DOP antagonist (naltriben) the endocytosis of the MOP/DOP heteromer complex is blocked. By contrast, the endocytosis of MOP receptor homomers and intracellular signalling are not altered (Milan-Lobo and Whistler, 2011). MOP/DOP receptor heteromers therefore persist in the cell membrane. The mice treated with naltriben and methadone, with the aim of stabilising the MOP/DOP heteromers in the cell membrane, developed increased analgesic tolerance when compared to those mice treated with methadone alone. This implicates MOP/DOP heteromers in analgesic tolerance (Milan-Lobo *et al.*, 2013).

Morphine analgesic tolerance is reduced in BAR2-/- mice and these animals exhibit basal analgesia. BAR2 is recruited to MOP receptors following phosphorylation by G protein receptor kinases (GRKs). It is currently unclear whether GRK mediated phosphorylation of MOP/DOP oligomers is required for basal BAR2 recruitment. Opioid receptor phosphorylation can also occur through a number of different pathways including second messenger dependent protein

kinases such as PKA and PKC (Ferguson, 2001). The BAR2 mediated pathway provides an alternative signalling mechanism that is thought to play a role in receptor desensitisation and endocytosis, in addition to the activation of alternative intracellular signalling pathways. The development in the understanding of this alternative signalling pathway has led to the concept of biased agonism or functional selectivity (Reiter and Lefkowitz, 2006, Shukla *et al*., 2011). This concept describes the ability of a ligand to selectively recruit one signalling pathway over another to produce differing intracellular effects (Urban *et al*., 2007, Kelly *et al*., 2008, Kenakin and Miller, 2010). Compounds that can selectively stabilise receptor conformation to produce signalling through either G protein mediated mechanisms or via BAR2 are now being developed. The differing receptor conformations are thought to produce recruitment of differing intracellular signalling pathways through a GRK mediated phosphorylation “barcode” at the receptor C terminus (Reiter *et al*., 2012).

Herkinorin, a derivative of Salvinorin A, has limited bioavailability and a bias towards G protein signalling. This compound produces analgesia with reduced tolerance (Groer *et al*., 2007). A compound called TRV130 has recently been developed by Trevena Inc. TRV130 produces analgesia at MOP receptors through the activation of G protein mediated signalling without affecting the BAR2 signalling pathway. In animal studies this compound produced analgesia with reduced gastric and respiratory side effects when compared to morphine (DeWire *et al*., 2013). During Phase I clinical trials in healthy volunteers it also appeared to produce improved analgesia with reduced side effects when

compared to equivalent doses of morphine and it has now entered Phase II clinical trials (Soergel *et al*., 2014).

Different GRKs are involved in rewarding processes and analgesic tolerance (Gluck *et al*., 2014). It has been demonstrated that c-Src is involved in GPCR desensitisation and internalisation (Walwyn *et al*., 2007, Gavi *et al*., 2006). The beta2-adrenergic receptor requires the recruitment and activation of Src to allow desensitisation and internalisation to occur (Fan *et al*., 2001, Huang *et al*., 2004). MOP receptors have not been studied in isolation but there is evidence for the requirement of c-Src for DOP receptor desensitisation, internalisation and signalling in recombinant cell models (Kramer *et al*., 2000a, Kramer *et al.*, 2000b, Gavi *et al*., 2006, Hong *et al*., 2009). Interestingly c-Src phosphorylates and activates GRK2, which is implicated in opioid receptor signalling and functional selectivity (Fan *et al*., 2001). Inhibition of c-Src using PP2 abolishes the phosphorylation of GRK2 and prevents the desensitisation of beta2-adrenergic receptor (Fan *et al*., 2001). While GRK2 knock-out is embryonically lethal, various studies have targeted siRNA knockdown of this protein. The results of these implicate both GRK2 and GRK3 in opioid receptor phosphorylation, and BAR recruitment (Whistler and von Zastrow, 1998, Bohn *et al*., 2004). GRK3 knock-out mice exhibit the development of normal morphine tolerance but reduced tolerance to fentanyl (Terman *et al*., 2004, Kuhar *et al*., 2015). Src has also been implicated in the recruitment of BAR1 and BAR2 to the plasma membrane as PP2 appears to reduce this in a recombinant cell model (Hong *et al*., 2009). Src has also been implicated in both the activation of protein tyrosine kinase receptors

such as PDGFRβ and the downstream effectors following activation of these receptors (Thomas and Brugge, 1997). Both the PDGFRα and the PDGFRβ couple to Src (Kypta *et al.*, 1990, Twamley *et al*., 1992), and exposure of fibroblast cells to the PDGFRβ agonist PDGFBB produces an upregulation of Src family activity (Kypta *et al*., 1990). This suggests that the reduction of tolerance seen with PDGFRβ inhibition (Wang *et al*., 2012) may actually be due to the inhibition of Src and this requires further exploration.

Costa and Herz (1989) were the first to demonstrate agonist independent constitutive DOP receptor activity in the NG108-15 neuroblastoma cell line. The level of constitutive activity of MOP receptors under normal circumstances is thought to be low, but it can be demonstrated when the receptors are overexpressed (Burford *et al*., 2000) or altered by specific mutations (Brillet *et al*., 2003). Long term exposure to opioid drugs such as morphine can also produce an increase in constitutive activity in both cell based systems (Wang *et al*., 2001, Sadee *et al*., 2005) and in the striatum of morphine dependent mice (Wang *et al* 2004). In DRG neurones from BAR2-/- mice it has been demonstrated that there is increased MOP receptor constitutive activity (Walwyn *et al*., 2007). Basal analgesia in the BAR2-/- mice appears to be the result of MOP receptor constitutive activity within the pain pathway because it is inhibited by inverse agonists but not neutral antagonists (Lam *et al*., 2011). As discussed previously we observe basal analgesia in both BAR2-/- and BAR2-/-//DOP-/- mice. This demonstrates that constitutive activity of MOP receptors occurs in DOP-/- mice, suggesting that DOP receptors do not influence this

process but BAR2 does, as it occurs in both the BAR2-/- and the BAR2-/-//DOP-/- mice.

There was no basal analgesia in mice treated with c-Src inhibitors. We do not know if there are differences in the level of c-Src in BAR2-/- neurones, the localisation of c-Src within the cell has been suggested to be disrupted in DRG neurones from BAR2-/- mice (Walwyn *et al.*, 2007). There may also be differences in pc-Src levels following opioid exposure when BAR2 is absent and these are questions that will need to be addressed in future experiments.

7.3 Genetic polymorphisms and opioid activity

Inter-individual differences in the response to opioids are well known. This includes both the analgesic response obtained from a single dose and the side effects that are experienced. Polymorphisms in the *Oprm1* gene have been suggested to be involved in these differences in both humans and rodents (Uhl *et al.*, 1999). One of the most studied is the A118G polymorphism where an adenine has been replaced by a guanine resulting in an amino acid switch from asparagine to aspartate in exon 1 of the Oprm1 gene at position 40 (so otherwise known as N40D) (Fillingim *et al.*, 2005). This polymorphism occurs in 0.8% of the Sub-Saharan population, 8 – 17% of Caucasians and 49% of Asians (Walter and Lotsch, 2009). It has been suggested that people who are homozygous for this polymorphism have a decreased sensitivity to pressure pain (Fillingim *et al.*, 2005). With varying reports of the decreased effectiveness of

opioid drugs, increased effect or no change (Diatchenko *et al*., 2011). A meta-analysis performed in 2009 suggested that at that point knowledge of the patient's genotype would not be useful for pain management, but there was a weak association with decreased nausea and increased opioid requirements in the homozygotes (Walter and Lotsch, 2009). There have also been several studies using mouse models and humanised mouse models, they reveal that MOP receptors containing the A118G polymorphism have a greater binding affinity for β-endorphin than the WT MOP receptors and that it is more potent at activating associated GIRK channels (Bond *et al*., 1998). The humanised mouse model suggested a decreased morphine potency with attendant decreased analgesia, but that the response to fentanyl was unchanged (Mahmoud *et al*., 2011). A recent study has revealed that these mice gain less of a reward from morphine and this is associated with decreased dopamine release in the nucleus accumbens, this is thought to be due to decreased receptor expression at the cell surface of the N40D containing receptors (Robinson *et al*., 2015). This is consistent with our work from the MOP+/- mice which exhibit reduced potency of morphine analgesia but increased susceptibility to tolerance. The demonstration of the importance of polymorphisms in receptor expression suggests that this is relevant to the clinical situation and may provide a mechanism for the observations of individual variability in the development of tolerance to morphine.

The second most common polymorphism in the *Oprm1* gene causes an A6V substitution, like N40D this occurs in the extracellular domain of the MOP

receptor (Ravindranathan *et al.*, 2009). The frequency of occurrence again depends on the population tested with identification of this polymorphism in <1% of Caucasians tested but 20% of African Americans and the Northern Indian population (Knapman and Connor, 2015). It appears that adenylyl cyclase activation of the ERK signalling pathway is disrupted following morphine and fentanyl exposure of cells containing MOP receptors with this mutation (Knapman *et al.*, 2015). Both N40D and A6V are associated with an increased risk of alcohol abuse (Rommelspacher *et al.*, 2001). This is interesting as ethanol has been demonstrated to reverse morphine tolerance in locus coeruleus neurones (Llorente *et al.*, 2013) and analgesic tolerance in a mouse model (Hull *et al.*, 2013).

7.4 The psychomotor effects of morphine

7.4.1 The role of MOP receptors

In rodents, morphine produces a robust dose-dependent locomotor activation, a phenomenon that exhibits sensitisation. This has been well described previously (Brase *et al.*, 1977) and is somewhat strain dependent (Mogil *et al.*, 1999, Leo *et al.*, 2008). MOP-/- mice do not develop locomotor activation by morphine and MOP+/- mice exhibit a reduction in morphine potency to produce locomotor activation. Interestingly MOP+/- fail to sensitise to locomotor activation even following morphine administration at 30 mg/Kg. Implicating MOP receptor number in this process.

Studies investigating differences in protein transcription within the nucleus accumbens core in response to both acute and chronic administration of morphine have identified differences in the level of chaperone proteins known as heat shock proteins (Hsp) (Salas *et al*., 2011). A link between behavioural sensitisation and Hsp70 expression has been identified in mice (Luo *et al*., 2011). Levels of Hsp70 expression in the nucleus accumbens core correlate with the degree of sensitisation of locomotor activation produced by a dose of morphine. This process appears to involve D1 receptors as the administration of D1 receptor antagonist prevents this sensitisation from occurring (Babovic *et al*., 2013). As previously discussed D1 receptors are also implicated in the direct activation of hyperactivity in mice after opioid administration and this has been associated with a BAR2/ERK signalling complex (Urs *et al*., 2011). Dopamine deficient mice exhibit reinforcement by morphine but the drug induced locomotor activation is significantly reduced (Hnasko *et al*., 2005) revealing that dopamine levels within the nucleus accumbens are not solely responsible for the reinforcing effects of these drugs but play a significant role in locomotor activation.

WT mice display a significant dose dependent preference for morphine following conditioning in a two chamber CPP model. MOP-/- do not display a preference for morphine as previously described (Matthes *et al.*, 1996). The MOP+/- mice do not exhibit a preference for morphine (10 mg/Kg) but do for morphine (30 mg/Kg) as for locomotor activation this suggests that the reduction in potency is greater for this aspect of morphine related behaviour than for analgesia.

Morphine and the selective MOP receptor agonist, DAMGO, caused a concentration dependent inhibition of sIPSC frequency in the VTA. Previous studies have been restricted to the use of morphine, DAMGO or met-enkephalin using single concentrations. In MOP+/- neurones morphine has a reduced potency which supports the results that we obtained for the CPP experiments. As does the fact that there is no significant inhibition of sIPSC frequency in MOP-/- neurones. MOP-/- mice do not display a preference for morphine or self-administer the drug (Matthes *et al*., 1996).

Very low concentrations of morphine (< 0.03 µM) inhibit IPSCs frequency in VTA slices. The presence of 5-HT_3 receptors within the VTA has been suggested (Cameron *et al*., 1997, Rodd *et al*., 2007). Morphine is a potent antagonist of 5-HT_3 receptors (Baptista-Hon *et al*., 2012). Ondansetron alone produced a significant inhibition of sIPSC frequency (23.1 ± 6.2%), and its actions were approximately additive with those of morphine indicating that these two drugs likely act through different sites to inhibit sIPSC frequency. These data suggest that while 5-HT_3 receptors are present within the VTA and influence sIPSC frequency they are probably not responsible for the effects of morphine. However, morphine can also activate DOP receptor signalling (Keith *et al*., 1996) and this may be the cause of this inhibition as it does not occur with DAMGO.

We have demonstrated that the sIPSCs that we have recorded are GABAergic, the events are abolished by the application of bicuculline a $GABA_A$ receptor

antagonist. Both $GABA_A$ and $GABA_B$ receptors are thought to be present within the VTA (Xi and Stein, 2002, Margolis *et al.*, 2012, Tan *et al.*, 2012, Ciccarelli *et al.*, 2012) it would be useful to perform further experiments to elucidate the effects of specific GABA receptor antagonist drugs on the response to opioid drugs in this brain area and to establish the $GABA_A$ receptor subtypes involved.

The control frequency of IPSC events and the event kinetics are not significantly different when TTX is present suggesting that the majority of GABA release is action potential independent spontaneous release. However, it will be important to establish whether higher concentrations of TTX reduce IPSC frequency. It is possible that VTA neurones express TTX resistant Na^+ channels. We have performed the majority of our experiments in the absence of TTX to enable the recording of sIPSC events. This allows for the possibility that there is an intact neuronal network present within the slice which is more like the situation in the whole brain. The nucleus accumbens is present in our slices and this raises the possibility that it may be possible to evoke IPSCs mediated by striatal inhibitory neurones projecting back to the VTA. It may be possible to record from presynaptic inhibitory neurones originating in the striatum or elsewhere, while simultaneously recording from postsynaptic dopaminergic neurones in the VTA. This may work if there is a direct connection between the two brain areas. Of note is the observation that introduction of MOP receptors into striatal GABAergic neurones reinstates opioid reinforcement in MOP-/- mice (Cui *et al.*, 2014). This implies that the inhibition from the striatum back to the VTA is instrumental in this behaviour. The ability to study the effects of conditional

opioid receptor KO or of knocking in MOP, DOP and BAR2 into specific neurones to establish the role of these components in the individual neuronal populations will be important in future studies.

To further address this issue, imaging of VTA neurones and their connectivity throughout the brain would be fascinating. It has been postulated that the properties of the dopaminergic neurones vary across the VTA depending on their target location (Ford *et al*., 2006). One way of addressing this would be through the use of retrograde tracers which could be injected either into the nucleus accumbens to investigate specific connectivity to the VTA. Another approach would be injection into the VTA to investigate neuronal projections into the VTA. A study by Matsui and Williams (2011) identified the rostromedial tegmental nucleus as an important input to the VTA utilising this technique. Understanding whether the roles of BAR2 and c-Src are specific to individual projection neurones would be very useful.

One of the issues that we have encountered has been unambiguous identification of the neurone involved in electrophysiological recording. With little consensus regarding electrophysiological markers of cell type we are dependent on secondary labelling techniques. While we have developed a robust protocol to identify the dopaminergic cells within the VTA in the slices used for recording, identifying the single biocytin labelled cell has been much less reliable. It would be very useful to whole cell patch a number of cells in a WT

brain slice with intracellular solution containing biocytin, process this slice for IHC immediately after recording and document the percentage of tyrosine hydroxylase cells were present that were co-labelled with biocytin. Another approach would be to utilise GAD67-GFP mice and patch cells that contained GFP and those that did not and compare the effects of morphine on these cell types. This approach would also allow the investigation of the effect of cell type on sIPSC activity. A longer term approach would be to create mouse lines that are both GAD67-GFP positive alongside the genetic models that we have used in this study, so for example GAD67-GFP/MOP-/- mice.

We have performed the electrophysiological experiments in brain slices from mice that were between 17 and 21 days old and the behavioural experiments in adult mice. This has been done due to the limitations of our current imaging system, which cannot visualise neurones in brain slices containing the VTA above post-natal day 21 due to the increasing myelination. It has been suggested that the opioid receptors may utilise differing mechanisms of phosphorylation and desensitisation at a young age (less than 20 days post-natal) compared to adult neurones with levels of GRK2 expression decreasing with increasing age (Llorente *et al*., 2012). This may account for some of our observed differences between the electrophysiological recordings and the behavioural responses to morphine.

7.4.2 DOP receptors in the psychomotor effects of morphine

WT mice do not exhibit significant locomotor activity when treated with 3 mg/Kg morphine, but do when treated with 10 mg/Kg morphine. DOP-/- mice exhibit no significant alterations in locomotor activity compared to WT mice following morphine (10 mg/Kg) but did exhibit an increased sensitivity to the locomotor activating effects at morphine (3 mg/Kg). This corresponds with previously published data that suggests that DOP-/- mice display an increased sensitivity to the locomotor activating aspects of morphine (Chefer and Shippenberg, 2009), although this study also observed an increased locomotor response when compared to the WT control, which we did not see.

DOP-/- mice also exhibit a dose dependent preference for morphine that is not significantly different from that which was observed in the WT mice. There have been conflicting reports of morphine reinforcement in this mouse model with some investigators not demonstrating reinforcement (Chefer and Shippenberg, 2009) and others reporting that it could be observed in these mice (Le Merrer *et al.*, 2011, Le Merrer *et al.*, 2012). As previously discussed in Chapter 4, all of CPP was performed in the same way with saline administered in the morning (AM) session and morphine in the afternoon (PM) session. It has been suggested that the DOP-/- mice require a cue to be able to develop CPP to morphine unlike the WT mice. The fact that we have used a specific time related administration protocol may have provided enough of a cue to establish the observed preference for morphine in the DOP-/- mice. However the preference scores that we have recorded for the DOP-/- mice are not significantly different from those

of the WT mice and the mice demonstrate a marked dose-dependent increase in preference for the morphine paired chamber. This, along with the fact that these mice will self-administer morphine (Lutz and Kieffer, 2013), suggests that morphine reinforcement is not significantly affected by the removal of DOP receptors from the signalling pathway. However, there were only eight mice of equal genders in each group in the current study, which is a relatively small sample size and an increased sample size will be required to make firm conclusions. It would be useful to be able to compare the effects of gender and genetic background on this aspect of behaviour as the previous studies used only male mice (Chefer and Shippenberg, 2009). They also maintained the mice on a different genetic background (129Sv/C57Bl/6 mixed background compared to our C57Bl/6J alone) (Le Merrer *et al*., 2011, Le Merrer *et al*., 2012). This may have implications for the differences in preference that we observed. It has been widely published that there are genetic variations in the results of nociceptive testing and response to morphine in mice (Mogil *et al*., 1999, Kest *et al*., 2000, Chesler *et al*., 2002). There are also variations in morphine preference and self-administration across different rodent genotypes (Korostynski *et al*., 2006).

7.4.3 The role of BAR2 in the psychomotor effects of morphine

BAR2-/- mice exhibit a significantly reduced locomotor response to morphine (10 mg/Kg) but still sensitise to this effect. We observe the same effects in the BAR2-/-//DOP-/- mice. Together with the DOP-/- mouse data this implicates the involvement of BAR2 in the development of locomotor activation following

morphine administration, but suggests that DOP receptors may also be involved in sensitisation. Locomotor activation following morphine administration is thought to be produced predominately through dopamine receptor signalling although there is a small component that occurs via a dopamine independent mechanism (Meye *et al.*, 2012). The dopaminergic dependent component of locomotor activation is mediated via a dopamine D1 receptor mechanism (Urs *et al.*, 2011). Activation of this pathway produces the formation of a BAR2/mitogen-activated protein kinase (MAPK) complex. Systemic administration of the MEK inhibitor, SL327, to WT mice inhibits morphine induced locomotion in a dose dependent manner (Urs *et al.*, 2011).

BAR2-/- mice exhibit an increased preference score for morphine compared to the WT mice at low concentrations of morphine (3 mg/Kg) suggesting that they experience a greater sensitivity to the reinforcing effects of morphine when compared to WT mice. This confirms a previous report (Bohn *et al.*, 2003). The preference score recorded for these mice was not different when either 3 or 10 mg/Kg of morphine was administered unlike the WT and DOP-/- mice which displayed a dose dependency in this range. This suggests that 3 mg/Kg morphine is at the top of the dose response curve for reinforcement in BAR2-/- mice and it would be interesting to repeat with lower doses to establish the potency of morphine. This increased sensitivity to morphine reinforcement appears to occur without constitutive activity of MOP receptors, a phenomenon observed in the pain pathway of BAR2-/- mice (Walwyn *et al.*, 2007, Lam *et al.*, 2011). We know that MOP receptors are involved in mediating hedonic tone as naloxone is

aversive in WT, but not in MOP-/- mice (Skoubis *et al*., 2001). BAR2-/- mice do not display any significant alteration in their aversive response to naloxone when compared to WT mice suggesting that there is no increase in “basal reward” in these animals (Lam *et al*., 2011).

In contrast to the reported increase in the preference of the BAR2-/- mice for morphine (Bohn *et al.*, 2003), it was initially reported that the degree of physical dependence produced by morphine was not significantly different between WT and BAR2-/- mice (Bohn *et al*., 2000). A further study utilising osmotic pumps for drug delivery instead of the pellet method that had been previously used produced slightly different results. It suggests that the withdrawal signs observed are dependent on the dose of morphine that is received. So BAR2-/- mice that receive high doses of morphine do not show differences in physical dependence when compared to WT mice. But for those mice that received a lower dose of morphine the signs of physical dependence and withdrawal were significantly reduced in the BAR2-/- mice compared to the WT mice (Raehal and Bohn, 2011). These results do not explain the increased preference for morphine that these mice display. It would be helpful to establish whether BAR2-/- exhibit greater morphine self-administration compared to WT mice for a more complete understanding of the role of BAR2 signalling in reward.

The BAR2-/-//DOP-/- mice exhibited a dose dependent preference for morphine in the two chamber CPP model, this was not significantly different to that

observed in the WT mice. Removing DOP receptors as well as BAR2 appears to have ameliorated the increased sensitivity to morphine preference observed in the BAR2-/- mice.

It would be very interesting to test CPP in mice that have been treated chronically with morphine and examine the effects of the different mouse strains. Particularly interesting would be the effect of the gene deletions in BAR2-/-, DOP-/- and the BAR2-/-//DOP-/- mice as they show a significantly reduced or abolished tolerance to the analgesic effects of morphine. It will be important to establish whether these genotypes have differences in morphine dependence following chronic exposure. This could be investigated using CPA to naloxone. Such an approach would also allow us to establish differences between the genotypes in withdrawal behaviours (for example vocalising, jumping and rearing). This may occur over a similar time course to the development of morphine analgesic tolerance.

7.4.4 The role of DOP receptors and BAR2 in VTA signalling

In the DOP-/-, BAR2-/- and the BAR2-/-//DOP-/- neurones there is a consistent reduction in the ability of morphine to inhibit sIPSC frequency compared to the WT neurones. The work that we have done also implicates BAR2 in the response to DAMGO. As we also observe a significant reduction in the ability of DAMGO to inhibit sIPSC frequency in the BAR2-/- neurones compared to the WT neurones.

In the DOP-/- mice this supports the previous reports of decreased CPP (Chefer and Shippenberg, 2009) but not our data of unchanged CPP and a report of unchanged morphine self-administration (Lutz and Kieffer, 2013). The dogma is that inhibition of sIPSC frequency within the VTA is responsible for the psychomotor effects of morphine through disinhibition of dopaminergic neurones in the mesocorticolimbic reward pathway. We also noted that naloxone, an antagonist/inverse agonist at MOP receptors, inhibits sIPSC frequency in WT, MOP+/- and MOP-/- neurones within the VTA. This MOP receptor independent phenomenon was unexpected. The lack of inhibition of sIPSC frequency by naloxone in the BAR2-/-, DOP-/- or BAR2-/-//DOP-/- neurones suggests that the effect is mediated through DOP receptors and BAR2. It has been previously reported that MOP receptor antagonists can increase dopamine in the striatum (Devine *et al.*, 1993b) an effect that is normally associated with MOP receptor agonists. Furthermore, a low dose of naltrexone has been reported to increase the duration of morphine CPP when administered with morphine during the conditioning phase of the protocol (Powell *et al.*, 2002).

The observation that DPDPE inhibits sIPSC frequency in WT but not DOP-/- VTA neurones demonstrates that DOP receptors are present within the VTA, in agreement with previous labelling studies (Erbs *et al.*, 2015). DPDPE also inhibits sIPSC frequency in MOP-/- neurones demonstrating that DOP receptors are able to function independent of MOP receptors in the VTA. This is consistent with

previously reported behavioural data that rodents will self-administer DPDPE directly into the VTA (Devine and Wise, 1994, McBride *et al*., 1999).

The observation that morphine has a reduced potency as an inhibitor of sIPSC frequency in VTA neurones from BAR2-/- mice is surprising as these mice display enhanced preference for morphine compared to WT mice. This suggests that inhibition of IPSCs in the VTA is not directly responsible for reward/reinforcement. We postulated that this synapse may be responsible for the locomotor activation rather than reward. As the BAR2-/- mice exhibit significantly decreased locomotor activation by morphine when compared to WT mice this would be consistent with a decrease in the ability of morphine to inhibit sIPSC frequency.

Removing either DOP receptors or BAR2 appears to have the same effect on reducing the ability of morphine to inhibit sIPSC frequency, suggesting the involvement of both of these factors in alterations in signalling produced by morphine within the VTA. It is possible that DOP receptors are required to allow the link between MOP receptors and BAR2 to occur perhaps by forming a MOP/DOP/BAR2 complex.

The VTA has also been implicated in the modulation of pain transmission (Bushnell *et al*., 2013, Niikura *et al*., 2010). Neuropathic pain models, such as sciatic nerve ligation, can decrease MOP receptor function within the VTA

resulting in a decrease in the rewarding effect of MOP agonist drugs (Ozaki *et al*., 2003). It is thought that these effects are mediated by separate mechanisms that are individually associated with reward and analgesia (Schifirnet *et al*., 2014).

7.4.5 The effects of c-Src inhibition on the psychomotor effects of morphine

It has been reported that dasatinib can cross the BBB (Porkka *et al*., 2008, Lagas *et al*., 2009). There were no significant alterations in reinforcement or locomotor activation when dasatinib was administered either alone or together with morphine to WT mice. PP2 directly applied to the VTA neurones of WT mice reduced the inhibition of sIPSC frequency by morphine, which resembled the reduced inhibitory effect of morphine in BAR2-/- neurones. This was unexpected given the behavioural findings. We also investigated the effects of a MEK inhibitor (SL327) on sIPSC frequency. Inhibition of MEK by SL327 significantly decreases locomotor activation in WT mice but does not alter CPP for morphine (Urs *et al*., 2011). We have demonstrated that MEK inhibition does not affect the ability of morphine to inhibit sIPSC frequency within the VTA. Taken together these data imply that MEK-ERK signalling is not involved in the vesicular release of GABA from presynaptic neurones within the VTA but that c-Src is involved in this process. We hypothesise that MOP receptors signal through BAR2 (perhaps with DOP receptors) to c-Src in which regulates the trafficking of GABAergic vesicles within the VTA. There are several experiments that need to be done to test this hypothesis. An obvious approach is to test whether c-Src inhibitors

reduce the inhibition of sIPSC frequency by morphine in BAR2-/- VTA neurones. An absence of an effect of c-Src inhibitors would support the hypothesis.

In DRG neurones c-Src inhibition or the removal of BAR2 (BAR2-/-) has been demonstrated to increase constitutive inhibitory coupling of MOP receptors to VACCs, reduce constitutive MOP receptor recycling and increase the cell surface expression of MOP receptors (Walwyn *et al.*, 2007). It may be that the role of c-Src is different within the VTA. There are a number of studies that implicate c-Src in membrane trafficking, receptor desensitisation and internalisation (Foster-Barber and Bishop, 1998, Hong *et al*., 2009).

It would be useful to confirm biochemically that dasatinib is crossing the BBB in our model and reaching a significant cerebral concentration. This experiment could be performed using western blots for c-Src and phosphorylated c-Src following administration of dasatinib to WT mice. This would reveal whether IP administered c-Src inhibitors (PP2 and dasatinib) inhibit the phosphorylation of c-Src within VTA and striatum and also DRG neurones and spinal cord.

We have implicated c-Src in the development of morphine tolerance. It would be interesting to pursue a health informatics approach to investigate whether differences in opioid prescribing are apparent in patients receiving dasatinib and opioid drugs compared to those that are receiving opioid drugs alone. Our data would suggest that patients receiving dasatinib alongside opioid drugs may not

experience tolerance to their analgesic effects and therefore would not require the same dose escalation likely to occur in cancer patients not receiving dasatinib.

These observations suggest that c-Src participates in the actions of morphine in analgesia and also within the VTA. The effects of inhibiting c-Src are similar to those observed in DOP-/- and BAR2-/- mice, in that there is a demonstrable reduction in analgesic tolerance. When PP2 is directly applied to WT VTA neurones the ability of morphine to inhibit sIPSC frequency resembles that within the BAR2-/- neurones. Inhibition of c-Src does not appear to affect locomotor activation produced by morphine or to alter morphine CPP. It will be important to determine whether the activation of c-Src is dependent on the MOP/DOP/BAR2 signalling pathway or if c-Src is acting in parallel as an independent mechanism. We have demonstrated in SW620 cells that dasatinib and PP2 inhibit the phosphorylation of c-Src. We plan to investigate the differences in the degree of morphine stimulated phosphorylation of c-Src in the VTA and DRG neurones of WT, MOP-/-, DOP-/- and BAR2-/- mice.

The tyrosine kinase system is a very complicated system that may provide multiple targets to modify the side effects of opioid drugs. We have demonstrated a role for the tyrosine kinase c-Src in the development of tolerance to the analgesic effects of morphine. It does not appear that inhibiting c-Src affects the rewarding properties of the drug. However this requires

verification by the introduction of c-Src inhibitors into specific brain nuclei such as the VTA and striatum. It also does not cause basal analgesia in mice or constitutive receptor activity like the removal of BAR2. It therefore presents us with a very interesting target to reduce opioid tolerance without affecting reward.

7.5 Conclusions

We hypothesised that MOP and DOP receptors interacted in the pain pathway to recruit BAR2 and c-Src. The finding that morphine analgesic tolerance is reduced in the DOP-/- and the BAR2-/- mice is consistent with the hypothesis. However, when both BAR2 and DOP receptors are removed, as in BAR2-/-//DOP-/- mice, then tolerance is completely abolished suggesting that both of these elements are independently involved in morphine analgesic tolerance. The implication is that the actions of DOP receptors and BAR2 are additive and therefore not be in a sequential pathway. The inhibition of c-Src also inhibits the development of morphine tolerance in both the WT and MOP+/- mice. DOP receptors also contribute to the analgesic effects of morphine supporting a role of MOP/DOP heteromers in signalling. While there appears to be a significant MOP receptor reserve, receptor number influences the development of tolerance.

Secondly we hypothesised that MOP/DOP interactions are not involved in signalling within the reward pathway. Our data suggest that this is may not be correct. DOP receptors are present within the VTA and they function not only in

response to selective agonists but they are also involved in morphine activated signalling. Both DOP and BAR2 are required for the full inhibitory effect of morphine on sIPSC frequency. The involvement of DOP receptors appears to be specific to morphine as their absence does not affect the ability of DAMGO to inhibit sIPSC frequency. However BAR2 is implicated in the response to both morphine and DAMGO and the inhibition of c-Src produces an effect similar to that seen in the BAR2-/- neurones with morphine.

The removal of MOP receptors abolishes the psychomotor effects of morphine. The electrophysiological results do not clearly correlate with the observed psychomotor differences observed in the DOP-/- and BAR2-/- mice. The absence of BAR2 increases the morphine preference score, this alteration does not appear to involve DOP receptor signalling as preference is unchanged in DOP-/- mice. While locomotor activation is reduced in BAR2-/- mice, sensitisation still occurs. The sensitisation of the locomotor response to morphine appears to be predominately mediated through MOP receptor signalling. The inhibition of c-Src does not appear to alter the psychomotor effects produced by morphine. As we have discussed it does reduce morphine analgesic tolerance. This suggests that c-Src is an attractive target to prevent the development of morphine analgesic tolerance without affecting hedonic homeostasis.

References

ADAMS, J. U., PARONIS, C. A. & HOLTZMAN, S. G. 1990. Assessment of relative intrinsic activity of mu-opioid analgesics in vivo by using beta-funaltrexamine. *The Journal of pharmacology and experimental therapeutics,* 255**,** 1027-32.

AGMO, A., FEDERMAN, I., NAVARRO, V., PADUA, M. & VELAZQUEZ, G. 1993. Reward and reinforcement produced by drinking water: role of opioids and dopamine receptor subtypes. *Pharmacology, biochemistry, and behavior,* 46**,** 183-94.

ALLOUCHE, S., NOBLE, F. & MARIE, N. 2014. Opioid receptor desensitization: mechanisms and its link to tolerance. *Frontiers in pharmacology,* 5**,** 280.

ALVAREZ, V. A., ARTTAMANGKUL, S., DANG, V., SALEM, A., WHISTLER, J. L., VON ZASTROW, M., GRANDY, D. K. & WILLIAMS, J. T. 2002. mu-Opioid receptors: Ligand-dependent activation of potassium conductance, desensitization, and internalization. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 22**,** 5769-76.

AMANCHY, R., ZHONG, J., HONG, R., KIM, J. H., GUCEK, M., COLE, R. N., MOLINA, H. & PANDEY, A. 2009. Identification of c-Src tyrosine kinase substrates in platelet-derived growth factor receptor signaling. *Mol Oncol,* 3**,** 439-50.

ARIAS-CARRION, O., STAMELOU, M., MURILLO-RODRIGUEZ, E., MENENDEZ-GONZALEZ, M. & POPPEL, E. 2010. Dopaminergic reward system: a short integrative review. *International archives of medicine,* 3**,** 24.

BABOVIC, D., JIANG, L., GOTO, S., GANTOIS, I., SCHUTZ, G., LAWRENCE, A. J., WADDINGTON, J. L. & DRAGO, J. 2013. Behavioural and anatomical characterization of mutant mice with targeted deletion of D1 dopamine receptor-expressing cells: response to acute morphine. *J Pharmacol Sci,* 121**,** 39-47.

BAILEY, C. P. 2004. Protein Kinase C Activation Enhances Morphine-Induced Rapid Desensitization of -Opioid Receptors in Mature Rat Locus Ceruleus Neurons. *Molecular pharmacology,* 66**,** 1592-1598.

BAILEY, C. P. & CONNOR, M. 2005. Opioids: cellular mechanisms of tolerance and physical dependence. *Current opinion in pharmacology,* 5**,** 60-8.

BAILEY, C. P., COUCH, D., JOHNSON, E., GRIFFITHS, K., KELLY, E. & HENDERSON, G. 2003. Mu-opioid receptor desensitization in mature rat neurons: lack

of interaction between DAMGO and morphine. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 23**,** 10515-20.

BAILEY, C. P., LLORENTE, J., GABRA, B. H., SMITH, F. L., DEWEY, W. L., KELLY, E. & HENDERSON, G. 2009a. Role of protein kinase C and μ-opioid receptor (MOPr) desensitization in tolerance to morphine in rat locus coeruleus neurons. *European Journal of Neuroscience,* 29**,** 307-318.

BAILEY, C. P., OLDFIELD, S., LLORENTE, J., CAUNT, C. J., TESCHEMACHER, A. G., ROBERTS, L., MCARDLE, C. A., SMITH, F. L., DEWEY, W. L., KELLY, E. & HENDERSON, G. 2009b. Involvement of PKCα and G-protein-coupled receptor kinase 2 in agonist-selective desensitization of μ-opioid receptors in mature brain neurons. *British journal of pharmacology,* 158**,** 157-164.

BAIN, J., PLATER, L., ELLIOTT, M., SHPIRO, N., HASTIE, C. J., MCLAUCHLAN, H., KLEVERNIC, I., ARTHUR, J. S., ALESSI, D. R. & COHEN, P. 2007. The selectivity of protein kinase inhibitors: a further update. *The Biochemical journal,* 408**,** 297-315.

BAPTISTA-HON, D., GALLAGHER, K., KING, F. & HALES, T. G. 2013. Examining the structural determinants for delta opioid receptor mediated basal recruitment of b-arrestin2 to mu opioid receptors. *Society of Neuroscience.* San Diego.

BAPTISTA-HON, D. T., DEEB, T. Z., OTHMAN, N. A., SHARP, D. & HALES, T. G. 2012. The 5-HT3B subunit affects high-potency inhibition of 5-HT3 receptors by morphine. *British journal of pharmacology,* 165**,** 693-704.

BARDO, M. T. & BEVINS, R. A. 2000. Conditioned place preference: what does it add to our preclinical understanding of drug reward? *Psychopharmacology,* 153**,** 31-43.

BARONE, M. V. & COURTNEIDGE, S. A. 1995. Myc but not Fos rescue of PDGF signalling block caused by kinase-inactive Src. *Nature,* 378**,** 509-12.

BARROT, M. 2012. Tests and models of nociception and pain in rodents. *Neuroscience,* 211**,** 39-50.

BARROT, M., SESACK, S. R., GEORGES, F., PISTIS, M., HONG, S. & JHOU, T. C. 2012. Braking dopamine systems: a new GABA master structure for mesolimbic and nigrostriatal functions. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 32**,** 14094-101.

BARTON, C., BASBAUM, A. I. & FIELDS, H. L. 1980. Dissociation of supraspinal and spinal actions of morphine: A quantitative evaluation. *Brain Research,* 188**,** 487-498.

BASBAUM, A. I. & FIELDS, H. L. 1984. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. *Annu Rev Neurosci,* 7**,** 309-38.

BEAULIEU, J.-M., SOTNIKOVA, T. D., GAINETDINOV, R. R. & CARON, M. G. 2006. Paradoxical Striatal Cellular Signaling Responses to Psychostimulants in Hyperactive Mice. *Journal of Biological Chemistry,* 281**,** 32072-32080.

BEAULIEU, J. M., SOTNIKOVA, T. D., MARION, S., LEFKOWITZ, R. J., GAINETDINOV, R. R. & CARON, M. G. 2005. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell,* 122**,** 261-73.

BECKER, A., GRECKSCH, G., BRODEMANN, R., KRAUS, J., PETERS, B., SCHROEDER, H., THIEMANN, W., LOH, H. H. & HOLLT, V. 2000. Morphine self-administration in mu-opioid receptor-deficient mice. *Naunyn-Schmiedeberg's archives of pharmacology,* 361**,** 584-9.

BECKER, A., GRECKSCH, G., KRAUS, J., LOH, H. H., SCHROEDER, H. & HOLLT, V. 2002. Rewarding effects of ethanol and cocaine in mu opioid receptor-deficient mice. *Naunyn-Schmiedeberg's archives of pharmacology,* 365**,** 296-302.

BECKETT, A. H. & CASY, A. F. 1954. Stereochemistry of Certain Analgesics. *Nature,* 173**,** 1231-1232.

BEFORT, K., FILLIOL, D., DECAILLOT, F. M., GAVERIAUX-RUFF, C., HOEHE, M. R. & KIEFFER, B. L. 2001. A single nucleotide polymorphic mutation in the human mu-opioid receptor severely impairs receptor signaling. *The Journal of biological chemistry,* 276**,** 3130-7.

BEIER, K. T., STEINBERG, E. E., DELOACH, K. E., XIE, S., MIYAMICHI, K., SCHWARZ, L., GAO, X. J., KREMER, E. J., MALENKA, R. C. & LUO, L. 2015. Circuit Architecture of VTA Dopamine Neurons Revealed by Systematic Input-Output Mapping. *Cell,* 162**,** 622-34.

BERGEVIN, A., GIRARDOT, D., BOURQUE, M. J. & TRUDEAU, L. E. 2002. Presynaptic mu-opioid receptors regulate a late step of the secretory process in rat ventral tegmental area GABAergic neurons. *Neuropharmacology,* 42**,** 1065-78.

BERRENDERO, F., KIEFFER, B. L. & MALDONADO, R. 2002. Attenuation of Nicotine-Induced Antinociception, Rewarding Effects, and Dependence in μ-Opioid Receptor Knock-Out Mice. *The Journal of Neuroscience,* 22**,** 10935-10940.

BERRIDGE, V. 1979. Opium in the Fens in Nineteenth-century England. *Journal of the History of Medicine and Allied Sciences,* XXXIV**,** 293-313.

BESSE, D., LOMBARD, M. C., ZAJAC, J. M., ROQUES, B. P. & BESSON, J. M. 1990. Pre- and postsynaptic location of mu, delta and kappa opioid receptors in the superficial layers of the dorsal horn of the rat spinal cord. *Prog Clin Biol Res,* 328**,** 183-6.

BILBEY, D. L., SALEM, H. & GROSSMAN, M. H. 1960. The anatomical basis of the straub phenomenon. *Br J Pharmacol Chemother,* 15**,** 540-3.

BILSKY, E. J., GIUVELIS, D., OSBORN, M. D., DERSCH, C. M., XU, H. & ROTHMAN, R. B. 2010. Chapter 21 - In Vitro and In Vivo Assessment of Mu Opioid Receptor Constitutive Activity. *Methods in Enzymology: Constitutive Activity in Receptors and Other Proteins, Part A*

BOHN, L. M., DYKSTRA, L. A., LEFKOWITZ, R. J., CARON, M. G. & BARAK, L. S. 2004. Relative Opioid Efficacy Is Determined by the Complements of the G Protein-Coupled Receptor Desensitization Machinery. *Molecular pharmacology,* 66**,** 106-112.

BOHN, L. M., GAINETDINOV, R. R., LIN, F.-T., LEFKOWITZ, R. J. & CARON, M. G. 2000. Mu-Opioid receptor desensitization by b-arrestin-2 determines morphine tolerance but not dependence. *Nature,* 408**,** 720-723.

BOHN, L. M., GAINETDINOV, R. R., SOTNIKOVA, T. D., MEDVEDEV, I. O., LEFKOWITZ, R. J., DYKSTRA, L. A. & CARON, M. G. 2003. Enhanced Rewarding Properties of Morphine, but not Cocaine, in βarrestin-2 Knock-Out Mice. *The Journal of Neuroscience,* 23**,** 10265-10273.

BOHN, L. M., LEFKOWITZ, R. J. & CARON, M. G. 2002. Differential Mechanisms of Morphine Antinociceptive Tolerance Revealed in beta-Arrestin-2 Knock-Out Mice. *Journal of Neuroscience,* 22**,** 10494-10500.

BOHN, L. M., LEFKOWITZ, R. J., GAINETDINOV, R. R., PEPPEL, K., CARON, M. G. & LIN, F. T. 1999. Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science,* 286**,** 2495-8.

BONCI, A. & WILLIAMS, J. T. 1997. Increased probability of GABA release during withdrawal from morphine. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 17**,** 796-803.

BOND, C., LAFORGE, K. S., TIAN, M., MELIA, D., ZHANG, S., BORG, L., GONG, J., SCHLUGER, J., STRONG, J. A., LEAL, S. M., TISCHFIELD, J. A., KREEK, M. J. & YU, L. 1998. Single-nucleotide polymorphism in the human mu opioid receptor gene alters beta-endorphin binding and activity: possible implications for opiate addiction. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 9608-13.

BOZARTH, M. A. & WISE, R. A. 1984. Anatomically distinct opiate receptor fields mediate reward and physical dependence. *Science,* 224**,** 516-7.

BRAITHWAITE, R. A. 2007. Heroin. *In:* WARING, R. H., STEVENSON, G. B. & MITCHELL, S. C. (eds.) *Molecules of Death.* 2 ed. London: Imperial College Press.

BRASE, D. A., LOH, H. H. & WAY, E. L. 1977. Comparison of the effects of morphine on locomotor activity, analgesia and primary and protracted physical dependence in six mouse strains. *The Journal of pharmacology and experimental therapeutics,* 201**,** 368-74.

BRILLET, K., KIEFFER, B. L. & MASSOTTE, D. 2003. Enhanced spontaneous activity of the mu opioid receptor by cysteine mutations: characterization of a tool for inverse agonist screening. *BMC Pharmacology,* 3**,** 14-14.

BROWNSTEIN, M. J. 1993. A brief history of opiates, opioid peptides, and opioid receptors. *PNAS,* 90**,** 5391-5393.

BURFORD, N. T., WANG, D. & SADÉE, W. 2000. G-protein coupling of mu-opioid receptors (OP3): elevated basal signalling activity. *Biochemical Journal,* 348**,** 531-537.

BUSHNELL, M. C., CEKO, M. & LOW, L. A. 2013. Cognitive and emotional control of pain and its disruption in chronic pain. *Nature reviews. Neuroscience,* 14**,** 502-11.

BUTCHER, A. J., PRIHANDOKO, R., KONG, K. C., MCWILLIAMS, P., EDWARDS, J. M., BOTTRILL, A., MISTRY, S. & TOBIN, A. B. 2011. Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code. *The Journal of biological chemistry,* 286**,** 11506-18.

BUTLER, R. K. & FINN, D. P. 2009. Stress-induced analgesia. *Prog Neurobiol,* 88**,** 184-202.

CAMERON, D. L., WESSENDORF, M. W. & WILLIAMS, J. T. 1997. A subset of ventral tegmental area neurons is inhibited by dopamine, 5-hydroxytryptamine and opioids. *Neuroscience,* 77**,** 155-66.

CARBONI, E., ACQUAS, E., LEONE, P. & DI CHIARA, G. 1989. 5HT3 receptor antagonists block morphine- and nicotine- but not amphetamine-induced reward. *Psychopharmacology,* 97**,** 175-8.

CARLEZON, W. A., JR., DUMAN, R. S. & NESTLER, E. J. 2005. The many faces of CREB. *Trends in neurosciences,* 28**,** 436-45.

CHARBOGNE, P., KIEFFER, B. L. & BEFORT, K. 2014. 15 years of genetic approaches in vivo for addiction research: Opioid receptor and peptide gene knockout in mouse models of drug abuse. *Neuropharmacology,* 76 Pt B**,** 204-17.

CHARLES, A. C. & HALES, T. G. 2004. From inhibition to excitation: Functional effects of interaction between opioid receptors. *Life sciences,* 76**,** 479-485.

CHARLES, A. C., MOSTOVSKAYA, N., ASAS, K., EVANS, C. J., DANKOVICH, M. L. & HALES, T. G. 2003. Coexpression of δ-Opioid Receptors with μ Receptors in GH3 Cells Changes the Functional Response to μ Agonists from Inhibitory to Excitatory. *Molecular pharmacology,* 63**,** 89-95.

CHAVKIN, C. & GOLDSTEIN, A. 1984. Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. *Proceedings of the National Academy of Sciences of the United States of America,* 81**,** 7253-7.

CHAVKIN, C., MCLAUGHLIN, J. P. & CELVER, J. P. 2001. Regulation of opioid receptor function by chronic agonist exposure: constitutive activity and desensitization. *Molecular pharmacology,* 60**,** 20-5.

CHEFER, V. I. & SHIPPENBERG, T. S. 2009. Augmentation of morphine-induced sensitization but reduction in morphine tolerance and reward in delta-opioid receptor knockout mice. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology,* 34**,** 887-98.

CHEN, Y., MESTEK, A., LIU, J. & YU, L. 1993. Molecular cloning of a rat kappa opioid receptor reveals sequence similarities to the mu and delta opioid receptors. *Biochemical Journal,* 295**,** 625-628.

CHESLER, E. J., WILSON, S. G., LARIVIERE, W. R., RODRIGUEZ-ZAS, S. L. & MOGIL, J. S. 2002. Identification and ranking of genetic and laboratory environment factors influencing a behavioral trait, thermal nociception, via computational analysis of a large data archive. *Neurosci Biobehav Rev,* 26**,** 907-23.

CHIENG, B., AZRIEL, Y., MOHAMMADI, S. & CHRISTIE, M. J. 2011. Distinct cellular properties of identified dopaminergic and GABAergic neurons in the mouse ventral tegmental area. *The Journal of physiology,* 589**,** 3775-87.

CHIU, T. T., YUNG, L. Y. & WONG, Y. H. 1996. Inverse agonistic effect of ICI-174,864 on the cloned delta-opioid receptor: role of G protein and adenylyl cyclase activation. *Molecular pharmacology,* 50**,** 1651-1657.

CHRISTIE, M. J. 2008. Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. *British journal of pharmacology,* 154**,** 384-396.

CHU SIN CHUNG, P. & KIEFFER, B. L. 2013. Delta opioid receptors in brain function and diseases. *Pharmacology & therapeutics,* 140**,** 112-20.

CICCARELLI, A., CALZA, A., PANZANELLI, P., CONCAS, A., GIUSTETTO, M. & SASSOE-POGNETTO, M. 2012. Organization of GABAergic synaptic circuits in the rat ventral tegmental area. *PloS one,* 7**,** 8.

CICERO, T. J., NOCK, B. & MEYER, E. R. 1996. Gender-related differences in the antinociceptive properties of morphine. *Journal of Pharmacology and Experimental Therapeutics,* 279**,** 767-773.

CONNOR, M., OSBORNE, P. B. & CHRISTIE, M. J. 2004. Mu-opioid receptor desensitization: is morphine different? *British journal of pharmacology,* 143**,** 685-96.

CONTARINO, A., PICETTI, R., MATTHES, H. W., KOOB, G. F., KIEFFER, B. L. & GOLD, L. H. 2002. Lack of reward and locomotor stimulation induced by heroin in μ-opioid receptor-deficient mice. *European Journal of Pharmacology,* 446**,** 103-109.

CONTET, C., GAVERIAUX-RUFF, C., MATIFAS, A., CARADEC, C., CHAMPY, M. F. & KIEFFER, B. L. 2006. Dissociation of analgesic and hormonal responses to forced swim stress using opioid receptor knockout mice. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology,* 31**,** 1733-44.

CORDER, G., DOOLEN, S., DONAHUE, R. R., WINTER, M. K., JUTRAS, B. L., HE, Y., HU, X., WIESKOPF, J. S., MOGIL, J. S., STORM, D. R., WANG, Z. J.,

MCCARSON, K. E. & TAYLOR, B. K. 2013. Constitutive mu-opioid receptor activity leads to long-term endogenous analgesia and dependence. *Science,* 341**,** 1394-9.

COSTA, T. & COTECCHIA, S. 2005. Historical review: Negative efficacy and the constitutive activity of G-protein-coupled receptors. *Trends in pharmacological sciences,* 26**,** 618-24.

COSTA, T. & HERZ, A. 1989. Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proceedings of the National Academy of Sciences of the United States of America,* 86**,** 7321-7325.

COSTANTINO, C. M., GOMES, I., STOCKTON, S. D., LIM, M. P. & DEVI, L. A. 2012. Opioid receptor heteromers in analgesia. *Expert reviews in molecular medicine,* 14**,** e9.

COX, B. M., CHRISTIE, M. J., DEVI, L., TOLL, L. & TRAYNOR, J. R. 2015. Challenges for opioid receptor nomenclature: IUPHAR Review 9. *British journal of pharmacology,* 172**,** 317-323.

CUI, Y., OSTLUND, S. B., JAMES, A. S., PARK, C. S., GE, W., ROBERTS, K. W., MITTAL, N., MURPHY, N. P., CEPEDA, C., KIEFFER, B. L., LEVINE, M. S., JENTSCH, J. D., WALWYN, W. M., SUN, Y. E., EVANS, C. J., MAIDMENT, N. T. & YANG, X. W. 2014. Targeted expression of mu-opioid receptors in a subset of striatal direct-pathway neurons restores opiate reward. *Nature neuroscience,* 17**,** 254-61.

CVEJIC, S. & DEVI, L. A. 1997. Dimerization of the δ Opioid Receptor:: IMPLICATION FOR A ROLE IN RECEPTOR INTERNALIZATION. *Journal of Biological Chemistry,* 272**,** 26959-26964.

D'AMOUR, F. E. & SMITH, D. L. 1941. A METHOD FOR DETERMINING LOSS OF PAIN SENSATION. *Journal of Pharmacology and Experimental Therapeutics,* 72**,** 74-79.

DANG, V. C., CHIENG, B., AZRIEL, Y. & CHRISTIE, M. J. 2011. Cellular morphine tolerance produced by betaarrestin-2-dependent impairment of mu-opioid receptor resensitization. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 31**,** 7122-30.

DANG, V. C. & WILLIAMS, J. T. 2005. Morphine-Induced mu-opioid receptor desensitization. *Molecular pharmacology,* 68**,** 1127-32.

DAVID, V., MATIFAS, A., GAVELLO-BAUDY, S., DECORTE, L., KIEFFER, B. L. & CAZALA, P. 2008. Brain regional Fos expression elicited by the activation of mu- but not delta-opioid receptors of the ventral tegmental area: evidence for an implication of the ventral thalamus in opiate reward. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology,* 33**,** 1746-59.

DAVIS, M. P. & PASTERNAK, G. W. 2009. Opioid receptors and opioid pharmacodynamics. *In:* DAVIS, M. P., GLARE, P. A., HARDY, J. & QUIGLEY, C. (eds.) *Opioids and cancer pain.* Oxford University Press.

DAY, J. J., CHILDS, D., GUZMAN-KARLSSON, M. C., KIBE, M., MOULDEN, J., SONG, E., TAHIR, A. & SWEATT, J. D. 2013. DNA methylation regulates associative reward learning. *Nature neuroscience,* 16**,** 1445-52.

DEVINE, D. P., LEONE, P., POCOCK, D. & WISE, R. A. 1993a. Differential involvement of ventral tegmental mu, delta and kappa opioid receptors in modulation of basal mesolimbic dopamine release: in vivo microdialysis studies. *The Journal of pharmacology and experimental therapeutics,* 266**,** 1236-46.

DEVINE, D. P., LEONE, P. & WISE, R. A. 1993b. Mesolimbic dopamine neurotransmission is increased by administration of mu-opioid receptor antagonists. *Eur J Pharmacol,* 243**,** 55-64.

DEVINE, D. P. & WISE, R. A. 1994. Self-administration of morphine, DAMGO, and DPDPE into the ventral tegmental area of rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 14**,** 1978-84.

DEWIRE, S. M., AHN, S., LEFKOWITZ, R. J. & SHENOY, S. K. 2007. β-Arrestins and Cell Signaling. *Annual Review of Physiology,* 69**,** 483-510.

DEWIRE, S. M., YAMASHITA, D. S., ROMINGER, D. H., LIU, G., COWAN, C. L., GRACZYK, T. M., CHEN, X. T., PITIS, P. M., GOTCHEV, D., YUAN, C., KOBLISH, M., LARK, M. W. & VIOLIN, J. D. 2013. A G protein-biased ligand at the mu-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *The Journal of pharmacology and experimental therapeutics,* 344**,** 708-17.

DIATCHENKO, L., ROBINSON, J. E. & MAIXNER, W. 2011. Elucidation of mu-Opioid Gene Structure: How Genetics Can Help Predict Responses to Opioids. *Eur J Pain Suppl,* 5**,** 433-438.

DORMANDY, T. 2012. *Opium: Reality's Dark Dream,* London, Yale University Press.

DUTTAROY, A. & YOBURN, B. C. 1995. The effect of intrinsic efficacy on opioid tolerance. *Anesthesiology,* 82**,** 1226-36.

ELMER, G. I., PIEPER, J. O., RUBINSTEIN, M., LOW, M. J., GRANDY, D. K. & WISE, R. A. 2002. Failure of intravenous morphine to serve as an effective instrumental reinforcer in dopamine D2 receptor knock-out mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 22**,** 10.

ERBS, E., FAGET, L., SCHERRER, G., MATIFAS, A., FILLIOL, D., VONESCH, J. L., KOCH, M., KESSLER, P., HENTSCH, D., BIRLING, M. C., KOUTSOURAKIS, M., VASSEUR, L., VEINANTE, P., KIEFFER, B. L. & MASSOTTE, D. 2015. A mu-delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks. *Brain structure & function,* 220**,** 677-702.

ERBS, E., FAGET, L., VEINANTE, P., KIEFFER, B. L. & MASSOTTE, D. 2014. In vivo neuronal co-expression of mu and delta opioid receptors uncovers new therapeutic perspectives. *Receptors & clinical investigation,* 1.

EVANS, C. J., KEITH, D. E., JR., MORRISON, H., MAGENDZO, K. & EDWARDS, R. H. 1992. Cloning of a delta opioid receptor by functional expression. *Science,* 258**,** 1952-5.

FAN, G., SHUMAY, E., MALBON, C. C. & WANG, H. 2001. c-Src tyrosine kinase binds the beta 2-adrenergic receptor via phospho-Tyr-350, phosphorylates G-protein-linked receptor kinase 2, and mediates agonist-induced receptor desensitization. *The Journal of biological chemistry,* 276**,** 13240-7.

FENALTI, G., GIGUERE, P. M., KATRITCH, V., HUANG, X. P., THOMPSON, A. A., CHEREZOV, V., ROTH, B. L. & STEVENS, R. C. 2014. Molecular control of delta-opioid receptor signalling. *Nature,* 506**,** 191-6.

FERGUSON, S. S. G. 2001. Evolving Concepts in G Protein-Coupled Receptor Endocytosis: The Role in Receptor Desensitization and Signaling. *Pharmacological Reviews,* 53**,** 1-24.

FIELDS, H. L. & MARGOLIS, E. B. 2015. Understanding opioid reward. *Trends in neurosciences,* 38**,** 217-25.

FILLINGIM, R. B., KAPLAN, L., STAUD, R., NESS, T. J., GLOVER, T. L., CAMPBELL, C. M., MOGIL, J. S. & WALLACE, M. R. 2005. The A118G single nucleotide

polymorphism of the mu-opioid receptor gene (OPRM1) is associated with pressure pain sensitivity in humans. *The journal of pain : official journal of the American Pain Society,* 6**,** 159-67.

FILLIOL, D., GHOZLAND, S., CHLUBA, J., MARTIN, M., MATTHES, H. W., SIMONIN, F., BEFORT, K., GAVERIAUX-RUFF, C., DIERICH, A., LEMEUR, M., VALVERDE, O., MALDONADO, R. & KIEFFER, B. L. 2000. Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat Genet,* 25**,** 195-200.

FINN, A. K. & WHISTLER, J. L. 2001. Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron,* 32**,** 829-39.

FORD, C. P., MARK, G. P. & WILLIAMS, J. T. 2006. Properties and opioid inhibition of mesolimbic dopamine neurons vary according to target location. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 26**,** 2788-97.

FOSTER-BARBER, A. & BISHOP, J. M. 1998. Src interacts with dynamin and synapsin in neuronal cells. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 4673-7.

FUCHS, P. N., ROZA, C., SORA, I., UHL, G. & RAJA, S. N. 1999. Characterization of mechanical withdrawal responses and effects of μ-, δ- and κ-opioid agonists in normal and μ-opioid receptor knockout mice. *Brain Research,* 821**,** 480-486.

FUJITA, W., GOMES, I. & DEVI, L. A. 2014. Revolution in GPCR signalling: opioid receptor heteromers as novel therapeutic targets: IUPHAR Review 10. *British journal of pharmacology,* 171**,** 4155-4176.

GABRA, B. H., BAILEY, C. P., KELLY, E., SANDERS, A. V., HENDERSON, G., SMITH, F. L. & DEWEY, W. L. 2007. Evidence for an important role of protein phosphatases in the mechanism of morphine tolerance. *Brain Research,* 1159**,** 86-93.

GAVÉRIAUX-RUFF, C., KARCHEWSKI, L. A., HEVER, X., MATIFAS, A. & KIEFFER, B. L. 2008. Inflammatory pain is enhanced in delta opioid receptor-knockout mice. *The European journal of neuroscience,* 27**,** 2558-2567.

GAVERIAUX-RUFF, C. & KIEFFER, B. L. 2011. Delta opioid receptor analgesia: recent contributions from pharmacology and molecular approaches. *Behavioural pharmacology,* 22**,** 405-14.

GAVÉRIAUX-RUFF, C., MATTHES, H. W. D., PELUSO, J. & KIEFFER, B. L. 1998. Abolition of morphine-immunosuppression in mice lacking the μ-opioid receptor gene. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 6326-6330.

GAVI, S., SHUMAY, E., WANG, H. Y. & MALBON, C. C. 2006. G-protein-coupled receptors and tyrosine kinases: crossroads in cell signaling and regulation. *Trends in endocrinology and metabolism: TEM,* 17**,** 48-54.

GENDRON, L., MITTAL, N., BEAUDRY, H. & WALWYN, W. 2015. Recent advances on the delta opioid receptor: from trafficking to function. *British journal of pharmacology,* 172**,** 403-19.

GEORGE, S. R., FAN, T., XIE, Z., TSE, R., TAM, V., VARGHESE, G. & O'DOWD, B. F. 2000. Oligomerization of μ- and δ-Opioid Receptors: GENERATION OF NOVEL FUNCTIONAL PROPERTIES. *Journal of Biological Chemistry,* 275**,** 26128-26135.

GHOZLAND, S., MATTHES, H. W. D., SIMONIN, F., FILLIOL, D., KIEFFER, B. L. & MALDONADO, R. 2002. Motivational Effects of Cannabinoids Are Mediated by μ-Opioid and κ-Opioid Receptors. *The Journal of Neuroscience,* 22**,** 1146-1154.

GLASS, M. J., BILLINGTON, C. J. & LEVINE, A. S. 1999. Opioids and food intake: distributed functional neural pathways? *Neuropeptides,* 33**,** 360-8.

GLUCK, L., LOKTEV, A., MOULEDOUS, L., MOLLEREAU, C., LAW, P. Y. & SCHULZ, S. 2014. Loss of morphine reward and dependence in mice lacking G protein-coupled receptor kinase 5. *Biological psychiatry,* 76**,** 767-74.

GOMES, I., GUPTA, A., FILIPOVSKA, J., SZETO, H. H., PINTAR, J. E. & DEVI, L. A. 2004. A role for heterodimerization of μ and δ opiate receptors in enhancing morphine analgesia. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 5135-5139.

GRANIER, S., MANGLIK, A., KRUSE, A. C., KOBILKA, T. S., THIAN, F. S., WEIS, W. I. & KOBILKA, B. K. 2012. Structure of the delta-opioid receptor bound to naltrindole. *Nature,* 485**,** 400-4.

GREGORY, N. S., HARRIS, A. L., ROBINSON, C. R., DOUGHERTY, P. M., FUCHS, P. N. & SLUKA, K. A. 2013. An overview of animal models of pain: disease models and outcome measures. *The journal of pain : official journal of the American Pain Society,* 14**,** 1255-69.

GRILLNER, P. & MERCURI, N. B. 2002. Intrinsic membrane properties and synaptic inputs regulating the firing activity of the dopamine neurons. *Behavioural brain research,* 130**,** 149-69.

GROER, C. E., TIDGEWELL, K., MOYER, R. A., HARDING, W. W., ROTHMAN, R. B., PRISINZANO, T. E. & BOHN, L. M. 2007. An opioid agonist that does not induce mu-opioid receptor--arrestin interactions or receptor internalization. *Molecular pharmacology,* 71**,** 549-57.

GUPTA, A., MULDER, J., GOMES, I., ROZENFELD, R., BUSHLIN, I., ONG, E., LIM, M., MAILLET, E., JUNEK, M., CAHILL, C. M., HARKANY, T. & DEVI, L. A. 2010. Increased abundance of opioid receptor heteromers after chronic morphine administration. *Science signaling,* 3**,** ra54.

HABERSTOCK-DEBIC, H., KIM, K.-A., YU, Y. J. & VON ZASTROW, M. 2005. Morphine Promotes Rapid, Arrestin-Dependent Endocytosis of μ-Opioid Receptors in Striatal Neurons. *The Journal of Neuroscience,* 25**,** 7847-7857.

HABERSTOCK-DEBIC, H., WEIN, M., BARROT, M., COLAGO, E. E. O., RAHMAN, Z., NEVE, R. L., PICKEL, V. M., NESTLER, E. J., VON ZASTROW, M. & SVINGOS, A. L. 2003. Morphine Acutely Regulates Opioid Receptor Trafficking Selectively in Dendrites of Nucleus Accumbens Neurons. *The Journal of Neuroscience,* 23**,** 4324-4332.

HALL, F. S., SORA, I. & UHL, G. R. 2001. Ethanol consumption and reward are decreased in µ-opiate receptor knockout mice. *Psychopharmacology,* 154**,** 43-49.

HANDAL, M., GRUNG, M., SKURTVEIT, S., RIPEL, A. & MORLAND, J. 2002. Pharmacokinetic differences of morphine and morphine-glucuronides are reflected in locomotor activity. *Pharmacology, biochemistry, and behavior,* 73**,** 883-92.

HASEGAWA, Y., KURACHI, M. & OTOMO, S. 1990. Dopamine D2 receptors and spinal cord excitation in mice. *Eur J Pharmacol,* 184**,** 207-12.

HAYWARD, M. D., HANSEN, S. T., PINTAR, J. E. & LOW, M. J. 2004. Operant self-administration of ethanol in C57BL/6 mice lacking β-endorphin and enkephalin. *Pharmacology Biochemistry and Behavior,* 79**,** 171-181.

HAYWARD, M. D., PINTAR, J. E. & LOW, M. J. 2002. Selective Reward Deficit in Mice Lacking β-Endorphin and Enkephalin. *The Journal of Neuroscience,* 22**,** 8251-8258.

HE, L., FONG, J., VON ZASTROW, M. & WHISTLER, J. L. 2002. Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell,* 108**,** 271-82.

HE, L. & WHISTLER, J. L. 2005. An opiate cocktail that reduces morphine tolerance and dependence. *Current biology : CB,* 15**,** 1028-33.

HE, S.-Q., ZHANG, Z.-N., GUAN, J.-S., LIU, H.-R., ZHAO, B., WANG, H.-B., LI, Q., YANG, H., LUO, J., LI, Z.-Y., WANG, Q., LU, Y.-J., BAO, L. & ZHANG, X. 2011. Facilitation of μ-Opioid Receptor Activity by Preventing δ-Opioid Receptor-Mediated Codegradation. *Neuron,* 69**,** 120-131.

HEINKE, B., GINGL, E. & SANDKÜHLER, J. 2011. Multiple Targets of μ-Opioid Receptor-Mediated Presynaptic Inhibition at Primary Afferent Aδ- and C-Fibers. *The Journal of Neuroscience,* 31**,** 1313-1322.

HNASKO, T. S., HJELMSTAD, G. O., FIELDS, H. L. & EDWARDS, R. H. 2012. Ventral tegmental area glutamate neurons: electrophysiological properties and projections. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 32**,** 15076-85.

HNASKO, T. S., SOTAK, B. N. & PALMITER, R. D. 2005. Morphine reward in dopamine-deficient mice. *Nature,* 438**,** 854-7.

HONG, M. H., XU, C., WANG, Y. J., JI, J. L., TAO, Y. M., XU, X. J., CHEN, J., XIE, X., CHI, Z. Q. & LIU, J. G. 2009. Role of Src in ligand-specific regulation of delta-opioid receptor desensitization and internalization. *Journal of neurochemistry,* 108**,** 102-14.

HUANG, J., SUN, Y. & HUANG, X. Y. 2004. Distinct roles for Src tyrosine kinase in beta2-adrenergic receptor signaling to MAPK and in receptor internalization. *The Journal of biological chemistry,* 279**,** 21637-42.

HUANG, W., MANGLIK, A., VENKATAKRISHNAN, A. J., LAEREMANS, T., FEINBERG, E. N., SANBORN, A. L., KATO, H. E., LIVINGSTON, K. E., THORSEN, T. S., KLING, R. C., GRANIER, S., GMEINER, P., HUSBANDS, S. M., TRAYNOR, J. R., WEIS, W. I., STEYAERT, J., DROR, R. O. & KOBILKA, B. K. 2015. Structural insights into μ-opioid receptor activation. *Nature,* 524**,** 315-321.

HUGHES, J., SMITH, T. W., KOSTERLITZ, H. W., FOTHERGILL, L. A., MORGAN, B. A. & MORRIS, H. R. 1975. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature,* 258**,** 577-80.

HUI, S. C., SEVILLA, E. L. & OGLE, C. W. 1993. 5-HT3 antagonists reduce morphine self-administration in rats. *British journal of pharmacology,* 110**,** 1341-6.

HUI, S. C., SEVILLA, E. L. & OGLE, C. W. 1996. Prevention by the 5-HT3 receptor antagonist, ondansetron, of morphine-dependence and tolerance in the rat. *British journal of pharmacology,* 118**,** 1044-50.

HULL, L. C., GABRA, B. H., BAILEY, C. P., HENDERSON, G. & DEWEY, W. L. 2013. Reversal of morphine analgesic tolerance by ethanol in the mouse. *The Journal of pharmacology and experimental therapeutics,* 345**,** 512-9.

HULL, L. C., LLORENTE, J., GABRA, B. H., SMITH, F. L., KELLY, E., BAILEY, C., HENDERSON, G. & DEWEY, W. L. 2010. The effect of protein kinase C and G protein-coupled receptor kinase inhibition on tolerance induced by mu-opioid agonists of different efficacy. *The Journal of pharmacology and experimental therapeutics,* 332**,** 1127-35.

IASP. 2014. *IASP Taxonomy* [Online]. IASP. Available: http://www.iasp-pain.org/Taxonomy [Accessed September 2015.

IRWIN, S., BENNETT, D. R., HENDERSHOT, L. C., SEEVERS, M. H. & HOUDE, R. W. 1951. THE EFFECTS OF MORPHINE, METHADONE AND MEPERIDINE ON SOME REFLEX RESPONSES OF SPINAL ANIMALS TO NOCICEPTIVE STIMULATION. *Journal of Pharmacology and Experimental Therapeutics,* 101**,** 132-143.

JAY, M. 2012. *High Society: Mind-Altering Drugs in History and Culture,* London, Thames and Hudson.

JOHNSON, E. A., OLDFIELD, S., BRAKSATOR, E., GONZALEZ-CUELLO, A., COUCH, D., HALL, K. J., MUNDELL, S. J., BAILEY, C. P., KELLY, E. & HENDERSON, G. 2006. Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. *Molecular pharmacology,* 70**,** 676-85.

JOHNSON, S. W. & NORTH, R. A. 1992a. Opioids excite dopamine neurons by hyperpolarization of local interneurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 12**,** 483-8.

JOHNSON, S. W. & NORTH, R. A. 1992b. Two types of neurone in the rat ventral tegmental area and their synaptic inputs. *The Journal of physiology,* 450**,** 455-68.

JORDAN, B. A. & DEVI, L. A. 1999. G-protein-coupled receptor heterodimerization modulates receptor function. *Nature,* 399**,** 697-700.

KAMATH, A. V., WANG, J., LEE, F. Y. & MARATHE, P. H. 2008. Preclinical pharmacokinetics and in vitro metabolism of dasatinib (BMS-354825): a potent oral multi-targeted kinase inhibitor against SRC and BCR-ABL. *Cancer Chemother Pharmacol,* 61**,** 365-76.

KARAMAN, M. W., HERRGARD, S., TREIBER, D. K., GALLANT, P., ATTERIDGE, C. E., CAMPBELL, B. T., CHAN, K. W., CICERI, P., DAVIS, M. I., EDEEN, P. T., FARAONI, R., FLOYD, M., HUNT, J. P., LOCKHART, D. J., MILANOV, Z. V., MORRISON, M. J., PALLARES, G., PATEL, H. K., PRITCHARD, S., WODICKA, L. M. & ZARRINKAR, P. P. 2008. A quantitative analysis of kinase inhibitor selectivity. *Nature biotechnology,* 26**,** 127-32.

KAS, M. J., VAN DEN BOS, R., BAARS, A. M., LUBBERS, M., LESSCHER, H. M., HILLEBRAND, J. J., SCHULLER, A. G., PINTAR, J. E. & SPRUIJT, B. M. 2004. Mu-opioid receptor knockout mice show diminished food-anticipatory activity. *The European journal of neuroscience,* 20**,** 1624-32.

KEENAN, S., LEWIS, P. A., WETHERILL, S. J., DUNNING, C. J. & EVANS, G. J. 2015. The N2-Src neuronal splice variant of C-Src has altered SH3 domain ligand specificity and a higher constitutive activity than N1-Src. *FEBS letters,* 589**,** 1995-2000.

KEITH, D. E., ANTON, B., MURRAY, S. R., ZAKI, P. A., CHU, P. C., LISSIN, D. V., MONTEILLET-AGIUS, G., STEWART, P. L., EVANS, C. J. & VON ZASTROW, M. 1998. mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Molecular pharmacology,* 53**,** 377-84.

KEITH, D. E., MURRAY, S. R., ZAKI, P. A., CHU, P. C., LISSIN, D. V., KANG, L., EVANS, C. J. & VON ZASTROW, M. 1996. Morphine activates opioid receptors without causing their rapid internalization. *The Journal of biological chemistry,* 271**,** 19021-4.

KELLEY, A. E., STINUS, L. & IVERSEN, S. D. 1980. Interactions between D-ala-met-enkephalin, A10 dopaminergic neurones, and spontaneous behaviour in the rat. *Behavioural brain research,* 1**,** 3-24.

KELLY, E., BAILEY, C. P. & HENDERSON, G. 2008. Agonist-selective mechanisms of GPCR desensitization. *British journal of pharmacology,* 153 Suppl 1**,** S379-88.

KENAKIN, T. & MILLER, L. J. 2010. Seven Transmembrane Receptors as Shapeshifting Proteins: The Impact of Allosteric Modulation and Functional Selectivity on New Drug Discovery. *Pharmacological Reviews,* 62**,** 265-304.

KEST, B., HOPKINS, E., PALMESE, C. A., ADLER, M. & MOGIL, J. S. 2002. Genetic variation in morphine analgesic tolerance: a survey of 11 inbred mouse strains. *Pharmacology, biochemistry, and behavior,* 73**,** 821-8.

KEST, B., PALMESE, C. & HOPKINS, E. 2000. A comparison of morphine analgesic tolerance in male and female mice. *Brain Res,* 879**,** 17-22.

KEST, B., WILSON, S. G. & MOGIL, J. S. 1999. Sex differences in supraspinal morphine analgesia are dependent on genotype. *The Journal of pharmacology and experimental therapeutics,* 289**,** 1370-5.

KIEFFER, B. L. 1999. Opioids: first lessons from knock out mice. *Trends in pharmacological sciences,* 20**,** 19-26.

KIEFFER, B. L., BEFORT, K., GAVERIAUX-RUFF, C. & HIRTH, C. G. 1992. The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proceedings of the National Academy of Sciences of the United States of America,* 89**,** 12048-12052.

KIM, J. A., BARTLETT, S., HE, L., NIELSEN, C. K., CHANG, A. M., KHARAZIA, V., WALDHOER, M., OU, C. J., TAYLOR, S., FERWERDA, M., CADO, D. & WHISTLER, J. L. 2008. Morphine-induced endocytosis of the mu opioid receptor in a novel knock in mouse enhances analgesia and reduces tolerance and dependence. *Current biology : CB,* 18**,** 129-135.

KIM, K. M., BARATTA, M. V., YANG, A., LEE, D., BOYDEN, E. S. & FIORILLO, C. D. 2012. Optogenetic mimicry of the transient activation of dopamine neurons by natural reward is sufficient for operant reinforcement. *PloS one,* 7**,** 10.

KITCHEN, I., SLOWE, S. J., MATTHES, H. W. D. & KIEFFER, B. 1997. Quantitative autoradiographic mapping of μ-, δ- and κ-opioid receptors in knockout mice lacking the μ-opioid receptor gene. *Brain Research,* 778**,** 73-88.

KNAPMAN, A. & CONNOR, M. 2015. Cellular signalling of non-synonymous single-nucleotide polymorphisms of the human mu-opioid receptor (OPRM1). *British journal of pharmacology,* 172**,** 349-63.

KNAPMAN, A., SANTIAGO, M. & CONNOR, M. 2015. A6V polymorphism of the human mu-opioid receptor decreases signalling of morphine and endogenous opioids in vitro. *British journal of pharmacology,* 172**,** 2258-72.

KOCH, T., WIDERA, A., BARTZSCH, K., SCHULZ, S., BRANDENBURG, L.-O., WUNDRACK, N., BEYER, A., GRECKSCH, G. & HÖLLT, V. 2005. Receptor

Endocytosis Counteracts the Development of Opioid Tolerance. *Molecular pharmacology,* 67**,** 280-287.

KONG, H., RAYNOR, K., YANO, H., TAKEDA, J., BELL, G. I. & REISINE, T. 1994. Agonists and antagonists bind to different domains of the cloned kappa opioid receptor. *Proceedings of the National Academy of Sciences of the United States of America,* 91**,** 8042-8046.

KONIG, M., ZIMMER, A. M., STEINER, H., HOLMES, P. V., CRAWLEY, J. N., BROWNSTEIN, M. J. & ZIMMER, A. 1996. Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. *Nature,* 383**,** 535-538.

KOOB, G. F., ARENDS, M. A. & MOAL, M. L. 2014. *Drugs, Addiction and the Brain,* Oxford, Elsevier.

KOOB, G. F. & BLOOM, F. E. 1988. Cellular and molecular mechanisms of drug dependence. *Science,* 242**,** 715-23.

KOOB, G. F., SANNA, P. P. & BLOOM, F. E. 1998. Neuroscience of addiction. *Neuron,* 21**,** 467-76.

KOROSTYNSKI, M., KAMINSKA-CHOWANIEC, D., PIECHOTA, M. & PRZEWLOCKI, R. 2006. Gene expression profiling in the striatum of inbred mouse strains with distinct opioid-related phenotypes. *BMC Genomics,* 7**,** 146.

KOROTKOVA, T. M., PONOMARENKO, A. A., BROWN, R. E. & HAAS, H. L. 2004. Functional diversity of ventral midbrain dopamine and GABAergic neurons. *Mol Neurobiol,* 29**,** 243-59.

KRAMER, H. K., ANDRIA, M. L., ESPOSITO, D. H. & SIMON, E. J. 2000a. Tyrosine phosphorylation of the delta-opioid receptor. Evidence for its role in mitogen-activated protein kinase activation and receptor internalization*. *Biochem Pharmacol,* 60**,** 781-92.

KRAMER, H. K., ANDRIA, M. L., KUSHNER, S. A., ESPOSITO, D. H., HILLER, J. M. & SIMON, E. J. 2000b. Mutation of tyrosine 318 (Y318F) in the delta-opioid receptor attenuates tyrosine phosphorylation, agonist-dependent receptor internalization, and mitogen-activated protein kinase activation. *Brain Res Mol Brain Res,* 79**,** 55-66.

KUHAR, J. R., BEDINI, A., MELIEF, E. J., CHIU, Y. C., STRIEGEL, H. N. & CHAVKIN, C. 2015. Mu opioid receptor stimulation activates c-Jun N-terminal kinase 2 by distinct arrestin-dependent and independent mechanisms. *Cellular signalling,* 27**,** 1799-806.

KUO, Y.-F., RAJI, M. A., CHEN, N.-W., HASAN, H. & GOODWIN, J. S. 2015. Trends in Opioid Prescriptions Among Part D Medicare Recipients From 2007 to 2012. *The American Journal of Medicine*.

KYPTA, R. M., GOLDBERG, Y., ULUG, E. T. & COURTNEIDGE, S. A. 1990. Association between the PDGF receptor and members of the src family of tyrosine kinases. *Cell,* 62**,** 481-92.

LACEY, M. G., MERCURI, N. B. & NORTH, R. A. 1989. Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 9**,** 1233-41.

LAGAS, J. S., VAN WATERSCHOOT, R. A., VAN TILBURG, V. A., HILLEBRAND, M. J., LANKHEET, N., ROSING, H., BEIJNEN, J. H. & SCHINKEL, A. H. 2009. Brain accumulation of dasatinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by elacridar treatment. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 15**,** 2344-51.

LAM, H., MAGA, M., PRADHAN, A., EVANS, C. J., MAIDMENT, N. T., HALES, T. G. & WALWYN, W. 2011. Analgesic tone conferred by constitutively active mu opioid receptors in mice lacking beta-arrestin 2. *Molecular pain,* 7**,** 24.

LAMMEL, S., ION, D. I., ROEPER, J. & MALENKA, R. C. 2011. Projection-specific modulation of dopamine neuron synapses by aversive and rewarding stimuli. *Neuron,* 70**,** 855-62.

LAMMEL, S., LIM, B. K. & MALENKA, R. C. 2014. Reward and aversion in a heterogeneous midbrain dopamine system. *Neuropharmacology,* 76 Pt B**,** 351-9.

LAMMEL, S., LIM, B. K., RAN, C., HUANG, K. W., BETLEY, M. J., TYE, K. M., DEISSEROTH, K. & MALENKA, R. C. 2012. Input-specific control of reward and aversion in the ventral tegmental area. *Nature,* 491**,** 212-7.

LAW, P.-Y., KOUHEN, O. M.-E., SOLBERG, J., WANG, W., ERICKSON, L. J. & LOH, H. H. 2000. Deltorphin II-induced Rapid Desensitization of δ-Opioid Receptor Requires Both Phosphorylation and Internalization of the Receptor. *Journal of Biological Chemistry,* 275**,** 32057-32065.

LE BARS, D., GOZARIU, M. & CADDEN, S. W. 2001. Animal models of nociception. *Pharmacological Reviews,* 53**,** 597-652.

LE MERRER, J., FAGET, L., MATIFAS, A. & KIEFFER, B. L. 2012. Cues predicting drug or food reward restore morphine-induced place conditioning in mice lacking delta opioid receptors. *Psychopharmacology,* 223**,** 99-106.

LE MERRER, J., PLAZA-ZABALA, A., DEL BOCA, C., MATIFAS, A., MALDONADO, R. & KIEFFER, B. L. 2011. Deletion of the delta opioid receptor gene impairs place conditioning but preserves morphine reinforcement. *Biological psychiatry,* 69**,** 700-3.

LE MERRER, J. L., BECKER, J. A. J., BEFORT, K. & KIEFFER, B. L. 2009. Reward Processing by the Opioid System in the Brain. *Physiological Reviews,* 89**,** 1379-1412.

LECCA, S., MELIS, M., LUCHICCHI, A., MUNTONI, A. L. & PISTIS, M. 2012. Inhibitory inputs from rostromedial tegmental neurons regulate spontaneous activity of midbrain dopamine cells and their responses to drugs of abuse. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology,* 37**,** 1164-76.

LEO, S., STRAETEMANS, R., D'HOOGE, R. & MEERT, T. 2008. Differences in nociceptive behavioral performance between C57BL/6J, 129S6/SvEv, B6 129 F1 and NMRI mice. *Behavioural brain research,* 190**,** 233-42.

LIANG, D. Y., LI, X. & CLARK, J. D. 2011. 5-hydroxytryptamine type 3 receptor modulates opioid-induced hyperalgesia and tolerance in mice. *Anesthesiology,* 114**,** 1180-9.

LIU, J.-G. & PRATHER, P. L. 2001. Chronic Exposure to μ-Opioid Agonists Produces Constitutive Activation of μ-Opioid Receptors in Direct Proportion to the Efficacy of the Agonist Used for Pretreatment. *Molecular pharmacology,* 60**,** 53-62.

LIU, J.-G., RUCKLE, M. B. & PRATHER, P. L. 2001. Constitutively active mu-opioid receptors inhibit adenylyl cyclase activity in intact cells and activate G-proteins differently than the agonist [D-Ala[super2},N-MePhe4,Gly-ol5]enkephalin. *Journal of Biological Chemistry*.

LLORENTE, J., LOWE, J. D., SANDERSON, H. S., TSISANOVA, E., KELLY, E., HENDERSON, G. & BAILEY, C. P. 2012. mu-Opioid receptor desensitization: homologous or heterologous? *The European journal of neuroscience,* 36**,** 3636-42.

LLORENTE, J., WITHEY, S., RIVERO, G., CUNNINGHAM, M., COOKE, A., SAXENA, K., MCPHERSON, J., OLDFIELD, S., DEWEY, W. L., BAILEY, C. P., KELLY, E. & HENDERSON, G. 2013. Ethanol Reversal of Cellular Tolerance to

Morphine in Rat Locus Coeruleus Neurons. *Molecular pharmacology,* 84**,** 252-260.

LOH, H. H., LIU, H. C., CAVALLI, A., YANG, W., CHEN, Y. F. & WEI, L. N. 1998. mu Opioid receptor knockout in mice: effects on ligand-induced analgesia and morphine lethality. *Brain Res Mol Brain Res,* 54**,** 321-6.

LOWE, J. D. & BAILEY, C. P. 2015. Functional selectivity and time-dependence of mu-opioid receptor desensitization at nerve terminals in the mouse ventral tegmental area. *British journal of pharmacology,* 172**,** 469-81.

LUO, J., JING, L., QIN, W. J., ZHANG, M., LAWRENCE, A. J., CHEN, F. & LIANG, J. H. 2011. Transcription and protein synthesis inhibitors reduce the induction of behavioural sensitization to a single morphine exposure and regulate Hsp70 expression in the mouse nucleus accumbens. *Int J Neuropsychopharmacol,* 14**,** 107-21.

LUSCHER, C. & SLESINGER, P. A. 2010. Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. *Nature reviews. Neuroscience,* 11**,** 301-15.

LUTTRELL, D. K. & LUTTRELL, L. M. 2004. Not so strange bedfellows: G-protein-coupled receptors and Src family kinases. *Oncogene,* 23**,** 7969-78.

LUTZ, P. E. & KIEFFER, B. L. 2013. The multiple facets of opioid receptor function: implications for addiction. *Current Opinion in Neurobiology,* 23**,** 473-9.

MADHAVAN, A., BONCI, A. & WHISTLER, J. L. 2010. Opioid-Induced GABA potentiation after chronic morphine attenuates the rewarding effects of opioids in the ventral tegmental area. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 30**,** 14029-35.

MAGUIRE, E. P., MACPHERSON, T., SWINNY, J. D., DIXON, C. I., HERD, M. B., BELELLI, D., STEPHENS, D. N., KING, S. L. & LAMBERT, J. J. 2014. Tonic inhibition of accumbal spiny neurons by extrasynaptic alpha4betadelta GABAA receptors modulates the actions of psychostimulants. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 34**,** 823-38.

MAHMOUD, S., THORSELL, A., SOMMER, W. H., HEILIG, M., HOLGATE, J. K., BARTLETT, S. E. & RUIZ-VELASCO, V. 2011. Pharmacological Consequence of the A118G Mu Opioid Receptor Polymorphism on Morphine- and Fentanyl-mediated Modulation of Ca(2+) Channels in Humanized Mouse Sensory Neurons. *Anesthesiology,* 115**,** 1054-1062.

MALDONADO, R., SAIARDI, A., VALVERDE, O., SAMAD, T. A., ROQUES, B. P. & BORRELLI, E. 1997. Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature,* 388**,** 586-9.

MANGLIK, A., KRUSE, A. C., KOBILKA, T. S., THIAN, F. S., MATHIESEN, J. M., SUNAHARA, R. K., PARDO, L., WEIS, W. I., KOBILKA, B. K. & GRANIER, S. 2012. Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. *Nature,* 485**,** 321-326.

MARGOLIS, E. B., LOCK, H., HJELMSTAD, G. O. & FIELDS, H. L. 2006. The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? *The Journal of physiology,* 577**,** 907-24.

MARGOLIS, E. B., TOY, B., HIMMELS, P., MORALES, M. & FIELDS, H. L. 2012. Identification of rat ventral tegmental area GABAergic neurons. *PloS one,* 7**,** 31.

MARTIN, M., MATIFAS, A., MALDONADO, R. & KIEFFER, B. L. 2003. Acute antinociceptive responses in single and combinatorial opioid receptor knockout mice: distinct mu, delta and kappa tones. *European Journal of Neuroscience,* 17**,** 701-708.

MATSUI, A., JARVIE, B. C., ROBINSON, B. G., HENTGES, S. T. & WILLIAMS, J. T. 2014. Separate GABA afferents to dopamine neurons mediate acute action of opioids, development of tolerance, and expression of withdrawal. *Neuron,* 82**,** 1346-56.

MATSUI, A. & WILLIAMS, J. T. 2011. Opioid-sensitive GABA inputs from rostromedial tegmental nucleus synapse onto midbrain dopamine neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 31**,** 17729-35.

MATTHES, H. W., MALDONADO, R., SIMONIN, F., VALVERDE, O., SLOWE, S., KITCHEN, I., BEFORT, K., DIERICH, A., LE MEUR, M., DOLLE, P., TZAVARA, E., HANOUNE, J., ROQUES, B. P. & KIEFFER, B. L. 1996. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature,* 383**,** 819-23.

MATTHES, H. W. D., SMADJA, C., VALVERDE, O., VONESCH, J.-L., FOUTZ, A. S., BOUDINOT, E., DENAVIT-SAUBIÉ, M., SEVERINI, C., NEGRI, L., ROQUES, B. P., MALDONADO, R. & KIEFFER, B. L. 1998. Activity of the δ-Opioid Receptor Is Partially Reduced, Whereas Activity of the κ-Receptor Is Maintained in Mice Lacking the μ-Receptor. *The Journal of Neuroscience,* 18**,** 7285-7295.

MCBRIDE, W. J., MURPHY, J. M. & IKEMOTO, S. 1999. Localization of brain reinforcement mechanisms: intracranial self-administration and intracranial place-conditioning studies. *Behavioural brain research,* 101**,** 129-52.

MCDONALD, J. & LAMBERT, D. 2005. Opioid receptors. *Continuing Education in Anaesthesia, Critical Care & Pain,* 5**,** 22-25.

MENG, F., XIE, G. X., THOMPSON, R. C., MANSOUR, A., GOLDSTEIN, A., WATSON, S. J. & AKIL, H. 1993. Cloning and pharmacological characterization of a rat kappa opioid receptor. *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 9954-9958.

MERCADANTE, S. & BRUERA, E. 2006. Opioid switching: a systematic and critical review. *Cancer Treat Rev,* 32**,** 304-15.

MERKOURIS, M., MULLANEY, I., GEORGOUSSI, Z. & MILLIGAN, G. 1997. Regulation of Spontaneous Activity of the δ-Opioid Receptor: Studies of Inverse Agonism in Intact Cells. *Journal of neurochemistry,* 69**,** 2115-2122.

MEUNIER, J.-C., MOLLEREAU, C., TOLL, L., SUAUDEAU, C., MOISAND, C., ALVINERIE, P., BUTOUR, J.-L., GUILLEMOT, J.-C., FERRARA, P., MONSARRAT, B., MAZARGUIL, H., VASSART, G., PARMENTIER, M. & COSTENTIN, J. 1995. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature,* 377**,** 532-535.

MEYE, F. J., VAN ZESSEN, R., SMIDT, M. P., ADAN, R. A. & RAMAKERS, G. M. 2012. Morphine withdrawal enhances constitutive mu-opioid receptor activity in the ventral tegmental area. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 32**,** 16120-8.

MILAN-LOBO, L., ENQUIST, J., VAN RIJN, R. M. & WHISTLER, J. L. 2013. Anti-analgesic effect of the mu/delta opioid receptor heteromer revealed by ligand-biased antagonism. *PloS one,* 8**,** e58362.

MILAN-LOBO, L. & WHISTLER, J. L. 2011. Heteromerization of the mu- and delta-opioid receptors produces ligand-biased antagonism and alters mu-receptor trafficking. *The Journal of pharmacology and experimental therapeutics,* 337**,** 868-75.

MINAMI, M., TOYA, T., KATAO, Y., MAEKAWA, K., NAKAMURA, S., ONOGI, T., KANEKO, S. & SATOH, M. 1993. Cloning and expression of a cDNA for the rat k-opioid receptor. *FEBS letters,* 329**,** 291-295.

MOGIL, J. S. 2009. Animal models of pain: progress and challenges. *Nature reviews. Neuroscience,* 10**,** 283-94.

MOGIL, J. S. & CHANDA, M. L. 2005. The case for the inclusion of female subjects in basic science studies of pain. *Pain,* 117**,** 1-5.

MOGIL, J. S., WILSON, S. G., BON, K., EUN LEE, S., CHUNG, K., RABER, P., PIEPER, J. O., HAIN, H. S., BELKNAP, J. K., HUBERT, L., ELMER, G. I., MO CHUNG, J. & DEVOR, M. 1999. Heritability of nociception I: Responses of 11 inbred mouse strains on 12 measures of nociception. *Pain,* 80**,** 67-82.

MOLES, A., KIEFFER, B. L. & D'AMATO, F. R. 2004. Deficit in attachment behavior in mice lacking the mu-opioid receptor gene. *Science,* 304**,** 1983-6.

MOLLEREAU, C., PARMENTIER, M., MAILLEUX, P., BUTOUR, J.-L., MOISAND, C., CHALON, P., CAPUT, D., VASSART, G. & MEUNIER, J.-C. 1994. ORL1, a novel member of the opioid receptor family: Cloning, functional expression and localization. *FEBS letters,* 341**,** 33-38.

MURPHY, N. P., LAM, H. A. & MAIDMENT, N. T. 2001. A comparison of morphine-induced locomotor activity and mesolimbic dopamine release in C57BL6, 129Sv and DBA2 mice. *Journal of neurochemistry,* 79**,** 626-35.

NADAL, X., BAÑOS, J.-E., KIEFFER, B. L. & MALDONADO, R. 2006. Neuropathic pain is enhanced in δ-opioid receptor knockout mice. *European Journal of Neuroscience,* 23**,** 830-834.

NESTLER, E. J. 1992. Molecular mechanisms of drug addiction. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 12**,** 2439-50.

NIIKURA, K., NARITA, M., BUTELMAN, E. R., KREEK, M. J. & SUZUKI, T. 2010. Neuropathic and chronic pain stimuli downregulate central mu-opioid and dopaminergic transmission. *Trends in pharmacological sciences,* 31**,** 299-305.

OBER, W. B. 1968. Drowsed with the fume of poppies: Opium and John Keats. *Bull. N. Y. Acad. Med.,* 44**,** 862-881.

OBLADEN, M. 2015. Lethal Lullabies: A History of Opium Use in Infants. *Journal of human lactation : official journal of International Lactation Consultant Association*.

OLMSTEAD, M. C., OUAGAZZAL, A.-M. & KIEFFER, B. L. 2009. Mu and Delta Opioid Receptors Oppositely Regulate Motor Impulsivity in the Signaled Nose Poke Task. *PloS one,* 4**,** e4410.

OSSIPOV, M. H., LAI, J., KING, T., VANDERAH, T. W., MALAN, T. P., JR., HRUBY, V. J. & PORRECA, F. 2004. Antinociceptive and nociceptive actions of opioids. *J Neurobiol,* 61**,** 126-48.

OZAKI, S., NARITA, M., IINO, M., MIYOSHI, K. & SUZUKI, T. 2003. Suppression of the morphine-induced rewarding effect and G-protein activation in the lower midbrain following nerve injury in the mouse: involvement of G-protein-coupled receptor kinase 2. *Neuroscience,* 116**,** 89-97.

PAPALEO, F., KIEFFER, B. L., TABARIN, A. & CONTARINO, A. 2007. Decreased motivation to eat in mu-opioid receptor-deficient mice. *The European journal of neuroscience,* 25**,** 3398-405.

PASTERNAK, G. W. 2014a. Opiate pharmacology and relief of pain. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* 32**,** 1655-61.

PASTERNAK, G. W. 2014b. Opioids and their receptors: Are we there yet? *Neuropharmacology,* 76**,** 198-203.

PASTERNAK, G. W. & PAN, Y. X. 2013. Mu opioids and their receptors: evolution of a concept. *Pharmacological Reviews,* 65**,** 1257-317.

PAXINOS, G. & FRANKLIN, K. B. J. 2013. *The Mouse Brain in Stereotaxic Coordinates,* London, Elsevier.

PEPPIN, J. F. & RAFFA, R. B. 2015. Delta opioid agonists: a concise update on potential therapeutic applications. *J Clin Pharm Ther,* 40**,** 155-66.

PERT, C. B. & SNYDER, S. H. 1973. Opiate receptor: demonstration in nervous tissue. *Science,* 179**,** 1011-4.

PETÄJÄ-REPO, U. E., HOGUE, M., LAPERRIÈRE, A., WALKER, P. & BOUVIER, M. 2000. Export from the Endoplasmic Reticulum Represents the Limiting Step in the Maturation and Cell Surface Expression of the Human δ Opioid Receptor. *Journal of Biological Chemistry,* 275**,** 13727-13736.

PIERCE, K. L. & LEFKOWITZ, R. J. 2001. CLASSICAL AND NEW ROLES OF β-ARRESTINS IN THE REGULATION OF G-PROTEIN-COUPLED RECEPTORS. *Nature reviews. Neuroscience,* 2**,** 727-733.

PORKKA, K., KOSKENVESA, P., LUNDAN, T., RIMPILAINEN, J., MUSTJOKI, S., SMYKLA, R., WILD, R., LUO, R., ARNAN, M., BRETHON, B., ECCERSLEY, L., HJORTH-HANSEN, H., HOGLUND, M., KLAMOVA, H., KNUTSEN, H., PARIKH, S., RAFFOUX, E., GRUBER, F., BRITO-BABAPULLE, F., DOMBRET, H., DUARTE, R. F., ELONEN, E., PAQUETTE, R., ZWAAN, C. M. & LEE, F. Y. 2008. Dasatinib crosses the blood-brain barrier and is an efficient therapy for central nervous system Philadelphia chromosome-positive leukemia. *Blood,* 112**,** 1005-12.

POWELL, K. J., ABUL-HUSN, N. S., JHAMANDAS, A., OLMSTEAD, M. C., BENINGER, R. J. & JHAMANDAS, K. 2002. Paradoxical effects of the opioid antagonist naltrexone on morphine analgesia, tolerance, and reward in rats. *The Journal of pharmacology and experimental therapeutics,* 300**,** 588-96.

PRADHAN, A., SMITH, M., MCGUIRE, B., EVANS, C. & WALWYN, W. 2013. Chronic inflammatory injury results in increased coupling of delta opioid receptors to voltage-gated Ca2+ channels. *Molecular pain,* 9**,** 1744-8069.

RAEHAL, K. M. & BOHN, L. M. 2011. The role of beta-arrestin2 in the severity of antinociceptive tolerance and physical dependence induced by different opioid pain therapeutics. *Neuropharmacology,* 60**,** 58-65.

RAEHAL, K. M., SCHMID, C. L., MEDVEDEV, I. O., GAINETDINOV, R. R., PREMONT, R. T. & BOHN, L. M. 2009. Morphine-induced physiological and behavioral responses in mice lacking G protein-coupled receptor kinase 6. *Drug and Alcohol Dependence,* 104**,** 187-96.

RAEHAL, K. M., WALKER, J. K. & BOHN, L. M. 2005. Morphine side effects in beta-arrestin 2 knockout mice. *The Journal of pharmacology and experimental therapeutics,* 314**,** 1195-201.

RAGNAUTH, A., SCHULLER, A., MORGAN, M., CHAN, J., OGAWA, S., PINTAR, J., BODNAR, R. J. & PFAFF, D. W. 2001. Female preproenkephalin-knockout mice display altered emotional responses. *Proceedings of the National Academy of Sciences of the United States of America,* 98**,** 1958-1963.

RANG, H. P., DALE, M. M. & RITTER, J. M. 2000. *Pharmacology.* Four ed. London: Churchill Livingstone.

RAVINDRANATHAN, A., JOSLYN, G., ROBERTSON, M., SCHUCKIT, M. A., WHISTLER, J. L. & WHITE, R. L. 2009. Functional characterization of human variants of the mu-opioid receptor gene. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 10811-10816.

REITER, E., AHN, S., SHUKLA, A. K. & LEFKOWITZ, R. J. 2012. Molecular mechanism of beta-arrestin-biased agonism at seven-transmembrane receptors. *Annual review of pharmacology and toxicology,* 52**,** 179-97.

REITER, E. & LEFKOWITZ, R. J. 2006. GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends in endocrinology and metabolism: TEM,* 17**,** 159-65.

ROBERTS, A. J., GOLD, L. H., POLIS, I., MCDONALD, J. S., FILLIOL, D., KIEFFER, B. L. & KOOB, G. F. 2001. Increased Ethanol Self-Administration in δ-Opioid Receptor Knockout Mice. *Alcoholism: Clinical and Experimental Research,* 25**,** 1249-1256.

ROBERTS, A. J., MCDONALD, J. S., HEYSER, C. J., KIEFFER, B. L., MATTHES, H. W. D., KOOB, G. F. & GOLD, L. H. 2000. μ-Opioid Receptor Knockout Mice Do Not Self-Administer Alcohol. *Journal of Pharmacology and Experimental Therapeutics,* 293**,** 1002-1008.

ROBINSON, J. E., VARDY, E., DIBERTO, J. F., CHEFER, V. I., WHITE, K. L., FISH, E. W., CHEN, M., GIGANTE, E., KROUSE, M. C., SUN, H., THORSELL, A., ROTH, B. L., HEILIG, M. & MALANGA, C. J. 2015. Receptor Reserve Moderates Mesolimbic Responses to Opioids in a Humanized Mouse Model of the OPRM1 A118G Polymorphism. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*.

ROBINSON, T. E. & BERRIDGE, K. C. 2000. The psychology and neurobiology of addiction: an incentive-sensitization view. *Addiction,* 95**,** S91-117.

RODD, Z. A., GRYSZOWKA, V. E., TOALSTON, J. E., OSTER, S. M., JI, D., BELL, R. L. & MCBRIDE, W. J. 2007. The reinforcing actions of a serotonin-3 receptor agonist within the ventral tegmental area: evidence for subregional and genetic differences and involvement of dopamine neurons. *The Journal of pharmacology and experimental therapeutics,* 321**,** 1003-12.

ROMMELSPACHER, H., SMOLKA, M., SCHMIDT, L. G., SAMOCHOWIEC, J. & HOEHE, M. R. 2001. Genetic analysis of the mu-opioid receptor in alcohol-dependent individuals. *Alcohol,* 24**,** 129-35.

ROSKOSKI JR, R. 2015. Src protein-tyrosine kinase structure, mechanism, and small molecule inhibitors. *Pharmacological Research,* 94**,** 9-25.

ROZENFELD, R. & DEVI, L. A. 2007. Receptor heterodimerization leads to a switch in signaling: beta-arrestin2-mediated ERK activation by mu-delta opioid receptor heterodimers. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 21**,** 2455-65.

RUBINSTEIN, M., MOGIL, J. S., JAPÓN, M., CHAN, E. C., ALLEN, R. G. & LOW, M. J. 1996. Absence of opioid stress-induced analgesia in mice lacking beta-endorphin by site-directed mutagenesis. *Proceedings of the National Academy of Sciences of the United States of America,* 93**,** 3995-4000.

RUSIN, K. I. & MOISES, H. C. 1995. mu-Opioid receptor activation reduces multiple components of high-threshold calcium current in rat sensory neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 15**,** 4315-27.

RUSSO, S. J. & NESTLER, E. J. 2013. The brain reward circuitry in mood disorders. *Nature reviews. Neuroscience,* 14**,** 609-25.

SACKS, D. B. 2006. The role of scaffold proteins in MEK/ERK signalling. *Biochem Soc Trans,* 34**,** 833-6.

SADEE, W., WANG, D. & BILSKY, E. J. 2005. Basal opioid receptor activity, neutral antagonists, and therapeutic opportunities. *Life sciences,* 76**,** 1427-37.

SALAS, E., BOCOS, C., DEL CASTILLO, C., PÉREZ-GARCÍA, C., MORALES, L. & ALGUACIL, L. F. 2011. Gene expression analysis of heat shock proteins in the nucleus accumbens of rats with different morphine seeking behaviours. *Behavioural brain research,* 225**,** 71-76.

SALTER, M. W. & KALIA, L. V. 2004. Src kinases: a hub for NMDA receptor regulation. *Nature reviews. Neuroscience,* 5**,** 317-28.

SANCHEZ-CATALAN, M. J., KAUFLING, J., GEORGES, F., VEINANTE, P. & BARROT, M. 2014. The antero-posterior heterogeneity of the ventral tegmental area. *Neuroscience,* 282**,** 198-216.

SCHIFF, P. L. 2002. Opium and Its Alkaloids. *American Journal of Pharmaceutical Education,* 66**,** 186-194.

SCHIFIRNET, E., BOWEN, S. E. & BORSZCZ, G. S. 2014. Separating analgesia from reward within the ventral tegmental area. *Neuroscience,* 263**,** 72-87.

SCHMID, C. L. & BOHN, L. M. 2009. Physiological and pharmacological implications of beta-arrestin regulation. *Pharmacology & therapeutics,* 121**,** 285-93.

SCHROEDER, J. E. & MCCLESKEY, E. W. 1993. Inhibition of Ca2+ currents by a mu-opioid in a defined subset of rat sensory neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 13**,** 867-73.

SCHULLER, A. G., KING, M. A., ZHANG, J., BOLAN, E., PAN, Y. X., MORGAN, D. J., CHANG, A., CZICK, M. E., UNTERWALD, E. M., PASTERNAK, G. W. & PINTAR, J. E. 1999. Retention of heroin and morphine-6 beta-glucuronide analgesia in a new line of mice lacking exon 1 of MOR-1. *Nature neuroscience,* 2**,** 151-6.

SCOTLAND, N. R. O. 2015. Drugs related deaths Scotland 2014, Statistics of drug-related deaths in 2014 and earlier years, broken down by age, sex, selected drugs reported, underlying cause of death and NHS Board and Council areas.

SEELIGER, M. A., NAGAR, B., FRANK, F., CAO, X., HENDERSON, M. N. & KURIYAN, J. 2007. c-Src binds to the cancer drug imatinib with an inactive Abl/c-Kit conformation and a distributed thermodynamic penalty. *Structure,* 15**,** 299-311.

SHARIFI, N., DIEHL, N., YASWEN, L., BRENNAN, M. B. & HOCHGESCHWENDER, U. 2001. Generation of dynorphin knockout mice. *Molecular Brain Research,* 86**,** 70-75.

SHARMA, M. R., WROBLEWSKI, K., POLITE, B. N., KNOST, J. A., WALLACE, J. A., MODI, S., SLECKMAN, B. G., TABER, D., VOKES, E. E., STADLER, W. M. & KINDLER, H. L. 2012. Dasatinib in previously treated metastatic colorectal cancer: a phase II trial of the University of Chicago Phase II Consortium. *Invest New Drugs,* 30**,** 1211-5.

SHENOY, S. K. & LEFKOWITZ, R. J. 2003. Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *Biochemical Journal,* 375**,** 503-515.

SHIPPENBERG, T. S. & ELMER, G. I. 1998. The neurobiology of opiate reinforcement. *Crit Rev Neurobiol,* 12**,** 267-303.

SHIPPENBERG, T. S., HERZ, A., SPANAGEL, R., BALS-KUBIK, R. & STEIN, C. 1992. Conditioning of opioid reinforcement: neuroanatomical and neurochemical substrates. *Ann N Y Acad Sci,* 654**,** 347-56.

SHOBLOCK, J. R. & MAIDMENT, N. T. 2006. Constitutively active micro opioid receptors mediate the enhanced conditioned aversive effect of naloxone in morphine-dependent mice. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology,* 31**,** 171-7.

SHOBLOCK, J. R. & MAIDMENT, N. T. 2007. Enkephalin release promotes homeostatic increases in constitutively active mu opioid receptors during morphine withdrawal. *Neuroscience,* 149**,** 642-649.

SHUKLA, A. K., XIAO, K. & LEFKOWITZ, R. J. 2011. Emerging paradigms of beta-arrestin-dependent seven transmembrane receptor signaling. *Trends in biochemical sciences,* 36**,** 457-69.

SIM-SELLEY, L. J., SELLEY, D. E., VOGT, L. J., CHILDERS, S. R. & MARTIN, T. J. 2000. Chronic heroin self-administration desensitizes mu opioid receptor-activated G-proteins in specific regions of rat brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 20**,** 4555-62.

SIMONIN, F., VALVERDE, O., SMADJA, C., SLOWE, S., KITCHEN, I., DIERICH, A., LE MEUR, M., ROQUES, B. P., MALDONADO, R. & KIEFFER, B. L. 1998. Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *The EMBO Journal,* 17**,** 886-897.

SKOUBIS, P. D., LAM, H. A., SHOBLOCK, J., NARAYANAN, S. & MAIDMENT, N. T. 2005. Endogenous enkephalins, not endorphins, modulate basal hedonic state in mice. *The European journal of neuroscience,* 21**,** 1379-84.

SKOUBIS, P. D., MATTHES, H. W., WALWYN, W. M., KIEFFER, B. L. & MAIDMENT, N. T. 2001. Naloxone fails to produce conditioned place aversion in mu-opioid receptor knock-out mice. *Neuroscience,* 106**,** 757-63.

SOERGEL, D. G., SUBACH, R. A., BURNHAM, N., LARK, M. W., JAMES, I. E., SADLER, B. M., SKOBIERANDA, F., VIOLIN, J. D. & WEBSTER, L. R. 2014. Biased agonism of the mu-opioid receptor by TRV130 increases analgesia and reduces on-target adverse effects versus morphine: A randomized, double-blind, placebo-controlled, crossover study in healthy volunteers. *Pain,* 155**,** 1829-35.

SORA, I., ELMER, G., FUNADA, M., PIEPER, J., LI, X.-F., SCOTT HALL, F. & UHL, G. R. 2001. Mu-Opiate Receptor Gene Dose Effects on Different Morphine Actions: Evidence for Differential in vivo Mu Receptor Reserve. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology,* 25**,** 41-54.

SORA, I., TAKAHASHI, N., FUNADA, M., UJIKE, H., REVAY, R. S., DONOVAN, D. M., MINER, L. L. & UHL, G. R. 1997. Opiate receptor knockout mice define μ receptor roles in endogenous nociceptive responses and morphine-

induced analgesia. *Proceedings of the National Academy of Sciences of the United States of America,* 94**,** 1544-1549.

STEIN, C., SCHÄFER, M. & MACHELSKA, H. 2000. Why is morphine not the ultimate analgesic and what can be done to improve it? *The Journal of Pain,* 1**,** 51-56.

STRANGE, P. G. 2002. Mechanisms of inverse agonism at G-protein-coupled receptors. *Trends in pharmacological sciences,* 23**,** 89-95.

STUBER, G. D., HNASKO, T. S., BRITT, J. P., EDWARDS, R. H. & BONCI, A. 2010. Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 30**,** 8229-33.

SZEKERES, P. G. & TRAYNOR, J. R. 1997. Delta Opioid Modulation of the Binding of Guanosine-5′-O-(3-[35S]thio)triphosphate to NG108–15 Cell Membranes: Characterization of Agonist and Inverse Agonist Effects. *Journal of Pharmacology and Experimental Therapeutics,* 283**,** 1276-1284.

TAN, K. R., BROWN, M., LABOUEBE, G., YVON, C., CRETON, C., FRITSCHY, J. M., RUDOLPH, U. & LUSCHER, C. 2010. Neural bases for addictive properties of benzodiazepines. *Nature,* 463**,** 769-74.

TAN, K. R., YVON, C., TURIAULT, M., MIRZABEKOV, J. J., DOEHNER, J., LABOUEBE, G., DEISSEROTH, K., TYE, K. M. & LUSCHER, C. 2012. GABA neurons of the VTA drive conditioned place aversion. *Neuron,* 73**,** 1173-83.

TANIMOTO, T., JIN, Z. G. & BERK, B. C. 2002. Transactivation of vascular endothelial growth factor (VEGF) receptor Flk-1/KDR is involved in sphingosine 1-phosphate-stimulated phosphorylation of Akt and endothelial nitric-oxide synthase (eNOS). *The Journal of biological chemistry,* 277**,** 42997-3001.

TERMAN, G. W., JIN, W., CHEONG, Y. P., LOWE, J., CARON, M. G., LEFKOWITZ, R. J. & CHAVKIN, C. 2004. G-protein receptor kinase 3 (GRK3) influences opioid analgesic tolerance but not opioid withdrawal. *British journal of pharmacology,* 141**,** 55-64.

THOMAS, S. M. & BRUGGE, J. S. 1997. Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol,* 13**,** 513-609.

THOMPSON, R. C., MANSOUR, A., AKIL, H. & WATSON, S. J. 1993. Cloning and pharmacological characterization of a rat μ opioid receptor. *Neuron,* 11**,** 903-913.

TIAN, M., BROXMEYER, H. E., FAN, Y., LAI, Z., ZHANG, S., ARONICA, S., COOPER, S., BIGSBY, R. M., STEINMETZ, R., ENGLE, S. J., MESTEK, A., POLLOCK, J. D., LEHMAN, M. N., JANSEN, H. T., YING, M., STAMBROOK, P. J., TISCHFIELD, J. A. & YU, L. 1997. Altered Hematopoiesis, Behavior, and Sexual Function in μ Opioid Receptor–deficient Mice. *The Journal of Experimental Medicine,* 185**,** 1517-1522.

TJØLSEN, A. & HOLE, K. 1993. The tail-flick latency is influenced by skin temperature. *APS Journal,* 2**,** 107-111.

TRESCOT, A., GLASER, S. E., HANSEN, H., BENYAMIN, R., PATEL, S. & MANCHIKANTI, L. 2008. Effectiveness of Opioids in the Treatment of Chronic Non-Cancer Pain. *Pain Physician,* 11**,** S181-S200.

TRIGO, J. M., MARTIN-GARCIA, E., BERRENDERO, F., ROBLEDO, P. & MALDONADO, R. 2010. The endogenous opioid system: a common substrate in drug addiction. *Drug and Alcohol Dependence,* 108**,** 183-94.

TSAI, H.-C., ZHANG, F., ADAMANTIDIS, A., STUBER, G. D., BONCI, A., DE LECEA, L. & DEISSEROTH, K. 2009. Phasic Firing in Dopaminergic Neurons Is Sufficient for Behavioral Conditioning. *Science,* 324**,** 1080-1084.

TWAMLEY, G. M., KYPTA, R. M., HALL, B. & COURTNEIDGE, S. A. 1992. Association of Fyn with the activated platelet-derived growth factor receptor: requirements for binding and phosphorylation. *Oncogene,* 7**,** 1893-901.

UHL, G. R., SORA, I. & WANG, Z. 1999. The mu opiate receptor as a candidate gene for pain: polymorphisms, variations in expression, nociception, and opiate responses. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 7752-5.

UITDEHAAG, J. C., VERKAAR, F., ALWAN, H., DE MAN, J., BUIJSMAN, R. C. & ZAMAN, G. J. 2012. A guide to picking the most selective kinase inhibitor tool compounds for pharmacological validation of drug targets. *British journal of pharmacology,* 166**,** 858-76.

UNGLESS, M. A. & GRACE, A. A. 2012. Are you or aren't you? Challenges associated with physiologically identifying dopamine neurons. *Trends in neurosciences,* 35**,** 422-30.

URBAN, J. D., CLARKE, W. P., VON ZASTROW, M., NICHOLS, D. E., KOBILKA, B., WEINSTEIN, H., JAVITCH, J. A., ROTH, B. L., CHRISTOPOULOS, A., SEXTON, P. M., MILLER, K. J., SPEDDING, M. & MAILMAN, R. B. 2007. Functional

selectivity and classical concepts of quantitative pharmacology. *The Journal of pharmacology and experimental therapeutics,* 320**,** 1-13.

URS, N. M., DAIGLE, T. L. & CARON, M. G. 2011. A dopamine D1 receptor-dependent beta-arrestin signaling complex potentially regulates morphine-induced psychomotor activation but not reward in mice. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology,* 36**,** 551-8.

VAN REE, J. M., GERRITS, M. A. F. M. & VANDERSCHUREN, L. J. M. J. 1999. Opioids, Reward and Addiction: An Encounter of biology, psychology and medicine. *Pharmacological Reviews,* 51(2)**,** 341-396.

WALDHOER, M., BARTLETT, S. E. & WHISTLER, J. L. 2004. Opioid receptors. *Annual review of biochemistry,* 73**,** 953-90.

WALTER, C. & LOTSCH, J. 2009. Meta-analysis of the relevance of the OPRM1 118A>G genetic variant for pain treatment. *Pain,* 146**,** 270-5.

WALWYN, W., EVANS, C. J. & HALES, T. G. 2007. Beta-arrestin2 and c-Src regulate the constitutive activity and recycling of mu opioid receptors in dorsal root ganglion neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 27**,** 5092-104.

WALWYN, W., JOHN, S., MAGA, M., EVANS, C. J. & HALES, T. G. 2009. Delta receptors are required for full inhibitory coupling of mu-receptors to voltage-dependent Ca(2+) channels in dorsal root ganglion neurons. *Molecular pharmacology,* 76**,** 134-43.

WALWYN, W., MAIDMENT, N. T., SANDERS, M., EVANS, C. J., KIEFFER, B. L. & HALES, T. G. 2005. Induction of delta opioid receptor function by up-regulation of membrane receptors in mouse primary afferent neurons. *Molecular pharmacology,* 68**,** 1688-98.

WALWYN, W. M., WEI, W., XIE, C. W., CHIU, K., KIEFFER, B. L., EVANS, C. J. & MAIDMENT, N. T. 2006. Mu opioid receptor-effector coupling and trafficking in dorsal root ganglia neurons. *Neuroscience,* 142**,** 493-503.

WANG, D., RAEHAL, K. M., BILSKY, E. J. & SADÉE, W. 2001. Inverse agonists and neutral antagonists at μ opioid receptor (MOR): possible role of basal receptor signaling in narcotic dependence. *Journal of neurochemistry,* 77**,** 1590-1600.

WANG, D., RAEHAL, K. M., LIN, E. T., LOWERY, J. J., KIEFFER, B. L., BILSKY, E. J. & SADEE, W. 2004. Basal signaling activity of mu opioid receptor in mouse

brain: role in narcotic dependence. *The Journal of pharmacology and experimental therapeutics,* 308**,** 512-20.

WANG, J. B., IMAI, Y., EPPLER, C. M., GREGOR, P., SPIVAK, C. E. & UHL, G. R. 1993. mu opiate receptor: cDNA cloning and expression. *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 10230-10234.

WANG, Y., BARKER, K., SHI, S., DIAZ, M., MO, B. & GUTSTEIN, H. B. 2012. Blockade of PDGFR-beta activation eliminates morphine analgesic tolerance. *Nature medicine,* 18**,** 385-7.

WATARI, K., NAKAYA, M. & KUROSE, H. 2014. Multiple functions of G protein-coupled receptor kinases. *Journal of Molecular Signaling,* 9**,** 1-1.

WAY, E. L., KEMP, J. W., YOUNG, J. M. & GRASSETTI, D. R. 1960. THE PHARMACOLOGIC EFFECTS OF HEROIN IX RELATIONSHIP TO ITS RATE OF BIOTRANSFORMATION. *Journal of Pharmacology and Experimental Therapeutics,* 129**,** 144-154.

WAY, E. L., LOH, H. H. & SHEN, F. H. 1969. Simultaneous quantitative assessment of morphine tolerance and physical dependence. *The Journal of pharmacology and experimental therapeutics,* 167**,** 1-8.

WEISBERG, D. F., BECKER, W. C., FIELLIN, D. A. & STANNARD, C. 2014. Prescription opioid misuse in the United States and the United Kingdom: Cautionary lessons. *International Journal of Drug Policy,* 25**,** 1124-1130.

WHISTLER, J. L., CHUANG, H. H., CHU, P., JAN, L. Y. & VON ZASTROW, M. 1999. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron,* 23**,** 737-46.

WHISTLER, J. L. & VON ZASTROW, M. 1998. Morphine-activated opioid receptors elude desensitization by β-arrestin. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 9914-9919.

WHO. 2015. *The ICD-10 Classification of Mental and Behavioural Disorders: Clinical descriptions and diagnostic guidelines* [Online]. WHO. Available: http://www.who.int/substance_abuse/terminology/ICD10ClinicalDiagnosis.pdf [Accessed September 2015].

WILLIAMS, J., CHRISTIE, M. J. & MANZONI, O. J. 2001. Cellular and synaptic adaptations mediating opioid dependence. *Physiological Reviews,* 81**,** 299-343.

WILLIAMS, J. T., INGRAM, S. L., HENDERSON, G., CHAVKIN, C., VON ZASTROW, M., SCHULZ, S., KOCH, T., EVANS, C. J. & CHRISTIE, M. J. 2013. Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacological Reviews,* 65**,** 223-54.

WILSON, S. G. & MOGIL, J. S. 2001. Measuring pain in the (knockout) mouse: big challenges in a small mammal. *Behavioural brain research,* 125**,** 65-73.

WISE, R. A. & BOZARTH, M. A. 1987. A psychomotor stimulant theory of addiction. *Psychol Rev,* 94**,** 469-92.

WITTWER, E. & KERN, S. E. 2006. Role of morphine's metabolites in analgesia: concepts and controversies. *Aaps J,* 8**,** E348-52.

WOOLFE, G. & MACDONALD, A. D. 1944. THE EVALUATION OF THE ANALGESIC ACTION OF PETHIDINE HYDROCHLORIDE (DEMEROL). *Journal of Pharmacology and Experimental Therapeutics,* 80**,** 300-307.

WU, H., WACKER, D., MILENI, M., KATRITCH, V., HAN, G. W., VARDY, E., LIU, W., THOMPSON, A. A., HUANG, X. P., CARROLL, F. I., MASCARELLA, S. W., WESTKAEMPER, R. B., MOSIER, P. D., ROTH, B. L., CHEREZOV, V. & STEVENS, R. C. 2012. Structure of the human kappa-opioid receptor in complex with JDTic. *Nature,* 485**,** 327-32.

XI, Z. X. & STEIN, E. A. 2002. GABAergic mechanisms of opiate reinforcement. *Alcohol Alcohol,* 37**,** 485-94.

YAKSH, T. L. & RUDY, T. A. 1976. Analgesia mediated by a direct spinal action of narcotics. *Science,* 192**,** 1357-8.

YAMAGUCHI, T., QI, J., WANG, H. L., ZHANG, S. & MORALES, M. 2015. Glutamatergic and dopaminergic neurons in the mouse ventral tegmental area. *The European journal of neuroscience,* 41**,** 760-72.

YANO, A., TSUTSUMI, S., SOGA, S., LEE, M. J., TREPEL, J., OSADA, H. & NECKERS, L. 2008. Inhibition of Hsp90 activates osteoclast c-Src signaling and promotes growth of prostate carcinoma cells in bone. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 15541-6.

ZADINA, J. E., HACKLER, L., GE, L. J. & KASTIN, A. J. 1997. A potent and selective endogenous agonist for the mu-opiate receptor. *Nature,* 386**,** 499-502.

ZHANG, L., LOH, H. H. & LAW, P. Y. 2013. A novel noncanonical signaling pathway for the mu-opioid receptor. *Molecular pharmacology,* 84**,** 844-53.

ZHANG, L., ZHAO, H., QIU, Y., LOH, H. H. & LAW, P. Y. 2009. Src phosphorylation of micro-receptor is responsible for the receptor switching from an inhibitory to a stimulatory signal. *The Journal of biological chemistry,* 284**,** 1990-2000.

ZHANG, T. A., PLACZEK, A. N. & DANI, J. A. 2010. In vitro identification and electrophysiological characterization of dopamine neurons in the ventral tegmental area. *Neuropharmacology,* 59**,** 431-6.

ZHU, Y., KING, M. A., SCHULLER, A. G. P., NITSCHE, J. F., REIDL, M., ELDE, R. P., UNTERWALD, E., PASTERNAK, G. W. & PINTAR, J. E. 1999. Retention of Supraspinal Delta-like Analgesia and Loss of Morphine Tolerance in δ Opioid Receptor Knockout Mice. *Neuron,* 24**,** 243-252.

ZIMMER, A., VALJENT, E., KÖNIG, M., ZIMMER, A. M., ROBLEDO, P., HAHN, H., VALVERDE, O. & MALDONADO, R. 2001. Absence of Δ-9-Tetrahydrocannabinol Dysphoric Effects in Dynorphin-Deficient Mice. *The Journal of Neuroscience,* 21**,** 9499-9505.