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Mitogen and Stress-activated Protein Kinase 1 Negatively Regulates Hippocampal Neurogenesis

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Abstract—Neurogenesis in the subgranular zone (SGZ) of the adult hippocampus can be stimulated by a variety of means, including via exposure of experimental animals to an enriched environment that provides additional sensory, social, and motor stimulation. Tangible health and cognitive benefits accrue in enriched animals, including the amelioration of signs modelling psychiatric, neurological and neurodegenerative conditions that affect humans, which may in part be due to enhanced production of neurons. A key factor in the neuronal response to enrichment is the release of brain-derived neurotrophic factor (BDNF) and the activation of the Mitogen-Activated Protein Kinase (MAPK) cascade, which can lead to the stimulation of neurogenesis. Mitogen and Stress-Activated protein Kinase 1 (MSK1) is a nuclear enzyme downstream of BDNF and MAPK that regulates transcription. MSK1 has previously been implicated in both basal and stimulated neurogenesis on the basis of studies with mice lacking MSK1 protein. In the present study, using mice in which only the kinase activity of MSK1 is lacking, we show that the rate of cellular proliferation in the SGZ (Ki-67 staining) is unaffected by the MSK1 kinase-dead (KD) mutation, and no different from controls levels after five weeks of enrichment. However, compared to wild-type mice, the number of doublecortin (DCX)-positive cells was greater in both standard-housed and enriched MSK1 KD mice. These observations suggest that, while MSK1 does not influence the basal rate of proliferation of neuronal precursors, MSK1 negatively regulates the number of cells destined to become neurons, potentially as a homeostatic control on the number of new neurons integrating into the dentate gyrus (DG).

Keywords: MSK1, BDNF, neurogenesis, SGZ, environmental enrichment, hippocampus.

INTRODUCTION

Progenitor cells located in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) contribute to the diverse roles the hippocampus plays through the integration of the newly differentiated cells into the hippocampal circuitry and the formation of new functional neuronal networks with other brain areas (Aimone et al., 2011; Cameron and Glover, 2015; Vicidomini et al., 2020). Several factors such as teratogens, injury, environmental enrichment, exercise, and stress have been shown to impact on the hippocampal neurogenic rate (Shors et al., 2012; Song et al., 2012; Cameron and Glover, 2015; Opendak et al., 2016). This suggests that the hippocampus is tuned to adjust its neurogenic capability to prevailing synaptic and environmental influences in order to maintain its functionality. These observations have provoked investigations into the mechanisms that convert extrinsic influences into the genomic changes that underpin the regulation of neurogenesis (Poiana et al., 2020).

Prominent among the regulators of neurogenesis in response to exercise and environmental enrichment are the neurotrophins, and in particular brain-derived neurotrophic factor (BDNF) (Vilar and Mira, 2016; Numakawa et al., 2018). BDNF activates the Mitogen-Activated Protein Kinase (MAPK) pathway leading to the activation of Extracellular Signal-Regulated Kinases (ERK1/2), which have repeatedly been implicated in neurogenesis (Chen et al., 1992; Sugino et al., 2000; Nozaki et al., 2001; Lennmyr et al., 2002; Choi et al., 2008; Li et al., 2010). ERK1/2 regulate transcription leading to

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Abbreviations: BDNF, brain-derived neurotrophic factor; DCX, doublecortin; DG, dentate gyrus; MAPK, Mitogen-Activated Protein Kinase; MSK1, Mitogen and Stress-Activated protein Kinase 1; SGZ, subgranular zone.

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neurogenesis (Okuyama et al., 2004; Choi et al., 2008; Tian et al., 2009), either by direct interaction with transcription factors, or via the activation of a series of downstream kinase pathways including Mitogen and Stress-Activated protein Kinase 1 and 2 (MSK1/2) (Deak et al., 1998; Wiggin et al., 2002; Hauge and Frodin, 2006; Reyskens and Arthur, 2016).

MSKs are prominent regulators of gene transcription through the phosphorylation of both CREB and histone H3 (Deak et al., 1998; Wiggin et al., 2002; Soloaga et al., 2003; Chwang et al., 2007; Reyskens and Arthur, 2016), and indeed, play a major role in CREB phosphorylation at the primary activating serine residue (S133) in response to BDNF (Arthur et al., 2004). MSK1's presence in DG granule cells (Choi et al., 2012; Karelina et al., 2012) activation by BDNF, phosphorylation of CREB (Arthur et al., 2004; Daumas et al., 2017) and regulation of transcription (Reyskens and Arthur, 2016; Privitera et al., 2020) likely explain why MSK1/2 have been previously shown to regulate neurogenesis. Studies using either single MSK1 knockouts, or double knockouts of both MSK1 and MSK2 have shown that loss of MSKs results in reduced numbers of BrdU-, Ki-67-, and DCX-positive cells in the SGZ under basal conditions (Choi et al., 2012; Karelina et al., 2012) and in response to status epilepticus (Choi et al., 2012), environmental enrichment (Karelina et al., 2012), and cerebral ischemia (Karelina et al., 2015). These observations suggest an important role for MSKs in basal and responsive neurogenesis, and may explain the reduced volume of the granule cell layer in MSK1/2 double knockout mice (Choi et al., 2012).

These neurogenic deficits are associated in MSK1 knockouts with aberrant developing and mature hippocampal neuron morphology and dendritic spine count (Choi et al., 2012; Karelina et al., 2012, 2015), and impairments in various tests of learning and memory, including the Barnes maze, novel object recognition (Karelina et al., 2012), fear conditioning and the Morris water maze (Chwang et al., 2007), implicating MSK1 in a range of crucial cellular and cognitive functions.

However, a subsequent mutant of MSK1, in which the kinase domain of the enzyme was selectively targeted leading to an extant, but kinase dead (KD) enzyme, revealed a different phenotype in terms of increased basal hippocampal CA1 spine density (Corrêa et al., 2012; Privitera et al., 2020), and the absence of cognitive impairment in tests of spatial working memory (spontaneous alternation) and spatial reference memory (Morris water maze) (Daumas et al., 2017; Privitera et al., 2020). This discrepancy in the phenotypes of knockout vs kinase-dead MSK1 mutants may reflect a structural role for MSK1 in forming a transcriptional regulation complex with ERK1/2 and the glucocorticoid receptor (Gutierrez-Mecinas et al., 2011). The absence of MSK1 protein in the single MSK1 or double MSK1/2 knockouts could therefore lead to dysregulation of this complex and impaired transcriptional regulation, independent of MSK1′s kinase-dependent influence on the genome, cellular morphology and cognition.

Accordingly, because of the dual structural and kinase functions of MSK1, the role that MSK1 plays in neurogenesis is unclear. To address this, we have examined neurogenesis in the hippocampal SGZ in MSK1 kinase-dead (MSK1 KD) and wild-type (WT) mice under conditions of standard housing and in response to 5 weeks of environmental enrichment. We show that the number of Ki-67-positive cells is unaffected by either enrichment or the loss of MSK1 kinase activity, suggesting both the absence of active cellular division at this time point, and the lack of involvement of MSK1 kinase activity in neuronal precursor proliferation. Intriguingly, greater numbers of DCX-positive cells were seen in the MSK1 KD mutant mice under both basal and enriched conditions. These observations suggest that the kinase activity of MSK1 negatively regulates the maturation of neuronal precursors, and is therefore necessary to maintain the homeostatic balance of SGZ neurogenesis in the integration of new neurons into the hippocampal DG.

EXPERIMENTAL PROCEDURES

Wild-type and MSK1 Kinase-Dead mice

The experimental animals were used according to the guidelines of the United Kingdom Animals (Scientific Procedures) Act 1986 and the University of Warwick's ethical review procedures. The MSK1 kinase-dead (MSK1 KD) mouse has been described previously (Corrêa et al., 2012; Daumas et al., 2017; Privitera et al., 2020). All the mice used in this study were on a C57-Bl/6J genetic background. WT and homozygous MSK1 KD mutant mice were maintained as separate lines that were initially derived from founder homozygous and WT breeders from heterozygote crosses. To avoid genetic divergence, backcrossing occurred when the founder mice reached the end of their reproductive life cycle (typically three litters) (Privitera et al., 2020). The MSK1 KD mutant mice were healthy, viable, fertile and had a similar age-span to that of WT mice, from which they were indistinguishable by appearance. They were neurologically intact, and their sensorimotor behaviour was identical to that of WT mice (Daumas et al., 2017; Privitera et al., 2020). Confirmation of genotype was performed routinely by PCR using the primers 5′-CGGCCA TGTGGTGCTGACAGC-3′ and 5′-GGGTCAGAGGCCTG CACTAGG-3′ which resulted in 378 and 529 bp products for both the wild-type and targeted alleles, respectively. Mice were maintained under a 12/12 light dark cycle (lights on at 7.00 am) in a facility kept at 20–24 °C and were given ad libitum access to standard mouse chow and water.

Housing condition

At 4 weeks of age (one week after weaning) male WT and MSK1 KD mutant mice either remained in standard housing (SH; Tecniplast 1284L; 365 × 207 × 140 mm; 530 cm^2 floor area; 2–3 mice per cage, with bedding material and a cardboard tube), or were transferred to

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environmentally-enriched (EE) cages (Tecniplast 1500U; 480 × 375 × 210 mm; 1500 cm^2 floor area). Enriched cages housed 4–5 mice per cage, and cages contained bedding material, a cardboard tube, one running wheel, a number of plastic toys (e.g. tunnels, platforms, see-saws), and a metal ladder. As a further enrichment and to provide novelty, the toys were moved around twice per week and new toys introduced once per week when cages were cleaned (Privitera et al., 2020). Mice remained in either the EE or SH for a further 5 weeks until the brains were processed for immunohistochemistry. Twenty mice were used in this study: 5 WT SH mice, 4 WT EE mice, 6 MSK1 KD SH mice, and 5 MSK1 KD EE mice.

Immunohistochemistry

The mice were killed by cervical dislocation and the brains were rapidly removed and placed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 24 h at 4 °C. The brains were subsequently cryoprotected by immersion in 30% buffered sucrose solution in 0.1 M PB at 4 °C until they equilibrated. Cryoprotected left hemispheres (n = 20) were sectioned at 40 μm in the sagittal plane. Endogenous peroxidase activity was reduced by pre-incubating the sections in an endogenous peroxidase inhibitor solution (1.6% of 30% H_2O_2, 49.2% methanol, 49.2% 0.1 M PB) for 30 min. Unspecific binding sites in the sections were blocked by pre-incubating in blocking buffer (2% bovine serum albumin and 0.25% Triton X-100 in 0.1 M PB). Thereafter, every fourth section from the same animal was immunolabelled with either anti-Ki-67 (for immunolabelling proliferative cells) (1:1000 rabbit anti-Ki-67; Abcam ab15580) or anti-doublecortin (DCX) (for immunolabelling newly formed cells) (1:300 rabbit anti-DCX; Abcam ab18723) for 48 h at 4 °C under gentle agitation. The sections were then incubated in a secondary antibody solution (1:1000 goat anti-rabbit IgG (biotinylated); Abcam ab6720) for 2 h at room temperature. This was followed by incubation for 1 h in an avidin–biotin solution (1:125; Vector Labs). Sections were placed in a solution of 0.05% diaminobenzidine (DAB) in 0.1 M PB for 5 min, followed by the addition of 3.3 μL of 30% hydrogen peroxide per 1 mL of DAB solution for 2 min for section developments. Finally, sections were mounted on 0.5% gelatin-coated glass slides, dried overnight, dehydrated in alcohol series, cleared in xylene and coverslipped with DPX. Non-specific immunolabelling in sections was ruled out by omitting either the primary or the secondary antibody, no staining was observed in both cases (results not shown).

Quantification of Ki-67 and DCX positive cells in the DG

Positively immunolabelled Ki-67- and DCX-positive cells (identified as those having dark nuclear staining) in the SGZ were counted along the whole rostro-caudal length of the suprapyramidal blade of the DG at ×40 magnification using an Axiovision light microscope. Cells were counted if they lay within or touched the SGZ. For all the experimental groups, between 3 and 7 (typically 5) sections/brain/immunostain (i.e. between 21 and 33 sections per each of the four experimental groups) were analysed in the medio-lateral plane. To determine the distribution of positive cells in the suprapyramidal blade in each section, the rostro-caudal length of the suprapyramidal blade was photographed with a camera attached to an Axiovision light microscope at ×5 magnification. From each photograph with its inscribed scale bar (i.e. for scale setting), the length of the blade was then carefully measured using ImageJ 1.47v software (NIH, USA). The distribution of positive cells in each section was calculated by dividing the total number of Ki-67- or DCX-positive cells counted by the measured length of the corresponding suprapyramidal blade of the DG. The data points for each of these sections, and when aggregated on a per animal basis, are provided in Fig. 1 with respect to stain and experimental group. Throughout the preparation of the tissue, the counting of cells and analyses, the experimenter was completely blinded as to which of the four experimental groups each animal belonged.

Statistical analysis

Once the cell counting was complete for all sections, the data were assigned to four experimental groups, at which point the housing condition and genotype of each of the four groups were revealed. Statistical analysis was performed using SPSS 27. Data were subject to tests of normality (Shapiro–Wilk test) and equivalence of variance (Levene's test). For normally-distributed data displaying equivalent variances, a two-way ANOVA, utilising a General Linear Model (Fixed effects), was employed, with genotype and housing condition as the two between-group factors. Post-hoc comparisons of the Simple Main Effects were conducted when appropriate. When necessary the data were analysed with non-parametric statistics (Kruskal–Wallis and Mann–Whitney *U* tests). Alpha was 0.05 for every analysis. Data points presented in Fig. 1 represent the counts from individual sections for each stain and experimental group, or the aggregate data from individual animals, with measures of the median, and 10–90 % and 25–75 % range indicated. Statistical significance was assumed when $p < 0.05$.

RESULTS

There were no observable differences in the general morphology or the neuronal distribution patterns in the brains of all the experimental mice (genotypes and housing) as described previously (Corrêa et al., 2012), and specifically so in the DG of the hippocampus as the Ki-67 and DCX immunopositive cells were present in the distinct proliferative region i.e. SGZ of the DG (Fig. 1A, B). In addition, the suprapyramidal and infrapyramidal blades of the DG were present in all the mice assessed, with distinct boundaries in both immunostains used. There was no observable difference in their patterns (Fig. 1A, B).

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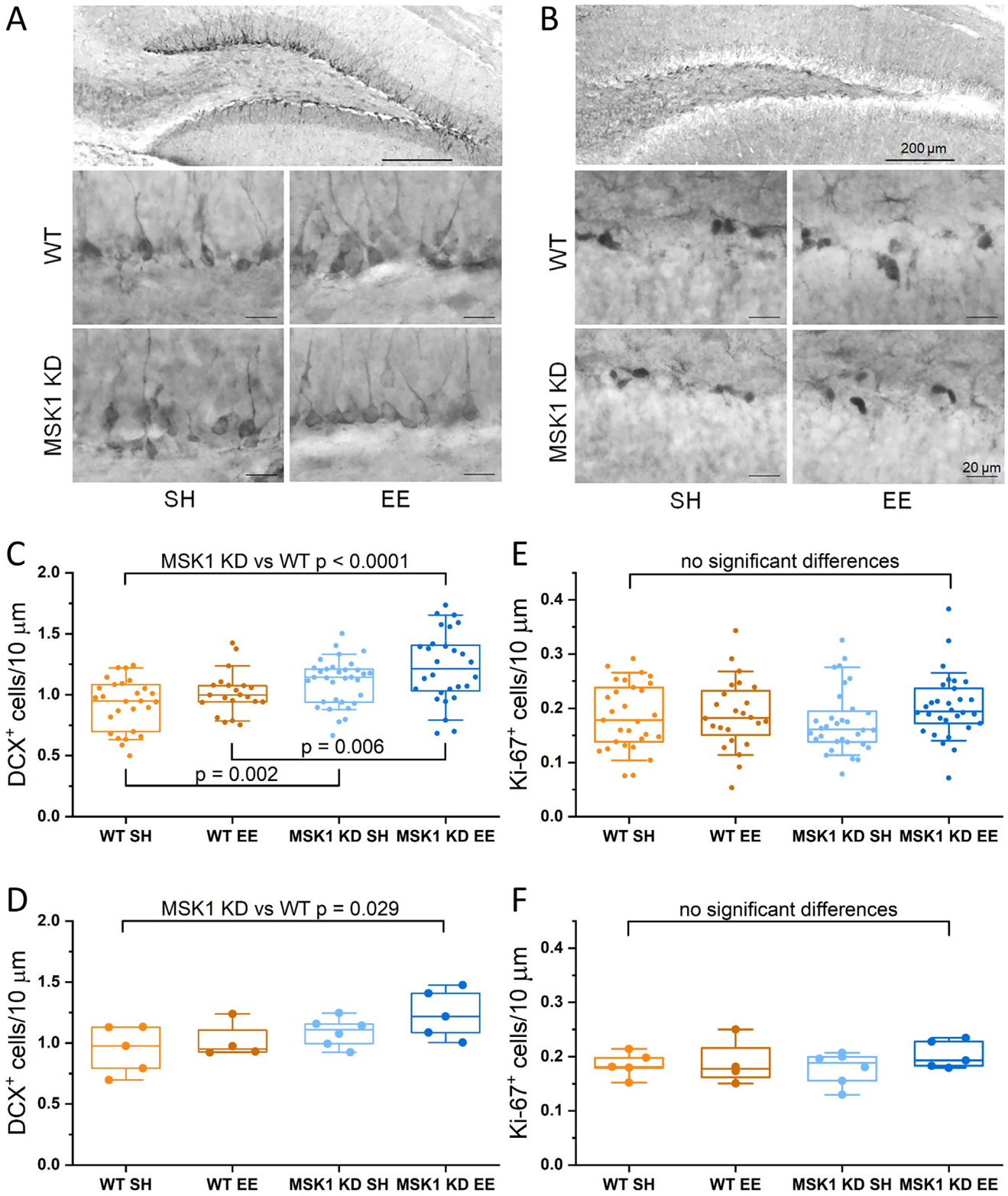

Fig. 1. MSK1 and experience differentially affect hippocampal neurogenesis. Top photomicrographs represent the dentate gyri of the hippocampi for **(A)** DCX and **(B)** Ki-67 immunostaining. The photographs of higher magnification show **(A)** the newly formed neurons immunolabelled with anti-DCX and **(B)** the proliferative cells immunolabelled with anti-Ki-67 within the subgranular layer of the suprapyramidal blade. Significant differences were observed between genotypes for the number of DCX-positive cells when assessed on a per histological section basis **(C)** or when assessed on a per animal basis **(D)**, but not for the number of Ki-67-positive cells in either individual histological sections (**E**) or when aggregated on a per animal basis **(F)**. Only a trend ($p = 0.077$) was observed for the effects of enrichment on DCX expression in **C**. Individual data points presented in **(C, E)** represent the number of DCX- or Ki-67-positive cells per histological section (between 21 and 33 sections) and in **(D, F)** from 4 to 6 animals per group, normalised to the length of dentate gyrus examined. The box represents 25–75 % of the range; the whiskers represent the 10–90% range, and the median is given by the horizontal bar.

The number of Ki-67- or DCX-positive cells in the suprapyramidal blade of the DG in the WT or MSK1 KD mice housed in SH or EE was quantified. The data were analysed in two ways: on a per histological section basis (n = 21–33 across each of the four groups), and, noting concerns regarding the non-independent nature of the data points in such an approach (Aarts et al., 2014), on a per animal basis (n = 4–6). The results of both approaches yielded identical conclusions.

In the analysis of DCX$^+$ cells in individual sections (Fig. 1C), the data across the four groups were normally distributed (Shapiro Wilk), but a test of equality of variance (Levene) indicated unequal variance across the four groups ($F_{(3,105)}$ = 4.216, p = 0.007), necessitating the use of the non-parametric Kruskal-Wallis (KW) test. The KW analysis showed a significant difference in the number of DCX$^+$ cells between groups ($H_{(3)}$ = 19.77, p = 0.000). In order to investigate the effect of the two between-group variables, genotype and housing conditions, on DCX expression, the Mann–Whitney (MW) U test was employed. There was a significant effect of genotype (U = 790.0, p = 0.000, r = 0.39) where the MSK1 KD had a greater number of DCX$^+$ cells, with a trend towards significance for housing conditions with a tendency for the enriched groups to have more DCX$^+$ cells (U = 1180.0, p = 0.077, r = 0.17). A further MW analysis on the individual groups showed that, while there was no significant effect due to housing within the same genotype (WT SH vs. WT EE; U = 229.0, p = 0.257, r = 0.16 and MSK1 KD SH vs. MSK1 KD EE; U = 346.0, p = 0.093, r = 0.21), there was a significant difference between genotypes in each housing condition, with the MSK1 KD mice having more DCX$^+$ cells than their comparably housed WT counterparts (WT SH vs MSK1 KD SH; U = 239.0, p = 0.002, r = 0.40 and WT EE vs. MSK1 KD EE; U = 159.0, p = 0.006, r = 0.39). The greater numbers of DCX-positive cells in both SH and EE MSK1 KD mice persisted when the data was aggregated on a per animal basis, in which the groups did not deviate from a normal distribution, and showed equivalent variance (Two-way ANOVA; WT vs. MSK1 KD: $F_{(1,16)}$ = 5.77, p = 0.029; Fig. 1D). These data suggest that the kinase activity of MSK1 exerts an inhibitory regulatory influence on neurogenesis, potentially to avoid the over-population of the DG with new neurons.

For the Ki-67 analysis of individual histological sections (Fig. 1E), the variance in the data was equivalent across groups (Levene test; $F_{(3,111)}$ = 0.435, p = 0.729), but one group (MSK1 KD SH) failed the Shapiro–Wilk test for normality ($W_{(32)}$ = 0.920, p = 0.021). Accordingly, the data were analysed with non-parametric tests. The number of Ki-67-positive cells was not significantly different across the four groups (KS; $F_{(3)}$ = 4.575, p = 0.206). There were no differences between the WT and MSK1 KD mice (MW; U = 1633.0, p = 0.955, r = 0.005), and no significant difference in the distribution of Ki-67$^+$ cells between the two housing conditions (MW; U = 1342.0, p = 0.087, r = 0.16). When the data were aggregated on a per animal basis (Fig. 1F), where normality and equivalence of variance of data were satisfied, no differences in Ki-67 expression across the four groups were observed (Two-way ANOVA; Genotype effect: $F_{(1,16)}$ = 0.076, p = 0.786; Housing effect: $F_{(1,16)}$ = 1.144, p = 0.301; Genotype × Housing interaction: $F_{(1,16)}$ = 0.623, p = 0.441). These data suggest that at this time point, after 5 weeks of enrichment, active proliferation of cells in the DG is not observed, but equally that the kinase activity of MSK1 is not required for the maintenance of neurogenic potential.

DISCUSSION

The factors influencing the experience- and exercise-dependent stimulation of neurogenesis have been intensively pursued given the potential to harness this knowledge for therapeutic benefit in conditions associated with cognitive decline such as Alzheimer's disease (Chohan, 2020; Mihardja et al., 2020). One potential approach is to develop exercise- or environment-mimetic drugs that activate the pathways recruited by enrichment for clinical benefits in patients (Guerrieri et al., 2017; Shepherd et al., 2018). Of note, BDNF-based approaches, such as the small molecule BDNF mimetic 7,8-dihydroxyflavone, have been shown to stimulate neurogenesis and reverse cellular and cognitive impairments in animal models of traumatic brain injury (Wurzelmann et al., 2017), psychiatric conditions (Gudasheva et al., 2019), neurodevelopmental disorders (Du and Hill, 2015), and neurodegeneration (Kazim and Iqbal, 2016; Choi et al., 2018). In humans, the potential benefits of exercise and physical activity may be mediated through an increase in BDNF (Kazim and Iqbal, 2016). It is thus against this backdrop that the importance of BDNF-dependent signaling in the regulation of neurogenesis is appreciated.

Key to BDNF signaling is MSK1, a nuclear kinase downstream of the BDNF TrkB receptor and MAPK cascade, that regulates transcription through chromatin remodeling and the phosphorylation of CREB (Reyskens and Arthur, 2016). Using a kinase-dead mutant of MSK1, we have previously shown that the kinase activity of MSK1 is necessary for the transcriptional regulation of Arc/Arg3.1 (Hunter et al., 2017), support of basal synaptic transmission (Daumas et al., 2017), homeostatic synaptic plasticity, the experience-dependent enhancement of quantal synaptic transmission and the expansion of the dynamic range of synapses, cognitive flexibility and the persistence of memory, and the genomic downscaling of key plasticity-related proteins such as EGR1 and Arc/Arg3.1 (Corrêa et al., 2012; Privitera et al., 2020). MSK1 likely plays a role in sensing prevailing experience-driven neuronal activity, in response to the associated activity-dependent release of BDNF, and orchestrates a genomic program designed to allow neurons to respond to and adapt to a dynamic environment. It is thus not surprising that MSK1 has been implicated in basal and responsive neurogenesis, where its deletion results in impaired neurogenesis as assessed by reduced BrdU, DCX and Ki-67 labelling in the hip-

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pocampal SGZ (Choi et al., 2012; Karelina et al., 2012, 2015).

Specifically addressing the role the kinase function of MSK1 plays in neurogenesis using a kinase-dead MSK1 mutant mice, we have made observations at odds with previous reports in MSK knockout mice. We show that the neurogenic potential of the SGZ, as assessed through Ki-67, a marker for cellular proliferation, is not affected by the loss of MSK1′s kinase activity under basal standard housing conditions. While this suggests that MSK1 is not involved in determining the potential for subsequent neuronal differentiation, further analysis is required with additional markers of neurogenesis (e.g. Nestin) to establish the identity of these proliferating cells and the relative role of MSK. Indeed, such further analysis may determine if the MSK2 isoform was able to compensate for the lack of MSK1 kinase activity. That enrichment did not provoke an increase in Ki-67-reported cellular division may indicate that at this late time point (5 weeks of enrichment) any initial surge in cellular proliferation has occurred, and proliferation has returned to baseline levels. That an earlier surge in proliferation may have occurred in response to enrichment is suggested by the tendency towards increased number of DCX-positive cells in the SGZ of enriched mice. While there are ample precedents for the stimulation of neurogenesis by enrichment (Kempermann et al., 1997; Kempermann, 2019), this was not fully captured in the present study. This is potentially due to under-powering of the experimental groups, the single time point and age at which the analysis was conducted, and perhaps due to relatively enriched standard housing conditions, with litter mates, nesting material and cardboard tube, which may have contributed to the stimulation of neurogenesis and thus reducing differences between housing conditions.

Intriguingly, however, the number of DCX-positive cells were significantly greater in MSK1 KD mice raised under both standard and enriched conditions. These observations suggest that the kinase activity of MSK1 negatively regulates the extent of neurogenesis, and prevents an excess of newly-formed neurons. Whether this regulation of neurogenesis occurs by limiting production of new neurons, and/or limiting their persistence, remains to be addressed, but such over-production may have negative impacts for hippocampal function, and indeed increased numbers of neurons have been described in neurodevelopmental conditions such as the autism spectrum disorders (ASD) (Fan and Pang, 2017). MSK1, also negatively regulates dendritic spine density in CA1 pyramidal neurons (Corrêa et al., 2012; Privitera et al., 2020), an overabundance of which has also been implicated in ASD (Nakai et al., 2018). MSK1 may thus exert an important homeostatic function in regulating the number of neurons, the synaptic contacts between them, and the networks in which they participate.

In summary, the present observations, of enhanced neurogenesis in mice lacking the kinase activity of MSK1, compared to previous reports of reduced neurogenesis in mice lacking MSK1 protein, suggest differential roles of MSK1 as an enzyme, and as a structural component of a transcription-regulating complex. Our observations identify MSK1′s kinase activity as an important homeostatic mechanism in the regulation of hippocampal neurogenesis. These studies, together with previous observations in MSK1 KD mice, suggest that MSK1 senses prevailing environmental and synaptic influences and responds in an experience- and activity-dependent manner to influence a genomic program capable of allowing appropriate cellular, synaptic and cognitive adaptations, and to maintain the stability of neuronal networks.

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