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# Bace1 activity impairs neuronal glucose metabolism: rescue by beta-hydroxybutyrate and lipoic acid

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In review

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# **BACE1 activity impairs neuronal glucose oxidation: rescue by beta-hydroxybutyrate and lipoic acid**

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## **Abstract**

Glucose hypometabolism and impaired mitochondrial function in neurons have been suggested to play early and perhaps causative roles in Alzheimer's disease (AD) pathogenesis. Activity of the aspartic acid protease, beta-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1), responsible for beta amyloid peptide generation, has recently been demonstrated to modify glucose metabolism. We therefore examined, using a human neuroblastoma (SH-SY5Y) cell line, whether increased BACE1 activity is responsible for a reduction in cellular glucose metabolism. Overexpression of active BACE1, but not a protease-dead mutant BACE1, protein in SH-SY5Y cells reduced glucose oxidation and the basal oxygen consumption rate, which was associated with a compensatory increase in glycolysis. Increased BACE1 activity had no effect on the mitochondrial electron transfer process but was found to diminish substrate delivery to the mitochondria by inhibition of key mitochondrial decarboxylation reaction enzymes. This BACE1 activity-dependent deficit in glucose oxidation was alleviated by the presence of beta hydroxybutyrate or  $\alpha$ -lipoic acid. Consequently our data indicate that raised cellular BACE1 activity drives reduced glucose oxidation in a human neuronal cell line through impairments in the activity of specific tricarboxylic acid cycle enzymes. Because this bioenergetic deficit is recoverable by nutraceutical compounds we suggest that such agents, perhaps in conjunction with BACE1

38 inhibitors, may be an effective therapeutic strategy in the early-stage management or  
39 treatment of AD.

40

## 41 **Introduction**

42

43 Alzheimer's disease (AD) is an age-related neurodegenerative disease, with the vast majority  
44 of cases described as sporadic and not currently linked with a specific gene defect. There are  
45 approximately 44 million people living with AD in the world, and this number is predicted to  
46 increase dramatically over the next three decades, severely impacting on healthcare systems  
47 and the socio-economic environment. Presently, we have a limited understanding of the early  
48 events in sporadic AD disease aetiology. While the progression of AD is closely associated  
49 with dysfunctional tau protein and cholinergic deficits, it is generally observed by biomarker  
50 analysis that changes in the hallmark amyloid plaque pathology precedes these other  
51 pathogenic drivers [1-4]. There has also been increasing interest in the role of brain  
52 mitochondrial dysfunction and reduced metabolic activity [5-7].

53

54 It has long been recognised that glucose is the predominant substrate utilised by the adult  
55 brain under physiological conditions [8]. Indeed, while only constituting 2% to body weight,  
56 the brain consumes around 20 and 25% of the total body oxygen consumption and glucose  
57 respectively [9]. Furthermore, the respiratory quotient of the brain is almost exactly 1,  
58 indicating near universal carbohydrate metabolism [10]. Although glucose is the dominant  
59 substrate for brain metabolic activity, alternative substrates, such as glycogen and amino  
60 acids can play a role in central metabolism. However due to limited supply and storage  
61 capacity, this is thought to be relatively minor under physiological conditions [11]. Glucose  
62 metabolism in the brain is tightly coupled to the generation of intracellular adenosine  
63 triphosphate (ATP), largely to support neurotransmitter production and release.  
64 Consequently, this reliance on glucose coupled with a high-energy consumption makes the  
65 brain, and synaptic transmission in particular, vulnerable to events, which lead to diminished  
66 metabolism.

67

68 Brain hypometabolism is a universal change observed during Alzheimer's disease (AD)  
69 progression and recently, it has been proposed to have a major causative role in AD  
70 pathogenesis [12,13]. Through the use of neuroimaging techniques, impaired brain glucose  
71 metabolism has been demonstrated to occur: before atrophy in autosomal AD cases, during  
72 the progression towards non-familial AD and in individuals at high risk of AD (i.e. APOE  $\epsilon$ 4  
73 carriers) prior to symptom manifestation [14-18]. This reduced brain glucose metabolism has  
74 been postulated to result from impaired mitochondrial functioning [19-21], with similar  
75 changes observed in a variety of transgenic mouse models of AD [7,22-24]. Recent evidence  
76 suggests a putative link between neuronal glucose metabolism and the presence of beta  
77 amyloid ( $A\beta$ ) peptides, considered by many to be the primary pathogenic driver of AD.  $A\beta$   
78 peptides derive from increased cleavage of amyloid precursor protein (APP), which is  
79 ubiquitously expressed in tissues, with highest levels in the brain. APP is cleaved by two  
80 competing enzyme processes; an alpha secretase pathway, resulting in the soluble cleaved  
81 product sAPP $\alpha$  and no  $A\beta$  production and a beta secretase pathway, which releases sAPP $\beta$   
82 and following additional cleavage of the remaining protein by  $\gamma$ -secretase, releases  $A\beta$ .  $A\beta$   
83 accumulates in the mitochondria of AD patients and AD mouse models, prior to the  
84 appearance of amyloid deposits [25-27]. Indeed, exogenous  $A\beta$  has been reported to decrease

85 mitochondrial respiratory chain function and the activity of various mitochondrial  
86 dehydrogenase enzymes [28-31], indicative of an A $\beta$ -mediated bioenergetic deficit in cells.

87 Additionally, it has been shown that the hypometabolic state associated with AD may be  
88 driven, at least in part, by a region-specific shift in neuronal metabolism towards aerobic  
89 glycolysis (reduced oxidative metabolism in the presence of adequate oxygen supply; [32]).  
90 This switch in glucose metabolism was shown to closely correlate with A $\beta$  deposition and  
91 later vulnerability to cell death during progression towards AD [33]. These findings clearly  
92 implicate the modulation of APP processing as a driver of the altered metabolic state  
93 observed in early, pre-AD states. A key enzyme driving excess A $\beta$  production, as observed in  
94 AD, is the aspartyl protease,  $\beta$ -site APP cleaving enzyme 1 (BACE1). BACE1 was initially  
95 characterised as the enzyme controlling the rate-limiting step in A $\beta$  generation [34-36]. More  
96 recently however, it has also been proposed to play a role in glucose metabolism with whole  
97 body knock out of BACE1 resulting in improved insulin sensitivity and glucose homeostasis  
98 [37]. Furthermore, genetic and pharmacological manipulation of APP processing can directly  
99 alter glucose uptake and metabolism in the C2C12 skeletal muscle cell line [38].  
100 Importantly, BACE1 is a stress-sensitive protease, with oxidative, hypoxic, inflammatory and  
101 metabolic stress (all associated with AD initiation and/or progression) demonstrated to  
102 increase BACE1 levels and activity, causing APP processing to shift from the physiologically  
103 predominant alpha-secretase, to the beta-secretase, pathway and increasing A $\beta$  levels.  
104 Indeed, recent work has demonstrated a role for oxidative, lipid and metabolic stressors in  
105 regulating BACE1 gene and protein expression as well as activity [39-43]. Furthermore,  
106 alterations in its expression result from changes in micro RNA (miR) regulation of the 5'  
107 untranslated region (UTR) of BACE1 have been demonstrated in sporadic AD cases [44-46].  
108 Consequently, chronic stress events and altered translational regulation may in turn culminate  
109 in the consistent reports that BACE1 mRNA, protein and activity are elevated in AD brains  
110 [47-53].

111

112 Given that A $\beta$  directly impairs mitochondrial enzyme function and that AD is associated with  
113 impaired glucose metabolism we hypothesised that manipulating APP processing through  
114 BACE1 overexpression in SH-SY5Y neuroblastoma cells would phenocopy the defects in  
115 glucose metabolism at the cellular level, allowing us to explore in more detail this initial and  
116 potentially causative change in AD progression.

117

## 118 **Materials and Methods**

119

### 120 **Cell culture**

121 SH-SY5Y cells were cultured under aseptic conditions and maintained in a humidified  
122 atmosphere of 95% air and 5% CO<sub>2</sub>. Cells were maintained in Dulbecco's Modified Eagle's  
123 Medium (DMEM) F-12 media (Gibco Life Technologies, Paisley, UK) supplemented with  
124 10% fetal bovine serum (Sera Laboratories, West Sussex, UK), 4 mM L-glutamine (Gibco)  
125 and 2% Penicillin streptomycin (100 units/ml; Gibco). SH-SY5Y cells were transfected with  
126 12  $\mu$ g DNA of pcDNA3.1 containing empty vector (SH-SY5Y<sub>EV</sub>), full-length human BACE1  
127 (SH-SY5Y<sub>B1</sub>), or BACE1 active site mutant (SH-SY5Y<sub>mB1</sub>) using Lipofectamine 2000  
128 (Invitrogen Life Technologies, Paisley, UK). Stable cells were selected and lines maintained  
129 using 1 and 0.5 mg/ml G418 sulphate respectively (Sigma-Aldrich, Gillingham, UK) as  
130 selection antibiotic. A minimum of 2 independently generated stable cell lines, with

131 concurrently produced EV controls, were used for these studies. Prior to some glucose  
132 oxidation assays, cells were treated overnight, as indicated, with the pyruvate dehydrogenase  
133 inhibitor, dichloroacetate (DCA; Sigma-Aldrich) or growth media supplemented with  $\alpha$ -  
134 lipoic acid (Sigma-Aldrich) for 48 hours.

135

### 136 **Cloning**

137 Full length myc-his tagged human BACE1 in pcDNA3.1 was obtained from  
138 GlaxoSmithKline (GSK; Harlow, UK) and mBACE1 (a kind gift from Professor Wolfe  
139 (Brigham and Women's Hospital, Boston)) was sub-cloned into pcDNA3.1.

140

### 141 **Immunoblotting and gene expression**

142 Protein isolation and immunoblotting procedures were as described previously [54]. For  
143 relative quantification of APP cleavage by alpha-secretase (sAPP $\alpha$ ) versus beta-secretase  
144 (sAPP $\beta$ ) pathways, cells were incubated for between 20-24 hours in Optimem (Gibco), and  
145 media concentrated (using 30 kDa Amicon Ultra 15 ml filters; Merck Millipore, Livingston,  
146 UK) by centrifugation (4000 x g) and subjected to SDS-PAGE with amounts presented  
147 relative to total protein. Protein antibodies used were: anti-Actin (Sigma-Aldrich; 1:5000),  
148 anti-APP (Ab54, GSK; 1:4000), anti-sAPP $\alpha$  (Cambridge Bioscience, Cambridge, UK;  
149 1:1000), anti-sAPP $\beta$  (GSK; 1:1000), anti-BACE1 (Sigma-Aldrich; 1:1000), anti-BAD (New  
150 England Biolabs, Hitchin, UK; 1:1000), with anti-total PDH (1 mg/ml) and anti-pPDHe1 $\alpha$  (1  
151 mg/ml) from Drug Discovery Unit, University of Dundee.

152

### 153 **Glucose oxidation assay**

154 SH-SY5Y<sub>EV</sub>, SH-SY5Y<sub>B1</sub> or SH-SY5Y<sub>mB1</sub> cells were plated into 6-well cell culture plates  
155 and any pre-treatments carried out as described above and in the results section. To begin the  
156 assay, cells were washed twice with HEPES-buffered saline (HBS (in mM); 140 NaCl, 20  
157 HEPES, 5 KCl, 2.5 MgSO<sub>4</sub> and 1 CaCl<sub>2</sub>, pH 7.4) and incubated in HBS containing 2.5 mM  
158 glucose and 74 kBq/ml D-[U-<sup>14</sup>C]glucose (PerkinElmer) along with any relevant inhibitors or  
159 treatments indicated in the results section for 3 hours at 37 °C. Media were transferred and  
160 <sup>14</sup>CO<sub>2</sub> liberated by acidification with 60% perchloric acid, trapped by Whatman (GF/B) filter  
161 papers discs pre-soaked with 1 M KOH and radioactivity quantified by liquid-scintillation  
162 counting. Cells from the assay were washed twice with ice-cold 0.9 % NaCl and lysed with  
163 1ml of 50 mM NaOH, and the radioactivity contained within the lysate quantified by liquid-  
164 scintillation counting, which served as a measure of the <sup>14</sup>C incorporation into the cell during  
165 the assay period. Total protein content was determined in the lysate via the Bradford method  
166 and used to normalize glucose incorporation and oxidation rates for each sample.

167

### 168 **Cellular respiration**

169 The Seahorse Extracellular Flux Analyser utilises solid sensors that simultaneously monitor  
170 the oxygenation and pH of the media. The rate of oxygen consumption (OCR) and  
171 extracellular acidification rates (ECAR) can therefore be assessed in near real time allowing  
172 for high resolution changes in a range of metabolic parameters. SH-SY5Y<sub>EV</sub>, SH-SY5Y<sub>B1</sub> or  
173 SH-SY5Y<sub>mB1</sub> cell monolayers were seeded into XF 24-well culture microplates (Seahorse  
174 Bioscience, Copenhagen, Denmark) the day prior to treatment or assay as indicated in the

175 results section. Optimal cell number was determined following a cell titration assay taking  
176 into account oxygen consumption rate (OCR) and extracellular acidification rate (ECAR),  
177 oxygen tension values and the appearance of the cell monolayers and was determined to be  
178 40,000 cells. On the day of the assay, cells were placed in unbuffered DMEM with relevant  
179 inhibitors/treatments as indicated and placed in a non-CO<sub>2</sub> incubator for 1 hour (to de-gas  
180 solutions) prior to assay initiation. Standard 3 minute mix, 2 minute wait and 3 minute  
181 measure cycles were used; with 5 baseline measurements taken before, and a subsequent 3  
182 measurements acquired following, drug additions.

183

#### 184 **Enzyme activity assays**

185 Activity assays for pyruvate dehydrogenase (PDH; Abcam, Cambridge, UK),  $\alpha$ -ketoglutarate  
186 dehydrogenase ( $\alpha$ -KGDH; Antibodies Online, Aachen, Germany), isocitrate dehydrogenase  
187 (IDH; Sigma-Aldrich) and fumarase (Abcam) were performed according to the  
188 manufacturer's instructions.

#### 189 **Results**

##### 190 **Overexpression of BACE1 increases amyloidogenic APP processing and suppresses** 191 **glucose oxidation**

192 Control, empty vector-treated (SH-SY5Y<sub>EV</sub>) and BACE1 overexpressing (SH-SY5Y<sub>B1</sub>) cells  
193 displayed equivalent APP protein levels, showing the three predominant APP transcript  
194 protein isoforms present in neurons ([55]; Fig. 1A,B). As expected, SH-SY5Y<sub>B1</sub> cells  
195 exhibited significantly higher BACE1 protein levels (Fig. 1A,C), which resulted in altered  
196 APP cleavage; promoting a substantial shift from non-amyloidogenic to amyloidogenic  
197 processing (as denoted by increased sAPP $\beta$  and decreased sAPP $\alpha$  levels (Fig. 1A,D,E).  
198 Overexpression of BACE1 resulted in a marked reduction in the rate of <sup>14</sup>C-glucose  
199 oxidation, compared to SH-SY5Y<sub>EV</sub> cells (Fig. 1F), in agreement with recent findings in  
200 C<sub>2</sub>C<sub>12</sub> skeletal muscle cells [34]. The decrease in glucose oxidation is not due to impaired  
201 glucose incorporation into the cell (Fig. 1G), and suggests that increased BACE1 protein  
202 levels and activity cause a fundamental change in the ability of SH-SY5Y cells to oxidise  
203 glucose (Fig. 1H).

204 In an attempt to discern whether the changes in glucose oxidation rate were dependent upon  
205 the secretase activity of BACE1, a concomitant group of cells that overexpress a mutant form  
206 of BACE1, devoid of secretase activity (SH-SY5Y<sub>mB1</sub>; [56]) were examined. BACE1 protein  
207 levels in SH-SY5Y<sub>mB1</sub> cells were raised to a similar extent to that observed for SH-SY5Y<sub>B1</sub>  
208 cells, with no alteration in APP protein levels (Fig. 2A-C). Interestingly, the overexpression  
209 of the secretase-dead BACE1 mutant exerted a dominant negative effect over APP processing  
210 with reduced sAPP $\beta$  release, compared with SH-SY5Y<sub>B1</sub> and SH-SY5Y<sub>mB1</sub> cells, into the  
211 culture media (Fig. 2A,D). The reduced  $\beta$ -secretase cleavage of APP in the presence of  
212 mBACE1 was also mirrored in its effect on glucose oxidation rate, with no reduction in  
213 glucose oxidation concomitant with comparable glucose incorporation between cell types  
214 (Fig. 2E,F), indicating that increased BACE1 activity is required to depress glucose oxidation  
215 in SH-SY5Y cells (Fig. 2G).

216

##### 217 **Chronic elevation of BACE1 stimulates aerobic glycolysis in SH-SY5Y cells**

218 The cellular pathways that facilitate glucose metabolism are glycolysis (and the pentose  
219 phosphate pathway), which occurs in the cytoplasm and the TCA cycle and oxidative  
220 phosphorylation, which are present in the matrix and inner mitochondrial membrane,  
221 respectively. Actively respiring mitochondria consume oxygen and therefore oxygen  
222 consumption rate (OCR) can be taken as a measure of substrate flux through the oxidative  
223 phosphorylation pathway while extracellular acidification rate (ECAR) reflects the release of  
224 lactate (lactic acid) converted from pyruvate following glycolysis. SH-SY5Y<sub>B1</sub> cells exhibit  
225 decreased OCR concurrent with increased ECAR in comparison to SH-SY5Y<sub>EV</sub> cells (Fig.  
226 3A,B). Taken together these changes reflect a robust shift between these metabolic processes  
227 as shown by the change in the ratio of oxidative phosphorylation to glycolytic metabolism in  
228 cells with increased BACE1 activity (Fig. 3C).

229 To further investigate these changes, a glycolysis stress test (which assesses basal glycolysis,  
230 glycolytic capacity and glycolytic reserve) was performed (Fig. 3D). SH-SY5Y<sub>EV</sub> and SH-  
231 SY5Y<sub>B1</sub> cells were incubated in media containing zero glucose, 2.5 mM pyruvate and 4 mM  
232 L-glutamine for 100 minutes, following which stimulation of basal glycolysis was achieved  
233 by the injection of 2.5 mM glucose into the assay media. The increase in basal ECAR was  
234 significantly higher in SH-SY5Y<sub>B1</sub> cells, compared to control cells, indicating that the raised  
235 BACE1 activity augmented aerobic glycolysis (Fig. 3E). Inhibition of F<sub>1</sub>F<sub>0</sub> ATP synthase by  
236 oligomycin leaves cells wholly reliant on glycolysis for ATP generation (termed maximal  
237 glycolysis). This rate was also significantly increased in SH-SY5Y<sub>B1</sub>, compared to control,  
238 cells following BACE1 overexpression (Fig. 3F). Finally, the cellular glycolytic reserve is  
239 given by the difference between basal and maximal glycolysis, with a significantly higher  
240 glycolytic reserve observed in the SH-SY5Y<sub>B1</sub> cells (Fig. 3G). Collectively, these data  
241 indicate that raised BACE1 activity depresses glucose oxidation in SH-SY5Y cells and, as a  
242 result, there is a compensatory increase in glucose metabolism through aerobic glycolysis.

243

#### 244 **Mitochondrial efficiency is unaltered by raised BACE1 activity in SH-SY5Y cells**

245 The marked reduction in oxidative phosphorylation, concurrent with increased glycolytic  
246 metabolism in SH-SY5Y<sub>B1</sub> cells, indicated that increased BACE1 activity reduced substrate  
247 delivery to the mitochondria and/or impaired mitochondrial function. To test mitochondrial  
248 efficiency in these cells, a modified Mitochondrial Stress Test (Seahorse Bioscience) protocol  
249 was utilised. As previously reported, a limited maximal respiration rate is achievable in  
250 undifferentiated SH-SY5Y cells [57] in the absence of pyruvate in the assay media.  
251 Consequently, we therefore performed a split assay to investigate the effects of mitochondrial  
252 metabolism perturbation: the first part measuring the proportion of respiration dedicated to  
253 ATP generation (ATP synthase inhibition with oligomycin), the maintenance of  
254 mitochondrial leak (a combination of rotenone (complex I inhibition) and antimycin A  
255 (complex III inhibition) to minimize mitochondrial respiration) and the relative contribution  
256 of non-mitochondrial OCR (Fig. 4A). However, increased BACE1 activity in SH-SY5Y  
257 cells had no effect on any of these oxidative parameters (Fig. 4B-D). The second part  
258 encompasses a test of the cellular reserve capacity via the induction of maximal respiration  
259 through addition of the proton ionophore FCCP. This drug collapses the mitochondrial  
260 membrane potential, leading to uncoupled (no ATP generation) substrate flux through the  
261 mitochondria, promoting an increase in glycolysis in an attempt to maintain intracellular ATP  
262 levels. Raised BACE1 activity also had no effect on SH-SY5Y mitochondrial reserve  
263 capacity (Fig. 4E,F). The results from these assays show that chronic elevation of BACE1  
264 protein expression and activity does not impair mitochondrial electron transfer function.



265 Consequently, this result strongly indicated that the BACE1 activity-driven decrease in  
266 glucose oxidation was the result of diminished substrate delivery to the mitochondria.

267

### 268 **Raised BACE1 activity lesions key metabolic pathways in oxidative glucose metabolism**

269 There are three key control points involved in the regulation of neuronal metabolism, these  
270 are the generation of glucose-6-phosphate, pyruvate and acetyl CoA. In neurons, pyruvate is  
271 predominantly produced directly from metabolism of glucose or indirectly, through the  
272 provision of extracellular lactate via the astrocyte-neuron lactate shuttle [58,59]. Therefore,  
273 in an attempt to better understand the changes in cellular metabolism induced by increased  
274 BACE1 activity, lactate utilisation by SH-SY5Y cells was assessed. SH-SY5Y<sub>B1</sub> cells  
275 displayed significantly reduced lactate consumption as assessed by the ability of lactate to  
276 repress <sup>14</sup>C-labelled glucose oxidation (Fig. 5A). This deficit could also be observed as a  
277 reduction in OCR when cells were provided physiological concentrations (0.5 or 2 mM; [60])  
278 of lactate as sole substrate, although the reduction in lactate metabolism was overcome by the  
279 presentation of a higher (4 mM) lactate concentration (Fig. 5A,B). As the oxidation of both  
280 glucose and lactate was diminished, we hypothesised that raised BACE1 activity resulted in  
281 impairment of the pyruvate dehydrogenase complex activity (PDH). To address this we used  
282 an indirect PDH activity assay and demonstrated that SH-SY5Y<sub>B1</sub> cells displayed a marked  
283 reduction of PDH activity (Fig. 5C). Consistent with this outcome, SH-SY5Y<sub>B1</sub> cells  
284 displayed reduced OCR when provided with pyruvate (2.5 mM) as the sole substrate (Fig.  
285 5D).

286 In response to cellular injury or stress, compensatory metabolic strategies are employed in an  
287 attempt to bypass reduced glucose utilization associated with decreased PDH activity and  
288 obviate impaired mitochondrial TCA function. For example, glutamine metabolism is  
289 diverted from an oxidative to reductive route [61,62], causing increased conversion to  
290 glutamate and, through glutamate dehydrogenase, raised levels of  $\alpha$ -ketoglutarate. This  
291 additional source of  $\alpha$ -ketoglutarate should supplement ATP generation through the TCA  
292 cycle as it bypasses the block at PDH. Consequently, we examined whether such an  
293 alternative route for TCA substrate replenishment was capable of recovering oxidative  
294 metabolism. Thus OCR and ECAR were measured when SH-SY5Y<sub>EV</sub> and SH-SY5Y<sub>B1</sub> cells  
295 were provided glucose (2.5 mM) alone or glucose (2.5 mM) + glutamine (4 mM). However,  
296 the presence of glutamine did not affect the attenuation of OCR or enhancement of ECAR in  
297 SH-SY5Y<sub>B1</sub> cells (Fig. 5E). Moreover, OCR was reduced in SH-SY5Y<sub>B1</sub> cells when  
298 glutamine was provided as the exclusive substrate (Fig. 5F). These data indicate that either  
299 glutamine does not play a significant role in SH-SY5Y basal metabolism and/or that raised  
300 BACE1 activity blunts additional TCA enzymes, such as  $\alpha$ -ketoglutarate dehydrogenase ( $\alpha$ -  
301 KGDH), which converts  $\alpha$ -ketoglutarate to succinyl-CoA. Indeed, SH-SY5Y<sub>B1</sub> cells showed  
302 a large, BACE1 secretase activity-dependent, reduction in  $\alpha$ -KGDH activity, which is the  
303 rate-controlling step of the TCA cycle (Fig. 5G).

304 Because PDH and  $\alpha$ -KGDH catalyse decarboxylation reactions involved in mitochondrial  
305 bioenergetics, we assayed the activity of the third enzyme utilising this process, isocitrate  
306 dehydrogenase (IDH). Three isoforms of IDH are present in cells, IDH3 in the mitochondrial  
307 matrix, which reduces NAD<sup>+</sup> to NADH, and IDH1 (cytoplasmic and peroxisomal) and IDH2  
308 (mitochondrial) reducing NADP<sup>+</sup> to NADPH. Consequently, specific isoform function was  
309 determined by differential supplementation (NAD<sup>+</sup> or NADP<sup>+</sup>) of the reaction mixes. In  
310 agreement with the results for the decarboxylation enzymes above, raised BACE1 activity

311 resulted in depression of NAD<sup>+</sup>- and NADP<sup>+</sup>-dependent IHD activity (Fig. 5H). However  
312 impairment of TCA cycle enzyme activities by increased BACE1 activity was not universal.  
313 For example, although fumarase activity was reduced slightly in SH-SY5Y<sub>B1</sub> cells, this was  
314 matched in SH-SY5Y<sub>mB1</sub> cells, with the protein levels of fumarase also decreased in both  
315 these cell lines, resulting in unaltered specific activity of fumarase by raised BACE1 in SH-  
316 SY5Y cells (Fig. 5I). Thus manipulation of APP cleavage towards amyloidogenic processing  
317 by increased BACE1 activity in SH-SY5Y cells reduces the overall catabolic capacity of the  
318 TCA cycle through specific enzyme lesions rather than a wholesale down-regulation.

319

### 320 **Rescue of glucose oxidation in SH-SY5Y<sub>B1</sub> cells.**

321 As glutamine supplementation was unable to recover BACE1-mediated inhibition of glucose  
322 oxidation, we focused on PDH as a potential target for pharmacological or alternative nutrient  
323 interventions. A key regulator of PDH activity is by phosphorylation of its E1 $\alpha$  subunit [63],  
324 which is predominantly controlled by the inhibitory effect of pyruvate dehydrogenase kinases  
325 (PDKs) versus activation through de-phosphorylation by pyruvate dehydrogenase  
326 phosphatase (PDP). Dichloroacetate (DCA) is a PDK1 inhibitor, which enhances oxidative  
327 phosphorylation in the brain [58] and has been promoted clinically for its anti-neoplastic  
328 effects [64]. Consistent with PDH inhibition, SH-SY5Y<sub>B1</sub> cells exhibited increased  
329 phosphorylation of the e1 $\alpha$  subunit at serine 293 compared to SH-SY5Y<sub>EV</sub> cells (Fig. 6A).  
330 Treatment with DCA (10 and 100  $\mu$ M) resulted in decreased levels of Ser<sup>293</sup> e1 $\alpha$   
331 phosphorylation of both SH-SY5Y cell types, although Ser<sup>293</sup> e1 $\alpha$  phosphorylation remained  
332 significantly higher in SH-SY5Y<sub>B1</sub> cells (Fig. 6A,B). In agreement with the reduction in e1 $\alpha$   
333 phosphorylation, incubation of SH-SY5Y<sub>EV</sub> cells with DCA (100  $\mu$ M) increased the glucose  
334 oxidation rate (Fig. 6C). However, although glucose oxidation of SH-SY5Y<sub>B1</sub> cells was also  
335 enhanced by DCA treatment, this remained significantly lower than that of the SH-SY5Y<sub>EV</sub>  
336 cells, mirroring e1 $\alpha$  phosphorylation status.

337 An alternative means of bypassing PDH is through the application of ketones, such as beta-  
338 hydroxybutyrate (BHB), which is metabolised to acetyl-CoA and enters the TCA cycle at  
339 oxaloacetate. The presence of BHB (0.5 – 10  $\mu$ M) recovered the relative deficit in glucose  
340 oxidation of SH-SY5Y<sub>B1</sub> cells, compared to SH-SY5Y<sub>EV</sub> cells, in a concentration-dependent  
341 manner (Fig. 6D). Previous studies [65,66] have indicated that diminished mitochondrial  
342 consumption of glucose in neurons and the ability to switch substrate preference to ketones is  
343 dependent on the presence and activity of BAD (BCL-2-associated agonist of cell death), a  
344 member of the BCL-2 gene family member of apoptotic control proteins, with increased  
345 mitochondrial usage of BHB in *Bad*<sup>-/-</sup> cortical neurons. In agreement with this model, we  
346 find that the increased ability of SH-SY5Y<sub>B1</sub> cells to utilise BHB over SH-SY5Y<sub>EV</sub> cells and  
347 enhance glucose oxidation, is associated with a large reduction of BAD protein expression  
348 (Fig. 6E). Finally we also investigated the ability of the naturally occurring enzyme co-  
349 factor,  $\alpha$ -lipoic acid to modify glucose oxidation of SH-SY5Y<sub>B1</sub> cells as previous studies  
350 have shown this compound to up-regulate mitochondrial bioenergetics and promote glucose  
351 uptake [67,68]. Indeed PDH and  $\alpha$ -KGDH protein complexes require  $\alpha$ -lipoic acid as a co-  
352 factor for their acyl transferase activity. Supplementation of the growth media with  $\alpha$ -lipoic  
353 acid (25 or 50  $\mu$ M for 48 hours) resulted in a robust concentration-dependent attenuation of  
354 the impaired glucose oxidation rate displayed by SH-SY5Y<sub>B1</sub>, compared to SH-SY5Y<sub>EV</sub> cells  
355 (Fig. 6F). Taken together, these results demonstrate the applicability of alternative nutrient  
356 (BHB) or co-factor ( $\alpha$ -lipoic acid) supplementation to alleviate or by-pass the impaired  
357 glucose metabolism elicited by raised BACE1 activity in SH-SY5Y cells.

359 **Discussion**

360 In this study we demonstrate that raised BACE1 activity by overexpression in SH-SY5Y  
361 cells, and subsequent modification of APP metabolism, induces a shift from the  
362 physiologically predominant alpha-secretase cleavage pathway to the amyloidogenic beta-  
363 secretase cleavage pathway, which results in decreased glucose metabolism. This  
364 bioenergetic impairment was dependent upon the secretase activity of BACE1 and produced  
365 a fundamental shift in the cellular metabolic profile, characterised by reduced glucose  
366 oxidation in association with a compensatory increase in glycolysis, in an attempt to maintain  
367 ATP production.

368 Decreased brain glucose metabolism is an invariant pathophysiological event occurring in  
369 AD progression, and it has been hypothesised that this may occur years, and even decades  
370 prior to symptom presentation [15,16,69,70]. Impaired glucose metabolism has also been  
371 noted in central tissues taken from AD animal models [22,23,68]. Furthermore, despite the  
372 observation that reduced glucose metabolism is predictive of later cognitive decline and AD  
373 symptom presentation, relatively little is known about the cellular mechanisms underlying  
374 these changes. Interestingly, the metabolic shift we have observed in favour of aerobic  
375 glycolysis under conditions of increased BACE1 activity mirrors early and predictive  
376 changes occurring in the brains of people who later develop AD. The brain areas that display  
377 this profile overlap with regions showing the greatest prevalence of A $\beta$  pathology, future  
378 susceptibility to cell death and are predictive of cognitive decline [32,33].

379 The mechanisms underlying glucose hypometabolism in AD are not well understood.  
380 However mitochondrial dysfunction has been widely reported in clinical and experimental  
381 AD studies [19-21] and A $\beta$  has been reported to accumulate in the mitochondria of AD  
382 patients and transgenic AD mouse models prior to amyloid deposition [25-27,71]. Although  
383 there are numerous reports of impaired electron transfer in mitochondria, for example via  
384 diminished activity in complexes I to V in AD subjects [72-75], and diminished complex III  
385 and IV activity in a transgenic AD mouse model [76] we found no effect of raised BACE1  
386 activity on mitochondrial electron transfer function in SH-SY5Y cells. It may be that  
387 additional injurious processes, such as increased oxidative and/or inflammatory stress are  
388 required to act concurrently with raised BACE1 activity and increased levels of A $\beta$  to elicit  
389 this outcome.

390 Thus the driving force for decreased glucose oxidation in SH-SY5Y<sub>B1</sub> cells appears to be  
391 through reduced substrate delivery to mitochondria by a BACE1 activity-dependent  
392 impairment of specific TCA cycle enzymes; PDH,  $\alpha$ KGDH and IDH each of which utilise  
393 decarboxylation reactions. Previous studies on post-mortem AD brains indicate reduced  
394 levels and/or activity of these key enzymes: PDH,  $\alpha$ KGDH and IDH [20,77-81], with PDH  
395 and  $\alpha$ KGDH exhibiting the largest decreases in activity. Furthermore, A $\beta$  peptides have been  
396 demonstrated to inhibit PDH and  $\alpha$ KGDH directly [28,31,82]. In contrast we find no change  
397 in the activity of the TCA enzyme fumarase, the activity of which is also unaltered in AD  
398 brains [20]. Our results add to these findings by implicating raised BACE1 activity and  
399 manipulation of APP processing as central to these enzyme deficits and metabolic adaptations  
400 at the cellular level, indicating a key role for BACE1 and its up-regulation in response to  
401 oxidative and inflammatory stress, which are associated with the very early stages of AD  
402 [39,43,83]. Indeed, these results show that raised BACE1 activity effectively phenocopies

403 some of the earliest changes in glucose metabolism seen in the brain during the progression  
404 towards AD.

405 In situations of diminished glucose utilization, there are a number of strategies that may be  
406 engaged in an attempt to bypass this block in metabolism. For example, in brain ischemic-  
407 reperfusion injury there is also decline in glucose oxidation and PDH activity [84,85].  
408 Application of glutamine has been demonstrated to offer some protection against ischemic-  
409 reperfusion injury in peripheral tissues by providing an alternative energy source for the cell  
410 to maintain ATP content [86,87]. In addition to providing a source of carbon for  
411 neurotransmitter production, glutamine can also feed into the TCA cycle and be metabolised  
412 to increase levels of  $\alpha$ -ketoglutarate and succinate. However, our data demonstrate that  
413 glutamine supplementation is unable to restore oxidative glucose metabolism in cells  
414 overexpressing BACE1. Furthermore, SH-SY5Y<sub>B1</sub> cells exhibited diminished oxidation of  
415 glutamine when applied as sole substrate, in line with the reduced activity of  $\alpha$ -KGDH  
416 observed in these cells.

417 The inhibition of PDH activity in SH-SY5Y<sub>B1</sub> cells was associated with increased  
418 phosphorylation of the e1 $\alpha$  subunit at Ser<sup>293</sup>, which is the main inhibitory site for PDH  
419 activity [88]. Although PDK1 inhibition with dichloroacetate reduced e1 $\alpha$  phosphorylation  
420 levels, this was also observed in SH-SY5Y<sub>EV</sub> cells, in conjunction with a maintained relative  
421 lower glucose oxidation in SH-SY5Y<sub>B1</sub> cells. This outcome may be owing to the presence of  
422 BACE1 activity-sensitive DCA-resistant PDK isoenzyme, or PDP, or an alternative kinase  
423 that phosphorylates e1 $\alpha$  at Ser<sup>293</sup> (e.g. GSK3). In contrast, application of the ketone, BHB, to  
424 SH-SY5Y<sub>B1</sub> cells demonstrates complete recovery of glucose oxidation, relative to SH-  
425 SY5Y<sub>EV</sub> cells, presumably by directly increasing the availability of acetyl CoA to the TCA  
426 cycle, thus circumventing PDH. The enhanced ability to utilise ketone bodies to aid glucose  
427 oxidation in SH-SY5Y<sub>B1</sub> cells may result from their reduced BAD levels, compared to SH-  
428 SY5Y<sub>EV</sub> cells. This finding agrees with the work of Nikita Danial's group showing that  
429 neurons derived from BAD knock out animals display an augmented bioenergetic profile in  
430 the presence of ketone bodies [66].

431  $\alpha$ -lipoic acid is synthesised in mitochondria and is a necessary cofactor for PDH and  $\alpha$ -  
432 KGDH activity. Our finding that exogenously applied  $\alpha$ -lipoic acid also increases glucose  
433 oxidation in SH-SY5Y<sub>B1</sub>, but not SH-SY5Y<sub>EV</sub> cells suggests that raised BACE1 activity  
434 either reduces mitochondrial  $\alpha$ -lipoic acid levels or lessens the ability of  $\alpha$ -lipoic acid to  
435 enhance PDH and  $\alpha$ -KGDH activity. The latter mechanism may be favoured as there is  
436 evidence that excess  $\alpha$ -lipoic acid provides protection to neuronal cells from the deleterious  
437 effects of exogenously applied A $\beta$  peptides on PDH activity [89] and A $\beta$ -induced toxicity  
438 [90]. Regarding the potential to reverse the metabolic deficits associated with age-dependent  
439 cognitive decline and early-stage or mild AD, the data presented herein demonstrate the  
440 capacity of  $\alpha$ -lipoic acid and BHB to attenuate impaired glucose oxidation at a cellular level  
441 in face of chronically raised BACE1 activity and increased levels of A $\beta$  peptides. BHB and  
442  $\alpha$ -lipoic acid have been demonstrated to exhibit some positive effects on cognitive decline in  
443 elderly dementia and mild AD patients [11,13,91-93].

444 Taken together, our findings are supportive for the rationale of targeting these TCA enzyme  
445 deficits in early cognitive impairment and AD. However, so far clinical trials based on  
446 raising circulating levels of ketones or giving  $\alpha$ -lipoic acid as a dietary supplement have  
447 provided mixed outcomes and further trials examining  $\alpha$ -lipoic acid are currently in progress.  
448 Perhaps a future strategy for AD management and treatment may be through the combination

449 of this neutraceutical approach with new therapeutics, such as BACE1 inhibitors, currently in  
450 clinical trials [94].

451

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457

#### 458 **Author contributions**

459 JAF and DLH performed experiments and analysed data. JAF, DLH and MLJA contributed  
460 to the conception and design of experiments, interpretation of data and drafting and revising  
461 the manuscript. MLJA supervised the study and JAF and MLJA wrote the manuscript. All  
462 authors approved the final version. MLJA is the guarantor of this work

463

#### 464 **Conflict of interest statement**

465 The authors declare that the research was conducted in the absence of any commercial or  
466 financial relationships that could be construed as a potential conflict of interest.

467

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## 761 **Figure legends**

762

763 **Figure 1: BACE1 overexpression alters APP processing and glucose oxidation in SH-**  
764 **SY5Y cells. A.** Representative immunoblots showing stable overexpression of BACE1 and  
765 resultant changes in cellular APP and sAPP $\alpha$  and sAPP $\beta$  shed into the culture media,  
766 compared to empty vector (EV) treated controls. **B. – E.** SH-SY5Y BACE1 overexpression  
767 did not change APP protein levels (**B**; n=4), increased total BACE1 protein levels (**c**; n = 7)  
768 and induced a shift in APP processing, with a reduction in sAPP $\alpha$  (**D**; n = 4) and an increase  
769 in sAPP $\beta$  (**E**; n = 4). **F. – H.** SH-SY5Y BACE1 overexpression reduced <sup>14</sup>C-glucose  
770 oxidation rate (**F**; n = 7), with no reduction in <sup>14</sup>C-glucose incorporation rate (**G**; n = 9),  
771 giving an overall reduction in the ratio of <sup>14</sup>C-glucose oxidation to incorporation (**H**; n = 7).  
772 Values are means  $\pm$  SEM. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

773

774 **Figure 2: Glucose oxidation in SH-SY5Y cells is not impaired following overexpression**  
775 **of secretase-dead BACE1 protein. A.** Representative immunoblots showing stable  
776 overexpression of wild type BACE1 and a secretase-dead BACE1 mutant (mBACE1) and  
777 resultant changes in cellular APP and sAPP $\beta$  shed into the culture media. **B. – D.** SH-SY5Y  
778 mBACE1 overexpression increased total BACE1 to levels equivalent to BACE1  
779 overexpressed cells and ~3-4X greater than empty vector (EV) controls (**B**; n = 5), with no  
780 effect on APP levels (**C**; n = 3), but in contrast to BACE1 overexpression, mBACE1 reduced  
781 sAPP $\beta$  to levels below that of the EV controls (**D**; n = 6 – 10). **E. – G.** SH-SY5Y mBACE1  
782 overexpression had no effect on <sup>14</sup>C-glucose oxidation compared to EV controls, in contrast  
783 to the reduction in <sup>14</sup>C-glucose oxidation observed in BACE1 overexpressing cells (**E**; n = 5),  
784 with mBACE1 having no effect on <sup>14</sup>C-glucose incorporation (**F**; n = 5), resulting in the ratio  
785 of <sup>14</sup>C-glucose oxidation to incorporation being unchanged in mBACE1 cells (**G**; n = 5).  
786 Values are means  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

787

788 **Figure 3: BACE1 overexpression alters glucose metabolism in SH-SY5Y cells.**

789 **A. – C.** BACE1 overexpression reduces oxygen consumption rate (OCR) (**A**) and increases  
790 the extracellular acidification rate (ECAR) (**B**) resulting in a reduced OCR:ECAR ratio (**C**),  
791 compared to EV cells (n = 7 – 12). **D.** Glycolysis stress test temporal profile for EV and  
792 BACE1 SH-SY5Y cells showing the effects on ECAR of the sequential addition of 2.5 mM  
793 glucose, 1  $\mu$ M oligomycin (Oligo) and 25 mM 2-deoxyglucose (2DG). **E. – G.** BACE1  
794 overexpression increased basal glycolysis (**E**), maximal glycolysis (**F**) and glycolytic reserve  
795 (**G**) compared to EV-treated cells (n = 18 – 21). Values are means  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p <$   
796  $0.01$ , \*\*\*  $p < 0.001$ .

797

798 **Figure 4: BACE1 overexpression has no effect on ATP-linked, maximal or leak**  
799 **mitochondrial oxygen consumption.**

800 **A.** Modified mitochondrial stress temporal profile for EV and BACE1 SH-SY5Y cells  
801 showing the effects on the percentage change in normalised OCR of the sequential addition  
802 of 1  $\mu\text{M}$  oligomycin (oligo) and 2  $\mu\text{M}$  rotenone and antimycin (Rot + Ant). **B. – D.** BACE1  
803 overexpression had no effect on ATP-linked OCR (**B**), on the OCR required to maintain the  
804 mitochondrial leak (**C**) or the non-mitochondrial OCR (**D**) compared to EV cells (n = 8). **E.**  
805 Temporal profile showing the normalised baseline OCR for EV and BACE1 cells and the  
806 maximal OCR (reserve capacity) attained following addition of 0.2  $\mu\text{M}$  carbonyl cyanide-p-  
807 trifluoromethoxyphenylhydrazone (FCCP). **F.** BACE1 overexpression has no effect on the  
808 percentage OCR increase following FCCP addition compared to EV cells (n = 9-10). Values  
809 are means  $\pm$  SEM.

810

811 **Figure 5: BACE1 overexpression induces specific mitochondrial TCA cycle enzyme**  
812 **lesions. A, B.** SH-SY5Y cells overexpressing BACE1 exhibit reduced lactate usage at levels  
813  $\leq 2\text{mM}$  compared to EV cells as demonstrated by (**A**) reduced repression of  $^{14}\text{C}$ -glucose  
814 oxidation (n = 6) and (**B**) direct OCR when lactate provided as sole substrate (n = 6 – 11). **C.**  
815 Pyruvate dehydrogenase activity (PDH) is reduced in BACE1 overexpressing, compared to  
816 EV, cells (n = 5). **D.** OCR when EV and BACE1 cells provided 2.5 mM pyruvate as sole  
817 substrate (n = 5). **E.** OCR of EV and BACE1 SH-SY5Y cells when provided with 2.5 mM  
818 glucose alone or 2.5 mM glucose + 4 mM glutamine (n = 4 – 10). **F.** direct OCR when 4 mM  
819 glutamine provided as sole substrate (n = 8). **G.** BACE1, but not mBACE1, overexpression,  
820 reduced the enzyme activity of  $\alpha$ -KGDH, compared to EV cells (n = 6). **H.** IDH activity  
821 ( $\text{NAD}^+$  and  $\text{NADP}^+$  isoforms) is reduced by BACE1 overexpression compared to EV cells (n  
822 = 4). **I.** BACE1 or mBACE1 overexpression has no effect on fumarase enzyme activity,  
823 protein levels or specific activity compared to EV cells (n = 7). Values are means  $\pm$  SEM. \*  $p$   
824  $< 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

825

826 **Figure 6: Reversal of BACE1 mediated impaired glucose oxidation in SH-SY5Y cells.**

827 **A,B.** Representative immunoblots (**A**) of phosphorylated PDHe1 $\alpha$  (p-PDHe1 $\alpha$ ) subunit, total  
828 PDHe1 $\alpha$  and actin loading control in control (EV) and BACE1 overexpressing cells in the  
829 absence and presence of 10  $\mu\text{M}$  or 100  $\mu\text{M}$  dichloroacetate (DCA), with quantification of the  
830 immunoblot data shown in **B** (n = 4). **C.** DCA partially reverses BACE1 mediated  
831 impairment of glucose oxidation, but also increases OCR of EV cells (n = 5). **D.** reduced  
832 OCR associated with BACE1 cells is recovered to EV control levels by addition of 1  $\mu\text{M}$  and  
833 10  $\mu\text{M}$  beta hydroxybutyrate (n = 4 – 6). **E.** Representative immunoblots of BCL-2-  
834 associated agonist of cell death (BAD) and actin loading control with quantification of the  
835 immunoblot data (n = 4). **F.** BACE1-mediated reduction in OCR is recovered to EV control  
836 levels by addition of 50  $\mu\text{M}$   $\alpha$ -lipoic acid (n = 6). Values are means  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p$   
837  $< 0.01$ , \*\*\*  $p < 0.001$ .

838

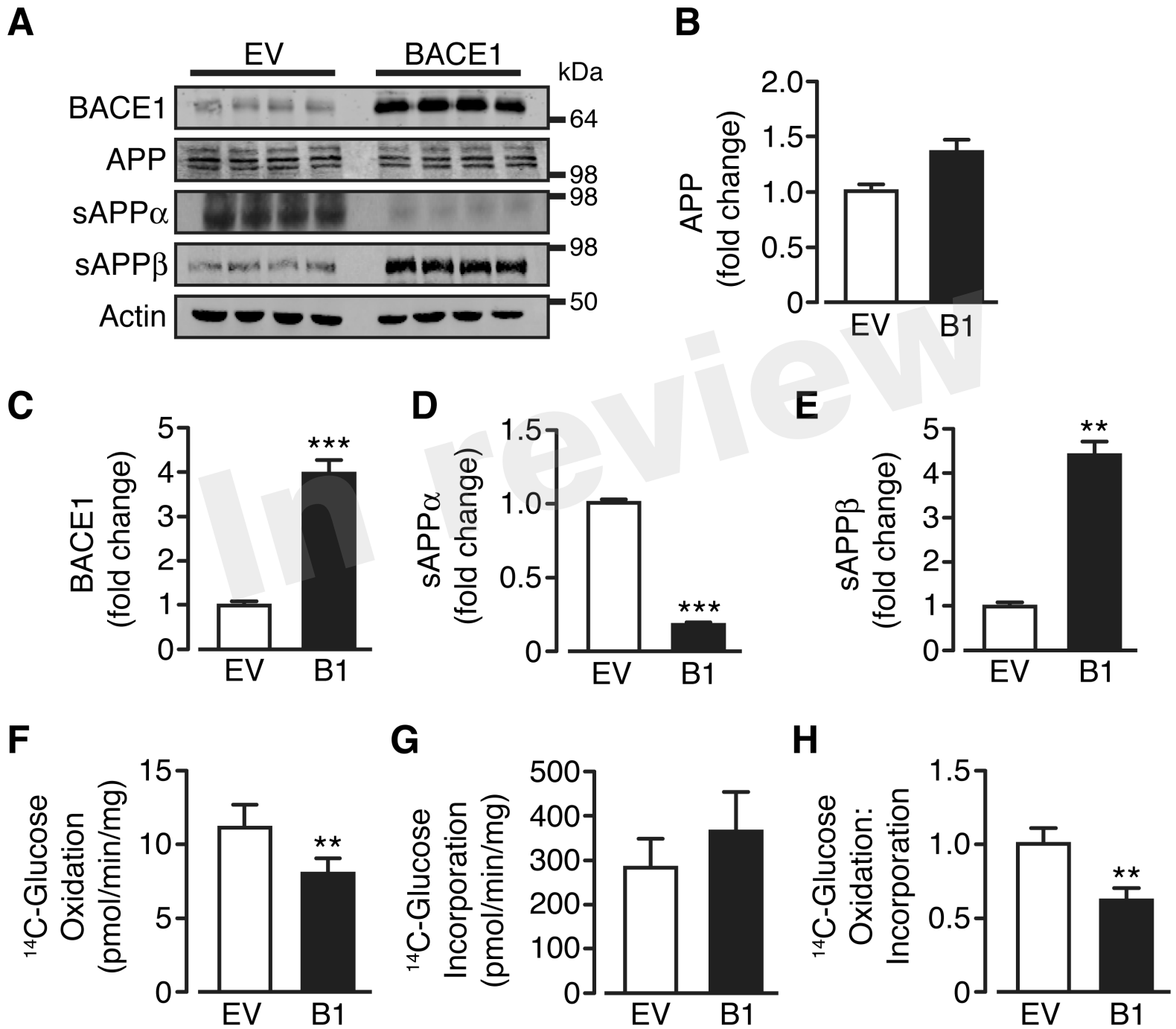


Figure 2.TIF

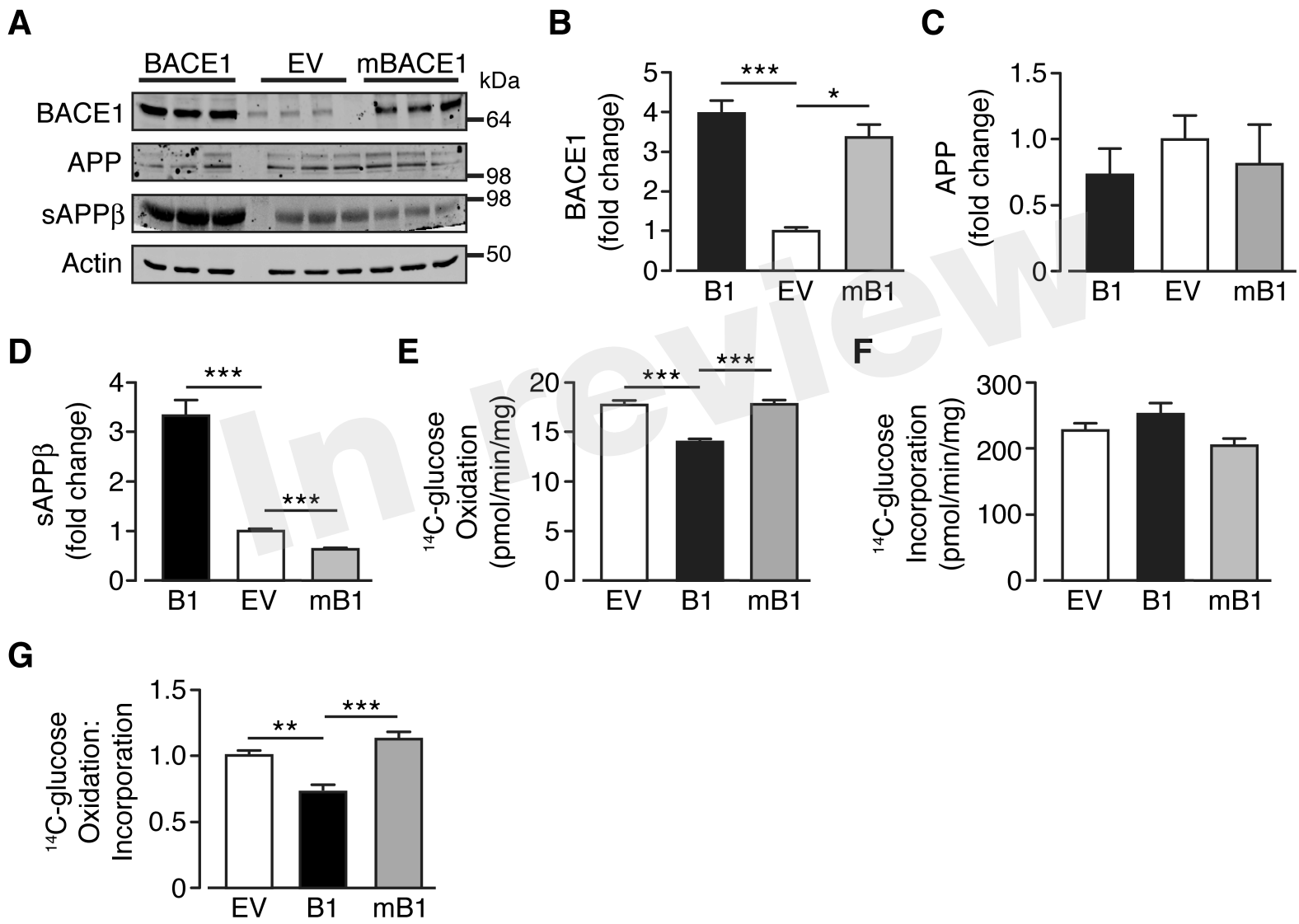


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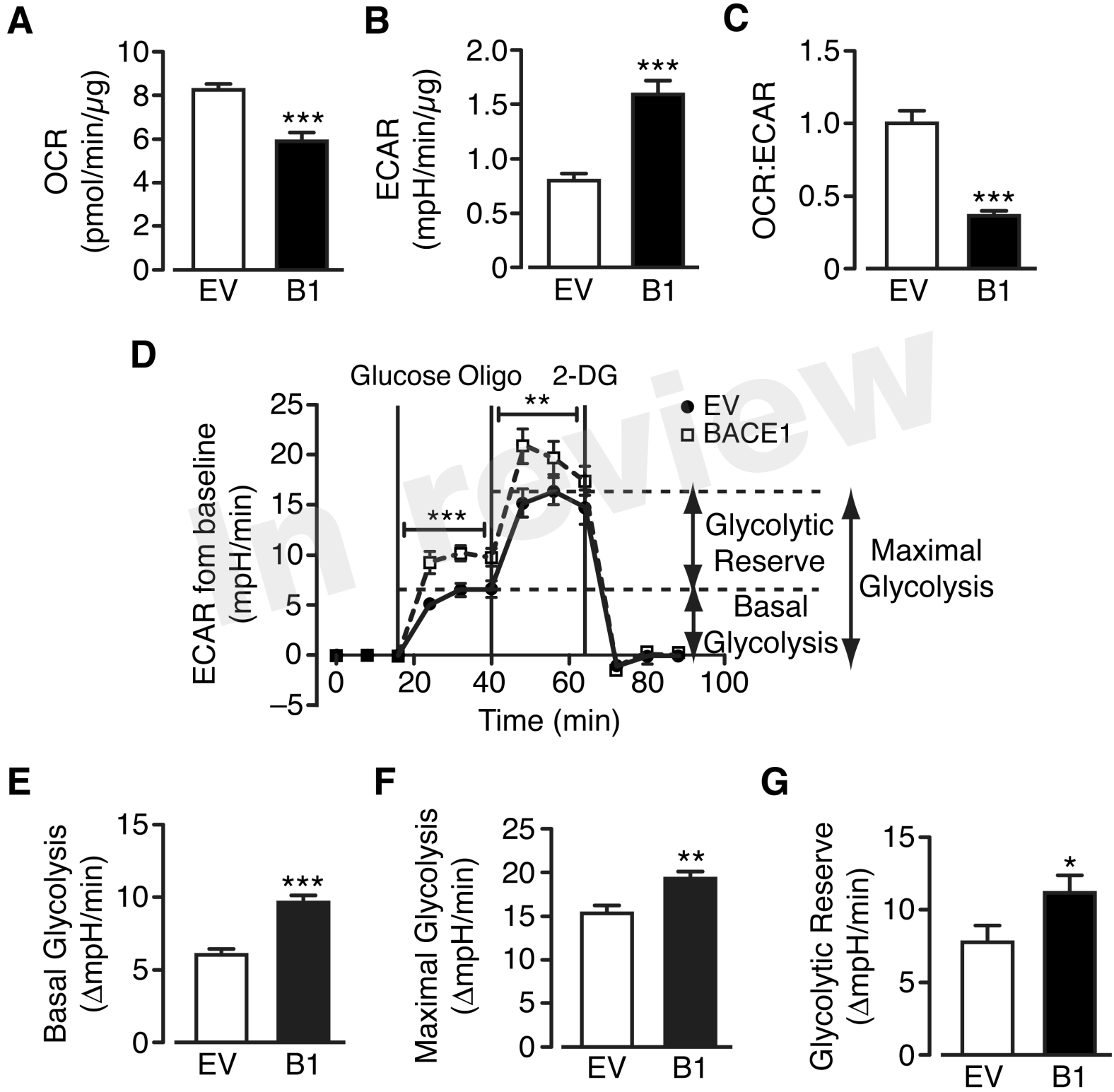


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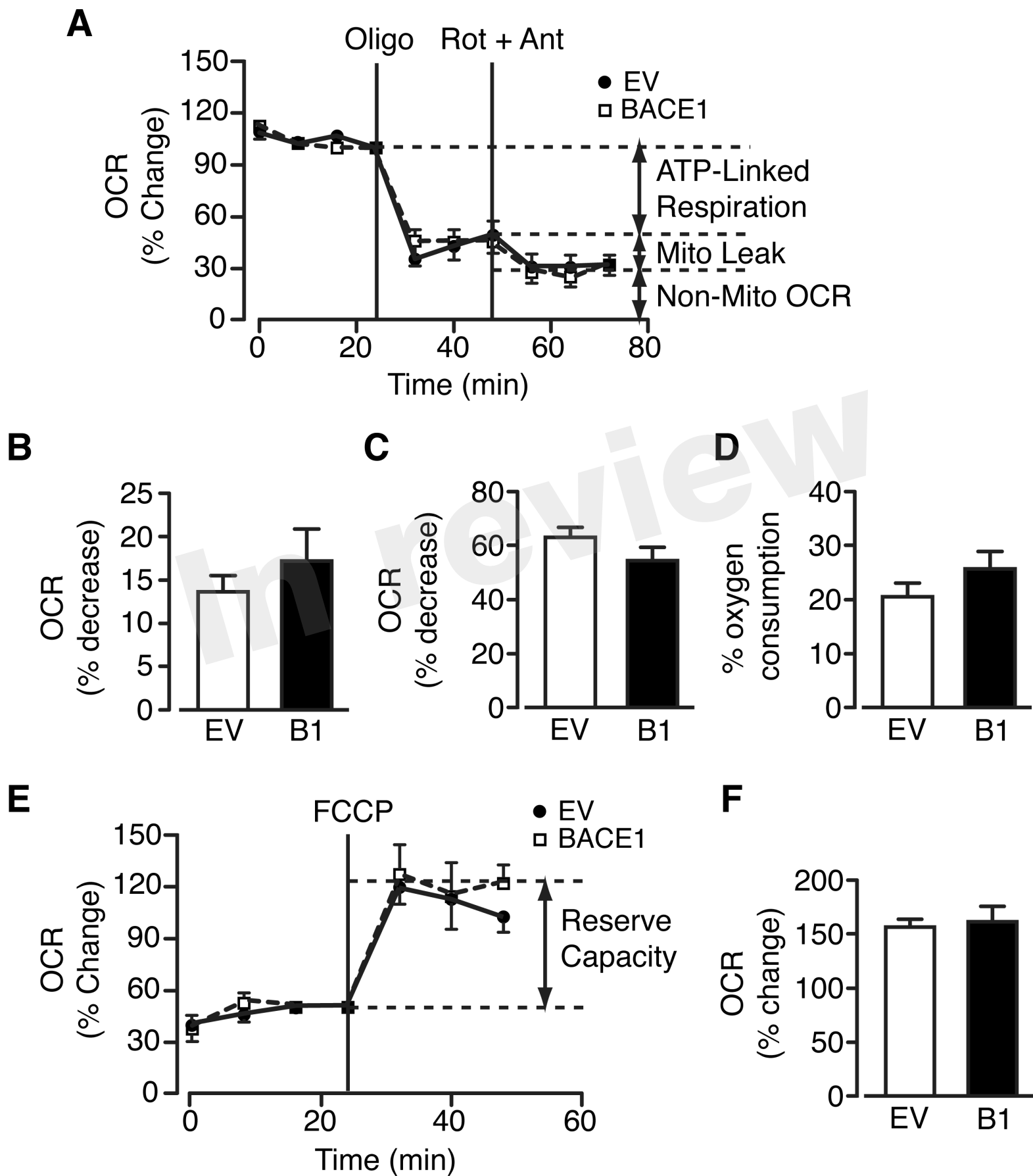




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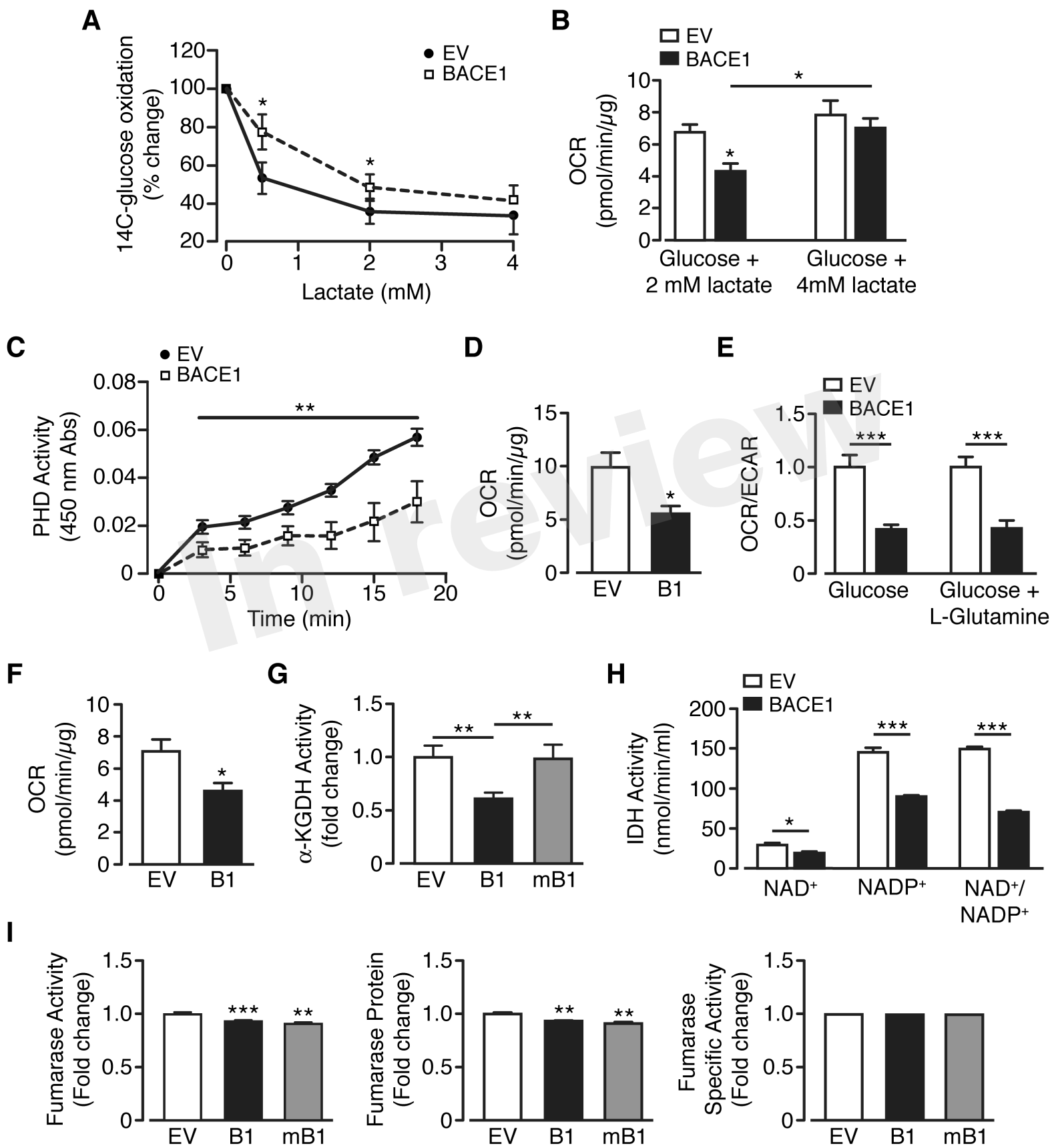
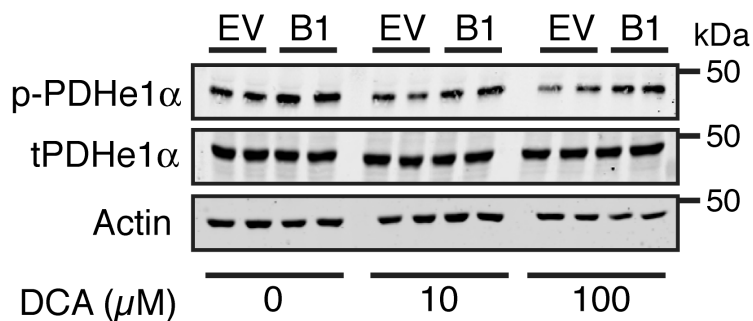
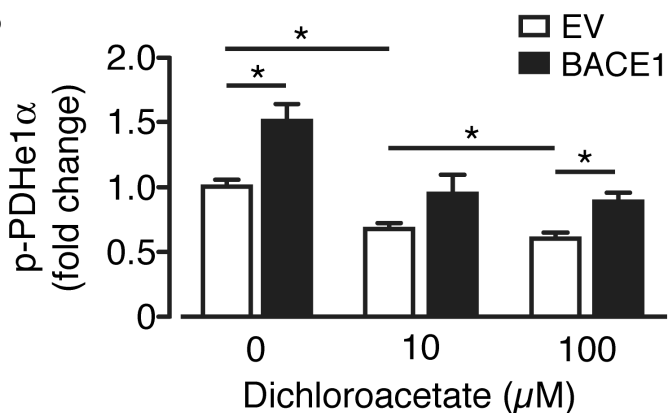


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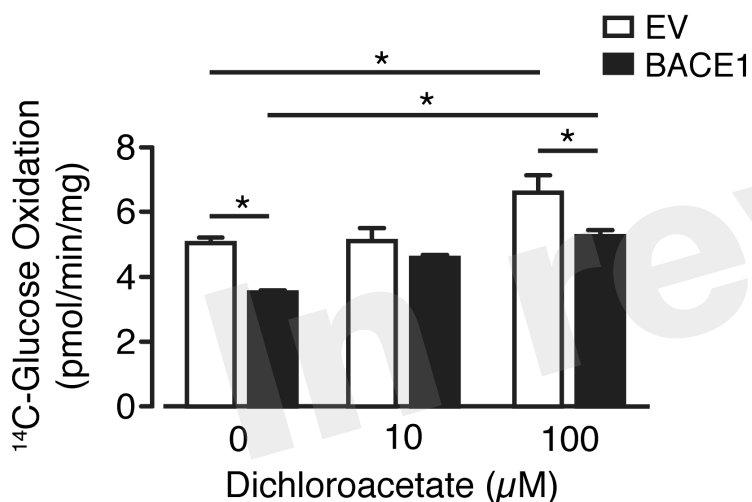
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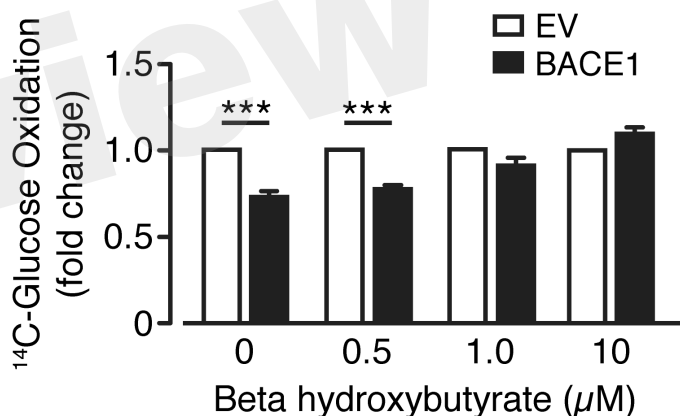
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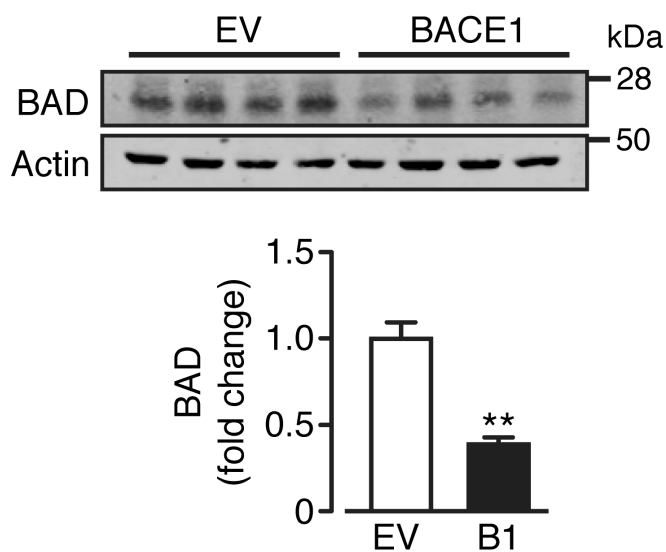
**C**



**D**



**E**



**F**

