1 **N-3 polyunsaturated fatty acids promote astrocyte differentiation and neurotrophin**
2 **production independent of cAMP in patient-derived neural stem cells**
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14 Running title: N-3 polyunsaturated fatty acids and astrocyte in depression

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29

30   Abstract

31   Evidence from epidemiological and laboratory studies, as well as randomized placebo-controlled

32   trials, suggests supplementation with n-3 polyunsaturated fatty acids (PUFAs) may be efficacious

33   for treatment of major depressive disorder (MDD). The mechanisms underlying n-3 PUFAs

34   potential therapeutic properties remain unknown. There are suggestions in the literature that glial

35   hypofunction is associated with depressive symptoms and that antidepressants may normalize

36   glial function. In this study, iPSC-derived neuronal stem cell lines were generated from

37   individuals with MDD. Astrocytes differentiated from patient-derived neuronal stem cells

38   (iNSCs) were verified by GFAP. Cells were treated with eicosapentaenoic acid (EPA),

39   docosahexaenoic acid (DHA) and stearic acid (SA). During astrocyte differentiation, we found

40   that n-3 PUFAs increased GFAP expression and GFAP positive cell formation. BDNF and

41   GDNF production were increased in the astrocytes derived from patients subsequent to n-3

42   PUFA treatment. Stearic Acid (SA) treatment did not have this effect.   CREB activity

43   (phosphorylated CREB) was also increased by DHA and EPA but not by SA. Furthermore, when

44   these astrocytes were treated with n-3 PUFAs, the cAMP antagonist, RP-cAMPs did not block n-

45   3 PUFA CREB activation. However, the CREB specific inhibitor (666-15) diminished BDNF

46   and GDNF production induced by n-3 PUFA, suggesting CREB dependence. Together, these

47   results suggested that n-3 PUFAs facilitate astrocyte differentiation, and may mimic effects of

48   some antidepressants by increasing production of neurotrophic factors. The CREB-dependence

49   and cAMP independence of this process suggests a manner in which n-3 PUFA could augment

50   antidepressant effects. These data also suggest a role for astrocytes in both MDD and

51   antidepressant action.

52

53   Introduction

54   Major depressive disorder (MDD) is the most common psychiatric disorder, with almost

55   one in six individuals experiencing at least one depressive episode at some point in their lifetime.

56   It is currently the leading cause of disability worldwide [1, 2]. While effective treatments exist,

57   about one third of patients treated with antidepressants do not reach symptomatic remission [3,

58   4].   While some of these non-remitters may respond to ketamine or other rescue strategies,

59   additional therapeutic options are needed.

60   Brain regions and even cell-types contributing to MDD are not well established [4].

61   Evidence from clinical, preclinical and post-mortem studies suggests that dysfunction and

62   degeneration of astrocytes may be one of potential candidates, and astrocytes may also represent

63   a potential therapeutic target for MDD [5-8]. Antidepressants, including SSRIs and ketamine,

64   increase brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor

65   (GDNF) expression in primary cultured animal astrocytes [9-11]. Substantial evidence suggested

66   that astrocytes might be the main resource of antidepressant-induced BDNF [8]. Furthermore,

67   overexpression of BDNF in astrocytes leads to antidepressant-like activity in mice [12].

68   N-3 polyunsaturated fatty acids (n-3 PUFAs) are found in fish oil, and include EPA and

69   DHA. Many studies have suggested antidepressant efficacy for omega-3 fatty acids (n-3 PUFA)

70   in MDD [13-17], and n-3 PUFAs might evoke an antidepressant paradigm in culture cells and

71   animal models [13].   The mechanisms of n-3 PUFA antidepressant action remain poorly

72   documented, but include inhibition of the production of proinflammatory cytokines and

73   maintenance of neuronal membrane stability and fluidity [13, 18]. Most efforts to characterize

74   the mechanism of action of n-3 PUFAs have been done in animals and cell lines.   Further,

75   probing the mechanisms of n-3 PUFA anti-depressant effects, especially in human cells, would

76   lead to better strategies for the use of n-3 PUFAs to improve outcome of MDD.

77   The restricted access to brain tissue and a variety of neuronal cell types in patients

78   represents a major hurdle in exploring neuropsychiatric diseases mechanisms and developing

79   therapeutic targets and approaches. Human-induced pluripotent stem cell (iPSC) technology

80   offers another means of accessing neuropsychiatric patient cells by enabling the differentiation of

81   patient iPSCs into relatively pure populations of neurons or glial cells, which may provide new

82   avenues to study neuropsychiatric disorders and potential treatments [19]. The neuronal or glial

83   cells resulting from patient-derived iPSC have been successfully used to investigate bipolar

84   disorder, schizophrenia and MDD[20] [19, 21-23].

85   To explore the potential mechanisms underlying the beneficial effects of n-3 PUFAs in

86   MDD, human astrocytes were obtained by differentiating MDD patient-derived iNSCs from

87   iPSCs. N-3 PUFAs facilitated astrocyte generation from iNSCs during differentiation. The

88   astrocytes also responded to n-3 PUFA with increased CREB activation and increased

89   neurotrophin production. These were cAMP-independent, even though evidence suggested the

90   importance of cAMP signaling in therapeutic effect of antidepressants[24]. This raises the

91   possibility that, while n-3 PUFA have a cellular hallmark similar to antidepressants, they achieve

92   this through a different pathway.

93

94 **Materials and methods.**

95 **Subject Recruitment**

96 Two patient iPSC lines were drawn from a large biobank collected between 2010 and 2018 at

97 Massachusetts General Hospital. Briefly, adult participants were recruited from outpatient

98 psychiatry clinics and signed written, informed consent approved by the Partners HealthCare

99 institutional review board. Diagnosis was confirmed by structured clinical interview by a trained

100 physician rater [25]. Current and lifetime treatment history was collected using the

101 Antidepressant Treatment Response Questionnaire[26] and the FAST[27], augmented with

102 clinician review of electronic health records. Selective serotonin reuptake inhibitor sensitive

103 (SSRI-sensitive) was defined as remission with one SSRI. SSRI-resistant was defined as lack of

104 remission with two adequate SSRI trials. Participants underwent a 3 or 4mm dermal punch

105 which was subsequently expanded into a fibroblast cell line. iPSC lines were generated from

106 cultured fibroblast lines as previously described [20].

107 **Human iNSC culture and astrocyte differentiation**

108 iNSCs from 1 SSRI-sensitive and 1 SSRI-resistant MDD patient were generated from iPSC lines

109 cultured feeder free in E8 medium (Gibco) on Geltrex (Gibco) coated 6-well plates and passaged

110 with Accutase (Sigma). iPSCs were cultured in E8 with 10uM Thiazovivin (Stemgent, Inc)

111 following passaging. Prior to neural induction, iPSCs were purified using magnetic-activated cell

112 sorting (MACS) with Tra1-60 microbeads (Miltenyi Biotec) on LS columns as described by

113 vendor and cultured to 10-20% confluence. Neural induction was initiated using Neurobasal

114 medium (Gibco) supplemented with 1X Neural Induction Supplement (Gibco). Induced cells

115 were cultured to confluence in neural expansion medium (NEM; 50% Neurobasal, 50%

116 Advanced DMEM/F-12, 1X Neural Induction Supplement; Gibco) and passaged using Accutase

117 with 10uM Thiazovivin for at least 6 passages. Putative iNSCs were further purified by

118 sequential MACS (PSA-NCAM^{+}, CD271^{-}, CD133^{+}; Miltenyi Biotec) according to the

119 manufacturer's protocol.

120

121 Glial fibrillary acidic protein (GFAP)-expressing astrocytes were differentiated from iNSCs as

122 reported with modifications [28, 29]. Briefly, iNSC were seeded in 6-well dishes coated with

123 Geltrex at a seeding density of 5×10^{5}/ml in NEM. On the second day, NEM was replaced by

124 astrocyte differentiation medium (DMEM/F-12, 5ng/ml BMP2, 5ng/ml CNTF, and 10 ng/ml

125 bFGF (PeproTech, Rock Hill, NJ)). Medium was exchanged every other day.

126

127 For testing the effect of n-3 PUFAs on astrocyte differentiation from iNSC, cells were seeded in

128 NEM on Geltrex-coated 24-well plates or culture dishes. After 24 hours, the medium was

129 changed to astrocyte differentiation medium containing vehicle (ethanol, 0.15%),

130 docosahexaenoic acid (DHA, 50 μM), eicosapentaenoic acid (EPA, 50μM) or stearic acid (SA,

131 50 μM), prepared as previously reported [30]. After 5 days, cells were processed for qPCR,

132 western blot assay or immunostaining. For studying the impact of n-3 PUFAs on neurotrophin

133 production, astrocytes were differentiated for 30 days from iNSCs and then treated with vehicle,

134 DHA, EPA or SA (same concentration as above described) for 3 days in 6–well plates and then

135 processed to designed experiments.

136 **Immunocytochemistry**

137 After fixation with cold 100% methanol (–20 °C) for 10 min, cells were incubated with blocking

138 buffer (PBS, 5% goat serum, 0.3% Triton X-100) for 60 min, then incubated in 1:100 dilution of

139 anti-PAX6 (Cell Signaling, Danvers, MA), 1:200 anti-Sox2 (DSHB, Iowa, IW) or 1:150 anti-

140 GFAP (Cell signaling, Danvers, MA) antibodies in antibody dilution buffer (PBS, 1% BSA,

141 0.3% Triton X-100) buffer for overnight at 4 $^{\circ}$C. Subsequently, the dishes were washed with

142 PBS four times and incubated with corresponding fluorochrome-conjugated secondary

143 antibodies in antibody dilution buffer (1:100) for 30 min. Mounting medium with DAPI (Vector)

144 was added to the dishes which were then imaged using confocal (LSM880) or epifluorescence

145 microscopy (Nikon). GFAP positive cells were counted under a 40X amplification field either

146 manually or in a Keyence (Itasca, IL) microscopy system.

147 **Reverse transcription qualitative polymerase chain reaction**

148 Total RNA from cells in 24-well cell culture plates was isolated using TRIZOL (Life

149 Technologies, Grand Island, NY, USA) following the manufacturer's instructions. Total RNA

150 (500 ng) was converted to cDNA by Superscript III Direct cDNA Synthesis System (Life

151 Technologies). Primers were designed to span exon-exon junction in order to preclude the

152 amplification of genomic DNA; primers for Human *GFAP*: forward, 5'-

153 AGGGGGCAAAAGCACCAAAGA-3', reverse 5'CTGGGAAAATGA CGCAGTCCAG-3'; for

154 *GDNF*: forward, 5'-CCCGCCGCAAATATGCCAGA-3', reverse, 5'-GTTCCTCCTTGGTTT

155 CATAGCCC-3'; for *BDNF*, forward, 5'-GCCATCCCAAGGTCTAGGTG-3', reverse, 5'-

156 GTGGGATGGTGGGCATAAGT-3'; for *GAPDH*; forward 5'-AACAGC GACACCC ACTCC

157 TC-3', reverse 5'-AGCCAAATTCGTTGTCATACCAGG-3'. The target amplicon sizes were

158 274, 208, 261 and 100 bp for *GDNF, BDNF*, *GFAP* and *GAPDH*, respectively. QPCR was done

159 with ViiA Real-time PCR system (ThemoFisher). Data was analyzed with ViiA 7 software with

160 *GAPDH* as internal control. Gene expression was normalized to vehicle control.

161 **Capillary Western Assays**

162

163   After 30 days of differentiation, Astrocytes were treated with vehicle, DHA, EPA and SA as

164   described above for three days and then washed twice with ice-cold PBS prior to lysis. For the

165   standard method, cells were incubated on ice for 15 min in RIPA lysis buffer (Cell Signaling,

166   Danvers, MA) supplemented with Complete Protease Inhibitor Cocktail (Roche) and

167   phosphatase inhibitor (Thermo Scientific). Lysates were scraped into tubes and clarified by

168   centrifugation at 15000 x g for 10 min at 4 °C. Total protein concentrations of the supernatants

169   were determined by Pierce BCA Protein Assay (Thermo Scientific).

170   Reagents and equipment were purchased from Protein-Simple (San Jose, CA) unless stated

171   otherwise. All experiments followed the manufacturer's instructions. 1 µl 5X Fluorescent Master

172   Mix was added to each sample (4 µl). Samples were incubated at 95 °C for 5 min to denature.

173   Three microliters of each sample were loaded into the top-row wells of plates that were designed

174   to separate proteins of 12-230 kDa. Primary antibodies were diluted in Antibody Diluent as

175   follows: anti-BDNF (1:50), anti-GDNF (1:50) (Santa Cruz Biotechnology, Dallas, TX), anti-

176   GPADH (1:10000) (Protein Tech, Rosemont, IL), anti-CREB (1:50), anti-pCREB (1:50)

177   (Invitrogen, Madison, WI) and anti-GFAP (1:50) were diluted. Secondary antibodies, Anti-

178   Mouse Secondary HRP Conjugate and Anti-Rabbit Secondary HRP Conjugate (Protein Simple).

179   Peak area calculations were conducted with companying Compass software as indicated by

180   manufacture (Protein Simple).

181   **cAMP measurement in living cells**

182   Astrocytes differentiated for 30 days were cultured on glass bottom dishes coated with Geltrex

183   and infected with cADDIS BacMam virus encoding the green upward cAMP sensor at

184   1.00×10^9 VG/mL (Montana molecular, Bozeman, MT) with sodium butyrate at a final

185   concentration of 1mM for 24 h, then replaced by astrocyte medium. Live cell images were

186   acquired under a 20× objective on a Zeiss 880 microscope. The acute effect of n-3 PUFA on

187   cAMP formation was evaluated for acute (20 min) DHA treatment. Every 5 min, the average

188   fluorescent density from each cell selected from the visual field was collected. The effect of n-3

189   PUFAs treatment on the potential of Gαs-stimulated cAMP production was determined as

190   previous reported [31]. Astrocytes derived from the SSRI-sensitive patient were treated for 3

191   days, The average fluorescent density of selected cells (5-12 cell in each dish) were first

192   recorded, then cells were challenged with forskolin (2μM), and recording was continued.

193   Fluorescence density was normalized to baseline fluorescence for each experiment.

194   **Statistical analysis**

195   Statistical analyses were performed using the Prism 5 statistical package (GraphPad Software,

196   Inc., La Jolla, CA). All data were analyzed by Student's t-test or one-way analysis of variance

197   (ANOVA) with a Tukey's post hoc test. Data were expressed as Mean ± SD. A P value of<0.05

198   was considered statistically significant.

199   **Results**.

200   n-3 PUFAs facilitate astrocyte differentiation from iNSCs.

201   Decrease in astrocyte count and GFAP expression are seen at many brain regions in MDD post-

202   mortem studies [32-35] and animal models of depression [8]. A recent reprot suggested that

203   antidepressants could induce recovery of astrocytes in MDD, which could be a potential

204   mechanism for their anti-depression effect [8]. To test whether n-3 PUFAs affect astrocyte

205   formation, we observed the effect of n-3 PUFAs on astrocyte differentiation from iNSCs derived

206   from SSRI-sensitive or -resistant MDD subjects. First, patient-derived iNSCs were confirmed

207   with Sox2 and Pax6 co-immunostaining (Supplemental figure 1 A). The iNSCs were cultured

208   with astrocyte differentiation medium that contained DHA, EPA, SA or vehicle for 5 days, after

209   which cells underwent immunostaining with GFAP antibody. Note that GFAP staining is weak

210   in some cells after 5 day differentiation, but positive staining can be identified compared to cells

211   without expression of GFAP in the same field (Figure 1B). Cells with positive GFAP staining

212   were counted using confocal microscopy with 40x magnification. Four fields in each dish were

213   selected, randomly, for cell counting. Data indicated that the percentage of GFAP positive cells

214   in each 40X field,   DHA or EPA treatment significantly increased the percentage of GFAP

215   positive cells compared to vehicle or SA treatment (Figure 1B). *GFAP* mRNA expression in

216   cells treated with DHA or EPA was almost two times higher than in vehicle control cells (Figure

217   2 A).   Furthermore, GFAP protein expression also increased in DHA or EPA treated cells

218   (Figure 2 B and C). We observed that differences in astrocyte differentiation between cells from

219   SSRI-sensitive and SSRI-resistant patients were not associated with n-3 PUFA treatment (Figure

220   1 and 2).

221

222   <u>N-3 PUFAs increased BDNF and GDNF production in astrocytes from patient-derived iNSC</u>

223   Therapeutic effects of antidepressants on MDD by modulation of astrocytic function (such as

224   producing BDNF and GDNF) have been reported [36-39]. Though animal and clinical studies

225   on MDD showed increased BDNF with n-3 PUFA treatment [40-42], the relevant cell types were

226   not delineated. Here, astrocytes were generated with patient-derived iNSCs. After 30 days of

227   differentiation in astrocyte differentiation medium, about 80-90% of cells were GFAP positive

228   (supplemental figure 1 B). The astrocytes were cultured with vehicle control, DHA, EPA and SA

229   for an additional 3 days. mRNA expression for BDNF or GDNF were tested. DHA and EPA

230   significantly increased the mRNA of BDNF and GDNF in astrocytes derived from SSRI-

231   sensitive and resistant patients, SA was without effect (Figure 3 A and B). The results from

232   capillary western assay further demonstrated that DHA and EPA elevated BDNF and GDNF

233   protein in the astrocytes from both SSRI-sensitive and SSRI-resistant subjects (Figure 3 C). This

234   increase in BDNF and GDNF induced by DHA and EPA required sustained n-3 PUFA treatment

235   and was not evident after a 24 h treatment. (Supplemental figure 2).

236

237   <u>PUFAs increased phosphorylated CREB in astrocytes in a cAMP independent manner.</u>

238   CREB is an important regulator for BDNF and GDNF expression [43-45]. Chronic

239   antidepressant treatment enhances CREB activation, suggesting CREB as an important mediator

240   for antidepressant treatment [24]. CREB must be phosphorylated to pCREB in order to transcribe

241   CREB-regulated genes, including BDNF and GDNF [46].To examine whether n-3 PUFAs alter

242   the activity of CREB in patient-derived astrocytes, CREB and pCREB were measured using a

243   capillary western assay in 30-day differentiated astrocytes. After 3 days treatment with DHA,

244   EPA, SA and vehicle control, CREB expression did not show significant alteration, but pCREB

245   was increased in both astrocytes derived from SSRI-sensitive and -resistant subjects (figure 4),

246   suggesting that DHA and EPA elevated CREB activity. cAMP activates CREB through protein

247   kinase A (PKA). Antidepressants, like escitalopram, evoke a sustained increase in cAMP [47].

248   The patient-derived astrocytes were treated with RP-cAMP (150 uM) +vehicle, DHA, EPA or

249   SA, respectively for 3 days. RP-cAMP, a potent and specific competitive inhibitor of the

250   activation of PKA by cAMP, did not diminish the increase of pCREB induced by DHA or EPA

251   (Figure 4), suggesting that cAMP cascades may not be involved in activation of CREB by n-3

252   PUFAs. With RP-cAMP treatment, DHA and EPA still elevated the BDNF and GDNF

253   production (Supplemental figure 3). Antidepressants including ketamine, translocate $G\alpha_s$ from

254   lipid rafts, which increases association of $G\alpha_s$ with adenylyl cyclase, increasing cellular cAMP

255   production [31, 47]. To test if n-3 PUFAs have similar effect, we measured the cAMP with a

256   fluorescent sensor in astrocytes from SSRI-sensitive and -resistant subjects. After three days of

257   treatment, no difference was seen among baselines of each group. However, DHA and EPA

258   increased Gαs-activated cAMP production stimulated by forskolin, while SA did not show the

259   effect in SSRI-sensitive cells (Supplemental figure 4). Similar to antidepressants, after 20 min

260   treatment with n-3 PUFAs, no change in cAMP was seen (Supplemental figure 5)

261

262   4. Increased expression of BDNF or GDNF by n-3 PUFAs in patient-derived astrocytes is

263   mediated by CREB.

264   CREB/pCREB and BDNF appear to be diminished in animal models and in patients suffering

265   from depression [37]. In a CREB-deficient mouse, the upregulation of BDNF induced by

266   antidepressant was abrogated [48]. To explore whether CREB mediates the elevated BDNF and

267   GDNF production induced by n-3 PUFAs, astrocytes were treated with vehicle, DHA, EPA and

268 SA for two days, then 666-15 (10 μM), a specific inhibitor of CREB [49] , was added and cells

269 were cultured for an additional 24 hours. The cellular lysate was subjected to capillary western

270 assay. BDNF or GDNF production induced by DHA or EPA was attenuated by 666-15 in

271 astrocytes that were derived from SSRI-sensitive or SSRI-resistant subjects. (Figure 5).

272

273   Discussion.

274   N-3 PUFAs play fundamental roles in brain structure and function. Accumulating evidence has

275   implicated deficiency of n-3 PUFA (EPA and DHA) in the etiology of depression and n-3 PUFA

276   in the treatment of MDD [13, 15]. Multiple mechanisms might underlie the

277   protective/therapeutic effect of n-3 PUFAs on MDD [13-17]. Decrease in astrocyte count and

278   GFAP expression are often seen in MDD post-mortem studies [32, 33] [34, 35] [50]. Astrocyte

279   reduction was also observed in a variety of animal models of depression [8]. Antidepressants,

280   including SSRIs, could increase/restore the astrocyte counts or GFAP staining, which positively

281   correlated with attenuated "depression-indicators" in rodents [8]. These suggested a mechanism

282   for relief of depression by restoring astrocyte function. Some reports have indicated that n-3

283   PUFA treatment could impact the neuronal differentiation in primary cultured animal NSCs [51].

284   A recent report indicated that DHA could enhance astrocyte-like morphologic formation in rat

285   astrocytes [52]. Here, taking the advantages of iPSC technology, the effect of n-3 PUFAs on

286   astrocyte differentiation from patient-derived iNSCs was tested. These data suggested that n-3

287   PUFAs could help restore the astrocyte population in MDD since n-3 PUFAs facilitated

288   astrocyte differentiation from patient-derived iNSC, as measured by an increase in GFAP

289   expression and GFAP positive cell counts compared to cells without n-3 PUFAs treatment. Thus,

290   gliogenesis may be relevant to therapeutic effects of n-3 PUFAs.

291   Human studies and animal models support relevance of neurotrophins to depression,

292   proposing that MDD is associated with decreased expression and/or function of BDNF and

293   GDNF, which can be alleviated with antidepressant therapy [53, 54] (though there are some

294   inconsistencies [38, 55]). Furthermore, overexpression of BDNF in astrocytes leads to

295   antidepressant-like activity in mice [12]. Ketamine elevated BDNF in cultured mouse astrocyte,

296 and did this along a more rapid time course than monoamine-centric antidepressants [31]. N-3

297 PUFAs appeared to have similar effect on neurotrophin production as antidepressants. DHA and

298 EPA elevated BDNF and GDNF expression after three day treatment, but the saturated fatty acid,

299 SA, did not. This is similar to the treatment time required for antidepressants to translocate Gsα

300 from lipid rafts in C6 cells [18].

301 It is worthy to mention that many (but not all) of these studies show greater efficacy of

302 EPA than DHA, while our studies suggest equal potency and efficacy for the two compounds

303 [14, 52, 56-58]. Note, however, that 1) the EPA- or DHA-"enriched" preparations used in

304 clinical studies are 80:20 mixtures of the two n-3 PUFA species and 2) orally-ingested

305 preparations are metabolized, something that does not occur in cell-culture medium

306 Several reports have shown that n-3 PUFAs could increase CREB expression and pCREB

307 in depression animal models [59]. In agreement with these reports, DHA and EPA increased

308 pCREB in patient-derived astrocytes, except that CREB expression was not altered by n-3

309 PUFAs in this study. The difference may result from using a different experimental model

310 (patient-derived astrocytes versus animal brain). CREB can be activated by cAMP-PKA

311 signaling pathway. The data from us and others have shown that chronic antidepressant treatment

312 increased adenylyl cyclase (AC) activity and cAMP in cells [31, 47, 60]. Phosphodiesterase 4 (a

313 cAMP-specific isoform) inhibitors show antidepressant effects in both patient and rodent models

314 [61-64]. Activation of CREB via the cAMP-PKA signaling pathway is thought to mediate the

315 therapeutic effects of antidepressants [24]. However, results from this study suggest that cAMP-

316 PKA does not participate in n-3 PUFAs-activated CREB in patient-derived astrocytes (figure 4).

317 Recently, a study reported an increase of cAMP subsequent to brief EPA treatment in epithelial

318 cells [65]. We did not observe this in the human astrocyte preparation, so a cAMP increase

319 induced by n-3 PUFAs might be cell type-dependent. Notably, data have indicated that

320 antidepressants, including ketamine, could increase cAMP by regulating interaction of Gαs with

321 adenylyl cyclase [31, 47]. N-3 PUFAS have similar effect; after 3-day treatment with n-3

322 PUFAs, Gαs-stimulated cAMP production was increased.

323   Consensus-binding sequences for CREB were identified in the promoter sequence of

324 either *BDNF* or *GDNF* genes [66]. Several studies suggested that elevation of expression of

325 BDNF or GDNF induced by antidepressants was dependent on CREB activity [37, 48], however,

326 this has not been documented for n-3 PUFAs. In this study, the cell permeable CREB specific

327 inhibitor[49], indicated that pCREB mediated the elevation of BDNF and GDNF induced by

328 DHA or EPA in the astrocytes, even as the process was cAMP-independent.

329   Note that there are some limits to data interpretation in this study. Firstly, astrocytes

330 were derived from stem cells obtained from two subjects, one SSRI-sensitive and the other,

331 SSRI-resistant. Both appear to have the same response to n-3 PUFA treatment in astrocyte

332 differentiation, production of BDNF and GDNF, and activation of pCREB. It is possible that

333 these subjects would have both responded positively to n-3 PUFA, but this cannot be established.

334 In addition, though astrocytes derived from iPSCs provide many advantages for *in vitro*

335 depression studies, the induced astrocytes are generally considered developmentally immature.

336 These cells cannot predict the behavior of the subjects from whom they were derived.

337 In summary, this study suggests that DHA and EPA could facilitate astrocyte differentiation

338 from iNSCs and like antidepressants, increase BDNF and GDNF production in those cells. While

339 pCREB mediates this effect, it is cAMP-independent. The study further adds to a growing body

340 of work suggesting that iPSC technology is a useful tool to probe the cellular basis of MDD and

341   antidepressant response. This study also further establishes a role for astrocytes in depression

342   and antidepressant therapy.

343

351 **Conflict of interest:** The authors declare that they have no conflict of interest.

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611

612

613   Figure legends

614

615   **Figure 1. DHA and EPA treatment increased astrocyte differentiation from patient-derived**

616   **iNSC.** iNSC were cultured with astrocyte medium in presence of vehicle control (ethanol), DHA

617   (docosahexaenoic acid), EPA(eicosapentaenoic acid) and SA (stearic acid) respectively for 5

618   days. Cells were fixed and immunostained with GFAP (Red). Arrows indicate positive cells. **A**.

619   cell images and counts from SSRI-sensitive subject (N=3, cells in 16 fields were counted). **B**.

620   cell images and counts from SSRI-resistant subject (N=3, cells in 16 fields were counted). *

621   p<0.05, compared to vehicle control.

622

623

624   Figure 2. **GFAP expression was increased by n-3 PUFAs treatment during astrocyte**

625   **differentiation from patient-derived iNSC.** iNSC were treated with DHA, EPA, SA and

626   vehicle control for 5 days in astrocyte medium, and then tested for GFAP mRNA and protein

627   expression. **A**, mRNA expression of GFAP was quantified using qPCR (N=3). The white bar

628   represents data from SSRI sensitive subject and the gray bar from the SSRI resistant subject.

629   GFAP protein in cells from SSRI-sensitive subject (**B**) and SSRI-resistant subject (**C**) was

630   determined with immunoblot using capillary western blots (N=5) * P<0.05 compared to vehicle

631   control.

632

633

634

635 **Figure 3. N-3 PUFAs elevated BDNF and GDNF production in astrocytes from patient-**

636 **derived iNSC.** After treatment, as indicated, for 3 days. BDNF (A) and GDNF (B) mRNA

637 expression were measured with qPCR. White bar represents astrocytes derived from SSRI-

638 sensitive patient and gray bar, from SSRI-resistant patient (N=6). * $p<0.05$, compared to vehicle

639 control. C, Astrocytes were treated with vehicle control, DHA, EPA, and SA, as indicated, for 3

640 days. Cell lysates were subjected to capillary western assay. Data were processed with software

641 provided by the manufacturer (ProteinSimple). The expression of BDNF or GDNF (represented

642 by peak area) was normalized to vehicle control. Each experiment was repeated at least 4 times

643 *$p<0.05$, compared to vehicle control

644

645

646 **Figure 4.   CREB was activated by DHA or EPA treatment, and this was cAMP**

647 **independent. A**, Astrocytes from patient-derived iNSC were treated with vehicle control, DHA,

648 EPA, and SA for 3 days. Cell lysates were used for capillary western blots and a representative

649 image is shown. **RP-cAMPS**, a cAMP antagonist, was used at 150 µM. **B**, Quantified data from

650 capillary western assays. Cells were treated with vehicle control, DHA, EPA and SA as indicated.

651 **C**, Quantified data for phosphorylated CREB. Cells were treated with vehicle control, DHA, EPA

652 and SA ± RP-cAMP for 3 days. White bar, SSRI sensitive subject, Gray bar, SSRI resistant

653 subject (N=4). The ratio of pCREB with CREB was normalized to vehicle control. *$p<0.05$,

654 compared to vehicle control.

655

656 **Figure 5. CREB inhibitor attenuated the BDNF and GDNF increase induced by n-3 PUFAs**

657 **in astrocytes**. Astrocytes were treated with vehicle, DHA, EPA and SA for 2 days, then the

658   CREB inhibitor, 666-15 (10 μM), was applied for 1 day. The 3-day treated cells were collected

659   and analyzed in capillary western blots. Expression of BDNF and GDNF showed no significant

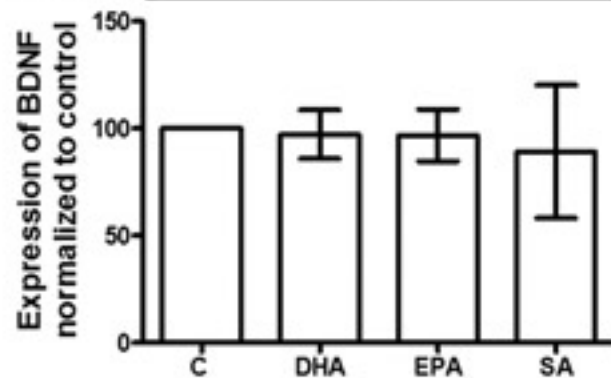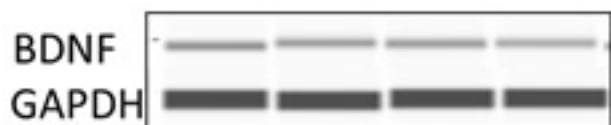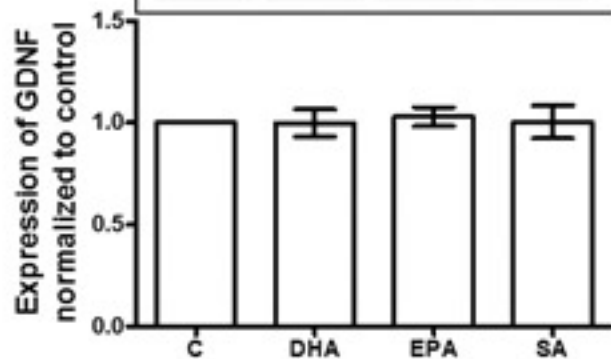660   alteration among different treatments. Each experiment was repeated 4 times.
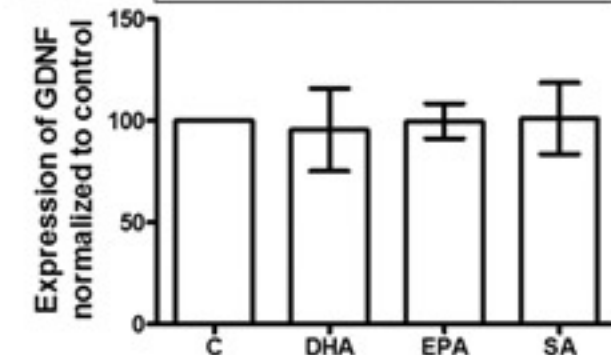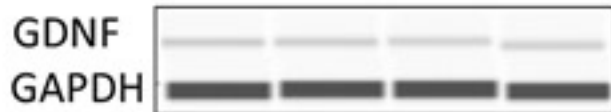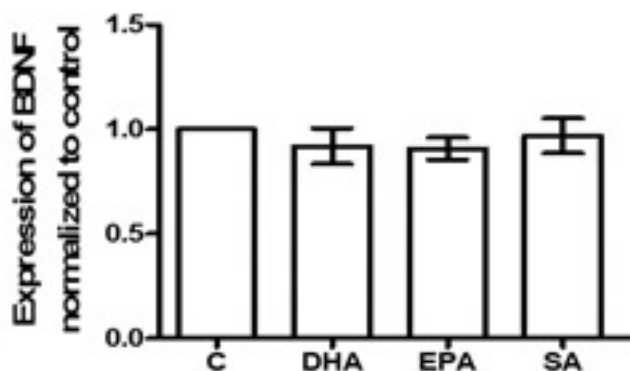
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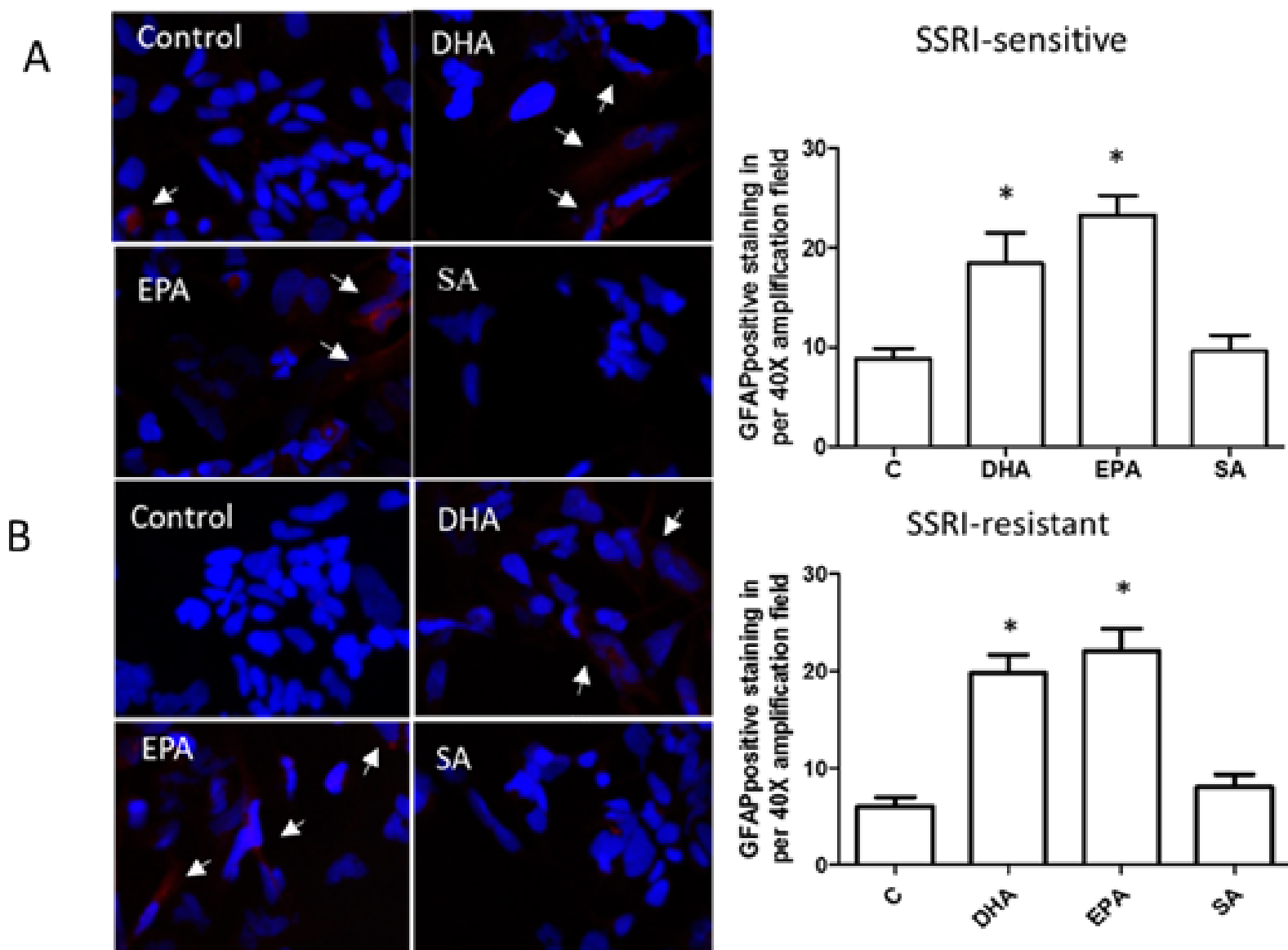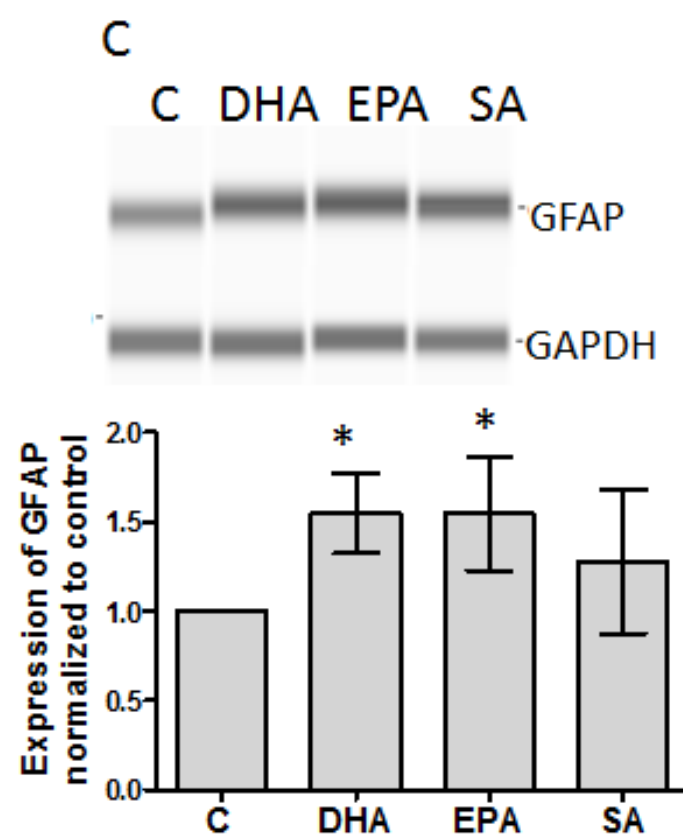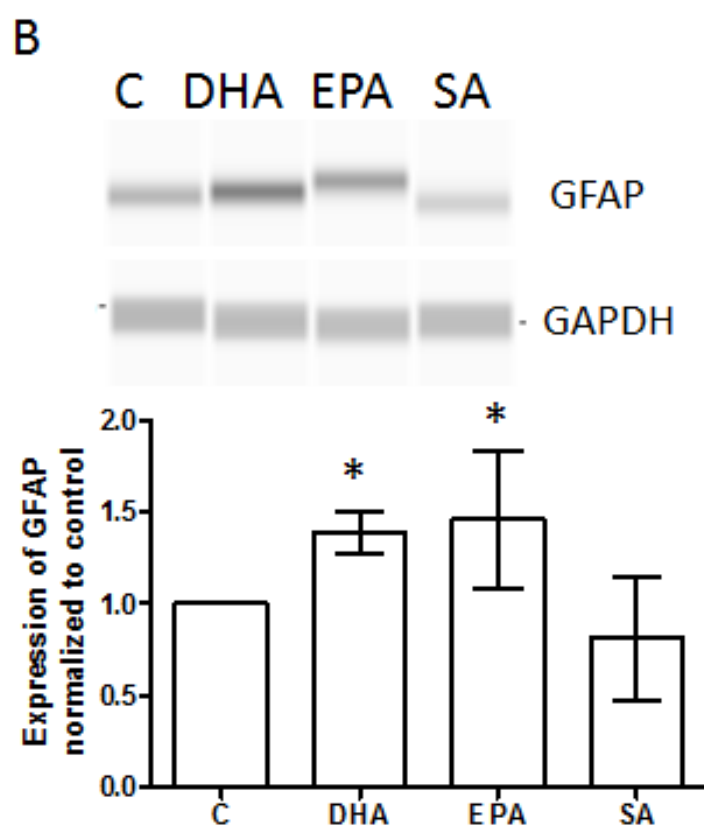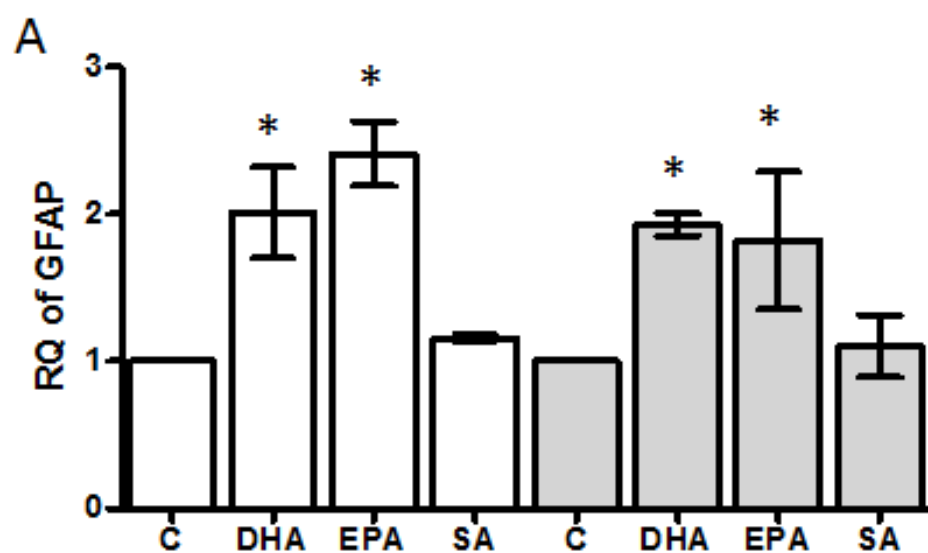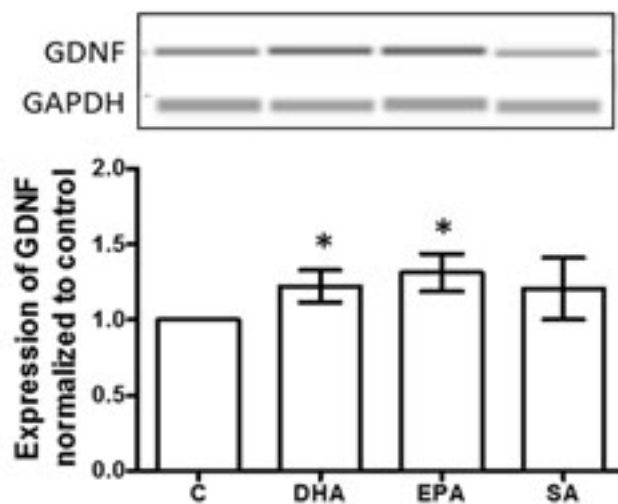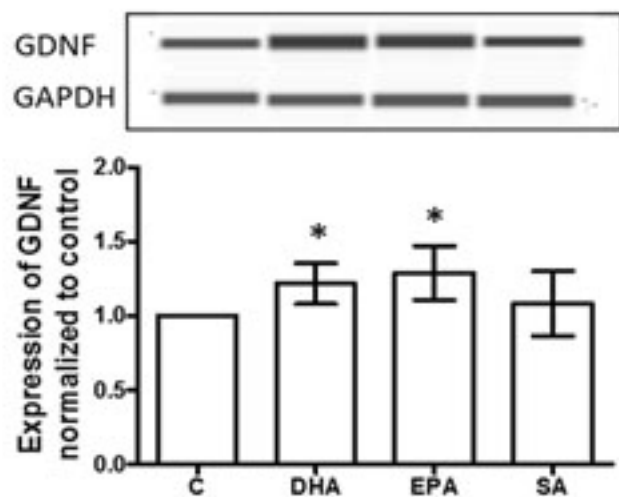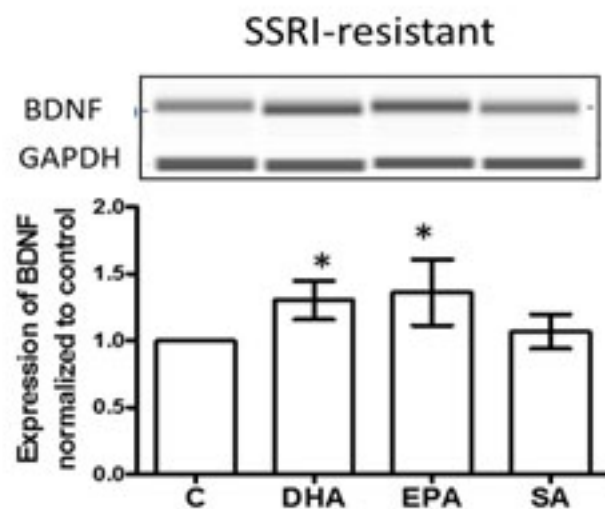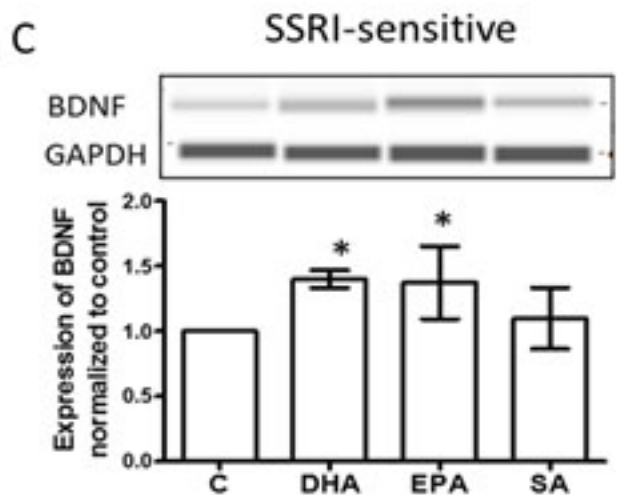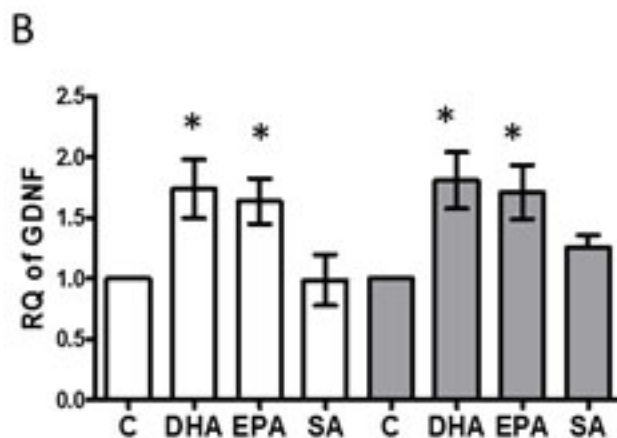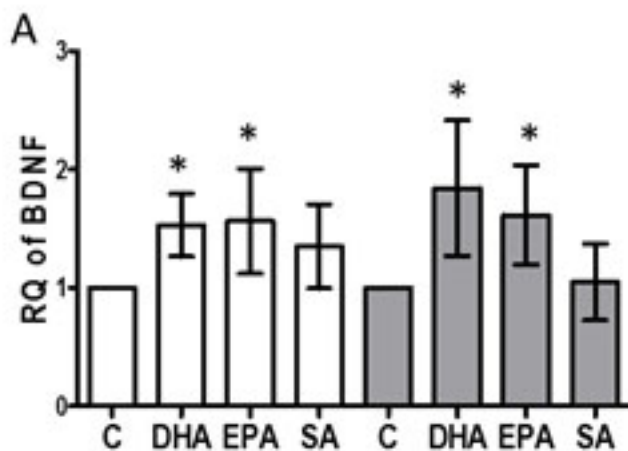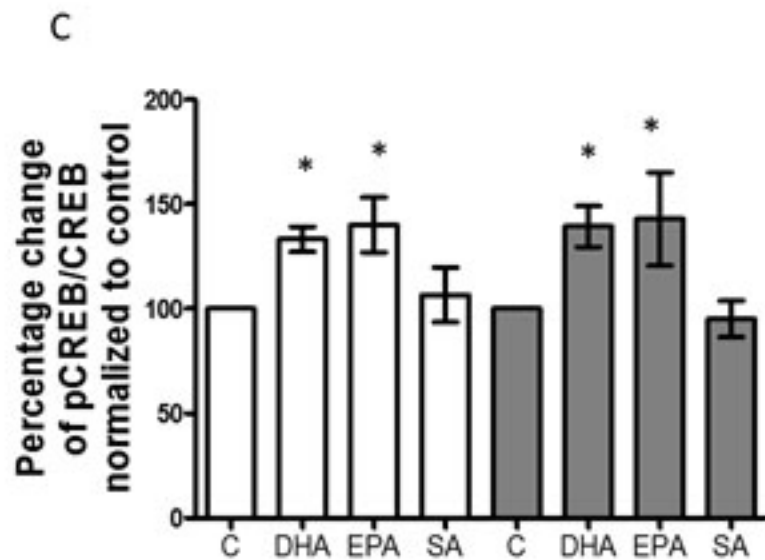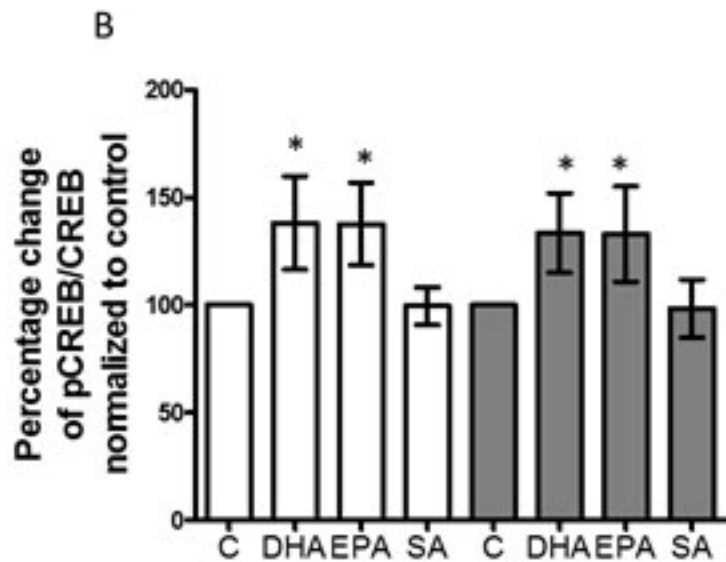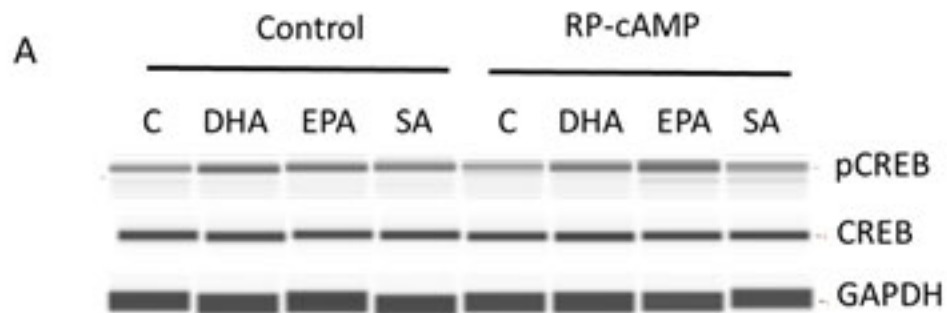
1   Figure legend for supplemental data

2   **Supplemental Figure 1. Characterization of patient-derived iNSC and astrocytes. A.**

3   representative images for PAX6 (green) and SOX2 (red) immunostaining of iNSC. Nuclei are

4   labeled with DAPI (blue). Image was taken with LSM 880 confocal microscopy using 20x

5   magnification. **B**. GFAP immunostaining for astrocytes.   Red indicates GFAP positive, blue is

6   DAPI staining for nuclei. Image was obtained with epifluorescence microscopy.

7

8   **Supplemental Figure 2. Brief (24h) n-3 PUFAs treatment did not alter BDNF and GDNF**

9   **production**

10   Astrocytes from SSRI-sensitive subject-derived iNSC were treated with n-3 PUFAs for 1 day,

11   and cells were lysed and processed for capillary western assay. DHA, EPA and SA did not alter

12   BDNF and GDNF production, compared to vehicle control (n=3).

13

14   **Supplemental Figure 3. RP-cAMPS did not affect BDNF or GDNF expression induced by**

15   **n-3 PUFAs in astrocytes.** Astrocytes from iNSC derived from an SSRI-responsive patient

16   treated with reagents as indicated in figure 4 and RP-cAMP for 3 days. Cell lysates were used

17   for capillary western assay. **A**, Quantified data for BDNF in astrocytes derived from an SSRI-

18   responsive subject (N=4). **B,** Quantified data for GDNF in cells from SSRI-resistant subject

19   (N=4). *$p < 0.05$, compared to vehicle control,

20

21   **Supplemental Figure 4. Three day N-3 PUFAs treatment increased cAMP potential with**

22   **forskolin challenge,**   After 3-day treatment with vehicle (Control), DHA, EPA and SA,

23   Astrocytes were infected with (1.09×10^9 VG/mL) cADDIS virus. The basal florescent density
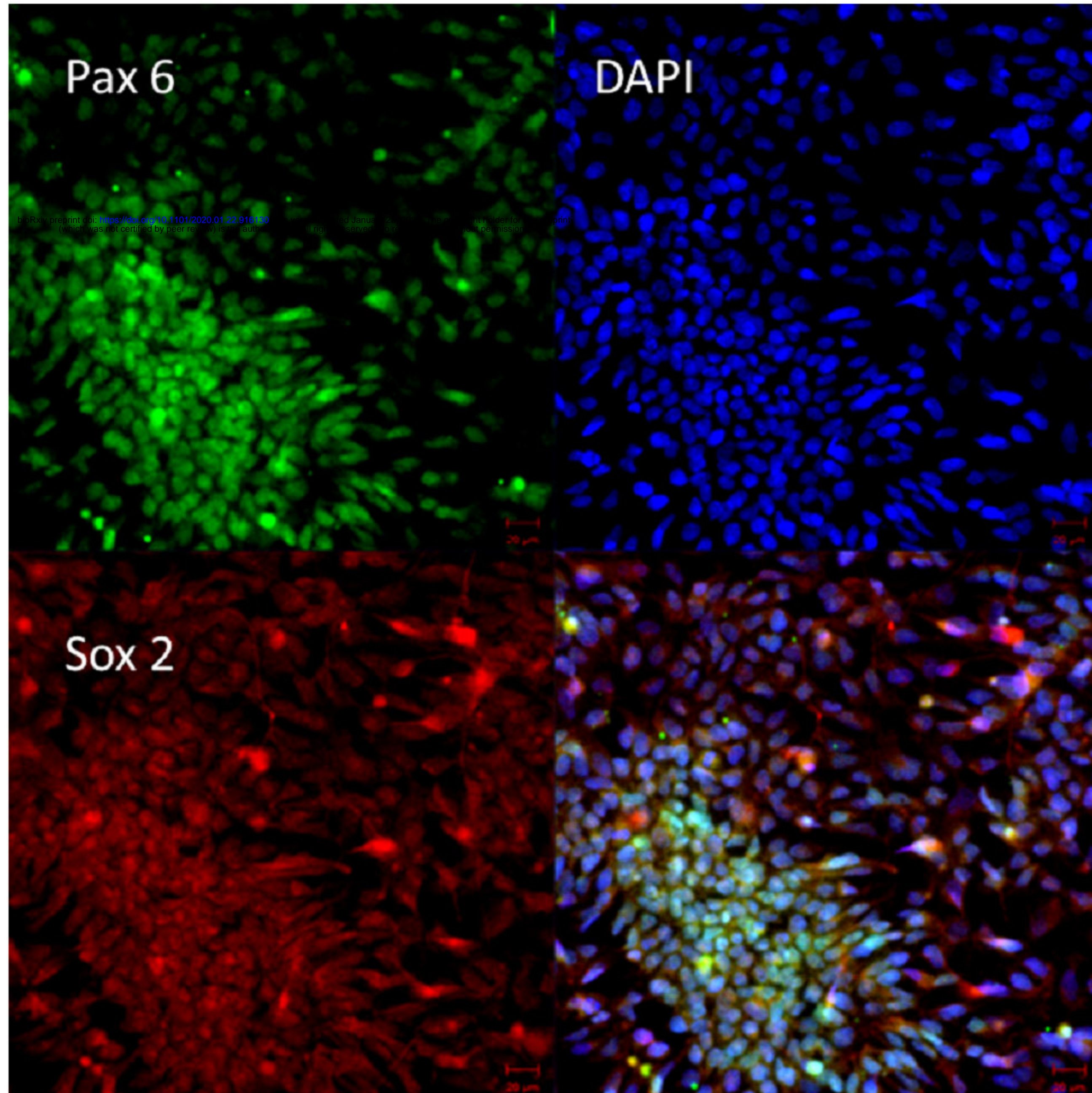
24   of the selected cells were recorded first, then cells were stimulated with forskolin (2 µM), and

25   immediately the fluorescent density of cell was determined. cAMP alteration in cell was

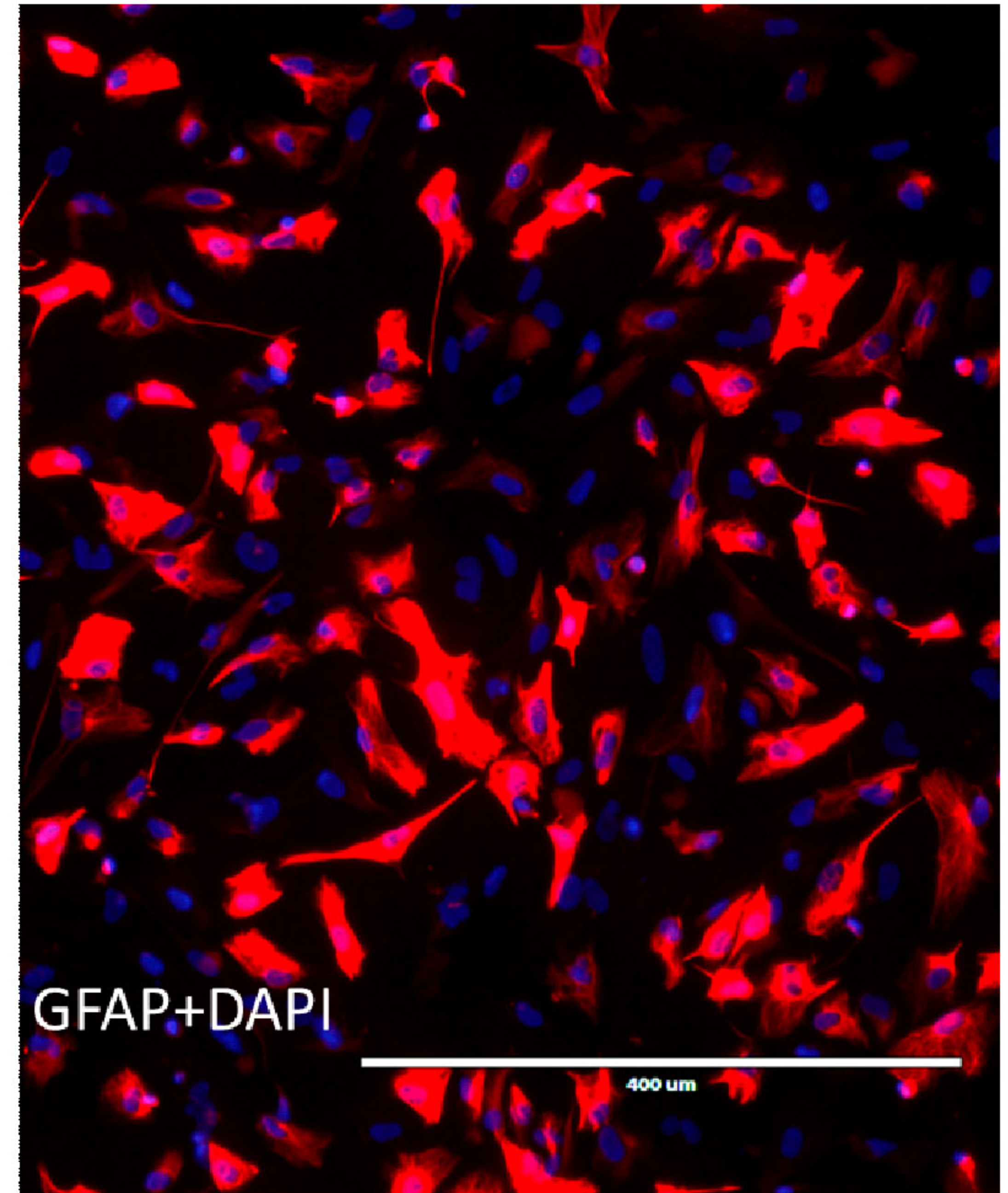26   represented by percentage of increase over baseline. *p<0.05. Compared to vehicle control, n=4.

27

28   **Supplemental Figure 5. DHA did not alter astrocyte cAMP production during 20 min**

29   **treatment.** Astrocytes were infected with $(1.09 \times 10^9$ VG/mL) cADDIS virus. The florescent

30   density of the selected cells were recorded with confocal microscopy (LSM-880) every 5 min.

31   The average fluorescent density at each time point measured (0, 5, 10, 15, 20 min) was roughly
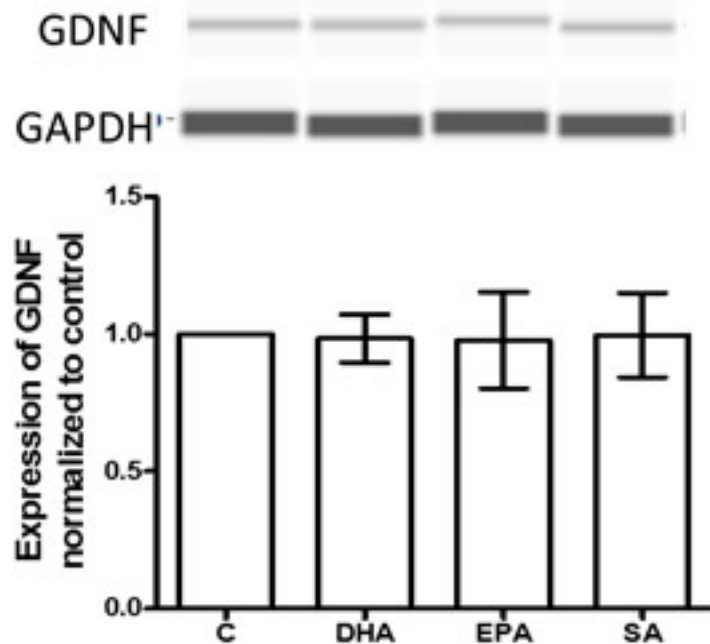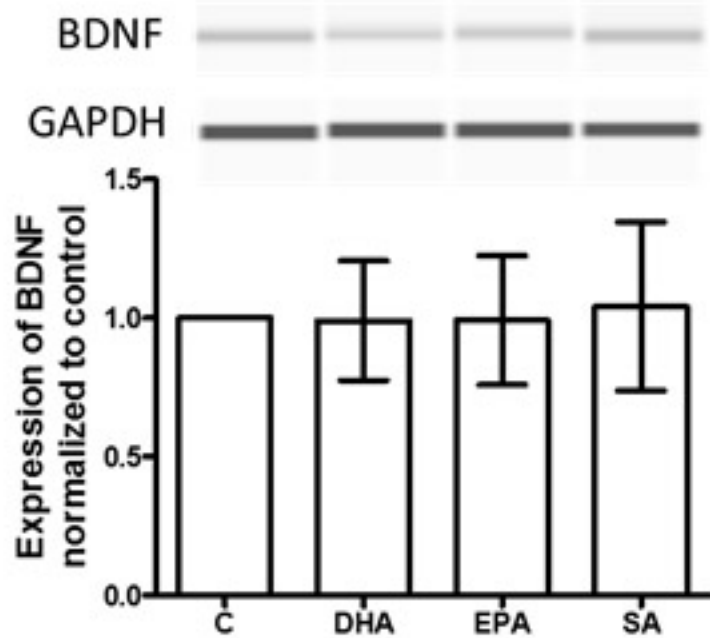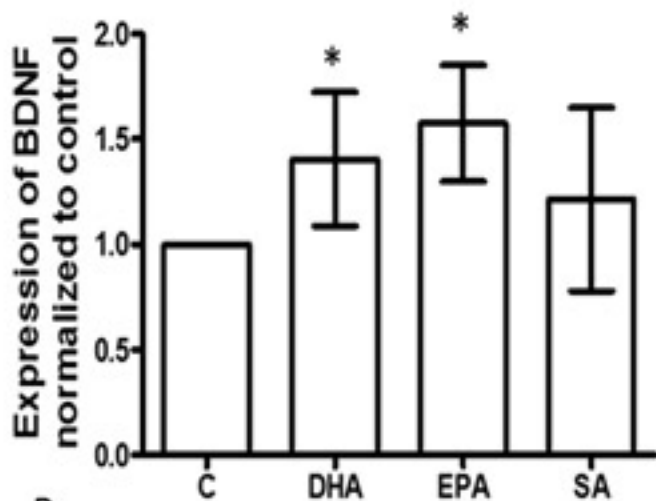
32   equal. (n=3)

33

A

B