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Neuronal adenosine A2A receptors are critical mediators of neurodegeneration triggered by convulsions

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57 **ABSTRACT**

58 Neurodegeneration is a process transversal to neuropsychiatric diseases and the
59 understanding of its mechanisms should allow devising strategies to prevent this irreversible step
60 in brain diseases. Neurodegeneration caused by seizures is a critical step in the aggravation of
61 temporal lobe epilepsy, but its mechanisms remain undetermined. Convulsions trigger an elevation
62 of extracellular adenosine and upregulate adenosine A_{2A} receptors ($A_{2A}R$), which have been
63 associated with the control of neurodegenerative diseases. Using the rat and mouse kainate model
64 of temporal lobe epilepsy, we now tested if $A_{2A}R$ control convulsions-induced hippocampal
65 neurodegeneration. The pharmacological or genetic blockade of $A_{2A}R$ did not affect kainate-
66 induced convulsions but dampened the subsequent neurotoxicity. This neurotoxicity began with a
67 rapid $A_{2A}R$ upregulation within glutamatergic synapses (within 2 hours), through local translation of
68 synaptic $A_{2A}R$ mRNA. This bolstered $A_{2A}R$-mediated facilitation of glutamate release and of long-
69 term potentiation (LTP) in CA1 synapses (4 hours), triggering a subsequent synaptotoxicity,
70 heralded by decreased synaptic plasticity and loss of synaptic markers coupled to calpain
71 activation (12 hours), that predated overt neuronal loss (24 hours). All modifications were
72 prevented by the deletion of $A_{2A}R$ selectively in forebrain neurons. This shows that synaptic $A_{2A}R$
73 critically controls synaptic excitotoxicity, which underlies the development of convulsions-induced
74 neurodegeneration.

75

76 **Significance statement**

77 Epilepsy is an evolving disease where neurodegeneration is associated with the aggravation of
78 subsequent convulsions. We now unveil that the up-regulation of adenosine A_{2A} receptors ($A_{2A}R$) is
79 paramount to link convulsions to neurodegeneration. This involves a translation of $A_{2A}R$ within
80 synapses to bolster the activity of excitatory glutamatergic synapses and triggers an excitotoxicity
81 first of synapses, that later evolves into neurodegeneration through increased calpain activity.
82 Accordingly, the pharmacological or genetic blockade of $A_{2A}R$ arrested neurodegeneration, thus
83 prompting $A_{2A}R$ as novel targets to alleviate neuronal damage associated with epilepsy.

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85 **INTRODUCTION**

86 Sclerotic temporal lobe epilepsy is the most common type of seizure disorder; it is an
87 evolving disease phenotypically characterized by episodes of tonic-clonic convulsions that trigger
88 adaptive changes and damage of brain tissue causing an aggravation of convulsions over time
89 (Pitkänen and Sutula, 2002). Although this seizure-induced neurodegeneration is a key component
90 of the pathophysiology of epilepsy, the underlying mechanisms are still unclear (Loscher and
91 Brandt, 2010). One proposed participant in epileptogenesis is the adenosine modulation system
92 (Dragunow, 1988; Dunwiddie, 1999; Boison, 2016): increased neuronal activity, and convulsions in
93 particular, trigger a robust (During and Spencer, 1992) and sustained (Berman et al., 2000)
94 elevation of the extracellular adenosine levels. Inhibitory adenosine A_1 receptors (A_1R) are
95 considered a major anti-convulsive system, since the acute administration of A_1R agonists
96 decreases seizures whereas the acute administration of A_1R antagonists worsens seizures and the
97 consequent neuronal damage (Dragunow, 1988; Dunwiddie, 1999). However, inhibitory A_1R
98 appear to have a limited time-window of effectiveness since there is a decreased density and a
99 reduced efficiency of A_1R in afflicted brain regions after the induction of seizures (Young and
100 Dragunow, 1994; Ochiishi et al., 1999; Rebola et al., 2003; but see Gouder et al., 2003). This
101 matches the role of A_1R as 'gate-keepers' of brain tissue viability, since their activation increases
102 the threshold for brain damage; however A_1R undergo a reduction of their density and efficiency
103 upon chronic brain dysfunction (Cunha, 2005). Accordingly, A_1R efficiently control the spreading of
104 ictal events to 'naïve' regions (Zeraati et al., 2006; Fedele et al., 2006) and lose efficiency over
105 time to control neurodegeneration (von Lubitz, 2001; Cunha, 2016).

106 By contrast, the density of adenosine A_{2A} receptors (A_{2A}R) increases in limbic regions in
107 different experimental models of epilepsy (Rebola et al., 2005a), as well as in sclerotic regions of
108 patients with temporal lobe epilepsy (Barros-Barbosa et al., 2016). Cortical A_{2A}R are mostly located
109 in synapses (Rebola et al., 2005b) but also in glia (Oor et al., 2009, 2015; Rebola et al., 2011), and
110 their activation increases glutamate release (Lopes et al., 2002: Marchi et al., 2002), enhances
111 NMDA receptor function (Rebola et al., 2008) and bolsters neuroinflammation (Rebola et al., 2011)
112 in the hippocampus. This provides a mechanistic basis for the robust neuroprotection afforded by

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113 $A_{2A}R$ antagonism in different noxious brain conditions (Chen et al., 2007; Cunha, 2016).
114 Accordingly, the genetic deletion of $A_{2A}R$ slows down epileptogenesis (El Yacoubi et al., 2001,
115 2009), but the role of $A_{2A}R$ in the control of convulsions-induced neurodegeneration is still unclear
116 (Lee et al., 2004; Rosim et al., 2011; Li et al., 2012).
117 This was now probed using kainate to trigger an acute convulsive period leading to
118 subsequent neurodegeneration (Sperk et al., 1983; Coyle, 1987), modelling temporal lobe epilepsy
119 (Ben-Ari, 1985). We report that the pharmacological or genetic blockade of $A_{2A}R$ did not affect
120 kainate-induced convulsions but dampened the subsequent neurotoxicity, which begins with
121 maladaptive alterations in synapses.
122

123 **MATERIALS AND METHODS**

124 *Animals*

125 Male Wistar rats and C57Bl/6 mice (8-10 weeks) were from Charles River (Barcelona, Spain).
126 C57BL6 global $A_{2A}R$-KO mice and forebrain $A_{2A}R$-KO mice were generated as previously
127 described (Shen et al., 2008). Rodents were handled following European Union Directives
128 (2010/63) upon approval by the Ethical Committee of the Center for Neuroscience and Cell Biology
129 (Orbea 78-2013).

130 *Kainate administration and evaluation of convulsions*

131 In rats, kainate (Tocris) was injected intraperitoneally (i.p.) at a dose of 10mg/kg, following
132 our previous experience (Rebola et al., 2005a). The selective $A_{2A}R$ antagonist SCH58261 (Tocris)
133 was used at an effective dose of 0.05mg/kg (Lopes et al., 2004), administered i.p. 30min before
134 kainate in most experiments, or 4 hours after the extinction of convulsions in the last experimental
135 protocol. $A_{2A}R$-KO and wild-type littermates or forebrain $A_{2A}R$-KO and floxed $A_{2A}R$-KO littermates
136 (designated as wild-type), all with a C57BL6 background, were injected subcutaneously with either
137 saline or kainate (35mg/kg). The animals were observed for 3h to score convulsions (Rebola et al.,
138 2005a) according to the original Racine scale for rats or the Racine scale adapted to mice, and
139 then maintained in groups of 3-4 per cage.

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140 *Amygdala kindling*

141 Amygdala kindling involved three groups: control, fully kindled and sham-operated rats. After 142 1-2 weeks of inserting an electrode in the amygdala, rats were stimulated twice a day (10AM and 143 4PM) with a 1s train at 50Hz with pulses of 1ms and 500μA, as previously described (Rebola et al., 144 2005a). After 24 days of stimulation, vehicle-treated rats were considered fully kindled (5 145 consecutive sessions reaching stage 4-5). SCH58261 (0.05mg/kg) was administered i.p. twice a 146 day, 30 min before each stimulation (or handling).

147 *Neuronal damage, astrogliosis and microgliosis*

148 The rodents' brain was sectioned into 20μm-thick coronal sections to analyze the general 149 neuronal morphology using a cresyl violet staining of Nissl bodies (Kaster et al., 2015), of cell 150 damage using FluoroJade-C staining (Kaster et al., 2015), astrocytosis using GFAP 151 immunoreactivity (Rebola et al., 2011; Kaster et al., 2015) with Cy3-conjugated anti-GFAP mouse 152 antibodies (Sigma, 1:500), and microgliosis using either OX-42/CD11b immunohistochemistry 153 (Rebola et al., 2011; Kaster et al., 2015) with mouse anti-CD11b antibodies (Serotec, 1:200) or 154 labeling with biotin-labeled tomato lectin (Dalmau et al., 2004) (Vector Laboratories, 1:250). The 155 integral of staining of FluoroJadeC (reporting degenerated neurons), CD11b (staining microglia) or 156 GFAP (staining astrocytes) were counted in 3 regions of 50x50μm in 3 sections per animal. These 157 windows were selected free hand and placed in the *stratum pyramidale* (FluoroJadeC) or *stratum* 158 *radiatum* (GFAP, CD11b) of the CA1 or CA3 regions, and a measure was taken in the anterior 159 commissure as background.

160 To assess the localization of $A_{2A}R$ in microglia-like cells, brain sections from saline- or 161 kainate-treated mice were collected 24h after kainate injection and incubated with a combination of 162 mouse anti-$A_{2A}R$ (1:500; Upstate Biotechnology) and rabbit anti-CD11b (1:100; Serotec) 163 antibodies, followed by incubation with AlexaFluor-labeled secondary antibodies (1:200; Molecular 164 Probes). Nuclei were stained with Hoechst33342 (2μg/mL) and sections were analyzed in a 165 confocal microscope (LSM510; Zeiss).

166 *Exposure of synaptosomes to kainate*

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167 Hippocampal synaptosomes (purified synapses), prepared as previously described (Rebola
168 et al., 2005b; Canas et al., 2014; Kaster et al., 2015), were incubated for 2h at 37ºC in the absence
169 or presence of kainate (5μM), without or together with cycloheximide (100μM).

170 *Receptor binding assay*

171 The density of $A_{2A}R$ was estimated by radioligand binding assays using a supra-maximal
172 concentration of ^{3}H-SCH58261 (6nM; offered by E.Ongini, Schering-Plough, Italy), as previously
173 described (Rebola et al., 2005b; Kaster et al., 2015). Specific binding was determined by
174 subtraction of non-specific binding, measured using 3μM XAC (Tocris).

175 *Immunocytochemistry*

176 The immunocytochemical detection of $A_{2A}R$ in individual glutamatergic and GABAergic nerve
177 terminals was carried out as previously described (Canas et al., 2014; Kaster et al., 2015) by
178 double-labeling with goat anti-$A_{2A}R$ (1:200, Santa Cruz Biotechnology), together with either guinea-
179 pig anti-vGluT1 (1:500, Synaptic Systems) or guinea-pig anti-vGAT (1:200, Synaptic Systems),
180 followed by incubation with AlexaFluor-labeled secondary antibodies (1:500, Molecular Probes).
181 The preparations were examined under a Zeiss Imager Z2 fluorescence microscope and each
182 coverslip was analyzed by counting three different fields and in each field an average of 500
183 individualized elements (Canas et al., 2014).

184 *Western blot analysis*

185 Western blot analysis was carried out by SDS-PAGE using synaptosomal membranes to
186 evaluate synaptic markers, using antibodies against syntaxin-I (1:5,000, Sigma), SNAP25 (1:2,000,
187 Sigma) and vGluT1 (1:5,000, Chemicon), as previously described (Canas et al., 2014; Kaster et
188 al., 2015). Membranes were re-probed for α-tubulin (1:1,000; Abcam) as a loading control.

189 *Glutamate release*

190 Rat hippocampal slices (Costenla et al., 2011) obtained at 3h after administering saline or
191 kainate (10mg/kg) to trigger Racine-stage 4-5 convulsions, recovered for 45min, before being
192 loaded for 15min at 37ºC with ^{3}H-glutamate (2μM; specific activity, 0.319Ci/mol) in the presence of
193 aminooxyacetic acid (100μM; Sigma). Slices were then superfused (0.7mL/min) for 10min and next

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194 stimulated for 3min with 20mM K^+. The tritium content of the collected effluent samples and of the
195 harvested slices was counted and the fractional release (FR%) calculated for each sample as the
196 percentage of ^{3}H-glutamate content in the slice (Lopes et al., 2002).

197 *Neuronal culture in microfluidic chambers*

198 Hippocampal neurons were cultured from 17-19-days old Wistar rat embryos and plated on
199 microfluid chambers as previously described (Taylor et al., 2005; Pinto et al., 2016). The
200 dissociated neurons were placed in one chamber and only the axons can grow (around DIV4/5)
201 through the microgrooves into the opposite chamber. Neurons were cultured at 37°C in a 5% CO_2
202 humidified atmosphere in Neurobasal medium with B-27 supplement, glutamate (25mM),
203 glutamine (0.5mM), and gentamicin (0.12mg/ml).

204 *PCR analysis*

205 Total RNA was extracted from the hippocampus with MagNA Lyser (Roche) to calculate the
206 mRNA expression of A_{2A}R, CD11b and Iba1 by real-time PCR using a SYBR Green I kit (Roche)
207 and the comparative cycle threshold method with glyceraldehide-3'-phosphate dehydrogenase as
208 housekeeping, as previously described (Costenla et al., 2011; Rebola et al., 2011). Non-
209 quantitative PCR to detect A_{2A}R mRNA was also carried out in cDNA samples from synaptosomes
210 (previously incubated with RNase for 30min at 37ºC) or striatal tissue from rats, or a scraped
211 collection of axon terminals or of cell bodies from rat hippocampal neurons cultured in different
212 microfluidic chambers, using histone-1 mRNA as nuclear control and β-actin mRNA as synaptic
213 control.

214 *Electrophysiological recordings*

215 Recordings of excitatory synaptic transmission and plasticity were performed in superfused
216 hippocampal slices (400µm thick), as previously described (Costenla et al., 2011; Kaster et al.,
217 2015). Briefly, Schaffer fibers were stimulated every 15s to evoked field excitatory postsynaptic
218 potentials (fEPSPs) recorded in the CA1 *stratum radiatum* to measure the fEPSP slope. LTP was
219 induced with a high-frequency train (100Hz for 1s) and was quantified as the percentage change
220 between the fEPSP slopes 60min after and 15min before the train. LTP amplitude was compared

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221 in different slices from the same animal in the absence and presence of a supra-maximal
222 concentration (50nM) of SCH58261.

223 *Experimental Design and Statistical Analyses*

224 Data are mean±SEM values. Data with one condition and one variable (effects of $A_{2A}R$
225 antagonist or of kainate treatment) were analyzed with Student's *t*-test. Data with more than one
226 variable (effect of $A_{2A}R$ blockade on kainate treatments) were analyzed with a two-way ANOVA
227 followed by Newman-Keuls *pos hoc* tests with a significance level was 95%.

228

229 **RESULTS**

230 *$A_{2A}R$ blockade attenuates epileptogenesis upon amygdala kindling*

231 Rats subject to daily electrical stimulation of the amygdala slowly began displaying
232 convulsions that escalated over continuous sessions of electrical stimulation (kindling; Figure 1a).
233 Both the onset and evolution of convulsions was attenuated in rats treated with the selective $A_{2A}R$
234 antagonist SCH58261 (0.05mg/kg) (Figure 1a). After 24 days of stimulation, the hippocampus
235 displayed neurodegenerative features, as heralded by the presence of cells stained with
236 FluoroJade-C in all sub-regions only in kindled but not in sham-operated (control) or SCH58261-
237 treated rats, irrespective of being kindled or not (Figure 1b). Thus $A_{2A}R$ blockade blunted
238 convulsions-induced neurodegeneration while it only attenuated the escalating convulsive profile;
239 this suggests that this prominent neuroprotection might underlie the anti-epileptogenic effect of
240 $A_{2A}R$ antagonists previously reported in other animal models of epilepsy (El Yacoubi et al., 2009; Li
241 et al., 2012). However, since tinkering with $A_{2A}R$ affected both abnormal excitability and neuronal
242 damage, which are tightly inter-twinned, we had to turn to another model of epilepsy allowing a
243 separation of abnormal excitability from the subsequent damage.

244 *$A_{2A}R$ blockade does not affect kainate-induced convulsions but prevents subsequent*
245 *neurodegeneration*

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246 To confirm this hypothesis, we used kainate administration, which triggers a period of intense
247 convulsions followed by neuronal damage evident after 24h and evolving with time (Sperk et al.,
248 1983). In contrast to the amygdala kindling model, the kainate model of convulsions, allows to
249 temporally disentangle convulsions and neurodegeneration. Kainate (10mg/kg, i.p.) triggered
250 convulsions reaching a maximal intensity of 4-5 in the Racine's scale within 40-60min, which had
251 similar intensities in saline- and SCH58261-treated rats (Figure 1c). This similar duration and
252 intensity of convulsions tentatively allows isolating the effect of $A_{2A}R$ on neurodegeneration.
253 Kainate damaged hippocampal pyramidal neurons, visible after 24h as a decreased Nissl staining
254 and appearance of FluoroJade-C-stained cells in CA1 area (Figure 1d), which was more evident
255 than in CA3 region (data not shown; see also Sperk et al., 1983), together with an astrogliosis (4-
256 fold more GFAP-stained profiles, *n*=9) and microgliosis (increased number of profiles labelled with
257 tomato lectin displaying thicker processes, i.e. 'activated' microglia (Dalmau et al., 2004)). These
258 modifications were more pronounced 7 days after kainate injection (data not shown), in
259 accordance with the evolution of limbic lesions following convulsions (Sperk et al., 1983).
260 SCH58261 (0.05mg/kg) virtually eliminated kainate-induced neuronal damage and glia-related
261 modifications, indicative of astrogliosis and microgliosis in the hippocampus (Figure 1d).

262 We next ascertained if $A_{2A}R$ also control convulsions-induced neurodegeneration in mice.
263 Differently from rats, mice treated subcutaneously with a higher dose of kainate (35mg/kg) to
264 convulse with a maximal intensity of 4-6 in the modified Racine's scale within 45-70min. Again, in
265 all experimental groups, we always confirmed that they displayed similar intensity and duration of
266 convulsions to tentatively isolate the effect of $A_{2A}R$ on neurodegeneration. Kainate damaged
267 hippocampal pyramidal neurons, visible after 24h as a decreased Nissl Mice with genetic $A_{2A}R$
268 deletion (global $A_{2A}R$ knockouts, KO) displayed a convulsive profile (4.30±0.30, *n*=10) similar
269 ($F_{1,37}$=2.62, *P*=0.11) to wild-type littermates (4.90±0.23, *n*=10), but did not display the kainate-
270 induced neuronal damage, astrogliosis or microgliosis that were present in wild-type mice (Figure
271 1e,f). Similarly, SCH58261-treated mice did not display kainate-induced damage although they
272 convulsed similarly to vehicle-treated mice (data not shown).

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273 *$A_{2A}R$ are upregulated in glutamatergic synapses through a local translation*

274 Neuroinflammation and glutamate excitotoxicity are involved in epilepsy-associated
275 neurodegeneration (Coyle, 1987; Devinsky et al., 2013) and brain insults can upregulate $A_{2A}R$ in
276 both microglia and synapses (Cunha, 2016), whereas the up-regulation of $A_{2A}R$ in astrocytes
277 occurs later in processes of neurodegeneration (Orr et al., 2015; Ogawa et al., 2018). Thus, we
278 tested if convulsions upregulated $A_{2A}R$ in microglia and/or in synapses. At 24h after kainate
279 administration, mouse hippocampal cellular elements labelled with the microglia marker CD11b
280 displayed increased immunoreactivity for both CD11b and $A_{2A}R$ (Figure 2a,b), whereas there was
281 no observable $A_{2A}R$ immunoreactivity in hippocampal sections collected from $A_{2A}R$-KO mice
282 challenged with kainate (data not shown). However, PCR analysis of hippocampal extracts from
283 kainate-treated mice revealed a different time course for the upregulation of $A_{2A}R$ and of the
284 microglia markers CD11b and Iba1 (Figure 2c,d), which are rapidly up-regulated by triggers of
285 neuro-inflammation such as LPS, to inform on dynamic adaptive changes of microglia function
286 (Rebola et al., 2011). CD11b and Iba1 mRNA levels only increased after 12-24h (Figure 2c),
287 whereas $A_{2A}R$ binding density increased in hippocampal synaptic membranes as early as 2h
288 (Figure 2d). This suggests that $A_{2A}R$ upregulation might occur in cellular compartments other than
289 microglia. In fact, 2h after kainate administration, an early $A_{2A}R$ upregulation was evident in
290 glutamatergic synapses, as gauged by increased $A_{2A}R$ immunoreactivity in vGluT1-positive
291 synaptosomes of kainate-treated *versus* saline-injected mice ($t=5.788$; $df=10$; $P=0.0002$) (Figure
292 2e,f). This $A_{2A}R$ upregulation did not occur in GABAergic synapses identified as vGAT-positive
293 ($t=0.232$; $df=10$; $P=0.82$).

294 The speed of this synaptic $A_{2A}R$ upregulation and the distance between synapses and the
295 nucleus in pyramidal hippocampal neurons prompted a possible involvement of local translation
296 within synapses. This would imply the presence of $A_{2A}R$ mRNA in synapses. This was identified by
297 qPCR in hippocampal synaptosomes (Figure 2g) and confirmed in axonal ends of hippocampal
298 neurons cultured in microfluidic chambers (Figure 2h), which physically separate cell bodies from
299 axon terminals (Taylor et al., 2005; Pinto et al., 2016). Moreover, exposure of synaptosomes

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300 (purified synapses, without nuclei) to kainate (5μM, for 2h) increased $A_{2A}R$ binding density
301 (t=2.609; df=8; *P*=0.031), an effect prevented by 100μM cycloheximide, a protein synthesis
302 inhibitor (t=0.7845; df=6; *P*=0.463) (Figure 2i). This local synaptic translation of $A_{2A}R$ mRNA
303 provides a rationale for the rapid $A_{2A}R$ upregulation selectively in synapses shortly after noxious
304 stimuli.

305 *Convulsions bolster $A_{2A}R$-mediated potentiation of glutamatergic synapse function*

306 To probe the impact of $A_{2A}R$ upregulation, we first compared glutamate release from
307 hippocampal slices collected from rats sacrificed 3h after the administration of either vehicle or
308 kainate (Figure 3a). SCH58261 (50nM) inhibited the K^+ (20mM)-induced glutamate release in the
309 kainate-treated group (t=2.735; df=7; *P*=0.029), but not in the saline group (t=1.435; df=7;
310 *P*=0.195) (Figure 3a,b), indicating a greater tonic activation of $A_{2A}R$ bolstering glutamate release
311 after kainate-induced convulsions. We next evaluated the $A_{2A}R$-mediated selective control of long-
312 term potentiation (LTP) in CA1 pyramid synapses (Costenla et al., 2011). LTP amplitude was
313 larger in slices 4h after kainate treatment than in saline-treated mice ($F_{1,20}$=11.65; *P*=0.0028)
314 (Figure 3c,d). Moreover, $A_{2A}R$ blockade caused a larger decrease of LTP amplitude (37.6±4.4%) in
315 slices from kainate- than saline-treated mice (25.6±3.9%; interaction: $F_{1,20}$=5.01; *P*=0.048) (Figure
316 3d), bringing LTP amplitude in slices from kainate-treated mice (38.30±3.35% above baseline) to
317 values close to control (43.78±2.08%). This indicates that convulsions trigger a rapid glutamatergic
318 hyperfunction through an increased $A_{2A}R$ modulation.

319 To confirm the selective involvement of neuronal $A_{2A}R$, we exploited forebrain $A_{2A}R$-KO mice,
320 which lack $A_{2A}R$ selectively in principal neurons of the forebrain (Shen et al., 2008). In forebrain
321 $A_{2A}R$-KO mice, kainate failed to alter LTP amplitude ($F_{1,17}$=0.028; *P*=0.86) and SCH58261 (50nM)
322 was also devoid of effects on LTP amplitude ($F_{1,17}$=0.457; *P*=0.50) (Figure 4a). Additionally,
323 hippocampal cellular damage, astrogliosis and microgliosis, were present in wild-type littermates
324 but were not observed in forebrain $A_{2A}R$-KO mice 24h after kainate administration (Figure 4b,c),
325 although the intensity of convulsions in wild-type mice (4.80±0.20, Racine's modified scale) was
326 similar ($F_{1,38}$=0.1293, *P*=0.72) to that of forebrain $A_{2A}R$-KO mice (4.70±0.20). These findings

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327 directly support a key role of neuronal $A_{2A}R$ in the control of neurodegeneration triggered by a
328 convulsive episode.

329 *$A_{2A}R$-induced glutamate hyperfunction triggers a subsequent dysfunction of glutamate synapses*

330 To clarify the evolution from an initial $A_{2A}R$-mediated bolstering of glutamatergic activity (at
331 4h) into subsequent neuronal damage (at 24h), we characterized alterations present 12h after
332 kainate administration. The kainate-induced increase of LTP amplitude at 4h (Figure 3d) was
333 transformed into an inhibitory effect at 12h (t=2.887; df=14; P=0.012) (Figure 4d,f), which was also
334 abolished (t=0.1666; df=10; P=0.87) in forebrain $A_{2A}R$-KO mice (interaction kainate x genotype:
335 $F_{1,24}$=4.588; P=0.040) (Figure 4e,f). In parallel, at 4h, there was no significant alteration in the
336 density of different synaptic markers (SNAP25: t=0.580; df=6; P=0.58; syntaxin: t=0.246; df=6;
337 P=0.81; vGluT1: t=1.423; df=6; P=0.20) (Figure 4g,h), contrasting to the decreased density at 12h
338 of SNAP25 (t=7.313; df=6; P=0.0003), syntaxin (t=11.37; df=6; P<0.0001) and vGluT1 (t=6.912;
339 df=6; P=0.0005) in the hippocampus of kainate- *versus* saline-treated mice (Figure 4g,h), which
340 was absent in forebrain $A_{2A}R$-KO mice (SNAP25: t=0.298; df=6; P=0.78; syntaxin: t=1.227; df=6;
341 P=0.27; vGluT1: t=1.524; df=6; P=0.18) (Figure 4h). However, although the neurochemical data
342 indicated the present of synaptotoxic alterations, there was no evidence of overt major
343 morphological changes in hippocampal section at 12h after kainate administration, which displayed
344 a lack of FluoroJadeC staining and no evident alteration of the pattern of GFAP or CD11b
345 immunoreactivity.

346 *$A_{2A}R$ control the activity of calpains*

347 Calpains are implicated in epilepsy-associated neurodegeneration (Araújo et al., 2008).
348 Indeed convulsions increased calpain activity in the hippocampus (Figure 4i), as gauged by the
349 decreased density of the calpain substrate spectrin (Figure 4i,j) ($F_{1,16}$=20.89, P=0.0003) paralleled
350 by the increased density of calpain-derived spectrin breakdown degradation products (SBDP-145-
351 150kDa) ($F_{1,16}$=8.838, P=0.009), rather than caspase-3-derived SBDP-120kDa (Figure 4i,k).
352 Importantly, both the decrease of spectrin (interaction kainate x genotype: $F_{1,16}$=10.98, P=0.006)

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353 and the increase of SBDP-145-150kDa densities (interaction kainate x genotype: $F_{1,16}$=36.41,
354 *P*<0.0001) were not observed in forebrain A_{2A}R-KO mice (Figure 4i,k).

355 *Therapeutic prospects of A_{2A}R antagonists to control convulsions-induced neurodegeneration*

356 The time course of A_{2A}R-mediated control of glutamate synapses and their subsequent
357 degeneration suggests that there might be a time window for intervention after convulsions to
358 mitigate neurodegeneration by blocking A_{2A}R. Indeed, SCH58261 (0.1mg/kg) administered 4h after
359 kainate-induced convulsions (all mice reaching stages 4-5), decreased neuronal damage and
360 microgliosis and attenuated astrogliosis (Figure 5a,b). This clearly indicates that A_{2A}R have a
361 particular role on the evolving processes of neurodegeneration after the convulsive period, which
362 was anticipated based on the lack of alteration of convulsive activity after kainate administration,
363 although the behavioural output of convulsions might not ensure a lack of alteration of any
364 neurophysiological mechanism involved in the insult.
365

366 **DISCUSSION**

367 The present study shows that A_{2A}R are paramount to link convulsions to neurodegeneration.
368 We temporally disentangled different events triggered by kainate-induced convulsions in
369 hippocampal tissue after the extinction of convulsions (Figure 5c): the first observed event was the
370 upregulation of A_{2A}R in synapses, namely in glutamatergic synapses (within 2h); this was
371 accompanied by an early increase of glutamatergic activity (within 4h), typified by increased
372 glutamate release and larger synaptic plasticity; in accordance with glutamate-mediated
373 excitotoxicity being a trigger of neurodegeneration (Lipton and Rosenberg, 1994), we observed a
374 later decrease of synaptic plasticity and loss of synaptic markers but no overt neurotoxicity (within
375 12h) coupled to a calpain activation and overt neuronal damage (within 24h). Our therapeutic-like
376 intervention after the termination of convulsions provides a proof-of-concept to re-enforce our
377 contention that A_{2A}R are selectively involved in the control of neurodegeneration after seizures.
378 However, we have not detailed the window of opportunity for intervention with A_{2A}R antagonists, to
379 define if A_{2A}R only control the initial process of excitotoxicity (seen at 6 h, without evidence of

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380 synaptotoxicity or overt neurotoxicity) and/or the process of synaptotoxicity (seen at 12h without
381 evidence of overt neurotoxicity) and the process of overt neurodegeneration (seen at 24h).
382 We evaluated kainate-induced toxicity using three different readouts previously described to
383 reflect kainate-induced hippocampal toxicity, namely cell damage, astrogliosis and microgliosis
384 (Sperk et al., 1983; Pitkänen and Sutula, 2002; Benkovic et al., 2004). All these degenerative
385 features were prevented by pharmacological or genetic $A_{2A}R$ inactivation in both rats and mice.
386 This provides strong evidence that the tonic $A_{2A}R$ activation by endogenous adenosine is crucial to
387 express hippocampal damage following kainate-induced convulsions. In contrast, the role of $A_{2A}R$
388 on behavioural seizures is, at best, disputable (El Yacoubi et al., 2001; Etherington and Frenguelli,
389 2004; Lee et al., 2004; Zeraati et al., 2006; Rosim et al., 2011; Li et al., 2012). Thus, similarly to the
390 effects of caffeine (Rigoulot et al., 2003), $A_{2A}R$ selectively control hippocampal damage
391 independently of their eventual ability to control the severity of convulsions. This is in accordance
392 with the established role of $A_{2A}R$ in the control of NMDA receptors and synaptic plasticity
393 processes, rather than to control excitability, which is a function instead fulfilled by A_1 receptors
394 (reviewed in Cunha 2016), This prompts $A_{2A}R$ antagonists as novel 'secondary neuroprotective
395 agents' (Meldrum, 2002) arresting the limbic maladaptive plasticity underlying the progressive
396 severity of epilepsy (Meldrum, 2002; Pitkänen and Sutula, 2002; Loscher and Brandt, 2010).
397 However, future studies should evaluate if the manipulation of $A_{2A}R$ might also alleviate
398 behavioural dysfunction often emerging after seizures, such as cognitive impairments that are
399 controlled by $A_{2A}R$ in different brain disorders (reviewed in Cunha, 2016).
400 Although $A_{2A}R$ are located in neurons as well as in glia (Cunha, 2016), the use of forebrain
401 $A_{2A}R$ knockout mice provided direct evidence that it is $A_{2A}R$ in neurons that critically control
402 kainate-induced neurodegeneration. Thus, the deletion of neuronal $A_{2A}R$ is sufficient to fully
403 account for the role of $A_{2A}R$ in the development of neurodegeneration following seizures and
404 further studies should clarify if $A_{2A}R$ in microglia and/or astrocytes might also play an ancillary role
405 in seizures-induced neurodegeneration. Cerebral cortical $A_{2A}R$ are most abundantly located in
406 synapses (Rebola et al., 2005b) with a low density in physiological conditions (Lopes et al., 2004),
407 as expected from a receptor selectively involved in bolstering synaptic plasticity (Rebola et al.,

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408 2008; Costenla et al., 2011). Probably as an attempt to increase adaptability after injury, $A_{2A}R$ are
409 upregulated after brain insults (Cunha, 2016), namely in epilepsy models (Rebola et al., 2005a)
410 and patients (Barros-Barbosa et al., 2016). The mechanisms linking brain insults to $A_{2A}R$
411 upregulation are unknown, since the regulation of the promoter(s) of the $A_{2A}R$ gene and of its
412 numerous transcripts encoding $A_{2A}R$ are still poorly understood (Lee et al., 2003; Yu et al., 2004).
413 The present study reveals some surprising novel findings: first, we identified $A_{2A}R$ transcripts in
414 synapses, where a local translation seems sufficient to account for synaptic $A_{2A}R$ upregulation;
415 second, we found that synaptic $A_{2A}R$ upregulation upon brain dysfunction is a rapid event,
416 occurring in less than 2h. $A_{2A}R$ upregulation is well positioned to trigger a transient hyperfunction of
417 glutamatergic synapses, which is involved in the pathophysiology of most neurodegenerative
418 disorders (Lipton and Rosenberg, 1994), since hippocampal $A_{2A}R$ bolster glutamate release (Lopes
419 et al., 2002), NMDA receptor function (Rebola et al., 2008) and calcium influx in synapses
420 (Gonçalves et al., 1997). Furthermore, we now show that $A_{2A}R$ activity is strictly required to trigger
421 calpain activity that was previously shown to mediate kainate-induced neurodegeneration (Araújo
422 et al., 2008).

423 These early synaptic modifications match the recognition of synapses as initial triggers of
424 neurodegeneration in other neurodegenerative conditions such as Alzheimer's disease (Selkoe,
425 2002), with a predominant early alteration of glutamatergic synapses (Kirvell et al., 2006; Canas et
426 al., 2014). Accordingly, animal models and epileptic patients with sclerosis display a loss of
427 synaptic markers (Looney et al., 1999; Zhang et al., 2014), in particular of glutamatergic markers
428 (Alonso-Nanclares and De Felipe, 2005; van der Hel et al., 2009). This is compatible with the
429 engagement of glutamate-mediated excitotoxicity, involving NMDA receptor activation and
430 excessive calcium influx to activate calpains to destroy glutamatergic synapses before the
431 emergence of overt neuronal death (Cunha, 2016), through mechanisms where glia cells likely
432 participate (Pitkänen and Sutula, 2002; Devinsky et al., 2013).

433 This conclusion that $A_{2A}R$ activation is paramount for the development of convulsions-
434 induced neurodegeneration prompts re-evaluating the concept of adenosine as an anti-epileptic
435 agent (Dragunow, 1988, Dunwiddie, 1999). The action of adenosine through A_1R lowers seizure

436 onset acting as anti-convulsive (Dragunow, 1988, Dunwiddie, 1999), but A_1R undergo a rapid
437 desensitization after convulsions (Young and Dragunow, 1994; Ochiishi et al., 1999; Rebola et al.,
438 2003). The present observation that the tonic $A_{2A}R$ activation by endogenous adenosine plays a
439 pivotal role for the expression of damage in hippocampal tissue after a convulsive period, shows
440 that the role of adenosine in the control of epilepsy may be more complex than previously
441 proposed. Thus, adenosine plays a bi-phasic role in the control of epilepsy, lessening convulsive
442 episodes but bolstering subsequent damage. This newly identified selective role of $A_{2A}R$ in the
443 control of the neurodegeneration that develops as a consequence but after the convulsive period
444 (Pitkänen and Sutula, 2002; Sperk et al., 1983) heralds the concepts of $A_{2A}R$ blockade as a new
445 therapeutic strategy to arrest the evolution of epilepsy. Indeed, $A_{2A}R$ blockade attenuated
446 amygdala- or pentylenetetrazol-induced kindled seizures (El Yacoubi et al., 2009), two models of
447 slowly developing convulsions. Furthermore, we now showed that the administration of a selective
448 $A_{2A}R$ antagonist after the convulsive period was still effective to arrest the subsequent hippocampal
449 damage. This is in agreement with the reported ability of $A_{2A}R$ antagonists to prevent the long-term
450 development of behavioural abnormalities in adult rats after convulsions early in life (Cognato et
451 al., 2010). Also, genetic polymorphisms of $A_{2A}R$ (ADORA2A) are associated with childhood
452 encephalopathy resulting from biphasic seizures (Shinohara et al., 2013). Altogether this evidence
453 heralds the new concept that $A_{2A}R$ are paramount for the development of neurodegeneration after
454 convulsions.

455

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610 **FIGURE LEGENDS**

611 **Figure 1.** The pharmacological blockade or genetic elimination of adenosine A_{2A} receptors ($A_{2A}R$)
612 prevents hippocampal damage caused by either amygdala kindling or kainate administration,
613 while only attenuating the evolution of convulsions. (**a**) Male Wistar rats were implanted with
614 electrodes in the amygdala and stimulation twice daily progressively increased the severity of
615 convulsions until triggering a fully kindled state (n=7), which was reduced by a selective $A_{2A}R$
616 antagonist SCH58261 (0.05 mg/twice daily) (n=8). (**b**) Rats were sacrificed five days after
617 reaching fully kindled state and only the kindled rats treated with saline displayed degenerated
618 cells, identified by FluoroJade-C labelling, in all hippocampal fields, whereas neither control nor
619 SCH58261-treated rats, irrespective of being kindled or not, displayed FluoroJade-C staining. (**c**)
620 Upon ip administration of kainate (KAI, 10 mg/kg; n=11), rats treated with 0.05 mg/kg of
621 SCH58261 (KAI+SCH, n=12) displayed a similar pattern of acute convulsions (within 15 min and
622 lasting no more than 75 min after KAI); (**d**) however, SCH58261 prevented the histological
623 modifications observed in the hippocampus 24 hours after kainate administration, namely the
624 dispersion of pyramidal cell layer with Nissl staining, the appearance of ruptured cells stained
625 with FluoroJade-C (FJ-C), the modification of microglia staining with tomato lectin and the
626 increase of the number and density of GFAP-stained element compatible with astrogliosis. (**e,f**)
627 The administration of kainate (35 mg/kg sub-cutaneous) to wild-type (WT) C57Bl6 mice (n=11)
628 triggered a convulsive period followed by the appearance of degenerated cells stained with
629 FluoroJade-C together with a microgliosis and astrogliosis concluded from the altered staining of
630 hippocampal sections with CD11b and GFAP, respectively; notably, the same exposure to
631 kainate (+K) of littermates with a genetic deletion of $A_{2A}R$ ($A_{2A}R$ knockout, gKO) triggered a
632 similar intensity of convulsions, which did not evolve into an evident pattern of
633 neurodegeneration, microgliosis or astrogliosis in the hippocampus after 24 hours (n=10).
634 Calibration bars in each photograph are 100 μm, except the inserts, which display higher
635 magnifications of either astrocytes or microglia (calibration bar= 10 μm). Data are mean±SEM.
636 *p<0.05 between bars or *versus* control (saline).

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637 **Figure 2.** A convulsive period triggers an early upregulation of $A_{2A}R$ in glutamate synapses, likely
638 involving a local translation of synaptic $A_{2A}R$ mRNA, and a delayed upregulation of $A_{2A}R$ in
639 modified microglia cells in the hippocampus. (**a**) The immune-density of the microglia marker
640 CD11b and of $A_{2A}R$ were low in hippocampal sections from saline-treated mice, whereas they
641 were increased and co-located circa 22 hours after a convulsive period triggered by the sub-
642 cutaneous injection of 35 mg/kg kainate (**b**) (confocal images representative of n=3 mice per
643 group). (**c**) Kainate-induced convulsions triggered an increase of mRNA levels of CD11b and
644 Iba1, characteristic of reactive microglia, only after 12-24 hours in mouse hippocampal extracts
645 (n=6 for each time point). (**d**) In contrast, an increased $A_{2A}R$ density was observed in
646 synaptosomes (purified synapses) as soon as 2 hours after kainate injection, whereas an $A_{2A}R$
647 binding density was only upregulated in total membranes after 24 hours in the hippocampus (n=5
648 for each determination, except n=6 at 24 hours). (**e,f**) Kainate-induced $A_{2A}R$ upregulation was
649 evident in glutamate synapses, as gauged by the kainate-induced increased $A_{2A}R$
650 immunoreactivity in hippocampal synaptosomes immuno-positive for vesicular glutamate
651 transporter type 1 (vGluT1) (n=6). (**g**) $A_{2A}R$ mRNA was identified in purified synaptosomes from
652 the hippocampus (HIP), similarly to its presence in rat striatal extracts (STR) of the rat brain
653 (n=3). (**h**) The synaptic localization of $A_{2A}R$ mRNA was confirmed in a pure axonal preparation
654 from hippocampal neurons cultured in microfluidic chambers, which ensures a physical
655 segregation of axon terminals and cell bodies (n=2). (**i**) The contribution of the local translation of
656 synaptic $A_{2A}R$ mRNA for the kainate-induced upregulation of $A_{2A}R$ was concluded by the ability of
657 cycloheximide (n=4) to prevent the increase of $A_{2A}R$ binding density in synaptosomes exposed for
658 2 hours to 5 μM kainate (n=5). Data are mean±SEM. *p<0.05 between bars or *versus* control
659 (saline, sal).

660 **Figure 3**. A convulsive period bolsters the function of synaptic $A_{2A}R$ in glutamatergic synapses.
661 (**a,b**) $A_{2A}R$ blockade with SCH58261 (50 nM) did not modified the depolarization-evoked release
662 of glutamate (i.e. upon augmentation of extracellular K^+ as indicated by the horizontal insert line)
663 from hippocampal synaptosomes from saline-treated mice (left panel, n=8), but increased

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664 glutamate release from synaptosomes collected from convulsing rats 2 hours after kainate
665 administration (10 mg/kg, i.p.; right panel, n=6). (**c,d**) The amplitude of long-term potentiation
666 (LTP), triggered by high frequency stimulation (100Hz for 1s) of afferent Schaffer fibers, was
667 larger in CA1 synapses from hippocampal slices collected 4 hours after the administration of
668 kainate (n=6) than in saline-treated mice (n=5). Panel (**c**) shows pairs of superimposed field
669 excitatory postsynaptic potential (fEPSP) recorded 10 min before (black and red traces) and 60
670 min after the high frequency train (dark or light blue traces) in slices from saline-treated mice (left
671 pair) or 4 hours after kainate administration (right pair). Panel (**d**) shows the time-course of
672 average fEPSPs before and after application of the high frequency train (at time 0) in slices from
673 control mice (black symbols), the aberrantly large LTP in slices collected 4 hours after kainate
674 administration (red symbols) and the ability of 50 nM SCH58261 to bring LTP amplitude back to
675 control levels in these slices collected 4 hours after kainate injection (orange symbols). This
676 indicates that convulsions-induced aberrant synaptic plasticity is due to over-functioning of $A_{2A}R$.
677 Data are mean±SEM. *p<0.05 *versus* control (saline in (**b**); WT – wild type in (**d**)); *p<0.05
678 comparing kainate *versus* kainate+SCH58261 in (**d**).

679 **Figure 4**. Neuronal $A_{2A}R$ are critical to convert the initial convulsions-induced glutamate hyper-
680 functioning into a subsequent dysfunction and loss of glutamate synapses with the activation of
681 calpains. (**a,b**) Absence of alteration of the high frequency train (100 Hz for 1 s, applied at time
682 0)-induced long-term potentiation (LTP) of field excitatory postsynaptic potential (fEPSP)
683 recorded in the CA1 *stratum radiatum* upon stimulation of afferent Schaffer fibers in hippocampal
684 slices collected 4 hours after the induction of a convulsive period upon sub-cutaneous
685 administration of 35mg/kg kainate to mice with a genetic deletion of $A_{2A}R$ selectively in forebrain
686 neurons (fb-$A_{2A}R$-KO, n=6). (**b,c**) Likewise, fb-$A_{2A}R$-KO mice analysed 24 hours after the injection
687 of kainate did not display neuronal damage (FluoroJade-C, FJ-C), microgliosis (CD11b
688 immunoreactivity) or astrogliosis (GFAP immunoreactivity), which was present in 'wild-type'
689 littermates (n=8-10 mice per group). The conversion from the initial convulsions-induced hyper-
690 functioning of glutamate synapses into neurodegeneration first involved a synaptic dysfunction,

691 as shown (**d**) by the lower amplitude of LTP in hippocampal slices collected 12 hours after the
692 administration of kainate to trigger a convulsive period within the first 75 minutes (red symbols)
693 compared to saline-treated mice (black symbols) (n=10). (**e**) The kainate-induced reduction of
694 LTP amplitude at 12 hours is not present in fb-A_{2A}R-KO mice (n=7). (**f**) Summary of the time-
695 dependent evolution of the impact of a kainate-induced convulsive period on hippocampal
696 synaptic plasticity: LTP was first bolstered (at 4 hours) and later depressed (at 12 hours) in a
697 manner strictly dependent on neuronal A_{2A}R (lack of alterations in fb-A_{2A}R-KO). (**g,h**) The
698 evolution from over-excitation to decreased synaptic plasticity caused by kainate-induced
699 convulsions was associated with a loss of synaptic markers, namely SNAP-25, syntaxin-I and
700 vesicular glutamate transporters type 1 (vGluT1), which was seen 12 hours after kainate
701 administration (n=4), but not after 4 hours in wild type mice (WT, open symbols, n=4) or in fb-
702 A_{2A}R-KO mice (filled symbols, n=4). (**i-k**) This putative synaptotoxicity likely involved the
703 recruitment of calpains, which was strictly dependent on the presence of neuronal A_{2A}R: in fact,
704 kainate triggered a decreased immunoreactivity of the calpain substrate, spectrin (**i,j**) and a
705 parallel increase of the immunodensity of the calpain-derived spectrin breakdown degradation
706 products (SBDP-145-150 kDa) in 'wild-type' but not in fb-A_{2A}R-KO mice (**i,k**) (n=5 in each group).
707 Data are mean±SEM. *p<0.05 between bars or between kainate and saline (SAL) or *versus*
708 control (100%).

709 **Figure 5**. The time window between kainate-induced convulsions and synaptotoxicity and
710 neurodegeneration offers a therapeutic window for A_{2A}R antagonists to prevent convulsions-
711 induced neurodegeneration. (**a,b**) The selective A_{2A}R antagonist SCH58261 (SCH, 0.05 mg/kg)
712 applied i.p. 4 hours after kainate-induced convulsions was therapeutically effective to abrogate
713 the emergence of neuronal damage (Nissl staining and FluoroJade-C), microgliosis (CD11b
714 immunoreactivity) or astrogliosis (GFAP immunoreactivity) 24 hours after kainate (K)
715 administration (n=5 mice per group). The data are mean±SEM. *p<0.05 between the indicated
716 bars. (**c**) The pharmacological or genetic blockade of A_{2A}R did not affect kainate-induced
717 convulsions but dampened the subsequent neurotoxicity. This neurotoxicity began with a rapid

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718 $A_{2A}R$ upregulation in glutamatergic synapses (within 2 hours), through local translation of synaptic
719 $A_{2A}R$ mRNA. This bolstered $A_{2A}R$ facilitation of glutamate release and of long-term potentiation
720 (LTP) in CA1 synapses (4 hours), triggering a subsequent synaptotoxicity, heralded by decreased
721 synaptic plasticity and loss of synaptic markers coupled to calpain activation (12 hours), that
722 predated overt neuronal loss accompanied by astrogliosis and microgliosis (24 hours). All
723 modifications were prevented by selective $A_{2A}R$ deletion in forebrain neurons. Overall, this shows
724 that synaptic $A_{2A}R$ critically control the development of convulsions-induced neurodegeneration.
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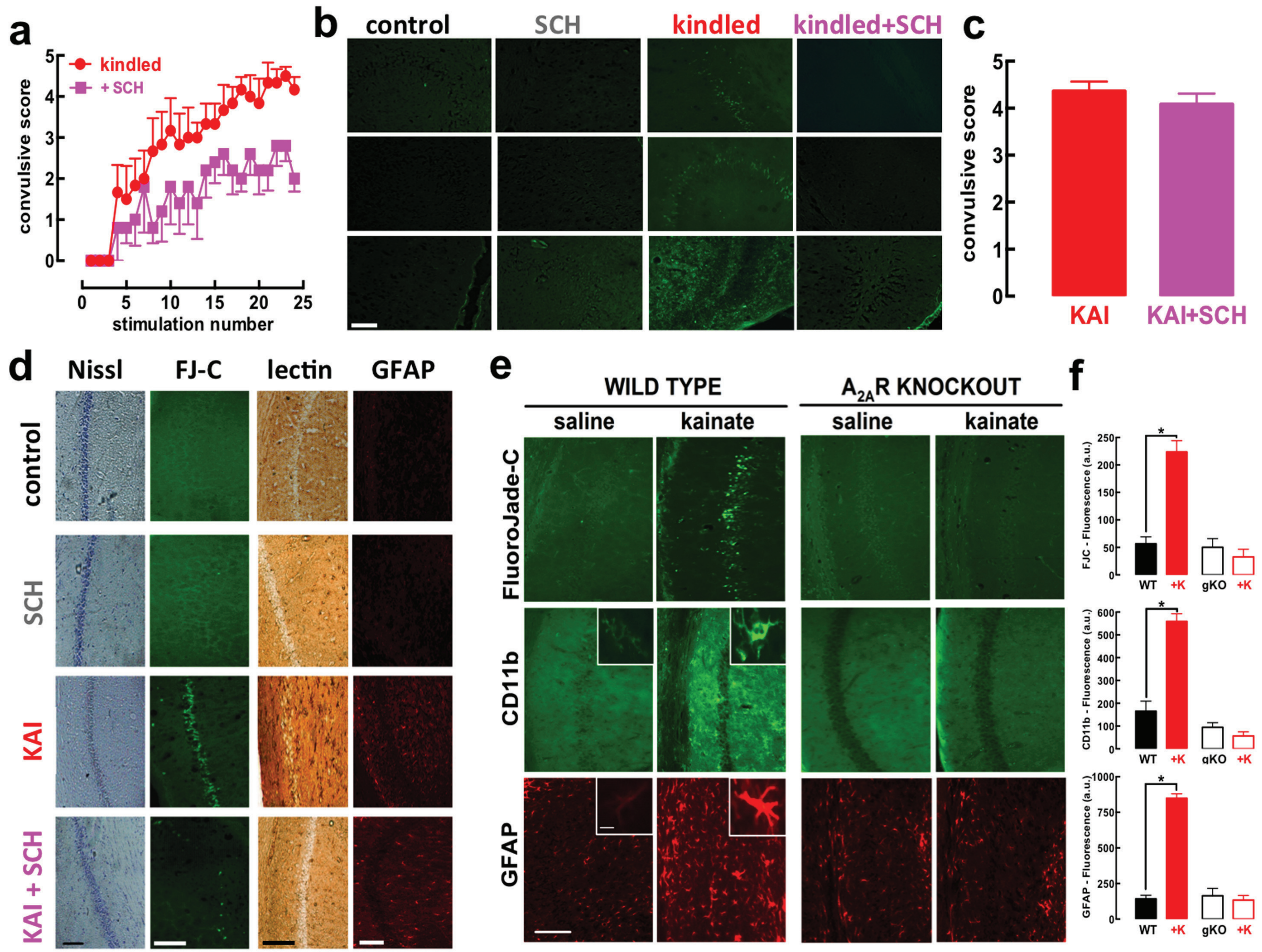

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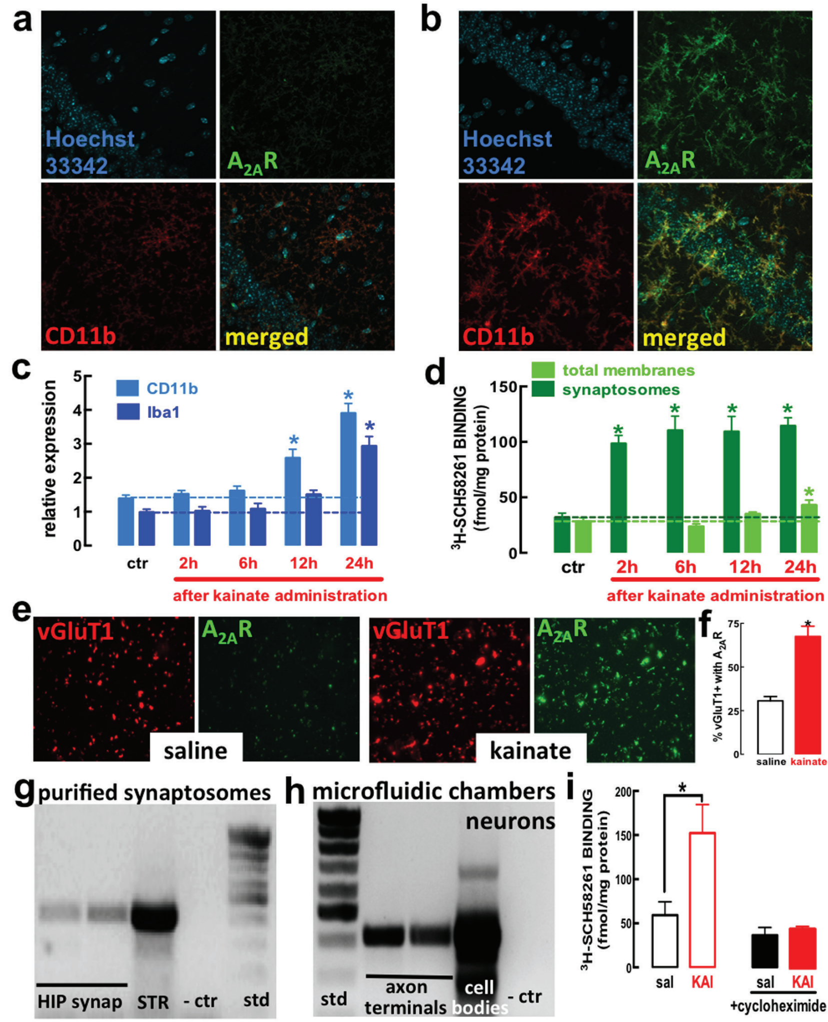

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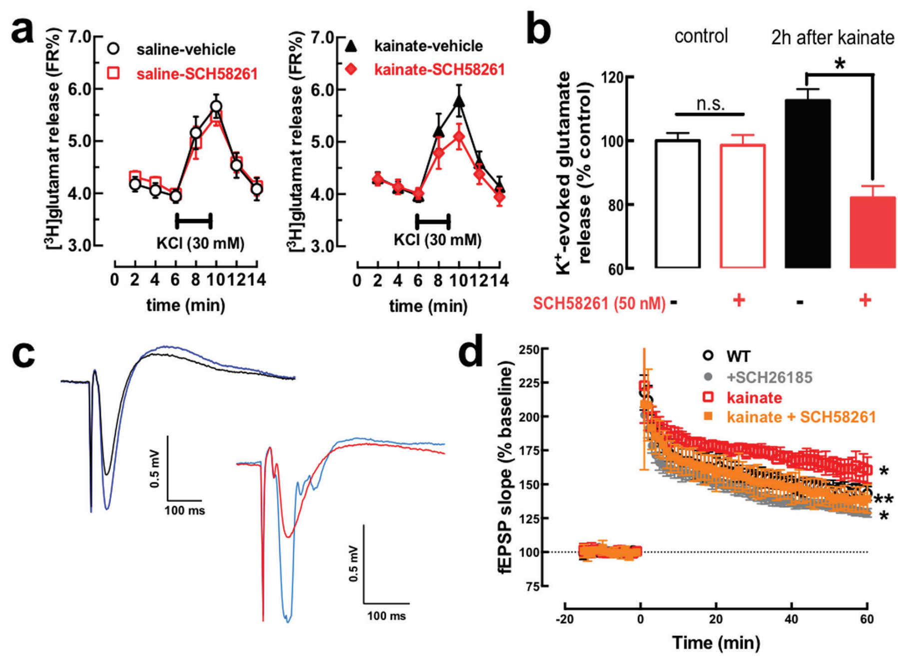

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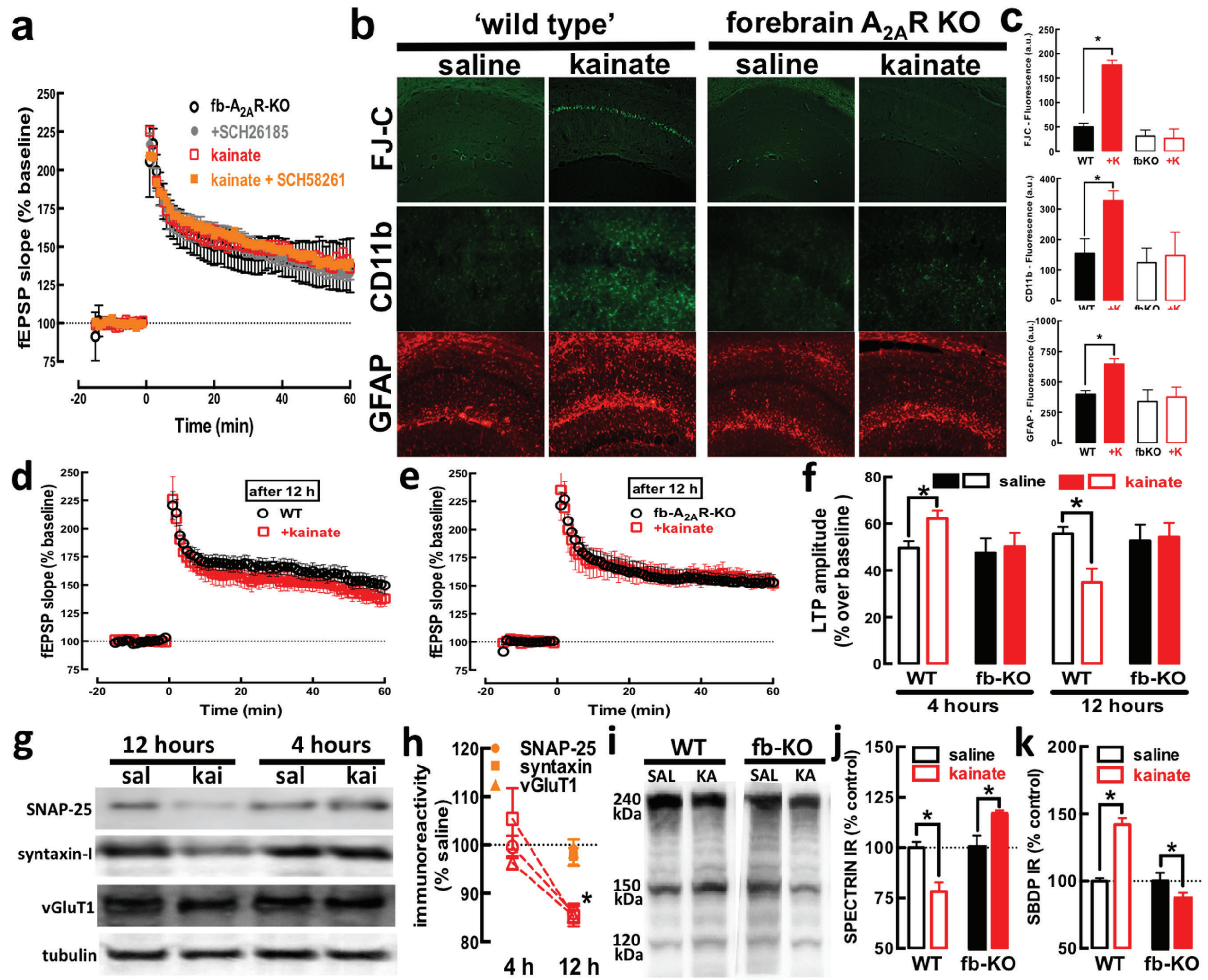

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