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Increased amygdalar metabotropic glutamate receptor 7 mRNA in a genetic mouse model of impaired fear extinction

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Abstract

Rationale—Post-traumatic stress disorder (PTSD) is a devastating anxiety-related disorder which develops subsequent to a severe psychologically traumatic event. Only ~ 9% of people who experience such a trauma develop PTSD. It is clear that a number of factors, including genetics, influence whether an individual will develop PTSD subsequent to a trauma. The 129S1/SvImJ (S1) inbred mouse strain displays poor fear extinction and may be useful to model this specific aspect of PTSD. The metabotropic glutamate receptor 7 ($mGlu_7$ receptor) has previously been shown to be involved in cognitive processes and anxiety-like behaviour placing it in a key position to regulate fear extinction processes. We sought to compare $mGlu_7$ receptor mRNA levels in the S1 strain with those in the robustly extinguishing C57BL/6J (B6) inbred strain using in situ hybridisation (ISH) in three brain regions associated with fear extinction: the amygdala, hippocampus and prefrontal cortex (PFC).

Results—Compared to the B6 strain, S1 mice had increased $mGlu_7$ receptor mRNA levels in the lateral amygdala (LA) and basolateral amygdala (BLA) subdivisions. An increase was also seen in the hippocampal CA1 and CA3 subregions of S1 mice. No difference in $mGlu_7$ receptor levels

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Compliance with ethical standards

All experiments were conducted in accordance with the European Directive 86/609/EEC and the local Animal Care and Use Committees.

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were seen in the central nucleus (CeA) of the amygdala, dentate gyrus (DG) of the hippocampus or prefrontal cortex.

Conclusions—These data show altered $mGlu_7$ receptor expression in key brain regions associated with fear extinction in two different inbred mouse strains which differ markedly in their fear extinction behaviour. Altered $mGlu_7$ receptor levels may contribute to the deficit fear extinction processes seen in fear extinction in the S1 strain.

Keywords

$mGlu_7$ receptor expression; Post-traumatic stress disorder; Trauma

Introduction

Post-traumatic stress disorder (PTSD) is a chronic anxiety disorder that develops subsequent to a severe trauma (DSM-V 2013). With only ~ 9% of individuals who experience a trauma developing full blown PTSD (Breslau et al. 1998; Kessler et al. 1995), it is clear that different susceptibility factors predispose individuals to PTSD with genetics likely to be influential (Kendler 2001; Maren and Holmes 2016; Stein et al. 2002). Our knowledge of the molecular underpinnings of PTSD remain incomplete with only a limited number of genetic models available (Depino and Gross 2007; Stiedl et al. 2003; Tovote et al. 2004). The inbred 129S1/SvImJ (S1) mouse strain has been shown to display impaired fear extinction without abnormal fear acquisition under most experimental conditions (Fitzgerald et al. 2014; Hefner et al. 2008; Whittle et al. 2013) and could be a potential model of the genetics underlying PTSD (Camp et al. 2009; Mi et al. 2018).

Recently, research on the metabotropic glutamate receptors (mGlus) has come to the fore in anxiety and cognition research (Peterlik et al. 2016) with early work on $mGlu_7$ receptor showing great promise (Gee et al. 2014; Goddyn et al. 2008; Moloney et al. 2015; O'Connor and Cryan 2010; O'Connor and Cryan 2013; O'Connor et al. 2010; O'Connor et al. 2013a; O'Connor et al. 2013b; O'Mahony et al. 2010; Palucha-Poniewiera and Pilc 2013; Toth et al. 2012). The advent of $mGlu_7$ receptor knockout mice paved the way for early research on $mGlu_7$ receptor's role in both anxiety and cognition. Interestingly, $mGlu_7$ receptor knockouts display impaired cognitive abilities in many paradigms including spatial memory acquisition (Callaerts-Vegh et al. 2006; Goddyn et al. 2015; Holscher et al. 2004), impaired extinction learning (Callaerts-Vegh et al. 2006; Goddyn et al. 2015; Goddyn et al. 2008) as well as deficits to acquiring conditioned responses (Goddyn et al. 2015; Goddyn et al. 2008). Furthermore, $mGlu_7$ receptor knockouts display an attenuated response to anxiogenic stimuli as measured in many different behavioural tests including the elevated-plus maze, staircase test, open-field test, marble-burying test and stress-induced hyperthermia (SIH) (Callaerts-Vegh et al. 2006; Cryan et al. 2003; Stachowicz et al. 2008).

The development of the first selective $mGlu_7$ receptor agonist, AMN082 (Mitsukawa et al. 2005), has allowed modulation of $mGlu_7$ receptor function in adult animals. However, the results obtained have been somewhat paradoxical when examined in light of some of the previous knockout studies. AMN082 administration impaired fear acquisition (Siegl et al. 2008) and the short-term extinction of fear memories (Toth et al. 2012) but interestingly

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facilitated the extinction of fear memories when tested over a longer time frame (Dobi et al. 2013; Fendt et al. 2008; Toth et al. 2012) possibly due to AMN082 induced impaired amygdalar plasticity (Fendt et al. 2013; Fendt et al. 2008). Moreover, AMN082 rescued the fear extinction deficits observed in the S1 mouse (Whittle et al. 2013). In relation to anxiety-like behaviour, AMN082 administration produced data similar to knockout animals with administration resulting in an anxiolytic phenotype in tests such as stress-induced hyperthermia (SIH) and the four-plate test (Stachowicz et al. 2008). Furthermore, siRNA-induced reduction of $mGlu_7$ receptor led to potent anxiolyisis in mice (O'Connor et al. 2013b). Interestingly, a negative allosteric modulator of the $mGlu_7$ receptor, ADX71743, induced anxiolysis as measured by the reduction of visceral hypersensitivity in a stress sensitive rat strain (Moloney et al. 2015). In contrast, neither genetic nor pharmacological reduction of $mGlu_7$ receptor activity impacts anxiety or fear learning in a mouse model of a neurodevelopmental disorder (*MECP2* Duplication syndrome) (Fisher et al. 2017).

Given the apparent similar effects resulting from $mGlu_7$ receptor agonism and inhibition it is worth noting reports that a metabolite of AMN082, N-benzhydrylethane-1,2-diamine (Met-1), at physiologically relevant levels, inhibits the serotonin transporter, noradrenaline transporter and dopamine transporter (Sukoff Rizzo et al. 2011) possibly inducing AMN082's observed effects in vivo. However, the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (Bradley et al. 2012) and an $mGlu_7$ receptor antagonist MMPIP both attenuated antidepressant-like effects of AMN082 (O'Connor and Cryan 2013) administration pointing to a glutamatergic mechanism.

Although much of the data has been somewhat paradoxical with knockout and pharmacological upregulation producing similar results, it is clear that $mGlu_7$ receptor is involved in regulating emotional behaviour and cognitive processes, including fear extinction. The S1 mouse strain represents a model of impaired regulation of fear memories seen in PTSD (Holmes and Singewald 2013). To assess whether differences in $mGlu_7$ receptor levels contribute to these fear extinction deficits, the present study used in situ hybridisation (ISH) to compare $mGlu_7$ receptor mRNA levels between this strain and the B6 strain, which shows robust fear extinction. Expression levels were analysed in three different brain regions critical for fear extinction, namely the prefrontal cortex (PFC), hippo-campus and amygdala.

Methods

Animals

Subjects were male S1 and B6 mice obtained at ~ 8 weeks of age from the Jackson Laboratory (Bar Harbour, ME). Animals were naïve from the same cohort tested in (Camp et al. 2009) where S1 mice showed robust deficits to fear extinction. Mice were housed by strain (2 per cage) in a temperature (22 ± 3 °C) and humidity (45 ± 15%) controlled vivarium under a 12-h light/dark cycle (lights on 0600 h). Cage dimensions were 37 cm × 16 cm × 13 cm. All experiments were conducted in accordance with the European Directive 86/609/EEC and the local Animal Care and Use Committees.

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Sample collection

Mice were sacrificed by decapitation. The brain was immediately extracted, snap-frozen in cold isopentane and stored at −80 °C before being processed for in situ hybridisation.

In situ hybridisation

The procedure was carried out using an oligodeoxynucleotide (cDNA) probe complementary to $mGlu_7$ receptor mRNA (1336–1292 pb access number NM_177328.3), labelled with a digoxigenin (DIG) oligonucleotide tailing kit (Roche, Molecular Biochemicals, Mannheim, Germany). Frozen 10-µm-thick sections were initially post-fixed in 4% paraformaldehyde and hybridised over night at 37 °C in formamide 50%, saline and sodium citrate buffer (SSC) 4×, sheared salmon DNA 6.25 mg/mL, tRNA 125 µg/mL and cDNA probe at fixed concentration of 100 pmol/mL. On the following day, sections were washed in saline-sodium citrate buffer, and the DIG molecules attached to the hybridised probes were detected with an anti-DIG antibody, conjugated with an alkaline phosphatase (Roche, Molecular Biochemicals, Mannheim, Germany). Finally, a substrate for the alkaline phosphatase (NBT/BCIP) (Sigma, St. Louis, MO, USA) was added. Specificity of the hybridization was evaluated by the use of 100-fold excess of the unlabelled oligodeoxynucleotide. For quantitative analysis, densitometric measurements of each hippocampal areas were analysed using FujiFilm's Science Lab Multi Gauge v2.2 software. All pictures were analysed in grey scale by measuring the density of pixels in a given area.

Statistical analysis

Data was analysed using Student's *t* test using GraphPad prism 6 software. A *p* value of 0.05 was chosen as the threshold for significance.

Results

Amygdala

$mGlu_7$ receptor mRNA expression was found to be significantly higher in the amygdala (LA + BLA) in the S1 strain when compared to B6 (Student's *t* test $p < 0.001$, $t = 3.557$, $df = 10$) (Fig. 1a, e, f). $mGlu_7$ receptor mRNA expression was found to be significantly higher in the LA in the S1 strain when compared to B6 (Student's *t* test $p < 0.001$, $t = 4.304$, $df = 10$) (Fig. 1b, e, f). $mGlu_7$ receptor mRNA expression was found to be significantly higher in the basolateral amygdala in the S1 strain when compared to B6 (Student's *t* test $p < 0.05$, $t = 2.318$, $df = 10$) (Fig. 1c, e, f). No significant difference in $mGlu_7$ receptor mRNAwas found in the shell of the CeA/medial intercalated cell clusters between the strains (Student's *t* test $p > 0.05$, $t = 1.604$, $df = 10$) (Fig. 1d, e, f).

Hippocampus

$mGlu_7$ receptor mRNA expression was found to be significantly higher in the hippocampus (CA1 + CA3) in the S1 strain when compared to B6 (Student's *t* test $p < 0.001$, $t = 2.770$, $df = 10$) (Fig. 2a, e, f). $mGlu_7$ receptor mRNA expression was found to be significantly higher in the CA1 region of the hippocampus in the S1 strain when compared to B6 (Student's *t* test $p < 0.05$, $t = 2.660$, $df = 10$) (Fig. 2b, e, f). $mGlu_7$ receptor mRNA expression was found to

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be significantly higher in the CA3 region of the hippocampus in the S1 strain when compared to B6 (Student's *t* test $p < 0.05$, $t = 3.839$, $df = 10$) (Fig. 2c, e, f). No significant difference in $mGlu_7$ receptor mRNA levels was found in the dentate gyrus of the hippocampus between the strains (Student's *t* test $p > 0.05$, $t = 0.4827$, $df = 10$) (Fig. 2d, e, f).

Infralimbic cortex, anterior cingulate gyrus and prelimbic region

No significant difference in $mGlu_7$ receptor mRNA was found in the infralimbic cortex (Student's *t* test $p > 0.05$, $t = 0.5937$, $df = 10$), anterior cingulate gyrus (Student's *t* test $p > 0.05$, $t = 0.5757$, $df = 10$) or the prelimbic region between strains (Student's *t* test $p > 0.05$, $t = 0.5496$, $df = 10$) (Fig. 3).

Discussion

The current study compared $mGlu_7$ receptor expression levels between the S1 inbred mouse strain, impaired in its ability to extinguish fear, and the B6 inbred strain which has normal extinction patterns (Hefner et al. 2008; Whittle et al. 2013) in key brain regions associated with fear extinction namely the amygdala, hippocampus, prefrontal cortex. In the amygdala, we found increased $mGlu_7$ receptor mRNA expression in the S1 strain and when further divided into different sub-regions, increased expression was found in the LA and the BLA, but not in the CeA. Similarly, increased expression levels of $mGlu_7$ receptor were also seen in the hippocampus of S1 mice compared to the B6 strain, with an increase seen in the CA1 and CA3 regions but not the DG. Interestingly, no difference in $mGlu_7$ receptor levels was seen in the medial prefrontal cortex, prelimbic region or anterior cingulated gyrus.

Previous work on $mGlu_7$ receptor has demonstrated its critical role in both maintaining correct cognitive functioning (Callaerts-Vegh et al. 2006; Dobi et al. 2013; Fendt et al. 2008; Goddyn et al. 2015; Goddyn et al. 2008; Holscher et al. 2004, 2005; Klakotskaia et al. 2013; Moloney et al. 2015; Toth et al. 2012), regulating anxiety-like behaviour (Callaerts-Vegh et al. 2006; Cryan et al. 2003; Moloney et al. 2015; O'Connor et al. 2013b; Stachowicz et al. 2008) and appropriately responding to psychologically stressful events (Gee et al. 2014; Moloney et al. 2015; Peterlik et al. 2016; Peterlik et al. 2017). Situated at the interface of cognition and emotion, $mGlu_7$ receptor is in a key position to regulate processes that may contribute to the pathophysiology underlying PTSD (O'Connor et al. 2010).

It is interesting to find that the S1 strain has increased $mGlu_7$ receptor mRNA in the amygdala and hippocampus since both global $mGlu_7$ receptor ablation and knockdown result in an attenuation to the extinction of aversive memories (Callaerts-Vegh et al. 2006; Fendt et al. 2008) and systemic pharmacological $mGlu_7$ receptor activation facilitates the extinction of aversive memories (Fendt et al. 2008; Toth et al. 2012). It might be expected that a strain showing poor extinction such as the S1 would have lower $mGlu_7$ receptor levels in neuronal circuits that regulate fear extinction. There are a number of different possibilities that may help explain elevated amygdalar and hippocampal $mGlu_7$ receptor levels including the $mGlu_7$ receptor's role as a presynaptic inhibitor of both GABA and glutamate release and possible increased compensatory $mGlu_7$ receptor expression in response to additional deficits in the signalling cascades that mediate the extinction of fear memories.

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Evidence to date has ascribed a role to many different members of the mGlu receptor family in the acquisition, extinction and recall of fear memories. However, each mGlu receptor seems to occupy a specific niche in these processes. For example, intra-amygdalar pharmacological activation of $mGlu_{2/3}$ receptors did not affect the rate of fear extinction acquisition but did enhance the retention of fear memories (Kim et al. 2015). Furthermore, systemically blocking $mGlu_5$ receptor activity facilitated the spontaneous recovery of fear memories but not extinction per se, whereas the other group 1 mGlu receptor member, $mGlu_1$ receptor, does not seem to play a role in fear extinction (Mao et al. 2013). The other group III mGlu receptors appear to be more involved in the acquisition of fear memories, given mutant mice lacking $mGlu_4$ receptor had enhanced acquisition of cued freezing responses (Davis et al. 2013) whereas $mGlu_8$ receptor knockout mice exhibit deficits in fear acquisition but no changes to fear extinction (Fendt et al. 2010). Thus, most members of the mGlu receptor family play key roles in fear memory formation but specifically regulating the extinction of such memories appears unique to $mGlu_7$ receptor based on the currently available evidence.

It is worth noting that the $mGlu_7$ receptor agonist AMN082 was able to rescue the fear extinction deficits seen in the S1 mouse (Whittle et al. 2013). The amygdala contains a large population of GABAergic neurons which are activated by glutamate (Rainnie et al. 1991) and it has been suggested that glutamate-induced increases in GABA release may contribute to the extinction of fear memories (Rainnie et al. 1991). Increased amygdalar $mGlu_7$ receptor levels would result in potentiation of the negative feedback that inhibits further release of glutamate (Summa et al. 2013). This would lead to a reduction in the glutamatergic innervation of GABAergic neurons, resulting in an overall decrease of inhibition levels, which may contribute to the poor extinction observed in the S1 strain.

Preceding extinction, the hippocampus contributes to the contextual retrieval of fear memory through its inputs to the BLA (Corcoran and Maren 2001; Herry et al. 2008). These inputs are coupled to a subset of neurons that are activated when a conditioned stimulus and an unconditioned stimulus are presented together. Following extinction, these neurons are no longer active. If the increased $mGlu_7$ receptor levels seen in the S1 strain are on hippocampal GABAergic neurons, the subsequent reduced GABA release could lead to an exaggerated activation of these fear neurons, preventing fear extinction. Recent data indicates that correct $mGlu_7$ receptor function is required for hippocampal plasticity (Klar et al. 2015). Currently, we can only speculate on the differing contributions of glutamatergic and GABAergic $mGlu_7$ receptor levels to fear extinction processes. Experiments localising $mGlu_7$ receptor on either GABAergic or glutamatergic neurons in different regions of the brain critical to fear extinction will reveal more about how $mGlu_7$ receptor influences the extinction of fear memories and may help to explain why a strain exhibiting profoundly impaired fear extinction displays increased $mGlu_7$ receptor in brain regions associated with fear extinction.

It has been well established that the mPFC plays a prominent role in both the acquisition and extinction of fear memories (Corcoran and Quirk 2007; Sotres-Bayon and Quirk 2010). Furthermore, there are reciprocal projections between the mPFC and the amygdala (Conde et al. 1995; Dzhaiani et al. 1987; Herry et al. 2008; McDonald et al. 1996) with cortical

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neurons providing inhibitory control over BLA neurons during the extinction of fear memories (Bukalo et al. 2015; Marek et al. 2013). Interestingly, in contrast to the hippocampus and the amygdala, no difference in $mGlu_7$ receptor levels was seen between the B6 and S1 strains in various subregions of the mPFC. Thus suggesting the impaired fear extinction seen in the S1 strain may not result from any differences in cortical derived inhibition of amygdalar activity driven via a mGlu7 receptor-dependent mechanism.

In sum, the current study shows $mGlu_7$ receptor mRNA levels to be different between two different inbred mouse strains that differ markedly in their ability to extinguish conditioned fear with the S1 mouse having increased $mGlu_7$ receptor mRNA levels in the amygdala and hippocampus. This strongly suggests a role for $mGlu_7$ receptor in the neuronal processes associated with fear extinction. Furthermore, based on pharmacological data showing activation of $mGlu_7$ receptor facilitates the extinction of aversive memories (Fendt et al. 2008; Toth et al. 2012), it is more likely, at least in C57 mice which display robust fear extinction, that changes to receptor function and not any changes to expression contributes to fear memory extinction. Moreover, it further confirms the utility of the S1 mouse as a tool to understand the molecular basis of extinction (Gunduz-Cinar et al. 2018) and ways to boost extinction memories (Bukalo et al. 2014; Singewald et al. 2015). This could be of great relevance to our understanding of the neuronal circuitry involved in PTSD and increases our knowledge of what may be the underlying molecular disturbances taking place in this devastating disorder.

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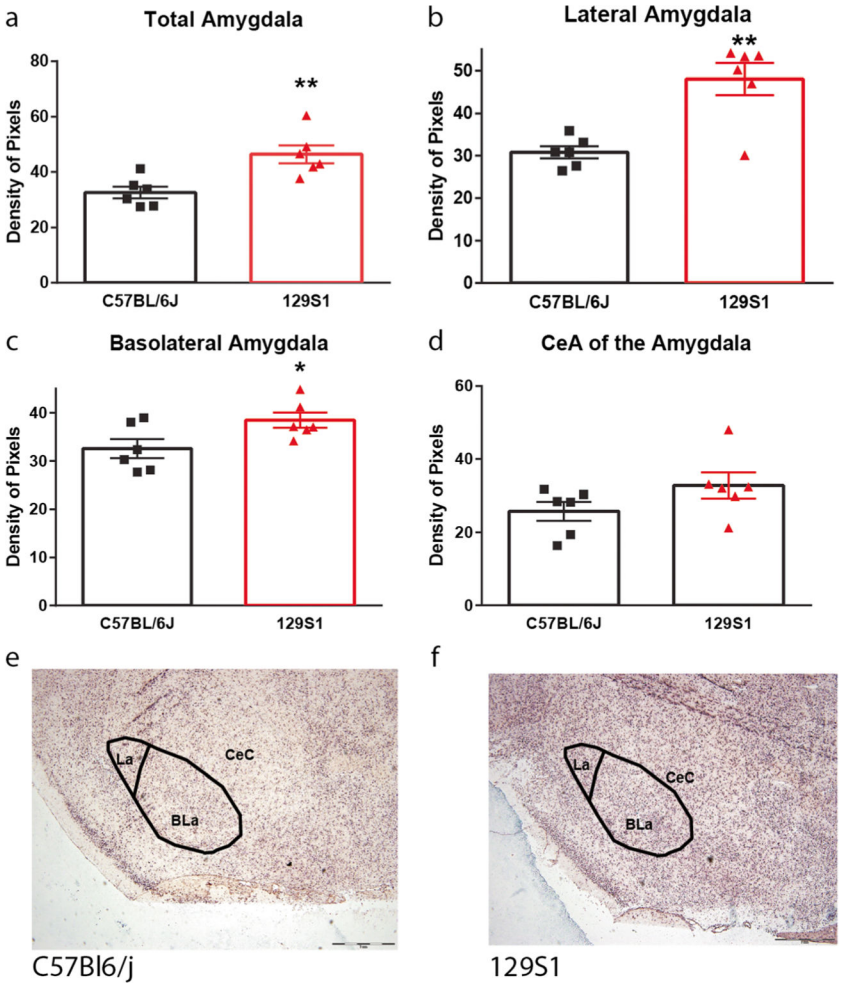


Fig. 1.

In situ hybridisation of $mGlu_7$ receptor in the amygdala of B6 and S1 mice. **a–c** Densitometric analysis showing the S1 mice had increased levels of $mGlu_7$ receptor in the amygdala (LA + BLA), lateral amygdala (LA) and basolateral amygdala (BLA), respectively. **d** No significant difference in $mGlu_7$ receptor expression of the CeA of the amygdala was found between the two strains. **e** Representative image of $mGlu_7$ receptor mRNA staining in the amygdala of B6 mice. **f** Representative image of $mGlu_7$ receptor mRNA staining in the amygdala of S1 mice.

*Significant using Student's *t* test when compared to B6. $n = 6$

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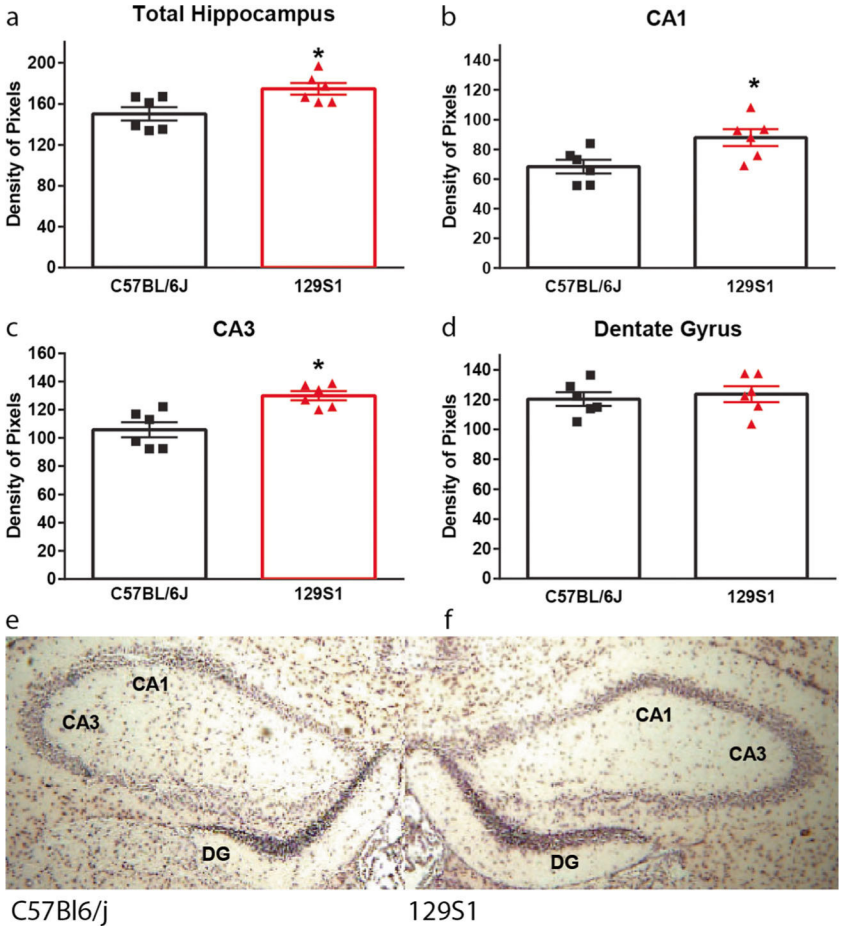


Fig. 2.

In situ hybridisation of $mGlu_7$ receptor in the hippocampus of B6 and S1 mice. **a–c** Densitometric analysis showing the S1 mice had increased levels of $mGlu_7$ receptor in the hippocampus (CA1 + CA3), CA1 and CA3 regions of the hippocampus. **d** No significant difference in $mGlu_7$ receptor expression of the dentate gyrus of the hippocampus was found between the two strains. **e** Representative image of $mGlu_7$ receptor mRNA staining in the hippocampus of B6 mice. **f** Representative image of $mGlu_7$ receptor mRNA staining in the hippocampus of S1 mice.

*Significant using Student's *t* test when compared to B6. $n = 6$

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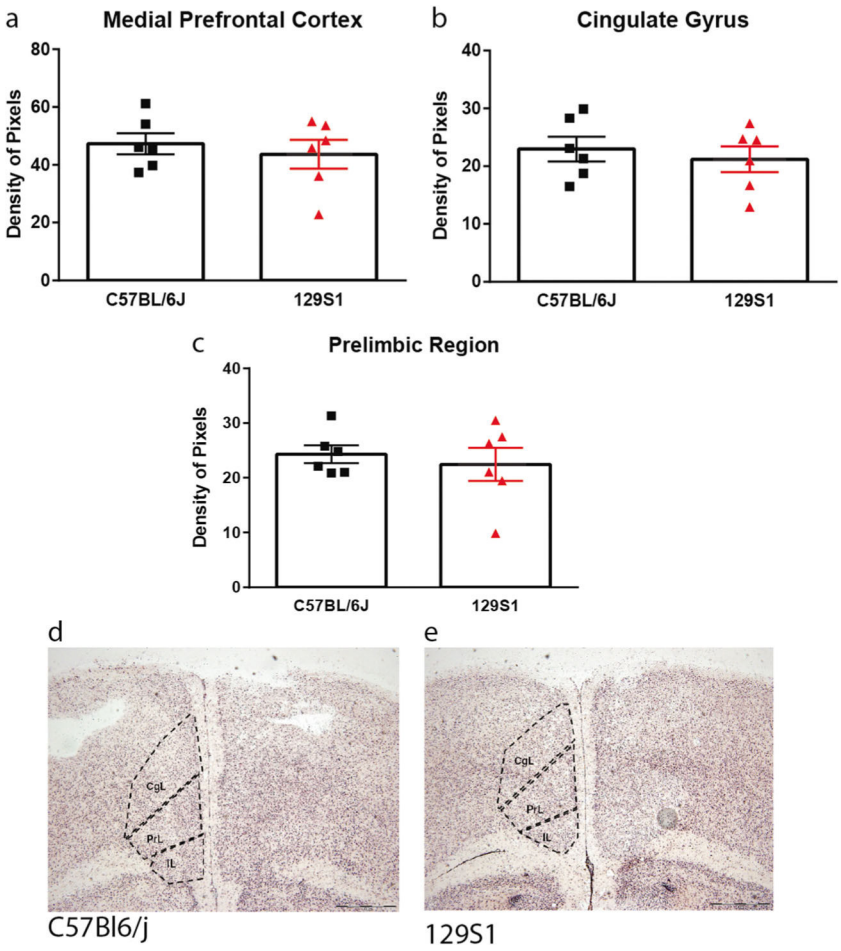


Fig. 3.

In situ hybridisation of mGlu7 receptor in the prefrontal cortex of B6 and S1 mice. **a–c** No significant difference in mGlu7 receptor expression in the prefrontal cortex was found between the two strains. **d** Representative image of mGlu7 receptor mRNA staining in the prefrontal cortex of B6 mice. **e** Representative image of mGlu7 receptor mRNA staining in the prefrontal cortex of S1 mice.

CgL = cingulate gyrus, PrL = prelimbic region, IL = infralimbic. $n = 6$