Reduced phosphorylation of GluA1 subunits relates to anxiety-like behaviours in mice

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Abstract

Anxiety and depression are highly prevalent and frequently co-morbid conditions. The ionotropic glutamate receptors N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) mediate actions of monoaminergic antidepressants and have been directly targeted by novel fast-acting antidepressants. Less is known about the role of these receptors in anxiety-like states. Here we investigate how two distinct anxiolytic agents, buspirone, a partial 5-HT1A agonist, and diazepam, a benzodiazepine, influence phosphorylation of GluA1 subunits of AMPA receptors at the potentiating residue Ser845 and Ser831 in corticostriatal regions. To test the functional relevance of these changes, phosphomutant GluA1 mice lacking phosphorylatable Ser845 and Ser831 were examined in relevant behavioural paradigms. These mutant mice exhibited a reduced anxiety-like phenotype in the light/dark exploration task and elevated plus maze, but not in the novelty induced hypophagia paradigm. These data indicate that reduced potentiation of the AMPA receptor signalling, via decreased GluA1 phosphorylation, is specifically involved in approach–avoidance based paradigms relevant for anxiety-like behaviours.

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Introduction

There is strong interest in the contribution of glutamate dysfunction to the pathophysiology and treatment of neuropsychiatric disorders including depression and anxiety (Skolnick et al. 2009). Glutamate exerts its actions via multiple metabotropic receptors and three families of ionotropic receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR); kainate receptors; N-methyl-D-aspartate receptors.

AMPA receptors are heteromeric postsynaptic receptors composed of a combination of four subunits, GluA1–4 and are known to play a major role in synaptic plasticity and various forms of behaviour. An emerging literature implicates AMPA in the regulation of anxiety. For example, administration of AMPAR antagonists, including LY326325 and GYKI 52466 has anxiolytic-like effects in rodent assays (Kapus et al. 2008; Kotlinska & Liljequist, 1998; Mathews & Guimaraes, 1997). In addition, increases in anxiety-like behaviour produced by benzodiazepine withdrawal correspond to increased levels of GluA1 in the cortex and hippocampus (Izzo et al. 2001) and increased amplitude of hippocampal AMPAR miniature excitatory postsynaptic currents (Xiang & Tietz, 2007). The anxiety-provoking effects of withdrawal in the elevated plus maze (EPM) are rescued by the AMPAR antagonist GYKI 52466 (Xiang & Tietz, 2007). Thus, AMPAR can bi-directionally mediate anxiety, possibly by modulating network excitability in corticostriatal regions such as the amygdala, prefrontal cortex (PFC) or hippocampus.

The function of AMPAR, including GluA1, is in part determined by phosphorylation. Phosphorylation of the Ser845 site by protein kinase (PK)A or at the Ser831 site by calcium kinase II or PKC increases synaptic expression of GluA1 and potentiates AMPAR signalling (Roche et al. 1996). Drugs with clinical efficacy as antidepressants and anxiolytics, including fluoxetine, tianeptine, imipramine and valproate, have been shown to increase GluA1 phosphorylation at Ser845 or Ser831 in the cortex or hippocampus (Du et al. 2007; Qi et al. 2009; Svenningsson et al. 2002; Svenningsson et al. 2007). These findings suggest phosphorylation as a potential mechanism for modulating GluA1-mediation of behaviours such as anxiety.

Mutant mice engineered with inactivated Ser831 and Ser845 phosphorylation sites have provided valuable insights into the consequences of loss of GluA1 phosphorylation for neural functions and behaviour.
Ser\textsuperscript{831} / Ser\textsuperscript{845} double phosphomutants have normal cell-surface expression of GluA1 in cultivated cortical neurons, but impaired hippocampal long-term potentiation and long-term depression (LTD; Lee et al. 2003). On tests for learning and reward-related behaviour, Ser\textsuperscript{831} / Ser\textsuperscript{845} double phosphomutants exhibit deficits in spatial memory (Lee et al. 2003), Pavlovian-to-instrumental transfer (Crombag et al. 2008a) and conditioned reinforcement (Crombag et al. 2008b). These mutants also fail to develop enhanced Pavlovian fear memories following exposure to predator threat (Hu et al. 2007).

The main aim of the present study was to clarify the role of AMPAR phosphorylation in anxiety-like behaviours. To this end, we conducted a comprehensive phenotypic characterization of Ser\textsuperscript{831} / Ser\textsuperscript{845} double phosphomutant mice on a battery of anxiety- and depression-related assays. This was complemented by examination of the effects of two anxiolytic treatments (diazepam, buspirone) on phosphorylation of Ser\textsuperscript{831} and Ser\textsuperscript{845} - GluA1 in corticolimbic regions.

Method and results

Subjects

Mutant mice lacking phosphorylatable Ser\textsuperscript{831} and Ser\textsuperscript{845} were generated as previously described via ‘knock-in’ replacement of the two sites with alanine substitutions and showed no gross behavioural or neurological deficits (Hu et al. 2007; Lee et al. 2003). Phosphomutant (MT) and corresponding wild type (WT) mice originally generated using embryonic stem cells from a 129S1/SvImJ donor were backcrossed >10 generations on to a C57BL/6 background at Johns Hopkins University as described previously (Hu et al. 2007; Lee et al. 2003). Phosphomutants were bred in-house and tested at 4–5 months of age. As there was no significant genotype × sex interactions, data were collapsed across sex to increase statistical power. Male C57BL/6j (Charles River, Sweden) mice were used for immunoblotting analysis of Ser\textsuperscript{831} and Ser\textsuperscript{845} after diazepam and buspirone treatment.

Mice were housed in a temperature- and humidity-controlled vivarium under a 12 h light/dark cycle (lights on 06:00 hours) and tested in the light phase. Mice were group-housed, by sex, except for sucrose preference and novelty-induced hypophagia, which necessitated single housing 24 h prior to testing. Experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and international (National Research Council, 1996 and 86/609/EEC) guidelines on the ethical use of animals and were approved by the local ethical committee at Karolinska Institute (N351/08; N366/10; N2/12). The number of mice used in each experiment is given in the Fig. legends.

Anxiety-related behaviours in Ser\textsuperscript{831} / Ser\textsuperscript{845} phosphomutants

A cohort of WT and MT was tested for anxiety-like behaviour and locomotor exploration in a novel open field test (OF). A separate cohort was tested in two classic approach-avoidance conflict tests, light–dark exploration (L/D) and EPM, with at least 5 d between assays.

The OF was conducted in the dark in a ventilated sound-attenuated box (outside size, 650 × 650 × 325; inside 500 × 500 × 225 mm) based on previously described methods (Svenningsson et al. 2007). Breaks on a lower row of infrared sensors 20 mm above the floor were taken as a measure of locomotor activity and beam breaks on an upper row of sensors 50 mm above the floor was taken as a measure of rearing. Infrared sensors were placed 31 mm apart, with the last photocell 17.5 mm from the wall. As previously reported (Svenningsson et al. 2007), genotypes did not differ in total distance travelled (Fig. 1a) but mutants did show significantly more rearing time than WT [t(22) = 2.14, p < 0.05; Fig. 1b]. There were no genotype differences in thigmotaxis, a measure of activity in the periphery of the maze compared to total horizontal activity (Fig. 1c) or in the time spent in the centre of the maze (Fig. 1d).

The L/D was performed as previously described (Inhe et al. 2012). Time spent and entries into the light (90 lux) vs. dark compartment over the first 5 min of a 15 min session were measured by the Anymaze videotracking system (UgoBasile, Italy). MT spent significantly more time [(30) = 5.21, p < 0.01; Fig. 1e] and made significantly more entries [(30) = 5.64, p < 0.01; Fig. 1f] into the light compartment than WT.

The EPM was conducted as previously described (Kindlundh-Hogberg et al. 2009). Mice were placed in the centre facing an open arm and allowed to explore the apparatus for 5 min. Entries into the open (90 lux) and closed (20 lux) arms were measured by the Anymaze videotracking system (Stoelting Co., USA). MT spent significantly more time [(37) = 4.37, p < 0.01; Fig. 1g] and made significantly more entries [(30) = 3.97, p < 0.01; Fig. 1h] into the closed compartment than WT. MT also made significantly more entries into the closed arms than WT [(37) = 3.22, p < 0.01; Fig. 1i].

Depression-related tests in Ser\textsuperscript{831} / Ser\textsuperscript{845} phosphomutants

Following L/D and EPM testing, mice were phenotyped on depression-related assays in the following order, with at least 5 d between assays: sucrose preference (SP), novelty-induced hypophagia (NIH) and forced swim stress-induced corticosterone.

SP over water provides a simple measure with relevance to the anhedonia seen in depressive patients and was measured as previously described (Krishnan et al. 2007). Mice drank from two bottles (1% sucrose solution or tap water) over 3 d (data from three mice were
removed due to bottle clogging). MT and WT had similar SP scores (~60%; Fig. 1j).

The NIH test is sensitive to both anxiolytic and anti-depressant treatments and was conducted as previously described (Karlsson et al. 2008). Mice were placed, in their home cage, under dim light (30 lux) and given access via a ‘sippy tube’ to diluted sweetened condensed milk (3:1 water:milk) for 15 min on four consecutive days. On day 5, mice were transferred to an empty novel cage under 120 lux lighting. Milk consumption was significantly lower in the novel cage relative to the last home cage day ($F_{1,16} = 87.78, p < 0.01$) irrespective of genotype (Fig. 1k).

Latency to first drink was also significantly higher in the novel cage than final home cage day ($F_{1,16} = 27.75, p < 0.01$; Fig. 1l) and genotypes did not differ (mice that did not drink were given a maximum latency score of 900 s).

Thirty minutes after exposure to 6-min forced swim (as previously described in Carroll et al. 2007), mice were killed via decapitation for serum corticosterone measurement from trunk blood. Samples from non-swum home cage mice were collected at the same time. After 1–2 h at room temperature, blood was centrifuged (4 °C; 30 s; 200 g). Serum was collected and run on Radioimmunoassay for Corticosterone (bound and free; MP Biomedicals, France). Corticosterone levels were significantly elevated after forced swim, relative to home cage ($F_{1,14} = 56.64, p < 0.01$) and genotypes did not differ (Fig. 1m).

**Anxiolytic-induced GluA1 phosphorylation**

GluA1 phosphorylation at Ser$^{831}$ and Ser$^{845}$ was measured following treatment with two anxiolytic drugs from different classes: the benzodiazepine diazepam and the 5-HT$_{1A}$ receptor agonist buspirone. Mice were injected i.p. (10 ml/kg body weight) with 0.9% saline vehicle or 0.25 mg/kg diazepam (Sigma, USA), while a separate cohort was treated with saline or 1.0 mg/kg buspirone (Sigma). Doses were based on previous studies showing anxiolytic-like effects in mice tested on anxiety-related
assays (Crawley & Davis, 1982; Garrett et al., 1998; Liu et al., 2007). Thirty minutes after injection, mice were killed by decapitation. Brains were quickly removed and snap frozen to prevent dephosphorylation.

The medial PFC, dorsal hippocampus (DH) and basolateral amygdala (BLA) were dissected and rapidly frozen at −80°C. Frozen tissue samples were sonicated in 1% SDS and boiled for 10 min. Small aliquots of the homogenate were retained for protein determination by the biocinchonic acid protein assay method. Immunoblotting was processed using 10% acrylamide gels as previously described (Svenningsson et al., 2002) using phosphospecific antibodies towards Ser\(^{845}\) (1:500; rabbit polyclonal; Millipore, USA) or Ser\(^{845}\) (1:1000; rabbit polyclonal; Upstate Biotechnology, USA) as well as total antibodies towards GluA1 (1:1000; rabbit polyclonal; Chemicon, USA) and β-actin (1:4000; rabbit polyclonal; Sigma), with the latter serving as a loading control. Antibody binding was detected by enhanced chemiluminescence (GE Healthcare, Sweden) and quantified by densitometry using NIH IMAGE 1.61 software. Phosphorylation levels at Ser\(^{845}\) and Ser\(^{845}\) of GluA1 subunits were normalized to total GluA1 protein levels. Values >2 S.D. from the mean were removed as outliers.

Treatment with either diazepam \([t(28) = 2.07; p < 0.05]\) or buspirone \([t(18) = 2.27; p < 0.05]\) significantly decreased Ser\(^{845}\) phosphorylation, relative to saline, in the DH and buspirone \([t(20) = 2.57; p < 0.05]\) produced significant Ser\(^{845}\) reductions in the BLA (Fig. 2a,b). There were no significant changes in Ser\(^{831}\) phosphorylation in any region after either treatment (Fig. 2c,d).

**Statistical analysis**

Genotypes were compared using Student’s \(t\) test, except for NIH and corticosterone data, which was analysed using a 2-factor (test × genotype) analysis of variance. Anxiolytic treatment effects on Ser\(^{831}\) and Ser\(^{845}\) activity were analysed by brain region using Student’s \(t\) test. The threshold for statistical significance was \(p < 0.05\).
Discussion

The first novel finding of the current study was that constitutive genetic inactivation of the Ser\textsuperscript{845} and Ser\textsuperscript{831} phosphorylation residues of GluA1 produced a test-specific decrease in anxiety-like behaviours. The second finding was that two structurally dissimilar anxiolytics produced decreases in Ser\textsuperscript{845}-GluA1 phosphorylation in the hippocampus. Taken together, these data suggest a role for Ser\textsuperscript{845} in anxiety-related behaviours.

Phosphomutations of both Ser\textsuperscript{831} and Ser\textsuperscript{845}-GluA1 had specific effects on measures of anxiety- and depression-related behaviours. We did not observe any phenotypic abnormality in three depression-related assays (SP, NIH, swim-induced corticosterone). Although previous assessment of these mice found an increased depression-like phenotype in the tail suspension task (Svenningsson et al. 2007) dissociations between these tests have been found previously (e.g. Lockridge et al. 2010).

On assays for anxiety-like behaviour, GluA1 phosphomutant mice showed general increases in exploratory locomotion (OF rearing, EPM closed entries) that could not be clearly dissociated from alterations in anxiety-related behaviour. This complex profile is reminiscent of the phenotype previously reported in mice with genetic deletion of GluA1, which was interpreted as a mania- or schizoaffective-like increase in approach behaviour and behavioural reactivity, given the reversal by anti-manic and antipsychotic drugs (Fitzgerald et al. 2010). In this context, it would be valuable to test whether drugs of this class also normalized the phenotype in the Ser\textsuperscript{831}/Ser\textsuperscript{845} phosphomutants.

To further investigate the possible role of Ser\textsuperscript{831} and Ser\textsuperscript{845} in anxiety-related phenotypes, we measured alterations of these sites in key corticolimbic regions after treatment with two common anxiolytics, diazepam and buspirone, with different neurotransmitter targets. We found both drugs decreased hippocampal Ser\textsuperscript{845}-GluA1 phosphorylation in C57BL/6j mice. This effect was not found in the BLA or PFC, where only buspirone had an effect, to decrease BLA phosphorylation. In contrast to effects on Ser\textsuperscript{845} phosphorylation, no changes in Ser\textsuperscript{831}-GluA1 phosphorylation were evident after either drug treatment.

These data suggest that Ser\textsuperscript{845}-GluA1 may be a target for the anxiolytic action of these drugs. This extends previous evidence that Ser\textsuperscript{845} and not Ser\textsuperscript{831}, GluA1 is involved in certain forms of hippocampal synaptic plasticity LTD (Lee et al. 2010) and fear extinction (Clem & Huganir, 2010). Earlier work has also shown that deletion of Ser\textsuperscript{831}, but not of Ser\textsuperscript{845}, GluA1 subunits alters incentive learning (Crombag et al. 2008b). Thus, these two residues appear to have domain-specific roles in behaviour. Additional studies are needed to clarify the role of specific phosphorylation sites in anxiety-related behaviours, for example by phenotyping mice with single Ser\textsuperscript{845} or Ser\textsuperscript{831} phosphomutations. The reduction of Ser\textsuperscript{845} phosphorylation after anxiolytic treatment provides a parallel to the anxiety-like phenotype of the GluA1 phosphomutants. However, the region-specific effects of the drug treatments suggest that they are unlikely to fully account for the region-wide phosphomutant deletion. Again, future experiments testing the effects of region-specific infusion of these drugs in the phosphomutant mice would help address which brain regions are key to the anxiolytic effect.

In summary, the current study found novel evidence for a role of GluA1 phosphorylation in anxiety-related behaviours. Future studies involving manipulations of Ser\textsuperscript{845}-GluA1 with more circuit specificity could dissociate roles in anxiety vs. novelty-seeking. Additionally, future studies administering buspirone or diazepam in single or double phosphomutant mice could demonstrate if these phosphorylation sites are necessary for the behavioural response to anxiolytics.

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Statement of Interest

None.

References


