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Behavioral and Physiological Characterization of PKC-dependent Phosphorylation in the *Grin2a* PKC mouse

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Abstract

Activity-dependent plasticity in NMDA receptor-containing synapses can be regulated by phosphorylation of serines and tyrosines in the C-terminal domain of the receptor subunits by various kinases. We have previously identified S1291/S1312 as important sites for PKC phosphorylation; while Y1292/Y1312 are the sites indirectly phosphorylated by PKC via Src kinase. In the oocyte expression system, mutation of those Serine sites to Alanine (that cannot be phosphorylated) in the GluN2A subunit, resulted in a decreased PKC stimulated current enhancement through the receptors compared to wild-type NMDA receptors. To investigate the behavioral and physiological significance of those PKC-mediated phosphorylation sites in vivo, the *Grin2a* PKC mouse expressing GluN2A with four mutated amino acids: S1291A, S1312A, Y1292F and Y1387F was generated using homologous recombination. The *Grin2a* PKC mice exhibit reduced anxiety in the open field test, light dark emergence test, and elevated plus maze. The mutant mice show reduced alternation in a Y maze spontaneous alternation task and a in a non-reinforced T maze alternation task. Interestingly, when the mutant mice were exposed to novel environments, there was no increase in context-induced Fos levels in hippocampal CA1 and CA3 compared to home-cage Fos levels, while the Fos increased in the WT mice in CA1, CA3 and DG. When the SC-CA1 synapses in slices from mutant mice were stimulated using a theta-burst protocol, there was no impairment in LTP. Overall, these results suggest that at least one of those PKC-mediated phosphorylation sites regulates NMDAR-mediated signaling that modulates anxiety.

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The authors declare no conflict of interest.

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Keywords

GluN2A; Grin2a; PKC; homologous recombination; mouse behavior; anxiety; spontaneous alternation; theta-burst stimulation; c-Fos; hippocampus

1. Introduction

NMDA receptors (NMDARs, are characterized by their Ca^{2+} permeability and unique gating, requiring both ligand binding and a depolarized membrane potential (Seeburg *et al.*, 2013). In vivo, these receptors form heterotetramers containing two GluN1 and two of 4 different GluN2 subunits (GluN2A-D) (Cull-Candy *et al.*, 2001, Dunah *et al.*, 1998). NMDAR channel properties such as maximal conductance, decay time, and sensitivity to Mg^{2+} are determined mainly by the GluN2 subunits of the receptor (Evans *et al.*, 2012). Certain forms of long-term potentiation and depression (LTP and LTD) and activity-dependent synapse remodeling may be dependent on the Ca^{2+} influx through the NMDARs (Eccles, 1983). Therefore, synaptic plasticity could be modulated by varying the calcium influx through the NMDAR, which in turn is determined by the GluN2 subunit. For example, calcium influx through the GluN2A-containing receptors at inter-stimulus interval more than 20 ms is much lower, compared to GluN2B-containing receptors (Evans *et al.*, 2012). The exact mechanistic role of GluN2 subunits in hippocampal synaptic plasticity, remains unknown despite several interesting studies (Berberich *et al.*, 2007, Foster *et al.*, 2010, Liu *et al.*, 2004, Tang *et al.*, 1999). Apart from subunit composition, phosphorylation of amino-acids in the C-terminal domain (CTD) of the GluN2 subunits by various kinases also regulates channel properties such as membrane trafficking, and synaptic localization (Salter *et al.*, 2009). For example, Protein Kinase C (PKC) activation increased the number of NMDARs inserted into the plasma membrane of oocytes and isolated hippocampal neurons (Lan *et al.*, 2001). PKC phosphorylates serines and also activates another kinase called Src which phosphorylates tyrosines in CTD of GluN2A (Lu *et al.*, 1999). Using HEK cells, a region in GluN2A CTD between amino-acids 1267 and 1406 was shown to be a key structural determinant for PKC-mediated current potentiation in NMDARs (Grant *et al.*, 1998). S1291 and S1312 have been identified as homologous sites for PKC phosphorylation on GluN2B, using oocytes (Liao *et al.*, 2001), while Y1292 and Y1387 in GluN2A have been identified as Src phosphorylation sites using HEK cells (Yang & Leonard, 2001). Although, a number of studies have established PKC-mediated modulation of NMDARS in neurons, the physiological relevance of the modulation in vivo remains largely unexplored. To address this, gene-targeted replacement was used to generate the *Grin2a* PKC mouse with specific serines and tyrosines mutated to unphosphorylatable alanines and phenylalanines (S1291A, Y1292F, S1312A, and Y1387F). Thus, the physiological significance of PKC-mediated phosphorylation of those specific sites was determined using different behavioral paradigms, immunostaining for the activity marker Fos, and electrophysiology. Specifically, the effects of those mutations were investigated using a variety of hippocampus-dependent behavioral tasks including measures of general activity, visual ability, spatial working memory, olfactory learning, novel object recognition, and anxiety-related behaviors exhibited in novel environments. Basal synaptic transmission and

TBS-induced synaptic plasticity were also examined in Schaffer-collateral (SC)-CA1 synapses in hippocampal slices obtained from WT and mutant mice.

2. Results

2.1 Generation and characterization of *PKC* mice

To study the effect of the PKC-mediated phosphorylation of S1291, Y1292, S1312 and Y1387 in GluN2A *in-vivo*, a gene-targeted replacement strategy using homologous recombination was used to insert mutated allele in a WT mouse genome. Although, a number of researchers who have used the selection markers have studied the resulting phenotype and physiology of the mutant mouse without deleting the Neo^r, there have been concerns that the PbGK-Neo cassette could influence the expression of several genes within a multi-gene locus (Pham et al., 1996). Thus, *PKC* mice were generated by breeding

PKC-Neo mice with EIIa-Cre mice and backcrossed with C57Bl6 mice [Fig. 1a]. The excision was successfully confirmed over four generations of pups using a PCR assay [data not shown]. Homozygous mutants were viable, and reproduced normally like their WT littermates. The mutant mice were also similar to their WT controls through all the developmental stages in body mass and brain weight [data not shown]. A heterozygous mating scheme provided littermate WT and homozygous male mice for all behavioral experiments. All animals were genotyped using a PCR assay followed by a restriction enzyme digest. The PCR product using the genomic DNA of WT and mutants as templates are 1590 bp, and the digestion of the PCR product containing the WT allele with a restriction enzyme results in a shorter product of 1328 bp [Fig. 1b]. Biochemical characterization of these mouse brains to determine the expression levels of Grin2a mRNA and protein levels of the GluN2A subunit in a whole brain was done using Northern and Western blotting respectively. There was no change in normalized Grin2a mRNA and GluN2A protein levels in the brain of mutant mice compared to their WT controls [Fig. 1c]. The intensities of bands on Northern and Western blots were quantified by densitometric analysis using Image Lab software. *Grin2a* mRNA levels were similar between WT and mutant mice ($t_4 = 0.42$, $p = 0.69$). There was also no significant difference in the quantities of protein expression levels of GluN2A subunits between the WT and *PKC* mouse brains ($t_4 = 1.52$, $p = 0.19$). No abnormalities in gross anatomical structures were observed in mutant mice brain slices stained using Cresyl-violet [Fig. 1d]. Overall, *PKC* mice could not be distinguished from the WT mice in appearance and gross brain morphology.

2.2 Behavioral characterization

Adult male *PKC* mice and their WT controls were used for all the behavioral experiments. The experiments and the analysis were done in a blind fashion to avoid bias towards a specific genotype, and both the groups of mice underwent a battery of behavioral tests. First, the general activity level of the mutant and WT mice was tested in a four-arm maze, Y maze, and an open field to ensure that the mutant mice have no motor deficits since any such deficits may influence the various parameters measured during all the behavioral tests. As shown in Fig. 2, *PKC* mice did not show any impairment in general exploratory activity compared to their littermate controls. The total arm entries made by both the groups of mice were similar in a four-arm maze ($t_{58} = 0.84$, $p = 0.40$), and in a Y maze ($t_{22} = 0.3$, $p = 0.76$). The

number of line crossing made by the two groups of mice ($n = 23$ in each group) during the entire test in an open field was not different ($t_{44} = 1.49$, $p = 0.16$). Both the groups of mice tested using the visual placement test had very similar placement scores (WT: 2.5 ± 0.1 ;

PKC: 2.6 ± 0.1 , $t_{37} = 0.82$, $p = 0.42$). Next, we tested the mice for any deficits in spatial working memory using a four-arm maze, Y maze and T maze. Spontaneous alternation in mazes has been used by a number of researchers as an indicator of exploratory activity and short-term spatial working memory. The performance of PKC mice was similar to the WT controls in the four-arm maze ($t_{58} = 0.51$, $p = 0.66$) [Figure 3a]. However, they show decreased alternation in a Y maze ($t_{22} = 2.22$, $p = .04$) [Figure 3b]. The alternation rate of

PKC mice was significant lower than WT mice in a delayed T maze alternation task. Irrespective of the inter-trial interval during the task, PKC mice alternated at significantly lower levels than the WT mice (ITI=15 s: $t_{58} = 10.12$, $p < .001$, ITI=60 s: $t_{28} = 3.87$, $p < 0.001$) [Fig. 3c]. There was no significant difference between the general activity levels (measured as total number of arms visited) while exploring mazes between the two groups of mice [data not shown]. Next, when the WT and mutant mice were subjected to anxiety-associated tasks, the mutants exhibited reduced anxiety-related behaviors including increased time spent in the center of an open field, increased time in light side of light/dark box, and increased dwell times in the open arms of an elevated plus maze [Fig. 4(a–c)]. Time spent by

PKC mice in the center of an open field was increased ($t_{44} = 3.4$, $p = .03$, $n = 23$ per group).

PKC mice spent significantly more time in the lit compartment of the light-dark box than their WT controls ($t_{12} = 3.9$, $p < .001$, $n = 23$ WT and 21 PKC mice). PKC mice show increased dwell times in the open arms of an elevated plus maze ($t_{48} = 3.31$, $p < .01$). The mutant mice also had increased number of open arm entries with no change in total arm entries compared to WT mice during the test [Fig. 4d–e)]. Finally, the performance of PKC mice in both the olfactory learning/retention test and the novel object recognition test was similar to WT mice. In the acquisition phase of the olfactory discrimination task, the number of trials to reach criteria, during each discrimination problem by both the groups of mice were similar. There was no main effect of genotype ($F_{1,110} = 0.48$, $p = 0.5$), but there was a main effect of odor pair ($F_{3,110} = 3.41$, $p < .05$; $n = 11, 17$) [Fig. 5a]. Furthermore, the interaction between genotype and odor pair was also not significant ($F_{3,110} = 1.3$, $p = 0.28$). Both WT and mutant mice retained the olfactory memory for the previously rewarded odor in the last discrimination problem, and the two groups did not differ in retention scores ($t_{26} = 0.71$, $p = 0.48$) [Fig. 5b]. PKC mice had similar recognition index compared to the WT controls when exposed to the novel object 10 minutes ($t_{26} = 0.36$, $p = .72$) and 24 hours ($t_{26} = 0.14$, $p = .76$; $n = 14$ each) after they were familiarized with an identical set of objects. Taken together, the data indicated that PKC mice may have an anxiolytic-like phenotype.

2.3 c-Fos immunostaining

c-Fos (Fos) an immediate early gene, has been used in a number of studies as a neuronal activation marker. Placement of a rat in a novel environment has been shown to elicit robust Fos expression in hippocampus, and in mice Fos protein levels peak within an hour after the placement in a novel environment (Wirtshafter and Sheppard, 2003; Montag-Salaz et al., 1998). PKC mice display reduced anxiety levels and have decreased Y/T maze alternation rate compared to their littermate controls. To detect if this behavioral phenotype correlates with Fos levels in WT and PKC mice hippocampus, the two groups of mice were exposed

to three different novel environments for 15 minutes each [Fig. 6a]. Exposure to the three novel environments for 15 minutes each, elicited a robust Fos expression in the Dentate Gyrus (DG), CA3 and CA1 regions of WT mouse hippocampus compared to its home-cage levels (WT-CA1: $F_{1,12}=7.54$, $p=.02$, WT-CA3: $F_{1,12}=6.10$, $p=.03$, WT-DG: $F_{1,12}=31.55$, $p<.001$) [Fig. 6b]. However, in mutant mouse hippocampus only the DG showed an increase in Fos expression upon placement in the three novel environments, while no change was observed in CA1 or CA3 (PKC -CA1: $F_{1,12}=0.05$, $p=.82$, PKC -CA3: $F_{1,12}=2.33$, $p=.15$, PKC -DG: $F_{1,12}=39.38$, $p<.001$) [Fig. 6b]. Interestingly, the magnitude of Fos increase in the DG of mutants was still significantly lower than the magnitude of Fos increase in DG of WT mice ($t_{14}=4.08$, $p<0.01$) [Fig. 6c]. Also a one-way ANOVA comparing the control Fos levels of WT and PKC , indicate that they were similar (Control-CA1: $F_{1,10}=4.52$, $p=.93$, Control-CA3: $F_{1,10}=2.15$, $p=.17$, Control-DG: $F_{1,10}=2.13$, $p=.18$). Thus, the lower Fos expression in the hippocampus of PKC mice was found to be consistent with the anxiolytic-like behavioral phenotype of the PKC mice.

2.4 Electrophysiology

Input-output curves were generated for one slice from each animal. Traces from representative WT and PKC mice are shown in Fig. 7a. The maximal evoked fEPSPs were not significantly different in WT and mutant mice ($t_{20}=0.93$, $p=0.35$) [Fig. 7b]. Paired-pulse curves were also generated for each of these slices. Representative traces from the same mice shown in Fig. 7a are displayed in Fig. 8c. No significant differences in the degree of facilitation were detected at inter-pulse intervals from 50 to 800 ms. Two-way, repeated measures analysis of variance yielded a significant main effect of IPI ($F_{4,80}=303.50$, $p<0.0001$) but no effect of genotype ($F_{1,20}=0.81$, $p=0.38$) or interaction ($F_{4,80}=0.38$, $p=0.82$) [Fig. 7d]. The results of LTP experiments are shown in Fig. 8. TBS induced an initial potentiation that decayed within 15–30 min to a level that remained stable for the rest of the recording session in slices from both WT and PKC mice [Fig. 8a and Fig. 8]. Measurements of the degree of potentiation of the initial slope of the fEPSPs were not significantly different for WT and mutant mice after either 5 or 10 theta bursts. Two-way analysis of variance yielded no significant effects of genotype ($F_{1,25}=0.11$, $p=0.74$), burst number ($F_{1,25}=2.48$, $p=0.13$), or interaction ($F_{1,25}=1.26$, $p=0.27$) [Fig. 8c]. The depolarization evoked during TBS was also not different. As shown in Fig. 8d, slices from both WT and PKC mice showed the same enhancement of burst responses 2–10, relative to the depolarization evoked by the first burst. The total area of negativity underlying the first burst was not different between WT and mutant mice (WT: 131.4 ± 8.7 mV-ms, $n=12$; PKC : 137.8 ± 10.9 mV-ms, $n=11$; $t_{21}=0.46$, $p=0.6$). Analysis of variance yielded a significant effect of burst number ($F_{8,168}=22.18$, $p<0.0001$) but no main effect of genotype ($F_{1,21}=1.21$, $p=0.25$). The interaction was significant ($F_{8,168}=2.07$, $p=0.04$) but none of the direct WT- PKC comparisons were significant.

3. Discussion

Phosphorylation of serines and tyrosines in the CTD of the receptor subunits introduces a negative charge in the protein, which may change the conformation of the receptor and also has been shown to be a critical factor regulating synaptic plasticity due to its time-course and

reversibility (Mammen et al., 1999; Chen and Roche, 2007; Choi et al., 2011). The GluN2A and GluN2B subunit of the NMDARs have serines that are phosphorylated by PKC and tyrosines that are indirectly phosphorylated by PKC via Src kinase (Chen et al., 2006; Liao et al., 2001, Lu et al., 1999). The present study using *PKC* mice, shows for the first time that PKC-mediated phosphorylation of at least one of these residues (S1291, Y1292, S1312, and Y1387) in the GluN2A subunit may be involved in regulation of anxiety-related behaviors.

The behavioral significance of the GluN2 subunits was evident in knockout and over-expression studies in mice. Over-expression of GluN2A in mouse forebrain has been shown to affect formation of long-term memories and not short term memories in various behavioral tests such as novel object recognition, spatial maze, and cued/contextual fear conditioning (Cui et al., 2012). The GluN2A knockout mice exhibit both reduced anxiety-like behavior and antidepressant-like behaviors in the tests similar to the ones performed on the *PKC* mice (Boyce-Rustay and Holmes, 2006). Mice expressing a truncated C-terminus in GluN2A show impaired LTP and cognition (Sprengel et al., 1998) and also decreased anxiety in stressful situations (Inta et al., 2013). The contribution of the CTD of GluN2 subunits was more evident when the CTDs were swapped in mice, where the authors proposed that differential interaction of the two subunits (GluN2A/N2B) with cytosolic proteins could contribute to different behaviors and also increased signaling complexity (Ryan et al., 2008; 2013). Specifically, the mice with CTD of GluN2B swapped with CTD of GluN2A showed reduced general activity, while the mice with CTD of GluN2A swapped with CTD of GluN2B showed reduced anxiety-like behavior, motor coordination and perceptual learning, establishing the subunit-specific CTD contribution to various behavioral phenotypes. Given that PKC modulates NMDARs by directly phosphorylating serine residues and indirectly phosphorylating tyrosine residues via Src kinase, it is important to study the behavioral consequence of mutating specific PKC-mediated phosphorylation sites on the GluN2A subunit. Hence, *PKC* mice with PKC-mediated phosphorylation site mutations (S1291A, Y1292F, S1312A, and Y1387F) were generated and characterized under various behavioral tests. These mice, like the GluN2A knockout mice also showed an anxiolytic-like phenotype in the appropriate behavioral tests. Interestingly, mice with a different tyrosine mutation on its GluN2A (Y1325F) show antidepressant-like behavior in forced swim test (Taniguchi et al., 2009). This behavior was not seen when the *PKC* mice were tested for depression-like behavior in the same test [data not shown]. Apart from the anxiolytic-like phenotype, the *PKC* mice also showed reduced spontaneous alternation in a Y maze and reduced delayed alternation in a T maze, although spontaneous alternation measured in a standard plus maze was normal. Although, a number of researchers have disputed the use of such mazes for detecting spatial working memory deficits, these mazes are more ethologically relevant than the Morris water-maze (Ehninger and Kempermann, 2006). Since the alternation of the mutant in a T Maze with an ITI of 60 seconds was higher than its alternation with an ITI of 10 seconds, it is also possible that the mutants were making more impulsive choices at lower ITIs. Also, the number of mutant mice that alternated correctly during every trial for both the ITIs was lower than the WT mice that made correct choices during the T maze alternation trials [data not shown]. Hence, increased impulsive choices made during the trials might result in reduced performance of the mice in

a T maze alternation task. Furthermore, in GluN2 subunits CTD-swap experiments, both the GluN2A and GluN2B subunits were shown to contribute to impulsive phenotype in mice (Ryan et al., 2013). However, analysis of traditional measures of anxiety and impulsivity in various mice strains showed that the parameters that predict anxiety-related behaviors had a low correlation with levels of impulsivity (Loos et al., 2009). There was also no impairment in short and long recognition memory of mutant mice in a novel-object recognition test. Finally, both the WT and *PKC* mice performed similarly in olfactory discrimination test. Prominently, *PKC* mice exhibited reduced anxiety. Thus, the present study using *PKC* mice may be the only study apart from the YF/YF mice from Yamamoto's group that reveals how phosphorylation of specific serines or tyrosines in GluN2A by various kinases could affect the behavior of mice (Taniguchi et al., 2009).

Fos, an immediate early gene (IEG), has been shown to be expressed in many regions of a rodent brain after exposure to various physiological conditions including exposure to a novel environment, traumatic brain injury, and inflammation (Herrera and Robertson, 2006; Kreiss et al., 2003; Wirtshafter, 2005). Fos expression in hippocampal subregions (DG, CA1, and CA3) has been studied to understand the hippocampal encoding of information from a novel context. Furthermore, calcium influx through both NMDARs and voltage-dependent calcium channels could cause an increase in intracellular calcium that result in enhanced Fos expression (Morgan et al., 1987). Fos expression levels in the WT mice exposed to novel environments are in accordance with a previously published studies that show a regional variation in Fos expression with the highest levels in DG followed by CA1 and CA3, although the paradigms used for Fos induction were different from the previously published studies (Barbosa et al., 2013; Satoko et al., 2013; Labrousse et al., 2012). The mutant mice exposed to novel environments showed Fos levels in DG, CA1, and CA3 that were significantly lower than the Fos levels measured in the same regions in WT mice exposed to the same novel environments. However, the mutant mice showed an increase in Fos levels in their DG compared to their home-cage levels, which might indicate that the cells in the DG may be more sensitive to the environment. Despite the increased Fos levels in the DG of mutants after exposure to novel environment, their increased Fos levels were still lower than the Fos levels in the DG of WT mice. Hence, the *PKC* mice show a decreased activation in the hippocampal areas known to be involved in anxiety-related pathways (Engin and Treit, 2007; Kheirbek et al., 2013; Zhang et al., 2014).

Even though Fos experiments indicated that there may be lower activation of neurons in CA3 and CA1, the basal synaptic transmission and LTP of the SC-CA1 synapses were found to be similar to WT mice in *PKC* mice. Previous work done in our lab showed that in oocytes injected with mutated Grin2a RNA (S1291A or S1312A), the current enhancement after PKC activation was reduced to about 60% compared to 120% in control WT Grin2a RNA. Hence, it was hypothesized that in hippocampus of the mice with those mutations, there might be altered synaptic transmission or synaptic plasticity due to the alanine that cannot be phosphorylated by PKC. However, since the mutated GluN2A subunit were present since E0 age of the mice, there might be some compensation due to other GluN2-containing or triheteromeric (GluN1/N2A/N2B) NMDARs trafficked onto the post-synaptic membrane of the hippocampus to maintain normal synaptic transmission, and current enhancement during the LTP-induction protocol (Tovar et al., 2013). There is also the

possibility that the mutated GluN2A containing NMDARs are trafficked onto the extra-synaptic region rather than the synaptic regions and hence may influence the opening of L-type Ca^{2+} channels contributing to LTP (Zhao et al., 2005). Thus, LTP induction in mature hippocampal synapses could be dependent on both GluN2A and GluN2B, wherein the GluN2A forms an ion channel which maybe modulated by PKC-mediated phosphorylation of specific amino acids, and the CTD of GluN2B subunit binds to other cytosolic proteins critical for LTP induction (such as PSD-95, β -catenin, and CRMP-2) (Al-Hallaq et al., 2007; Barria and Malinow, 2005; Foster et al., 2010; Mayadevi et al., 2002). The electrophysiology experiments done so far indicated that there were no alterations in LTP of hippocampal SC-CA1 synapses that were investigated using TBS protocol. Future whole-cell patch recordings and stimulations using a range of frequencies that could induce either potentiation or depression of hippocampal synapses may reveal if there were any other impairments in the SC-CA1 hippocampal synapses (or other synapses in the mutant brain). Hence, all those possibilities might explain the normal hippocampal synaptic electrophysiology observed in the *PKC* mice. In conclusion, PKC-mediated serine phosphorylation at S1291/S1312 or tyrosine phosphorylation at Y1312/Y1387 in the GluN2A subunit may play an important role modulating anxiety-related behaviors in an open field test, light dark box emergence test and elevated plus maze test, and performance in Y and T mazes. The mutants also show region-specific reduction in hippocampal Fos expression compared to WT mice after exposure to novel environments. Therefore, these mice could be used to design therapeutic interventions for anxiety-related disorders based on these specific phosphorylation sites.

4. Experimental Procedure

4.1 Animals

All experiments using animals were carried out in accordance with the guidelines for animal use issued by the Office of Animal Care and Institutional Biosafety at University of Illinois at Chicago, with group housing at maximum of 4 per cage. Heterozygous mice carrying alleles of *Grin2a* containing the phosphorylation mutations (S1291A, Y1292F, S1312A, and Y1387F) were bred to yield littermate controls (WT, *Grin2a* *PKC*) for all experiments. Adult male mice 3 to 6 months age were used for all experiments. All behavioral experiments were done between in light 9AM–12PM. The experiments were performed and analyzed by researchers blind to the genotype of the animal. All the apparatuses used for behavioral experiments were cleaned using 70% ethanol between individual mouse trials.

4.2 Targeting vector construction

Mutagenesis of the *Grin2a* phosphorylation sites was accomplished by using PCR to amplify a 1.8 kb fragment spanning exon 12 of the mouse *Grin2a* gene. This fragment was cloned into the pCR4Blunt-TOPO vector and used as a template for serial site-directed mutagenesis with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, CA, USA). The resulting plasmid pCR4Grin2a12-5'-AFAF contained the four desired amino acid substitutions: S1291A, Y1292F, S1312A, and Y1387F. For construction of the *Grin2a* targeting vector, DNA from bacterial artificial chromosome clones spanning the *Grin2a* gene was digested with *Sma*I, *Sna*BI, and *Bst*EII, and 8.1 kb and 1.8 kb fragments spanning the *Grin2a* locus

were isolated and cloned into pBKSII, generating plasmids pBSGrin2a12-5' and pBSGrin2a12-3'. These fragments represent the 5' and 3' arms of the Grin2a targeting vector, respectively. To replace the exon 12 region of the 5' targeting arm with the phosphorylation site mutant, plasmid pBSGrin2a12-5' was digested with EcoRV, releasing a 1.6 kb segment of exon 12. The mutant exon 12 was similarly released from the plasmid pCR4Grin2a12-5'-AFAF, and cloned into pBSGrin2a12-5', creating plasmid pBSGrin2a12-5'-AFAF. The 5' arm was then released from pBSGrin2a12-5'-AFAF with EcoRI and BamHI, and blunt-ended into the XhoI site of the targeting vector ploxPNT. Similarly, the 3' arm insert was released from pBSGrin2a12-3' with EcoRI and BamHI and blunt-ended into the KpnI site of ploxPNT, creating the targeting vector Grin2aKINR. The targeting vector therefore carries the 8.1 kb 5' arm containing exon 12 upstream of the *Neo* gene, and the 1.8 kb 3' arm downstream of the *Neo* gene. Accuracy and orientation of the cloned fragments was verified by sequencing across all cloning junctions.

4.3 Generation of *Grin2a* PKC mice

The targeting vector Grin2aKINR containing the substitutions (S1991A, Y1292F, S1312A and Y1472F) along with a *Neo^r* was designed to replace the exon 12 of *Grin2a* gene by homologous recombination [Fig. 1a]. The targeting vector Grin2aKINR also had a loxP sites flanking the *Neo^r* cassette. The Grin2aKINR vector was linearized with NotI, electroporated into J1 embryonic stem cells and 500 *Neo*-resistant ES cell colonies were selected and expanded. PCR genotyping employed a primer located within the targeting construct, and one in the *Grin2a* locus outside the targeted area. Twenty clones were found to have undergone homologous recombination, and the mutated region was verified by sequencing select clones. Three of the targeted clones were micro-injected into blastocysts from C57BL/6 mice, in collaboration with the University of Illinois at Chicago Transgenic Production Service. These blastocysts were returned to outbred surrogate mothers, yielding 14 chimeras that were up to 90% ES cell-derived. The best chimeras were crossed to C57BL/6 mice to obtain germline transmission of the targeted allele. Heterozygous animals were crossed over at least five generations to generate homozygous mice for the mutant *Grin2a* allele. These mutants were called *Grin2a* PKC-*Neo* mice (shortened as PKC-*Neo*). The *Neo^r* cassette was excised by crossing the *Grin2a* PKC-*Neo* with EIIa-Cre mice, and the F1 generation was then backcrossed to C57Bl6/6J strain (Lakso et al., 1996). The mutant homozygous mice strain was denoted as *Grin2a* PKC (shortened as PKC). The complete excision of the *Neo^r* cassette in heterozygotes was verified using a PCR assay.

4.4 Northern and Western blots

In order to determine the mRNA and protein levels of the NMDA receptor subunit type 2A, WT and PKC mice were deeply anesthetized using a mixture of 100 mg/kg of Ketamine and 16 mg/kg of Xylazine and their brains were rapidly dissected. mRNA levels of *Grin2a* were determined from RNA isolated from the whole brains of WT and PKC mice using probes labeled using the North2South Biotin Random Prime Labeling Kit (Life Technologies, CA, USA). The hybridized probes were detected using a Streptavidin-HRP solution, and were subsequently developed using a Luminol enhancer solution. Whole brains dissected from anesthetized mice were lysed using RIPA buffer (Sigma, MO, USA). Protein concentration in the lysate was determined using Bradford assay (Bio-Rad, CA, USA).

Protein levels were determined using primary antibodies against GluN2A, GAPDH, and HRP conjugate Goat anti-rabbit secondary antibody (EMD-Millipore, MA, USA) and the blots were developed using a chemiluminescent detection system (Clarity WB ECL, Bio-Rad) on a Bio-rad ChemiDocMP system. All blots were quantified using Image Lab software for ChemiDoc (Bio-Rad). Each animals' mRNA and protein intensity levels were normalized to its respective GAPDH intensity, and the values were averaged among three animals in both WT and *PKC* mice.

4.5 Behavioral experiments

4.5.1 General activity and visual test—Parameters from three different behavioral experiments (four arm-maze, Y maze, and open field) were used to determine the general activity levels of the mutant mice, to ensure that motor deficits do not influence the various parameters measured during all the behavioral tests. First, in a four-arm maze and Y maze the total arm entries made by the mouse were measured for 10 minutes. Next, the number of whole-body line crosses made by the mouse during the entire test duration of 10 minutes in an open field ($72.5 \times 72.5 \times 30.5$ cm) was measured as an indicator of general locomotor activity. To determine if the mice used for various behavioral and electrophysiological experiments were impaired in vision, they were tested using the visual placement test paradigm. In this test, the mouse was held using its tail, and was gradually lowered on to the wired-cage top from a height of 15 cm. The mouse was observed and scored on a 0–3 point scale based on its forelimb extension (0 - no forelimb extension; 1 - forelimb extension upon nose contact; 2 - forelimb extension upon vibrissae contact; 3 - vigorous forelimb extension).

4.5.2 Spontaneous alternation in four-arm and Y maze—Spontaneous alternation behavior was observed in the mice using a four-arm maze and a Y maze (Hughes, 2004). The arms of the maze, made of black plexiglass were 30.5 cm in length. The walls of the arm were 7.5 cm. The arms of the maze were arranged to form either a plus or a Y shape where each animal was allowed free access to explore the maze for 10 minutes and the arm entries were recorded by the experimenter. A set of unique entries into each of the four arms during a four-choice sequence in a four-arm maze or unique set of entries in a three-choice sequence in a Y maze was termed as an alternation. For example in a four-arm maze ADCB, BACD, CDAB, DBAC were considered as alternations while ADAB, BACA, CDBD, and DBAB were not considered as alternations (Lennartz, 2008). The performance of the mice in the mazes was expressed as a percentage of number of alternations made by the mice in 10 minutes to the maximum number of possible alternations. The number of arm entries made by WT and *PKC* mice while exploring the maze in 10 minutes was used to assess their activity levels.

4.5.3 Non-reinforced delayed alternation—In a non-reinforced T maze delayed alternation task, the mouse was placed at the stem of the T-shaped maze which acts as the start arm. The stem of the T maze was 69 cm, and the walls were 7.5 cm. The left and the right arms were 30.5 cm long. The entire test consisted of one forced choice trial and 14 free choice trials with an inter trial interval (ITI) of 15 or 60 seconds (Gerlai, 1998). After exploration of one arm, the mouse was returned to the start arm and was confined for 15 or

60 seconds in the holding area before it was allowed another free choice. The arm choice and the time taken to explore the arm were recorded during the experiment and the performance was measured as the percentage of alternations between the two arms.

4.5.4 Anxiety-related behaviors—All the anxiety-related behavioral experiments were based on natural aversion of mice to large areas, light, and open elevated places. Anxiety-related behaviors of mice were observed using open field (Prut and Belzung, 2003), light dark box emergence (Bourin and Hascoët, 2003), and elevated plus maze tests (Walf and Frye, 2007). The open field (OF) apparatus was constructed of a rectangular, clear plexiglass material ($72.5 \times 72.5 \times 30.5$ cm) comprising of four walls and an open roof. The box was divided into 9 smaller rectangles each measuring about 24×10 cm. During an open field test, the mouse was placed in a clear rectangular box and its activity was recorded for 10 minutes. This experiment was based on the fact that mice exhibit thigmotaxis, the predisposition of a mouse to explore the area closer to the walls of an open field. Reduced anxiety levels were indicated by increased dwell times in the center ($24 \text{ cm} \times 10 \text{ cm}$ square compartment marked below clear chamber) of the open field. The light dark box emergence test assesses the behavior of rodents in bright light that normally increases anxiety levels. The entire setup consists of large rectangular area ($75 \text{ cm} \times 50 \text{ cm} \times 30 \text{ cm}$), which was separated into two compartments - a relatively darker area that makes up a third of the box area and the remaining area that was illuminated using a 60 W incandescent bulb at 30 cm height to produce a brighter lit area. A small ($7 \text{ cm} \times 7 \text{ cm}$) opening on the wall separating the two compartments allowed the mice to freely move between lit and dark areas. The activity of mice was recorded for 10 minutes. Increased dwell times in the lit compartment indicated lower anxiety levels in mice. An elevated plus maze made of two open arms (arms without any walls) and two enclosed arms (arms with 15 cm high walls on both sides), was used to determine the behavioral response of individual mice to elevation and open area. The arms measured $30 \text{ cm} \times 5 \text{ cm}$, and the entire maze was elevated from the ground by 40 centimeters. The mouse was placed in the center of the maze and allowed to explore for 5 minutes. Increased time spent in open arms and increased entries into open arms indicate reduced anxiety-like behaviors.

4.5.5 Learning and memory

4.5.5.1 Olfactory discrimination learning: Both the WT and PKC mice were water-deprived to reduce their body weight by about 20% over five days with access to 1 ml water per day. This schedule was also used to stably maintain their reduced body weight (target weight) throughout the training. After all the animals reached their target weight, they were trained in an enclosed testing chamber made of black acrylic that consisted of an alley measuring $60 \text{ cm} \times 10 \text{ cm}$ as described previously (Larson et al., 2008). Briefly, a shaping procedure was used at the end of which a mouse was familiarized to the apparatus and also to reinforce nose-poke responses in either sniff-port. The nose-poke training consisted of two 20-trial sessions per day, in which the mouse was reinforced with a drop of water ($12.5 \mu\text{l}$) for a nose poke in either sniff port at one end of the alley. A single trial lasted 120 s with an ITI of 10 s for correct response (nose-poke) or an ITI of 30 s for an incorrect response (no nose-poke). The mouse was trained until it has made 90% reinforced response consecutively over three days. After the nose-poke shaping, each mouse was trained on a series of four

different two-odor discrimination problems. Each mouse was given a single session of 100 training trials per day. The performance criteria for successful completion of an odor discrimination problem were to obtain eight consecutively correct responses. When a mouse completes one problem, training on the next problem commenced with the following session. After each mouse reached criterion performance on the fourth (final) discrimination, and its memory retention was tested after 14 days. The retention test was composed of five probe trials, each of which was identical to a training trial in the final discrimination with no reinforcement. The ITI was 10 sec. Only the response in the first trial was used to determine the olfactory memory retention.

4.5.5.2 Recognition memory - Novel object recognition: Individual mice were habituated to the testing box (made of clear plexiglass material) for two consecutive days prior to testing by placing them in the test box for 10 minutes. On the testing day, each mouse was placed in the cage containing two identical objects and allowed to explore for 10 minutes (training phase). One of the objects was replaced with a novel (new) object and the mouse was allowed to explore for 5 minutes (testing phase). The two objects were similar size, but were different in texture, shape and color. The time between the object switch was 10 minutes for assessing short-term memory and 24 hours for long-term memory. The time spent by the animal interacting with the object such as sniffing, touching with paws and any activity done facing the object within 2–3 cm was analyzed. Thus, the novel object discrimination index was calculated as the ratio or percentage of time spent interacting with the novel object to total time spent interacting with both the familiar and novel object (Leger et al., 2013).

4.6 Behavioral induction of c-Fos expression

Fos levels in home-cage and after exposure to three novel environments (new cage in a different room, open field and an elevated plus maze) for 15 minutes each, were determined using immunostaining. Based on previously published protocols an hour after the mice were exposed to three different novel environments to induce robust Fos expression, the experimental animals were perfused transcardially using phosphate buffered saline (PBS) and 4% formaldehyde fixative (Wirtshafter, 2005). Brains were postfixed for one hour at 4°C in a high pH fixative solution (pH 9.5) and then transferred to 20% sucrose in PBS until slicing. Serial 35 µm slices were prepared from frozen slices and were stored in a cryopreservative until they were processed for Fos immunoreactivity using immunostaining methods described elsewhere (Wirtshafter and Sheppard, 2003; Wirtshafter, 2005). Fos immunoreactivity was measured after the tissues were processed using Vectastain Elite ABC Kit (Vector Labs, CA, USA). The tissues were then rinsed and stained using diaminobenzidine with nickel to obtain a contrasting image. After a final rinse, the slices were mounted in subbed slides and cover-slips were placed on them. Fos immunoreactivity was quantified as Fos(+) cells per 100 µm of the hippocampal regions CA1, CA3 and DG as they are relatively linear structures. Additionally, a few serial 40 µm slices were processed for cresyl-violet staining as described elsewhere (Paul et al., 2008).

4.7 Electrophysiology

WT and *PKC* mice were sacrificed at 3–4 months of age for preparation of hippocampal slices using standard methods (Larson et al., 1999; Hu et al., 2010). Both the preparation of slices and electrophysiological experiments were performed blind with respect to genotype of the mice. Slices were maintained at $35 \pm 1^\circ\text{C}$ in an interface chamber constantly perfused (1.0 ml/min) with medium containing 124 mM NaCl, 3 mM KCl, 1.2 mM KH_2PO_4 , 26 mM NaHCO_3 , 10 mM D-glucose, 2.5 mM CaCl_2 , 2.5 mM MgSO_4 , and 2 mM Na-ascorbate, gassed with 95% O_2 /5% CO_2 . The upper surfaces of slices were exposed to an atmosphere of 95% O_2 /5% CO_2 . Schaffer/commissural synapses were stimulated with twisted, bipolar electrodes placed in stratum radiatum (SR) of field CA1a or CA1c; field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass micropipette placed in SR of CA1b. Basal synaptic transmission was assessed by constructing input-output curves using stimulation currents ranging from 1.6 to 160 μA . Paired-pulse facilitation was assessed with inter-pulse intervals from 50 to 800 ms. LTP was induced by theta burst stimulation (TBS) consisting of five or ten high frequency bursts (100 Hz, 4 pulses) repeated at 200 ms intervals (5 Hz). fEPSP slope was monitored at 20 s intervals for at least ten minutes before and 60 minutes after TBS. LTP was assessed as the potentiation present 30 and 60 min after TBS.

4.8 Data analysis and statistics

All data from behavioral experiments were analyzed using QuickCalcs (an online GraphPad Software for statistical analysis) and Microsoft Excel, and compared using one-way ANOVA. For Fos immunostaining experiments, the novel environment-induced Fos levels in WT and *PKC* hippocampal regions were compared to home cage Fos levels in WT and *PKC* hippocampal regions respectively. All data represented in histograms are mean $\pm$ SEM.

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Highlights

- Mice with GluN2A receptors deficient in PKC phosphorylation were constructed.
- These *PKC* mice show reduced anxiety-like behavior in three types of tests.
- *PKC* mice abolished Fos signal in CA1 and CA3 after exposure to novel environments.
- *PKC* mice show reduced spontaneous alternation behavior.
- Field CA1 basal synaptic transmission, and TBS-induced LTP is normal in *PKC* mice.

Fig. 1.

General characterization of *PKC* mice. (a) A target vector with a *Neo^r* replaced the exon 12 of WT *Grin2a* gene by homologous recombination. *PKC-Neo* mice were crossed with EIIa-Cre mice to generate *PKC* mice. (b) 10 μ l of either PCR products or Restriction enzyme *RseI*(RE) digested PCR products obtained from mice tail snips DNA run on an agarose gel was used to determine the genotype of every mouse. (c) Representative Northern (top) and Western blot (bottom) showed similar mRNA and protein levels of the NMDAR subunit 2A in WT and *PKC* mice. (d) Cresyl-violet staining of the brain slices revealed that there was no difference in anatomical structures between WT and *PKC* mouse brains.

Fig. 2.

Similar activity levels of *PKC* and WT mice in four-arm maze, Y maze and Open field. General activity levels of the WT and *PKC* mice in (a) Four-arm maze ($n = 30$ in each group) and (b) Y maze ($n = 12$ in each group) were determined during the spontaneous alternation task. The total arm entries made by both the groups of mice in a 10 min trial were similar in a four-arm maze, and in a Y maze. (c) The number of line crossing made by the two groups of mice ($n = 23$ in each group) during the entire 5 min test in an open field was not different.

Fig. 3.

Decreased alternation of *PKC* mice in Y and T maze but not in four-arm maze.

Spontaneous alternation behavior (SAB) in a four-arm maze ($n = 30$ per group), Y maze ($n = 12$ per group), and delayed alternation behavior in a T maze ($n = 30$ in each group for 15 s ITI, $n = 15$ per group for 60 s ITI) was observed in both the groups of mice. (a) There was no difference in the spontaneous alternation levels of the two groups of mice in four-arm maze during the test duration of 10 minutes. (b) *PKC* mice alternated at lower levels than the WT mice in a Y maze. (c) The alternation rate of *PKC* mice was significant lower than WT mice in a delayed T maze alternation task. Irrespective of the inter-trial interval during the task, *PKC* mice alternated at significantly lower levels than the WT mice. The horizontal gray lines on the graphs represent alternation at chance levels in four-arm maze, Y maze, and T maze. *** $p < 0.001$, * $p < 0.05$

Fig. 4.

Reduced anxiety-like behaviors exhibited by *PKC* mice in an Open field, Light-dark box, and Elevated plus maze. (a) Time spent by *PKC* mice in the center of an open field was increased. (b) *PKC* mice spent significantly more time in the lit compartment of the light-dark box than their WT controls. (c) *PKC* mice show increased dwell times in the open arms of an elevated plus maze. (d, e) *PKC* mice show increased entries into open arms. However, the total arm entries made by both the groups were similar. *** $p < .001$, ** $p < .01$, * $p < .05$.

Fig. 5.

Normal olfactory discrimination learning and object recognition memory exhibited by

PKC mice in a Novel object recognition test and Olfactory discrimination learning task. (a) The number of trials to reach criteria (eight sequential correct choices) in a maximum of 100 trials per session during the acquisition trials was similar in WT and *PKC* for all the four odor discrimination problems. (b) The retention levels for the odor pair that the WT and *PKC* mice were trained to acquire on the last day of their odor discrimination acquisition trials (when they reached criteria) were also similar. (c) *PKC* mice also had a similar novel object recognition index compared to the WT controls when they were exposed to the novel object 10 minutes and 24 hours after they were familiarized with an identical set of objects.

Fig. 6. Differential Fos expression in hippocampal region of ΔPKC mouse brains after novel environment exposure. (a) Timeline of Fos induction and immunostaining experiment. (b) Representative images showing control Fos levels in CA1 region of WT and ΔPKC hippocampus from home-cage animals (WT-C and ΔPKC -C) and exposure induced Fos expression in experimental animals (WT-E and ΔPKC -E). $n = 6$ per group (home-cage), and $n = 8$ per group (novel environment), *** $p < .001$, * $p < .05$. (c) Control Fos level in brains of both the groups of mice in their home-cages was not significantly different in DG, CA1, and CA3. All the hippocampal regions of WT mice exposed to the novel environments (WT-E group) had a significant increase in Fos levels compared to its home-cage levels. However, the ΔPKC -E group had an increased Fos only in the DG, and not in CA1 or CA3, compared to its home-cage levels.

Fig. 7.

Basal synaptic transmission and paired-pulse facilitation do not differ in hippocampal field CA1 of WT and ΔPKC mice. (a) fEPSPs evoked by stimulus intensity ranging from 1.6–16 mA (WT) and 2.5–40 (ΔPKC) in typical slices (each trace is an average of four consecutive responses). Calibration bar: 2 mV, 10 ms. (b) Maximal fEPSP amplitudes in WT and ΔPKC slices were did not differ. (c) Recordings of first and second responses evoked in WT and ΔPKC mice at an inter-pulse interval (IPI) of 50 ms. Calibration bar: 1 mV, 10 ms. (d) Degree of facilitation of the second response relative to the first at intervals ranging from 50 to 800 ms in all WT and ΔPKC mice.

Fig. 8.

LTP induced by TBS is not different in hippocampal field CA1 of WT and ΔPKC mice. (a, b) Representative experiments showing LTP induced by 10 theta bursts. Each graph shows the fEPSP slope (each point is an average of four consecutive responses) throughout the experiment, expressed as a percentage of the average value during the pre-TBS baseline. TBS induced synaptic potentiation that stabilized in 15–30 minutes in both WT and ΔPKC mice. Traces show records obtained immediately before and 60 minutes after TBS in the same experiments (each trace is an average of four consecutive responses). Calibration bar: 1 mV, 10 ms. (c) The average degree of fEPSP potentiation measured 60 minutes after either 5 or 10 theta bursts were not different in WT and ΔPKC mice. (d) Waveforms evoked during TBS in WT and ΔPKC mice. Traces show the responses to the first and second burst superimposed (the second is larger in both cases). Graph shows the enhancement of burst 2–10 response area relative to the area evoked by the first burst. None of the direct WT- ΔPKC comparisons were significant. Calibration bar: 2 mV, 20 ms.