Slow in Motion but Smart in Learning and Memory: Behavioral Changes in Adult NR3A Knockout Mice

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Summary

The expression of NMDA receptor subunit NR3A is high in the neonatal brain but low in adults. However, its functional role in the adult brain is obscure. Using wild-type (WT) and NR3A knockout (KO) mice, we show here that NR3A plays imperative roles in multiple behavioral functions in adults. NR3A deletion produced a slow locomotor phenotype with enhanced memory capacities. Hippocampal slices from juvenile and adult NR3A KO mice showed greater long-term potentiation (LTP) compared to WT slices. NR3A deletion resulted in increased expression and phosphorylation of calmodulin-dependent kinase II (CaMKII). CaMKII inhibition abrogated the enhanced LTP in NR3A KO slices. NR3A KO mice were also more sensitive to acute and chronic pain. These data reveal for the first time that NR3A, despite its low expression, plays several critical roles in behavioral activities in adults and may be a therapeutic target for modulating behaviors under normal and pathological conditions.

Key words: NMDA receptors; AMPA receptors; LTP; NR3A subunit; knockout mice; hippocampus; learning and memory; locomotion; pain; CaMKII

NMDA receptors (NMDARs) are key components in neuronal development and functional activities in the nervous system including membrane excitability, synaptic plasticity, pain sensation and neuronal excitotoxicity [1]. NMDARs are hetero-multimers that are composed of at least one NR1 plus one or more NR2A-D subunits [2]. NR2 subunits, especially NR2B, play important roles in the induction and maintenance of long-term potentiation (LTP) and long-term depression (LTD) that are likely the underlying mechanisms of learning and memory [3]. The recently discovered and characterized NR3A (NMDAR-L or χ-1) and NR3B subunits are unique inhibitory subunits of NMDARs. NR3A is widely expressed in different brain regions such as the cortex, hippocampus, thalamus, hypothalamus, brainstem, and spinal cord [4]. Unlike NR2B whose expression gradually decreases after birth but remains highly expressed in adulthood [5], NR3A mRNA and protein levels peak at the end of the first postnatal week but decrease from P12 to very low levels (~15% of NR1) by adulthood [6]. This sharp reduction in NR3A expression seems to support the idea that NR3A plays a critical role in development but its function in adulthood could be negligible.

NR3A co-localizes with other subunits to form functional channel complexes [7]. Co-expression of NR3A with NR1 and NR2 reduces NMDAR-mediated Ca^{2+} influx, whole-cell currents and results in smaller unitary conductance of NMDAR channels. The smaller conductance is also seen in cortical neurons isolated from wild-type P8 rodents when compared to age-matched neurons from NR3A knockout (KO) mice [8]. These small NMDAR

inward currents are most likely due to the relatively reduced Ca^{2+} permeability and reduced Mg^{2+} sensitivity in the NR3A containing NMDARs [9]. Mice lacking NR3A have increased dendritic spine densities in cortical neurons. This difference is more pronounced in cortical neurons of P19 mice compared to the adults [10]. Supporting a role in early brain development, the elimination of NR3A from active synapses in the first two weeks in rodents is required to increase NMDAR conductance and to form stronger and larger synapses. At the behavioral level, NR3A down-regulation is required to form long-term memories [11]. Thus, NR3A down-regulation appears critical for synapse maturation and memory formation during development.

A previous investigation showed that enhanced LTP was only observed in hippocampal slices from P6-P8 NR3A KO mice but not in juvenile or adult slices [11]. These observations have enforced the general assumption that NR3A is not a major player in NMDAR-dependent activities in adult brains. Opposite to previous observations and assumptions, here we demonstrate for the first time that markedly significant differences in locomotor functions, learning and memory, and pain sensation can be detected between adult WT and NR3A KO mice. These findings indicate that the NR3A subunit, despite its relatively low expression, has a surprising imperative functional role in adult animals and might be a target for therapeutic interventions.

Results:

Glutamate receptor subunits and LTP regulators in the adult NR3A KO brain

Adult NR3A KO mice (3 - 4 months old) have normal body/brain weights and reproductive activity (Fig. 1a). They showed no increased seizures or convulsions. The expression of NR3A in the brain of WT mice increased from postnatal day 1 to day 10, reached a maximal level between day 10 and 20 and then significantly dropped in adulthood (Supplemental Fig. 1a) [12]. As a necessary control, we evaluated the expression levels of major ionotropic glutamate receptor subunits in NR3A KO mice. The expression of the AMPA receptor subunit GluR1 and NMDAR subunit NR3B was similar in the adult brains of WT and NR3A KO mice (Fig. 1b and 1c). Expression of GluR1 was low in postnatal day 10 and increased significantly in the adult brain for both WT and NR3A KO mice (Supplemental Fig. 1b). Moreover, there was no difference in the expression of other NMDAR subunits (NR1, NR2A and NR2B) in post-synaptic densities (PSD-2Ts) of adult WT and KO forebrains (Fig. 1d).

Western blot analysis of forebrain tissue lysates showed similar expression levels of LTP regulators including Akt, cAMP response element-binding (CREB), protein kinase A (PKA) and protein kinase C (PKC) (Fig. 1e and 1f). Moreover, there was no difference in the expression of phosphorylated Akt (p-Akt) and phosphorylated CREB (p-CREB) (Supplemental Fig. 1c).

(Figure 1 near here)

Impaired or slow locomotor activity and normal stress levels in adult NR3A KO mice

The role of NR3A in locomotion is completely unknown. In order to test for balance, coordination and motor learning abilities, animals were trained everyday for 5 days on the Rota-rod test. During the first few days, adult WT mice improved their performance better (stayed longer on the rotating beam) than NR3A KO mice. By day 5, however, NR3A KO mice caught up with their WT counterparts. The animals were then rested for a week before being tested again at days 12 and 16. The performance of KO mice declined at day 12, but improved again at day 16 (Fig. 2a). Similar results from the Rota-Rod test were also seen with juvenile (P20) mice (data not shown). These initial data indicated that while adult NR3A KO mice had locomotor difficulties compared to the WT controls, their motor learning was intact and their locomotor deficits could be compensated by training.

Consistent with the Rota-rod test, in a 5-day Morris water maze test adult NR3A KO mice swam slower than WT mice after day 1 during which WT and KO mice performed similarly in the Rota-rod and swimming tests (Fig. 2b). The average swimming speed of the KO mice during day 2 through 5 was significantly slower than that of WT mice (219 ± 6.7 and 193 ± 5.4 mm/sec for WT and KO mice, respectively, n = 10, $P<0.05$). This slow movement phenotype of the NR3A KO mice was verified in the open field test. Adult NR3A KO mice walked slower and traveled less distance than WT mice (Fig. 2c and 2d; Supplemental videos 1 and 2). They had fewer entries into the central zone and

fewer vertical rears (Fig. 2e and 2f). Moreover, NR3A KO mice took longer time to exit the central zone where they were initially placed indicating normal stress levels and reduced locomotor abilities. The total time NR3A KO mice spent in the central zone was likewise similar to WT mice during the 5-min open-field test (Fig. 2g and 2h). All these behaviors were consistent with a slow locomotion phenotype.

Since the above behaviors might be affected by anxiety or stress, defensive-withdrawal test was performed to specifically evaluate the anxiety level of these animals [13]. NR3A KO mice showed no difference in the time to leave the sheltered chamber and the time to cross into the central zone compared to WT mice (Fig. 2i and 2j). These data indicated that, although NR3A KO mice moved less and slower, these mice were not more stressed than their WT counterparts.

(Figure 2 near here)

Enhanced object and spatial memory in adult NR3A KO mice

Since learning and memory are NMDAR dependent, adult WT and NR3A KO mice were tested in the novel-object recognition and Morris water maze tests for assessing visual and spatial memory, respectively [14, 15]. Both types of memory are NMDAR and hippocampus dependant. In the novel-object recognition test which require acquisition, storage, and recall of features of familiar objects, WT and NR3A KO mice showed similar preference to the

"familiar" objects during the training session (Fig. 3a), indicating that these two strains had similar curiosity and motivation. During the retention tests where one of the objects was replaced with a novel object, both WT and NR3A KO mice showed more preference for the novel object 1 hr after training. NR3A KO mice, however, showed even more preference for novelty compared to the WT controls. Different animal groups were tested with the novel object 1 or 7 days after training, respectively. WT mice lost preference for novelty in these tests while NR3A KO mice still showed preference for the novel object even 7 days after training (Fig. 3a), indicating better recognition memory compared to WT mice.

In the Morris water maze test [15] in which animals use spatial cues to swim towards an escape platform, adult WT and NR3A KO mice were trained to find the submerged platform four times a day for five days (Supplemental videos 3 and 4). On day 1, NR3A KO mice needed more time to get to the platform (Fig. 3b) likely due to their locomotor deficit. The latency and distance traveled to escape to the platform decreased in both WT and NR3A KO mice during the 5-day training (Fig. 3b and 3c). NR3A KO mice not only caught up with the WT counterparts, they performed significantly better than WT controls during the last three training days (27.7 ± 3.0 and 19.0 ± 2.2 sec for WT and KO mice, respectively; n=10, P=0.022; Fig. 3d). When animals were tested after removal of the platform on day 6, NR3A KO mice spent significantly more time in the target quadrant than WT controls (41.9% ± 5.0 and 57.3% ± 4.6 for WT and KO mice, respectively; n=10, P=0.03; Fig. 3e), indicating better spatial memory of the KO mice.

(Figure 3 near here)

Enhanced long-term potentiation in the juvenile NR3A KO brain

LTP is a cellular and molecular event that is believed to be the electrophysiological basis of synaptic plasticity associated with learning and memory [3]. NMDA receptors are major regulators in the induction and maintenance of LTP [16]. Long-term potentiation (LTP) was examined in the CA1 region of hippocampal slices from juvenile (postnatal day 10 and 20) WT and NR3A KO mice. The field excitatory post-synaptic potentials (fEPSPs) were recorded before and after a single episode of high-frequency stimulation (HFS, 100 Hz, 1 sec) at the Schaffer collaterals. The average magnitude of HFS-induced LTP was similar between P10 WT and NR3A KO mice (Supplemental Fig. 2a) and between P20 WT and NR3A KO animals (Supplemental Fig. 2b). Western blot examination revealed that AMPAR expression in the developing brain is much lower than in adults (Supplemental Fig. 1b) [17]. Since LTP induction relies on sufficient activation of AMPARs that removes the voltage-dependent Mg^{2+} block on NMDARs [18] and since NMDARs without NR3A are more sensitive to the Mg^{2+} block than NR3A containing NMDARs [19], we hypothesized that LTP generation in P10 mice might be greatly affected by the nature of HFS. In other words, a single HFS could not induce sufficient membrane depolarization as it can do in adult brains. We further hypothesized that sufficient postsynaptic depolarization could reveal enhanced LTP capability in P10 and P20 NR3A KO slices.

To test our hypothesis in young animals, LTP was tested using a low Mg^{2+} external solution (0.2 mM Mg^{2+} vs. 1.0 mM Mg^{2+}) in P10 and P20 brain slices. In the low Mg^{2+} solution, fEPSPs were gradually potentiated in P10 and P20 slices from both WT and KO animals. However, the increases were greater in NR3A KO slices (Fig. 4a and 4b). Therefore, under a low Mg^{2+} condition that compensated for insufficient AMPAR expression/activation, NR3A showed a key regulatory role in synaptic plasticity of the developing brain.

Enhanced long-term potentiation in the adult NR3A KO brain

In adult brain slices from 3 - 4 months old mice, the role of NR3A in LTP regulation was studied using normal Mg^{2+} concentration (1 mM) since the normal level of AMPARs exists in the adult brain (Supplemental Fig. 1b) and HFS should induce sufficient post-synaptic depolarization. As expected, fEPSP recordings after HFS (100 Hz, 1 sec) showed significantly greater LTP in adult NR3A KO slices (Fig. 4c). We then measured paired-pulse facilitation (PPF) which is a specific indicator of pre-synaptic plasticity. Paired-pulse facilitation (PPF) induced by various inter-stimulus intervals (ranging between 10 to 500 ms) was similar between WT and NR3A KO hippocampal slices (Fig. 4d), indicating that the pre-synaptic function of NR3A KO neurons was not different from that of WT neurons. As a control, we checked whether the enhanced LTP in the adult KO mice was dependent on post-synaptic NMDA receptor activation. The NMDAR antagonist D(-)2-amino-5-phosphonovalerate (AP-5, 100 μM) was bath applied 20 min prior to HFS. LTP induction was almost fully blocked in both WT and NR3A KO slices

(Fig. 4e). On the other hand, LTP induction was not affected when AP-5 was applied after HFS (Supplemental Fig. 2c).

Long-term depression (LTD) is another form of synaptic plasticity. We observed, as others reported [20], that low frequency stimulation (LFS, 1 Hz, 900 ms) could not induce LTD in hippocampal slices of adult mice (data not shown) of either the WT or NR3A KO brain. Alternatively, we tested a chemical induction protocol to induce LTD in the CA1 region of the hippocampus [21]. A brief (4 min) bath application of NMDA (30 μM) transiently abolished the evoked field responses followed by stable responses that were depressed below the baseline (Fig. 4f). The magnitude of LTD in KO slices was not different from that of the WT controls.

(Figure 4 near here)

Enhanced CaMKII expression in the adult NR3A KO mice

The NMDA-dependent LTP induction requires NMDA-mediated Ca^{2+} influx and rapid rise in intracellular Ca^{2+} concentration, which serves to activate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) that acts as a major mediator in the induction of LTP [22, 23]. We, thus, sought to detect the expression of CaMKII in the brains of adult NR3A KO mice. The protein expression of CaMKII and phosphorylated CaMKII (p-CaMKII) in the cortical forebrain (Fig. 5a) and hippocampus (Fig. 5b) of adult NR3A KO mice was significantly higher than that in WT mice. We then induced HFS in hippocampal slices and detected the

expression level of CaMKII and p-CaMKII. Ten min after hippocampal stimulation, the expression levels of CaMKII and p-CaMKII were higher in NR3A KO slices compared to slices from WT controls (Fig. 5c). To understand whether the elevated CaMKII in the adult NR3A KO mice could explain the differences in LTP and the increased memory performance, the selective CaMKII inhibitor, 1-[NO-bis-1,5-isoquinolinesulfonyl]-N-methyl-L-tyrosyl- 4-phenylpiperazine (KN-62, 5 μM), was applied into the bathing solution 15 min prior to and during the first 5 min after HFS. KN-62 suppressed LTP induction in hippocampal slices from both WT and NR3A KO mice and abrogated the LTP difference between the WT and KO slices (Fig. 5d).

(Figure 5 near here)

Enhanced acute and chronic pain sensation in adult NR3A KO mice

NMDARs play a key regulatory role in pain sensation. Hot-plate and tail-flick tests were performed to elucidate whether adult NR3A KO mice had altered acute nociception. The hot-plate test reflects supraspinal sensory integration [24], while the tail-flick test is a measurement of spinal pathways of acute nociception [25]. Adult NR3A KO mice showed enhanced responses (shorter latency) on the hot-plate test (Fig. 6a). On the other hand, the tail-flick test showed no difference in the responses elicited in WT and NR3A KO mice (Fig. 6b). Taken together, these results are consistent with a role of NR3A in mediating an analgesic response in supraspinal but not spinal pain pathways.

The established formalin inflammatory pain model was used to investigate the response to inflammatory nociception and tissue injuries. Formalin was subcutaneously injected into the plantar side of the right forepaw and the pain score was determined [26]. There was no obvious difference in the forepaw edema between the WT and NR3A KO strains 1 day after the formalin insult (data not shown). We analyzed the duration of pain responses (licking and biting the injected forepaw) during the first hr and pooled the data into 2 distinct phases: phase I (0-5 min) and phase II (6-60 min). In phase I, there was no difference in the responses between WT and KO mice (Fig. 6c). In phase II, NR3A KO mice exhibited a more pronounced pain response compared to WT controls (Fig. 6d), indicating an enhanced response to inflammatory pain stimuli with NR3A deletion.

(Figure 6 near here)

Discussion:

The present investigation reveals a novel role of the inhibitory NMDAR subunit NR3A in locomotor and learning/memory functions and pain sensation in adult animals. Previous investigations on the functional role of the NR3A subunit have focused on neonates and brain development due to the high expression level of this subunit during the neonatal stage. Data from our study are a surprise considering the low expression level of NR3A in the adult brain. The data suggest that even at relatively low expression levels, functional impacts of NR3A in the adult brain should not be underestimated. Specifically, deletion of NR3A enhanced LTP in hippocampal slices from P10, P20 and adult mice. These ages were chosen to represent the time points when NR3A expression was maximal (P10), has just dropped to its lowest level (P20), and has been chronically very low (adults). The data demonstrate that NR3A regulation of LTP is present in immature as well as adult brains. A possibility that requires further investigation is a long-term effect inherited from the high expression of NR3A during the neonatal stage. Neonatal NR3A expression may cause subsequent gene regulation that affect brain function in adulthood. An acute deletion or a conditional knock-down in adult animals will help further confirm or exclude this possibility.

A previous study could not detect any LTP difference between adult WT and NR3A KO mice [11]. Since the NR3A subunit acts as a molecular brake on NMDAR activities, it is possible that the strong stimulation protocol (HFS x 3) used in the earlier study overcame the inhibiting effect of NR3A and thus

prevented the detection of LTP differences between the two strains. The same study detected a difference in LTP between WT and NR3A KO P6-8 mice with a 3x HFS protocol. It is possible that 3x HFSs are enough to stimulate the P6-8 slices despite the low GluR1 expression.

The enhancement of learning and memory in NR3A KO mice is similar to that in the “smart mice” over-expressing NR2B [27]. NR2B, however, has been identified as a pro-death NMDA receptor subunit [28], whereas deletion or down-regulation of NR3A in the adult brain does not increase the risk of excitotoxicity or ischemic injury [29]. In this respect, NR3A appears to be a more realistic target for learning and memory modulation than NR2B. Supporting our observations, prolonged NR3A expression in the forebrains of adult mice attenuates LTP and disrupts long-term memory [11]. It may be worth assuming that the actual memory enhancement is much greater than what we observed in the tests because of the reduced locomotion in NR3A KO mice. For example, despite their significantly reduced swim speed in the Morris maze memory test, NR3A KO mice still managed to reach the platform faster than WT controls. Moreover, the expression of NR3A in adult non-human primates is higher in contrast to the rodent brain [30] suggesting that the NR3A regulation observed in mice could be more prominent in primates.

NR3A is moderately expressed in the striatum and the cerebellum [12]; both regions heavily implicated in motor learning and coordination [31, 32]. Transgenic mice over expressing CaMKII in the forebrain show reduced locomotor activity in novel environments [33]. Thus, higher CaMKII expression in

adult NR3A KO mice might explain the reduction in locomotor activity. In contrast to the CaMKII over-expressing mice which displayed increased aggression and anxiety-like behaviors [33], NR3A KO mice exhibited un-altered anxiety levels. It is likely that the brain regions with enhanced CaMKII expression are different in NR3A KO and CaMKII transgenic mice leading, therefore, to different behavioral changes. CaMKII is a major constituent of the post-synaptic densities and plays an important role in initiating a cascade of events that regulate LTP and potentiate synaptic transmission [34]. Pharmacological inhibition of CaMKII blocks hippocampal LTP [35] and impairs hippocampus-dependant memory and learning [36]. Similar results were also found in transgenic mice with deletion of CaMKII [37]. CaMKII may also regulate nociception. Acute pharmacological inhibition of CaMKII with KN-62 reversed complete Freund's adjuvant (CFA)-induced inflammatory pain responses [38]. On the other hand, forebrain over-expression of CaMKII reduced CFA-induced allodynia and hyperalgesia without affecting pain responses to heat, mechanical pressure and formalin injection [39]. While the exact role of CaMKII in mediating LTP and pain sensation may be affected by multiple co-factors, the enhanced expression and phosphorylation of CaMKII in the hippocampus of the NR3A KO mice are likely the molecular mediators in enhanced LTP and nociceptive responses. On the other hand, we were not able to objectively study emotional memory using contextual and cued fear conditioning because of the difference in acute pain responses.

Patients suffering from schizophrenia exhibit impaired memory [40], pain insensitivity [41] and disrupted motor functions [42]. Animal models of

schizophrenia showed decreased expression of CaMKII [43, 44]. Interestingly, the schizophrenic symptoms and CaMKII expression in schizophrenia animal models are the opposite phenotype to what we observed in NR3A KO mice. Indeed, NR3A transcription level is elevated by 32% in schizophrenic patients and has been implicated in the pathogenesis of schizophrenia [45]. Thus, the present work may additionally identify a novel target whose modulation may reverse phenotypes in disorders that involve impaired synaptic structures and dysfunctions such as those found in schizophrenia patients.

Materials and Methods

Animals:

Animal handling and all experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University. The NR3A KO mice and WT counterparts were provided by Nobuki Nakanishi and Stuart A. Lipton at Sanford-Burnham Medical Research Institute (La Jolla, California, USA) [10].

Genotyping:

DNA for genotyping was extracted from tail snips. Two separate sets of primers were used for the NR3A KO and WT mice. For the WT reaction,

forward primer: 5'-CCACGGTGAGCTTGGGGAAG-3' and reverse primer 5'-TTGGGGAGCGCCCTGCATGG-3'

For the KO reaction, forward primer:

5'-CCACGGTGAGCTTGGGGAAG-3' and reverse primer 5'-GCCTGAAGAACGAGATCAGG-3'

DNA (2 µl) was amplified on a thermocycler (MJ Mini, Personal Thermal Cycler, BIORAD) for 40 cycles (2 min, 94 ^{o}C; 30 sec, 60 ^{o}C; 10 min, 72 ^{o}C for the WT reaction and 2 min, 94 ^{o}C; 30 sec, 58 ^{o}C; 10 min, 72 ^{o}C for the KO reaction).

Western Blot:

Western blot analysis was performed to analyze protein expression in brain tissues from WT and NR3A KO mice following previous procedures [46]. In brief, brain tissue was lysed in lysis buffer containing 25 mM Tris–HCL (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 2 mM sodium orthovanadate, 100 mM NaF, 1% triton, leupeptin, aprotinin, and pepstatin with continuous manual homogenization. Tissue lysate was then spun at 13,000 rpm for 15 minutes and supernatant was collected. Protein concentration was determined using BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein (30 μg) were resolved on SDS-PAGE using gradient gels (6-18%) and gels were blotted onto PVDF membranes (Amersham, Buckinghamshire, UK), blocked with 5% BSA in TBST buffer (20 mM Tris, 137 mM NaCl and 0.1% tween) and incubated overnight with primary antibodies against rabbit polyclonal anti-NR3A (1:1000, Millipore), rabbit anti-NR3B (1:1000, Millipore), mouse monoclonal anti-GluR1 (1:1000, Millipore), rabbit anti-CREB (1:1000, Cell Signaling), rabbit anti-phospho-CREB (1:1000, Cell Signaling), rabbit anti-Akt (1:1000, Cell Signaling), rabbit anti-phospho-Akt (1:1000, Cell Signaling), rabbit anti-PKCα (1:1000, Cell Signaling), rabbit anti-PKA C-α (1:1000, Cell Signaling), rabbit anti-CaMKII α (1:1000, Cell Signaling), rabbit anti-phospho-CaMKII (Thr286) (1:1000, Cell Signaling) and mouse anti-actin (1:5000, Sigma). After 3 washes with TBST, blots were incubated with HRP-conjugated secondary antibodies (anti-rabbit or anti-mouse, 1:2000, Bio-Rad, CA) in 5% BSA. Blots were developed using Pierce ECL Western Blotting Substrate (Thermo Scientific, IL). The level of protein expression was normalized to β-actin controls.

For synaptosomes and post-synaptic density preparations, we used a modification of previous protocols [47]. In brief, total forebrains were homogenized in 0.32 M sucrose solution using Teflon coated homogenizers. The homogenate was centrifuged to collect the supernatant S1 and the pellet P1. S1 was further centrifuged to collect S2 and P2. P2 was resuspended in Percoll and the P2/Percoll solution was differentially centrifuged in a Percoll gradient. The band corresponding to the heavy synaptosomes was resuspended in HEPES/EDTA/Triton-X buffer and centrifuged (22,000 rpm; 70.1 Ti rotor) to collect the first PSD fraction (PSD-1T). PSD-1T was resuspended in HEPES/EDTA/Triton-X buffer and centrifuged again (50,000 rpm; 70.1 Ti rotor) to collect the second PSD fraction (PSD-2T). The following antibodies were used: mouse monoclonal anti-NR1 (1:1000, Millipore), rabbit polyclonal anti-NR2B (1:1000, Millipore), rabbit polyclonal anti-NR2A (1:1000, Millipore), and rabbit anti-PSD95 (1:1000, Cell Signaling). The level of protein expression was normalized to PSD95 controls.

Hippocampal Slice Preparation and Recording:

WT and NR3A KO mice were anaesthetized with isoflurane, decapitated, and their brains dropped in ice-cold artificial cerebrospinal fluid (a-CSF) containing: 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 6.0 mM $MgCl_2$, 26 mM $NaHCO_3$, 2.0mM $CaCl_2$, and 10 mM glucose. a-CSF was saturated with 95% O_2 and 5% CO_2, at pH 7.4. The hippocampi were cut into 400-μm thick transverse slices with a vibratome (Vibratome 1000 Plus Sectioning System, St Louis, MO).

After incubation at room temperature (23-24°C) in a-CSF for 60-90 min, slices were placed in a recording chamber (RC-22C, Warner Instruments) on a stage of up-right microscope (Olympus CX-31) and perfused at a rate of 3 ml per min with a-CSF (1 mM $MgCl_2$) at 23-24°C. A 0.1 MΩ tungsten monopolar electrode was used to stimulate the Schaffer collaterals. The field excitatory post-synaptic potentials (f-EPSPs) were recorded in CA1 stratum radiatum by a glass microelectrode filled with a-CSF with resistance of 3-4 MΩ. The stimulation output (Master-8; AMPI, Jerusalem, Israel) was controlled by the trigger function of an EPC9 amplifier (HEKA Elektronik, Lambrecht, Germany). f-EPSPs were recorded in the current-clamp mode. Data were filtered at 3 KHz and digitized at sampling rates of 20 KHz using Pulse software (HEKA Elektronik). The stimulus intensity (0.2 ms duration, 16–30 μA) was set to evoke 40% of the maximum f-EPSP initial slope and the test pulse was applied at a rate of 0.033 Hz. Paired-pulse facilitation (PPF) was examined by applying pairs of pulses which were separated by 10-500 ms different intervals. To induce LTP, high frequency stimulation (HFS) was applied at 100 Hz for 1 second. Low frequency stimulation (LFS, 1 Hz, 900 ms) or NMDA (30 μM) application were used for the induction of LTD in both WT and KO slices. The magnitude of LTP and LTD was expressed as the mean percentage of baseline fEPSP initial slope.

Behavioral Tests:

Memory tests: The Novel Object Recognition test and Morris water maze tests were performed and analyzed as described previously [14, 48]. All trials were

videotaped and performance was analyzed using TopScan or MazeScan (Clever Sys, Inc., Reston, VA).

The Novel object recognition test was performed during the dark cycle. On the two days preceding the training session, mice were individually habituated to the testing environment (30cm x 30cm x 20 cm; polycarbonate box) for 10 minutes. During the training session, mice were allowed to explore the same box in which 2 identical objects were placed in adjacent corners for 5 minutes. During the retention sessions, one of the objects was replaced by a novel object and mice are allowed to explore for 5 minutes. The ratio of the time spent exploring any of the identical objects during the training session or the time spent exploring the novel object during the retention session to the time spent exploring the familiar object is calculated.

The water maze apparatus is a round, water-filled tub (52 inch diameter filled with white tempura paint) placed in a room rich with extra maze cues. Mice were placed in the maze starting from 4 different positions (N, S, E, and W). An invisible escape platform was located in the same spatial location 1 cm below the water surface independent of the starting position on a particular trial. In this manner, subjects were able to utilize extra maze cues to determine the platform's location. Each subject was given 4 trials per day (N, S, E, and W) for 5 days with a 15-min inter-trial interval. The maximum trial length was 60 seconds and subjects were manually guided to the platform if they do not reach it in the allotted time. Upon reaching the invisible escape platform, subjects were kept on it for an additional 5 seconds to allow for survey of the spatial cues in the

environment to guide future navigation to the platform. After 5 days of task acquisition, a probe trial was carried out during which the platform is removed. The time spent and distance travelled in the quadrant which previously contained the escape platform during task acquisition was measured over 60s.

Pain tests: To test acute pain, we measured the latency of pain responses on hot-plate and tail-flick tests. For the hot-plate test, mice were placed on a hot plate (55^{o}C) and the latency to forepaw licking or jumping was recorded for each mouse in 3 trials separated by at least 15 minutes. For the tail-flick test, the latency to withdraw the tail from water (50^{o}C) was recorded for three trials separated by 15 min. To test chronic or inflammatory pain, the response to subcutaneous formalin injection was recorded. In brief, 10 µl of 5% formalin were subcutaneously injected in the plantar surface of a forepaw. Pain scores were collected as detailed before [26]. The total time spent licking or biting the injected forepaw was recorded over a 3-min interval for each mouse for one hour.

Locomotor and anxiety tests: The Rota-Rod test was performed to assess the ability of mice to maintain balance and coordination on an accelerating rotating rod (UGO Bacile 7650). On each testing day, mice were tested 3 times separated by at least 15 min. In the open field locomotion test, mice were allowed to freely explore an open field container (50 cm x 30 cm x 30 cm height, divided into 12 equal segments) for five min. Activity was recorded on a video camera. The average distance and speed, the number of vertical rears (standing upright on the hind paws or leaning on the wall), number of central zone entries and the latency to leave the central zone were recorded. In the defensive withdrawal test,

animals were placed in a dark chamber (10 x 15 x 10 cm high) placed along the wall of a large box (50 x 50 x 50 cm high). Animals were habituated to the open field 24 hrs prior to behavioral testing by allowing them to explore for 15 min without access to the defensive withdrawal chamber. The "latency to exit" was measured as the time it takes the animal to place his four paws outside the small chamber. The "latency to cross" was measured as the time to cross a square drawn to represent the central zone of the box. The experiment was video-taped and analysis was performed by a researcher blinded to the identity of the groups.

Statistical Analysis:

Results are expressed as mean ± S.E.M. Statistical comparisons were made with Student's t-test, one- or two-way analysis of variance (ANOVA) with the Bonferroni's *post-hoc* analysis to identify significant differences. $P < 0.05$ was considered significant for all comparisons.

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Figure legends

Figure 1. Expression of NMDA and AMPA receptors and LTP regulators in the adult NR3A KO brain

(**a**) Adult WT and NR3A KO mice are visually indistinguishable from each other. (**b**) The expression of the AMPA receptor subunit GluR1 in the brain is similar between adult WT and NR3A KO mice. (**c**) The expression of the NMDA receptor subunit NR3B in the brain is similar between adult WT and NR3A KO mice. Protein expression in (**b – c**) was determined in whole tissue lysates with β-actin as the loading control. (**d**) The expression of the NMDA receptor subunits NR1, NR2A and NR2B in the brain is similar between adult WT and NR3A KO mice. Protein expression in (**d**) was determined in post-synaptic densities fractions (PSD-2T) with PSD-95 as the loading control. (**e – f**) Western blot analysis of LTP-related effectors in the forebrain of adult WT and NR3A KO mice. There is no difference in the forebrain expression of Akt (**e**), CREB (**e**), PKC (**f**), PKA (**f**) between adult WT and NR3A KO mice. Protein expression was determined in whole tissue lysates with β-actin as the loading control. n = 3 or 4 for each group; *. $P < 0.05$ vs. WT.

Figure. 2. Reduced locomotor functions without increased anxiety in adult NR3A KO mice

(**a**) WT and NR3A KO mice were evaluated for locomotor abilities using the Rota-Rod test. They were subjected to 5 training days and then tested at day 12 and 16 (7 and 11 days after training). Despite the difference during training,

both WT and KO mice performed similarly at the end of a 5-day training session. After a long resting interval (from day 5 to day 12), the performance of NR3A KO mice dropped but it soon recovered and remained similar to WT counterparts 4 days later (day 16). n = 8 for WT and n = 7 for KO; *. $P < 0.05$ vs. WT. (**b**) Morris water maze test demonstrated slower swim speeds of the NR3A KO mice during the 5 consecutive training days. n = 10; *. $P < 0.05$ vs. WT. (**c – h)** The locomotor ability of WT and NR3A KO mice was evaluated in the open field test. NR3A KO mice walked slower (**c**) and covered less distance (**d**) during the 5-min period. This was also reflected in the fewer times the NR3A KO mice crossed into the central zone (**e**) and their fewer vertical rears (**f**) compared to the WT mice. While NR3A KO mice needed more time to leave the central zone (**g**), they spent similar total time in the central zone as the WT mice (**h**). These data indicate that NR3A KO mice had reduced locomotor abilities but their locomotor learning was intact. n = 12 for WT and n = 10 for KO in all tests; *. $P < 0.05$ vs. WT. (**i – j)** WT and NR3A KO mice were evaluated for anxiety levels in the defensive withdrawal test. The latency to exit the sheltered chamber (**i**) and the latency to enter the central zone (**j**) were similar between the WT and KO mice, indicating unaltered anxiety levels between the two strains. n = 11 for WT and n = 9 for KO.

Figure 3. Enhanced recognition and spatial memory in adult NR3A KO mice

(**a**) WT and NR3A KO mice were evaluated for recognition memory in the novel object recognition test. Both strains had equal exploratory preference during training as they spent similar amount of time approaching, touching and smelling the similar objects during training. During the retention test (1 hr after

training), both WT and NR3A KO mice showed enhanced preference for the novel object. This enhanced preference for novelty was more pronounced in KO animals. 1 and 7 days after training, WT mice lost preference for novelty while NR3A KO mice still showed preference for the novel object. The exploratory behavior on the Y-axis represents the ratio of the time spent exploring the novel object to the time spent on the familiar object. n = 10 for all groups; *. $P < 0.05$ vs. WT; #. $P < 0.05$ vs. training day controls. (**b – e)** WT and KO mice were evaluated for spatial memory in the Morris water maze test. (**b**) Latency to escape to platform during the 5-day training session. On day 1, NR3A KO mice were slower to reach platform but their time improved at day 3. (**c**) Swim distance to reach platform during training. KO mice performed better at day 3. (**d**) Average latency to reach platform during the last 3 training days showing that KO mice reached the escape platform faster than WT mice. (**e**) The place preference test conducted at day 6 when the platform was removed after the last training day. NR3A KO mice spent more time in the platform (marked as * in the inset photos) quadrant compared to WT mice, indicating enhanced spatial memory. The insets show representative traces of the navigation of both WT and NR3A KO mice. n = 10 for both groups; *. $P < 0.05$ vs. WT.

Figure 4. Enhanced LTP in the adult NR3A KO brain

LTP induction was recorded in hippocampal slices from WT and NR3A KO mice of different ages. (**a – b**) Using 0.2 mM Mg^{2+} external solution, fEPSP slope were gradually and significantly increased in both WT and NR3A KO slices from P10 (**a**) and P20 (**b**) mice. The LTP event was greater in the NR3A KO slices. *P*

< 0.05 between the slopes of fEPSPs of WT and KO slices during time points 0 to 60 min. n = 6 in each group in (**a**) and n = 5 in each group in (**b**). Representative fEPSP traces recorded at the indicated time points are shown on top. (**c**) In slices from adult mice, LTP was induced by HFS (100Hz, 1s). The magnitude of LTP was more than doubled in NR3A KO slices. *P* < 0.05 between the slopes of fEPSPs of WT and KO slices during time points 0 to 60 min. n = 9 and 11 for WT and KO groups, respectively. (**d**) Paired pulse facilitation (PPF) of fEPSP induced by various interpulse intervals was similar between adult WT and NR3A KO slices. n = 4 for both groups. (**e**) Similar NMDA-induced chemical LTD was recorded in WT and NR3A KO slices from adult mice. N = 5 in each group. (**f**) LTP was recorded in WT and NR3A KO slices from adult animals in the presence of AP-5, an NMDA receptor antagonist. LTP was inhibited in both WT and NR3A KO slices when AP-5 was bath applied 20 min prior to HFS (100 Hz, 1 s) indicating that the difference in LTP between WT and NR3A KO slices is NMDAR dependent.

Figure 5. Enhanced CaMKII expression in the adult NR3A KO mice

The expression of CaMKII and phosphorylated CaMKII (p-CaMKII) was measured in the cortex, hippocampus and stimulated hippocampus. (**a**) CaMKII expression is higher in the forebrain cortex of adult NR3A KO mice compared to WT animals (n = 8 for both groups). (**b**) CaMKII and p-CaMKII expression is higher in the hippocampus of adult NR3A KO mice compared to WT animals. For CaMKII, n = 12 for both groups; for p-CaMKII, n = 6 and n = 4 for WT and KO, respectively; *. P<0.05 vs. WT. (**c**) Western blotting of CaMKII and p-CaMKII in

the hippocampus 10 min after HFS. CaMKII and p-CaMKII expression is higher in adult NR3A KO mice compared to WT animals. n = 6; *. P<0.05 vs. WT. Protein expression in (**a – c**) was determined in whole tissue lysates with actin as the loading control. (**d**) KN-62 (5 μM), a specific inhibitor of CaMKII, suppressed the induction of LTP in WT and KO slices (downward triangles), and abrogated the difference between them. n = 4 and n = 3 for WT and KO mice, respectively. Open and filled circles show LTP without KN-62.

Figure 6. Enhanced acute and chronic pain sensation in adult NR3A KO mice

Adult WT and NR3A KO mice were tested for pain sensation. (**a – b**) Acute pain perceptions of supraspinal and spinal pain pathways were tested in WT and NR3A KO mice in the hot-plate and tail-flick tests, respectively. Enhanced pain sensation (shorter pain latency) was seen in the hot-plate test with KO mice (**a**), but not in the tail-flick test (**b**). (**c – d**) Chronic pain perception (licking and biting the injected forepaw) triggered by formalin injection was evaluated in adult mice. Although there was no difference immediately (0-5 min) after the injection (**c**), significantly enhanced responses in the KO mice were seen 6-60 min after formalin injection (**d**).

Supplementary Figure 1.

(**a**) Levels of NR3A protein in the brain during development. NR3A expression in the forebrain peaks early during development and then reaches a minimum in adulthood. (**b**) Expression of the AMPA receptor subunit GluR1 is

higher in the adult forebrain of the WT and NR3A KO mice as compared to the 10-day old mice. (**c**) Western blot analysis of LTP-related effectors in the forebrain of adult WT and NR3A KO mice. There is no difference in the forebrain expression of phospho-Akt and phospho-CREB between adult WT and NR3A KO mice. Protein expression in (**a – c**) was determined in whole tissue lysates with actin as the loading control; *. $P<0.05$ vs. WT.

Supplementary Figure 2.

(**a – b**) LTP induction was recorded in hippocampal slices from P10 and P20 WT and NR3A KO mice. LTP was induced by HFS (100 Hz, 1 s). The magnitude of LTP was similar between WT and NR3A KO mice in the P10 (**a**) and P20 (**b**) age groups. Representative fEPSP traces recorded at the indicated time points are shown on top. n = 5 or 6 for all groups. (**c**) LTP was recorded in WT and NR3A KO slices from adult animals in the presence of AP-5, an NMDA receptor antagonist. LTP was not inhibited in both WT and NR3A KO slices when AP-5 was bath applied after HFS (100 Hz, 1 s).

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Figure 1

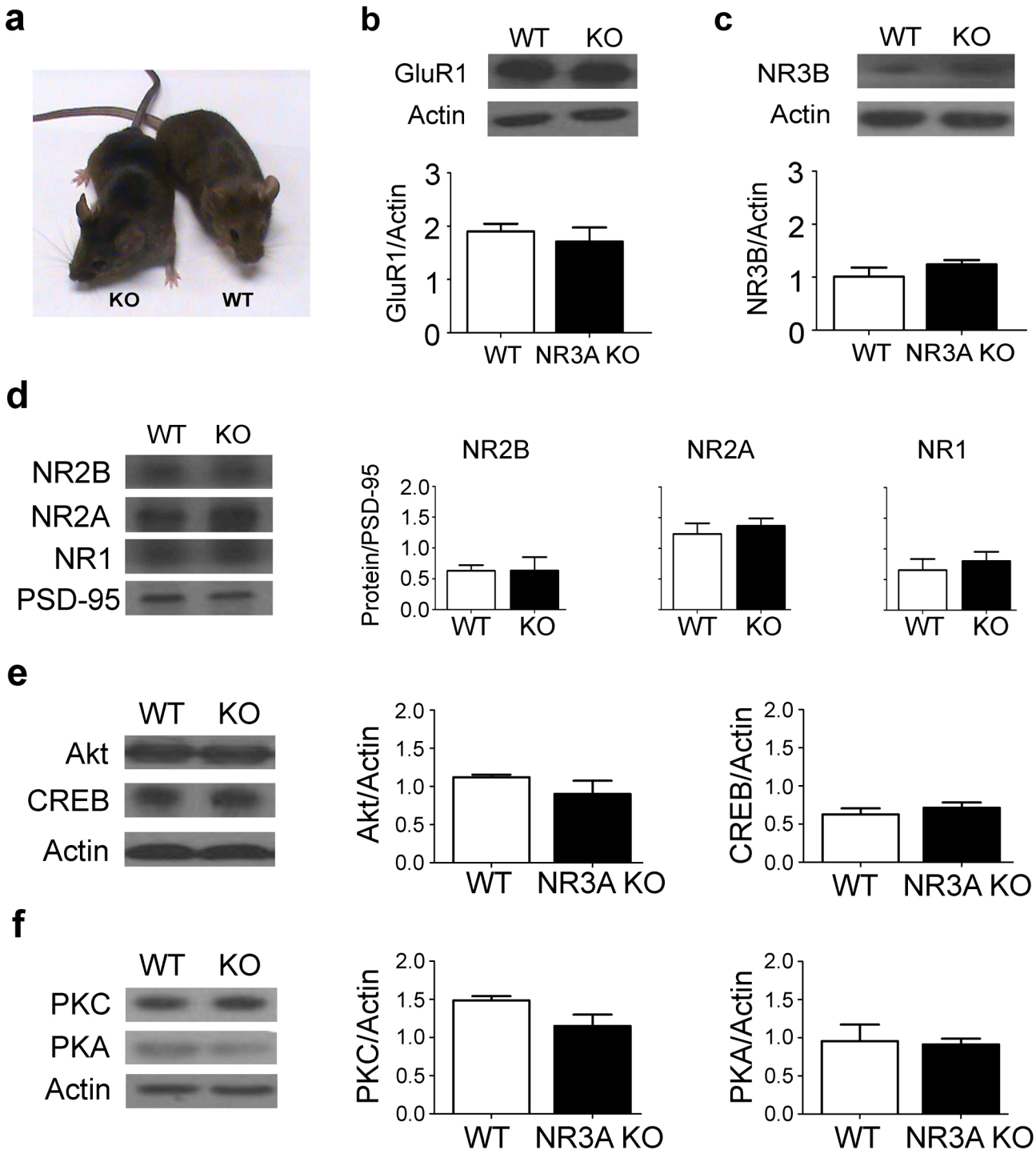

a
KO
WT
b
WT KO
GluR1
Actin
GluR1/Actin
WT NR3A KO
c
WT KO
NR3B
Actin
NR3B/Actin
WT NR3A KO
d
WT KO
NR2B
NR2A
NR1
PSD-95
NR2B
NR2A
NR1
Protein/PSD-95
WT KO
e
WT KO
Akt
CREB
Actin
Akt/Actin
CREB/Actin
WT NR3A KO
f
WT KO
PKC
PKA
Actin
PKC/Actin
PKA/Actin
WT NR3A KO

Figure 2

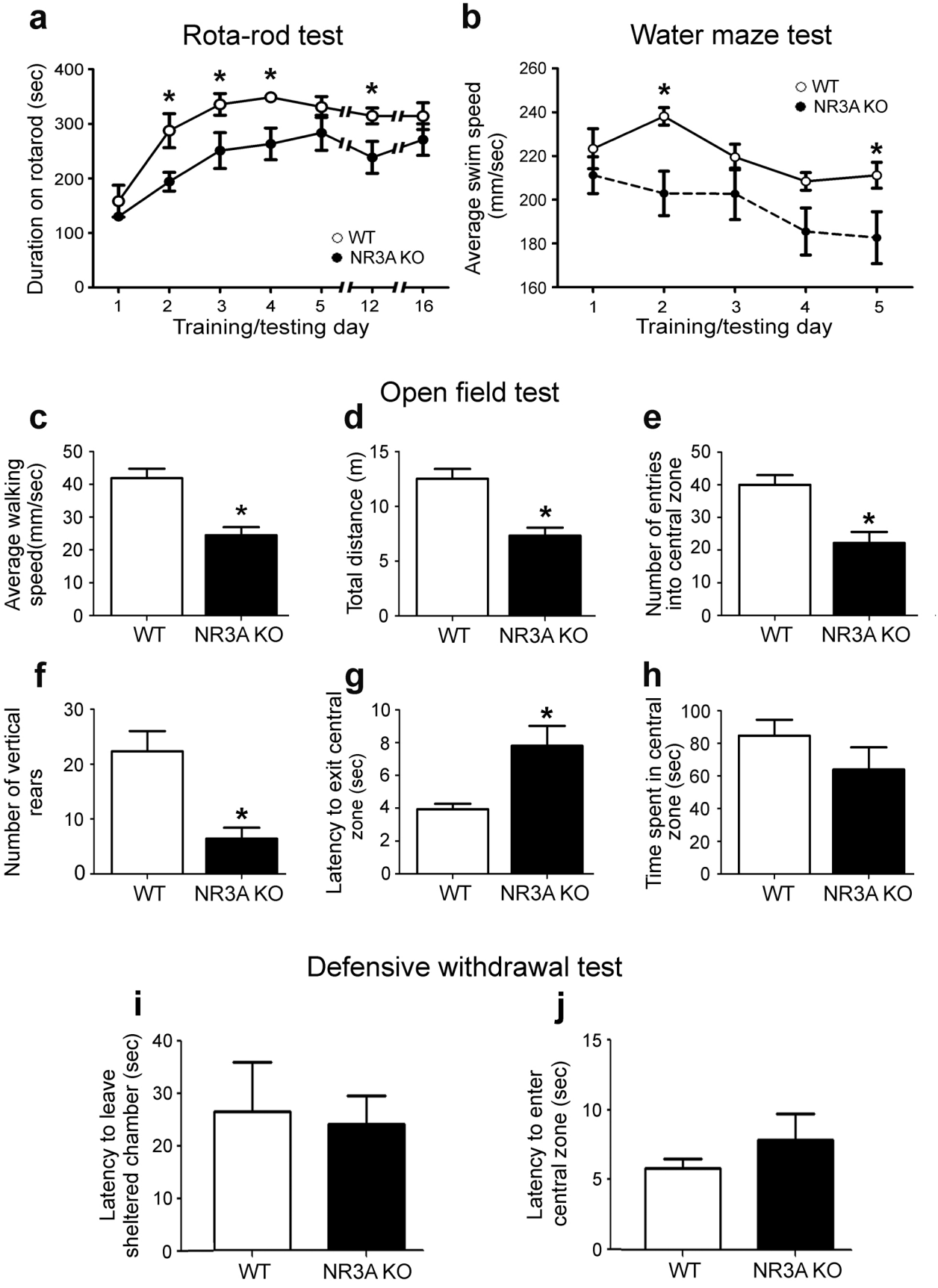

a
Rota-rod test
Duration on rotarod (sec)
400
300
200
100
0
1
2
3
4
5
12
16
Training/testing day
WT
NR3A KO
b
Water maze test
Average swim speed (mm/sec)
260
240
220
200
180
160
1
2
3
4
5
Training/testing day
WT
NR3A KO
Open field test
c
Average walking speed(mm/sec)
50
40
30
20
10
0
WT
NR3A KO
d
Total distance (m)
15
10
5
0
WT
NR3A KO
e
Number of entries into central zone
50
40
30
20
10
0
WT
NR3A KO
f
Number of vertical rears
30
20
10
0
WT
NR3A KO
g
Latency to exit central zone (sec)
10
8
6
4
2
0
WT
NR3A KO
h
Time spent in central zone (sec)
100
80
60
40
20
0
WT
NR3A KO
Defensive withdrawal test
i
Latency to leave sheltered chamber (sec)
40
30
20
10
0
WT
NR3A KO
j
Latency to enter central zone (sec)
15
10
5
0
WT
NR3A KO

Figure 3

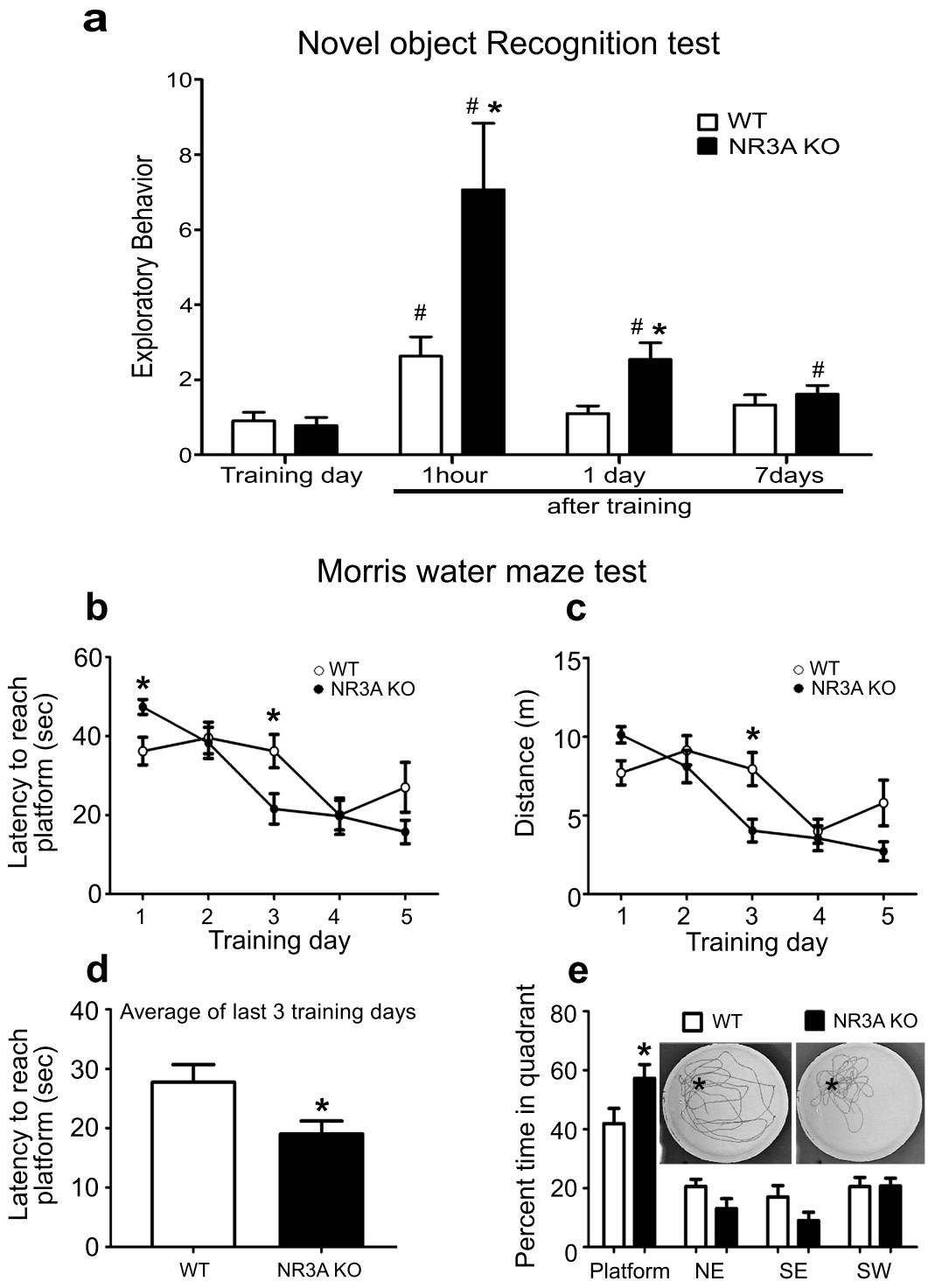

a
Novel object Recognition test
Exploratory Behavior
WT
NR3A KO
Training day
1hour
1 day
7days
after training
Morris water maze test
b
Latency to reach platform (sec)
Training day
c
Distance (m)
Training day
d
Average of last 3 training days
Latency to reach platform (sec)
WT
NR3A KO
e
Percent time in quadrant
Platform
NE
SE
SW

Figure 4

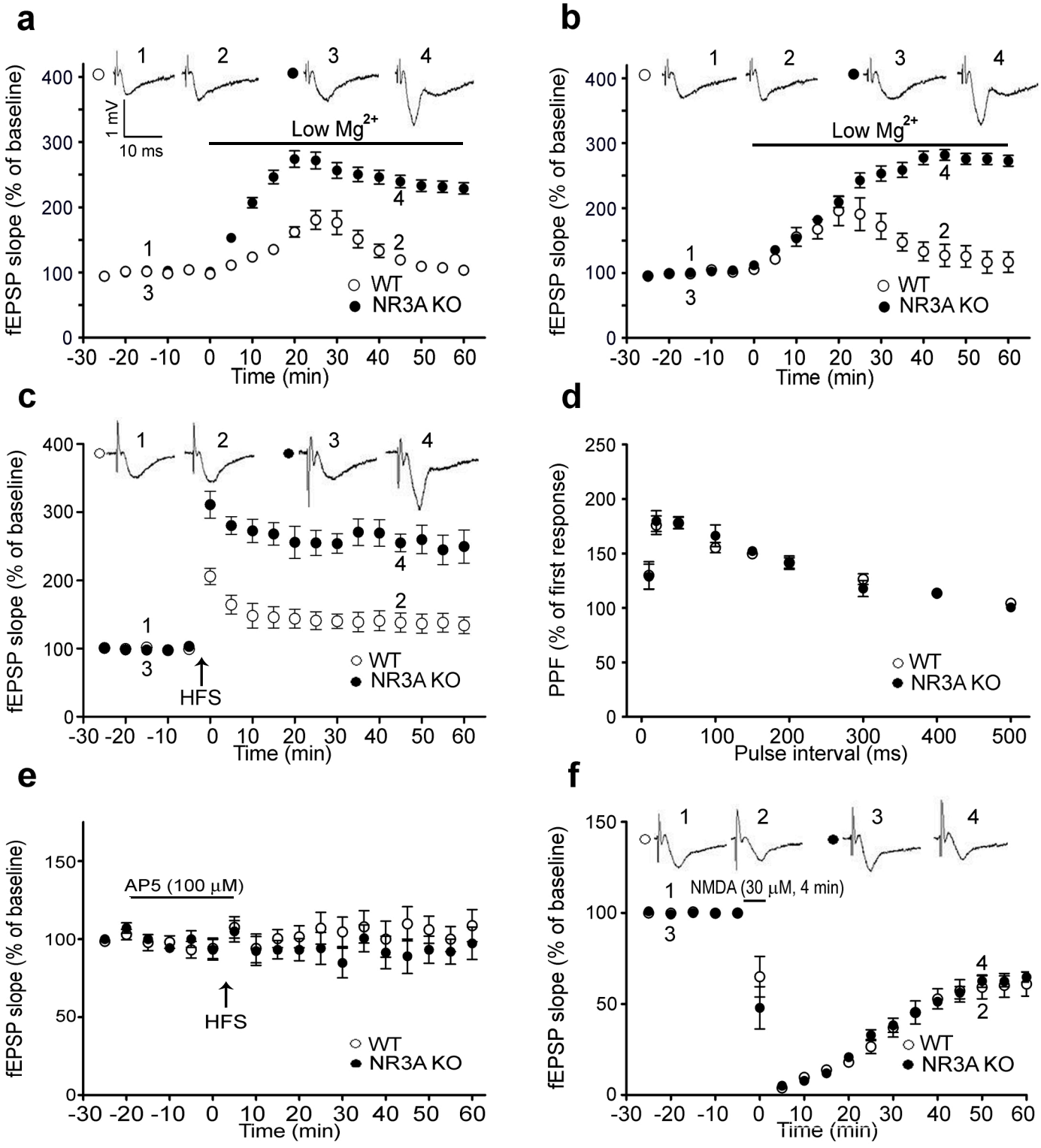

a
b
c
d
e
f
fEPSP slope (% of baseline)
Time (min)
Low Mg2+
1 mV
10 ms
WT
NR3A KO
HFS
PPF (% of first response)
Pulse interval (ms)
AP5 (100 μM)
NMDA (30 μM, 4 min)

Figure 5

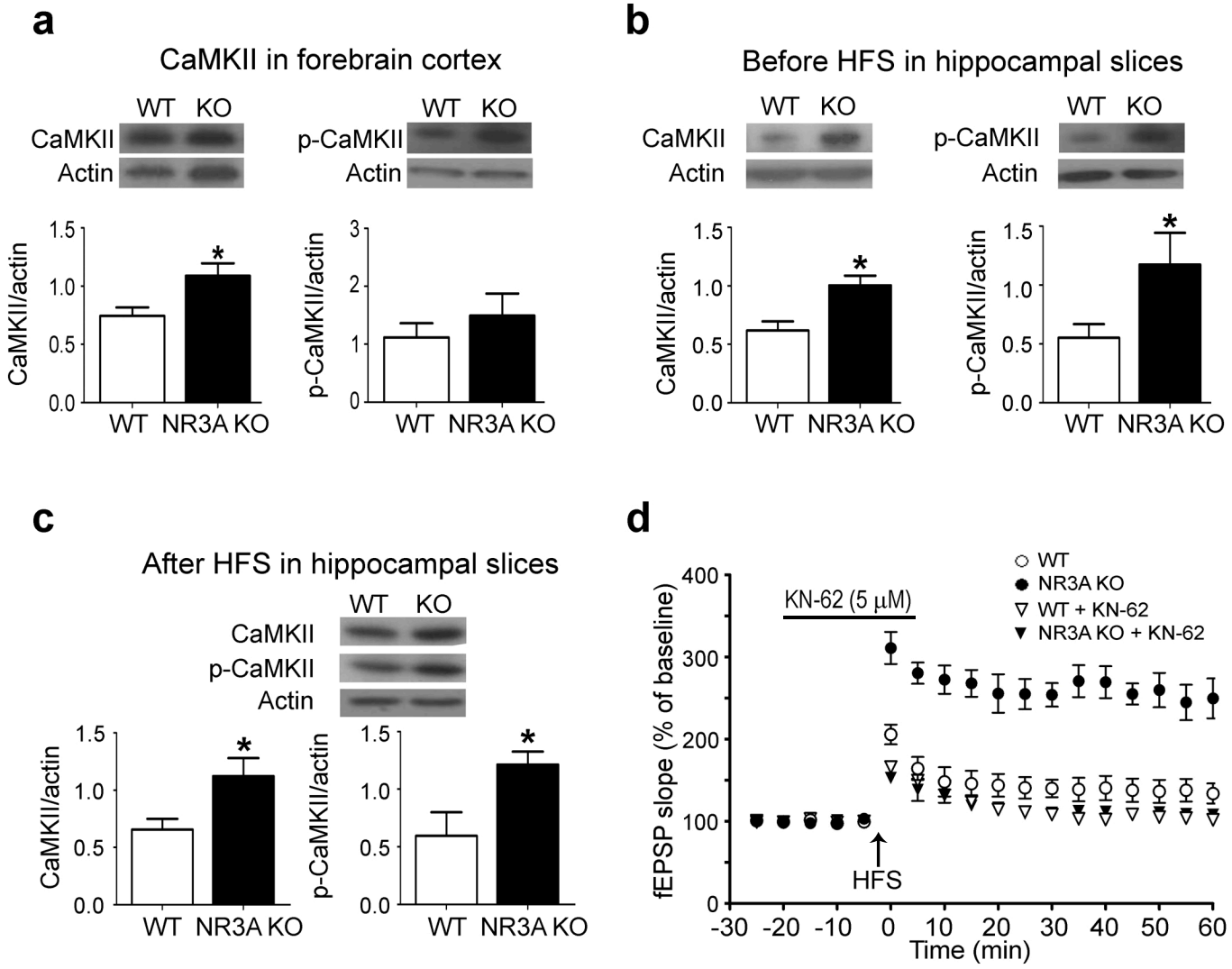
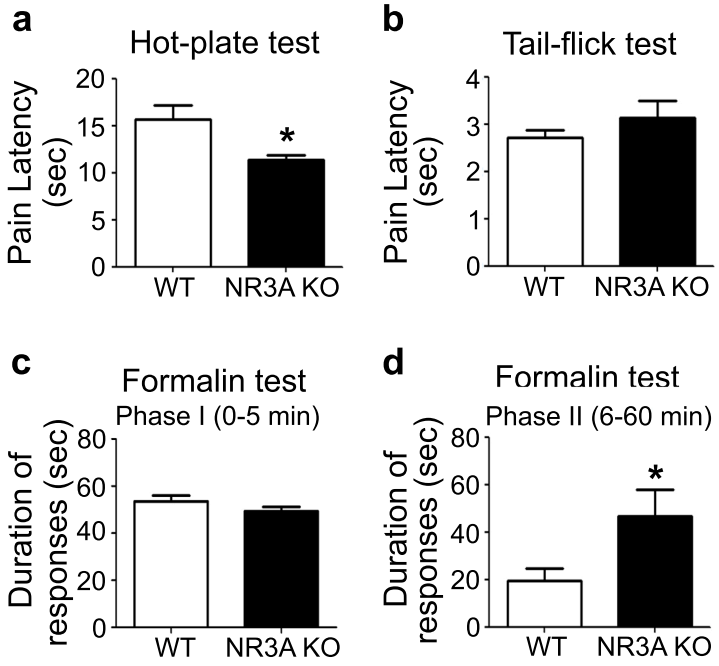

a
CaMKII in forebrain cortex
WT KO
CaMKII
Actin
p-CaMKII
CaMKII/actin
p-CaMKII/actin
WT NR3A KO
b
Before HFS in hippocampal slices
c
After HFS in hippocampal slices
d
KN-62 (5 μM)
WT
NR3A KO
WT + KN-62
NR3A KO + KN-62
fEPSP slope (% of baseline)
HFS
Time (min)

Figure 6


a
Hot-plate test
Pain Latency (sec)
20
15
10
5
0
*
WT
NR3A KO
b
Tail-flick test
Pain Latency (sec)
4
3
2
1
0
WT
NR3A KO
c
Formalin test
Phase I (0-5 min)
Duration of responses (sec)
80
60
40
20
0
WT
NR3A KO
d
Formalin test
Phase II (6-60 min)
Duration of responses (sec)
80
60
40
20
0
*
WT
NR3A KO