Title: Chronic impairment of ERK signaling in glutamatergic neurons of the forebrain does not affect spatial memory retention and LTP in the same manner as acute blockade of the ERK pathway.

Abbreviated Title: The effect of chronic disruption of ERK signaling on the hippocampus.

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ABSTRACT

The ERK/MAPK signaling pathway has been extensively studied in the context of learning and memory. Defects in this pathway underlie genetic diseases associated with intellectual disability, including impaired learning and memory. Numerous studies have investigated the impact of acute ERK/MAPK inhibition on long-term potentiation and spatial memory. However, genetic knockouts of the ERKs have not been utilized to determine whether developmental perturbations of ERK/MAPK signaling affect LTP and memory formation in postnatal life. In this study, two different ERK2 conditional knockout mice were generated that restrict loss of ERK2 to excitatory neurons in the forebrain, but at different time-points (embryonically and post-natally). We found that embryonic loss of ERK2 had minimal effect on spatial memory retention and novel object recognition, while loss of ERK2 post-natally had more pronounced effects in these behaviors. Loss of ERK2 in both models showed intact LTP compared to control animals, while loss of both ERK1 and ERK2 impaired late phase LTP. These findings indicate that ERK2 is not necessary for LTP and spatial memory retention and provide new insights into the functional deficits associated with the chronic impairment of ERK signaling.

INTRODUCTION

Neurocardiofacial cutaneous (NCFC) syndromes are congenital disorders, which include DiGeorge, Noonan, LEOPARD and Costello syndromes, that have many overlapping features, in particular variable degrees of cognitive impairment and intellectual disability. The disorders are genetically linked by mutations in the elements of the ERK/MAPK signaling pathway (Bentires-Alj et al., 2006; Samuels et al., 2009). ERK/MAPK signaling has been shown to be involved in

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various cognitive processes, such as learning and memory, through multiple mechanisms of synaptic plasticity, one of which is long-term potentiation (LTP) (Grant et al., 1992; English and Sweatt, 1996; Lynch, 1998; Zamanillo et al., 1999; Sweatt, 2004; Fedulov et al., 2007; Tidyman and Rauen, 2009; Kandel, 2012; Rauen, 2013; Nabavi et al., 2014).

The initial studies examining the role of ERK/MAPK signaling in memory and LTP utilized pharmacologic inhibitors of their upstream regulatory kinases, MEK1 and MEK2, which blocked signal transduction through ERK1 and ERK2, the terminal kinases of the ERK/MAPK pathway (English and Sweatt, 1997). Acute inhibition of MEK1/2 resulted in impaired LTP as well as impaired spatial memory when tested in the Morris water maze (MWM) (Atkins et al., 1998; Blum et al., 1999; Selcher et al., 1999; 2003). These results were further supported in a genetic mouse model that used the *CamK2a-Cre* transgene to conditionally express a dominant negative *MEK1* allele post-natally (Kelleher et al., 2004).

A persistent question has been whether there are isoform specific requirements for ERK1 or ERK2 in hippocampal-based learning and memory. Both isoforms are thought to have overlapping functions, but ERK2 is present at much higher levels than ERK1 in the brain and some studies suggest isoform specific differences (Ortiz et al., 1995; Indrigo et al., 2010; Roskoski, 2012). Loss of ERK1, in *Mapk3* (ERK1) null mice, had no effect on spatial memory or LTP (Selcher et al., 2001). ERK2, on the other hand, was more challenging to study because constitutive knockouts of ERK2 are embryonic lethal (Lefloch et al., 2008). Transgenic mice with hypomorphic ERK2 alleles, were able to learn the water maze task, but exhibited slightly delayed learning, however, LTP was not assessed in these animals (Satoh et al., 2007). ERK2 loss-of-function mouse models have been studied using conditional deletion strategies which have revealed more pronounced cognitive deficits compared to ERK1 knockouts, but spatial

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memory and LTP have not been assessed. Double knockouts of ERK1 and ERK2 have not been studied beyond the early post-natal period due to the lethal nature of embryonic loss of both ERK isoforms (Imamura et al., 2010; Pucilowska et al., 2012; Vithayathil et al., 2015).

In the present study, we utilize an Emx1-Cre knockout of ERK2 (CKO^{Emx}), which inactivates ERK2 at embryonic day 9.5, as well as a post-natal knockout of ERK2 using the CamK2a-Cre (CKO^{CamK}), which inactivates ERK2 in the third post-natal week (Tsien et al., 1996; Gorski et al., 2002). Using these models, we assessed how pre- and post-natal loss of ERK signaling differentially impacts learning and memory, as well as hippocampal LTP.

MATERIALS AND METHODS

Animals

Transgenic mouse lines carrying the ERK2 floxed alleles (Samuels et al., 2008) as well as ERK1 null mice were generated previously (Nekrasova et al., 2005; Samuels et al., 2008). Emx1-Cre and Camk2a-Cre mouse lines were obtained from Jackson Laboratories. All mice were maintained on C57/B6 background. Animals were housed in the Case Western Reserve University (CWRU) Animal Resource Center (ARC) on a 12 hour light-dark cycle, with regular cage changes and *ad libitum* food and water.

Immunohistochemistry and Histology

Brains from 12-14 week mice of mixed gender were removed and drop fixed in 4% paraformaldehyde (PFA) overnight. Tissue was sequentially incubated in 10%, 20% and 30% sucrose for 24hr at each concentration. Tissue was embedded in OCT and sectioned into 10µm coronal sections and collected onto glass slides. Tissue was stained using previously described protocols (Vithayathil et al. 2015). Immunostaining was performed by blocking sections in 10%

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normal goat serum (NGS) in 0.1% Triton X-100 in PBS. Sections were then incubated overnight in primary antibody at 4°C. Antibodies used: phospho-ERK (Cell Signaling, 1:500). Sections were then washed and incubated with the appropriate fluorophore-conjugated secondary antibodies for 1hr. The sections were washed and DAPI counterstained before mounting with Prolong Gold.

Hippocampal slices used for electrophysiological recordings were fixed in 4% PFA overnight after recording. The slices were then transferred and maintained in 30% sucrose. Immunostaining was performed by treating slices with heated antigen retrieval (Reveal Decloaker, Biocare) followed by overnight blocking and permeabilization in 10% NGS in 0.5% Triton X-100. Slices were then incubated in primary antibody overnight at 4°C. Slices were then washed and incubated in secondary antibody overnight at 4°C. The slices were then washed and mounted onto slides with Prolong Gold.

Western Analysis

Brains of mice were removed followed by dissection of the hippocampus, which were homogenized using a dounce homogenizer. Tissue extracts were sonicated and centrifuged at 1000rpm. Supernatants were collected and a BCA assay was performed to measure protein levels. Bis-Tris polyacrylamide gels (4-12%) were loaded with 15-20ug of protein. Protein was transferred to PVDF membranes overnight. Membranes were then blocked in 5% milk or 5% BSA for 1hr at room temperature. After blocking, the membranes were incubated in primary antibody overnight at 4°C. Antibodies used: phospho-ERK1/2 (Cell Signaling, 1:3000), ERK1/2 (Cell Signaling, 1:3000), PSD95 (Thermo, 1:5000), GluR1 (Millipore, 1:1000), GluR2 (Millipore, 1:1000), NR2A (Millipore, 1:1000), NR2B (Millipore, 1:1000). Blots were washed and then incubated in HRP-conjugated secondary antibodies. The blots were then washed,

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treated with chemiluminescent substrate and imaged on the LI-COR Odyssey Fc. Densitometry analysis was performed using Odyssey imaging software and statistically analyzed using Graphpad Prism. Densitometry values for each sample were normalized to the average of the control animals and average densitometry of CKO animals were compared to control animals using t-tests.

Golgi Staining

Golgi staining was performed using a Rapid Golgi stain kit (FD Neurotechnologies). Whole mouse brains from P90 mice were stored in impregnantion solution for 10 days, followed by a 5-day incubation in wash buffer. Tissue was frozen and then sectioned into 100μm sections on a cryostat. Sections were then treated and mounted using the FC Golgi stain kit reagents per the manufacturer's protocol. Dendrites and spines were traced using the Neurolucida program on a Zeiss axioplane microscope. 4-6 dendrites (at least 100μm in length) in the stratum radiatum (CA1 pyramidal neurons) were analyzed per mouse with a total of 4-5 mice per genotype. Average spine density, surface area, volume and length were calculated for each dendrite and then multiple dendrites averaged together for each mouse. Averages of each spine parameter in control and CKO mice were compared using t-test.

Electrophysiology

Male and female animals were sacrificed at 10-14 weeks and their brains were removed and placed in ice-cold dissection solution (211mM sucrose, 10mM glucose, 26mM sodium bicarbonate, 2.6mM potassium chloride, 1.25mM sodium monophosphate, 4mM magnesium chloride, 0.5mM calcium chloride). Transverse slices through the hippocampus were made using a vibratome. Slices were allowed to recover in a high magnesium and low calcium ACSF buffer (125mM sodium chloride, 11mM glucose, 25mM sodium bicarbonate, 3.25mM potassium

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chloride, 1.25mM sodium monophosphate, 4mM magnesium chloride, 0.5mM calcium chloride) for 20 minutes (at temp 30° C) and then transferred to regular ACSF (1mM magnesium chloride, 2mM calcium chloride) to recover for 60 minutes (at room temperature). Slices were then transferred to a submerged recording chamber with continuous superfusion and maintained at 30-31° C. A bipolar tungsten stereotrode (WPI) was placed into the stratum radiatum of CA1 for stimulation using constant current pulses of 0.08ms duration and a patch electrode containing ACSF was placed approximately 200um away in the stratum radiatum for measurement of field EPSPs (fEPSP). Test stimuli were administered until fEPSPs were stable. Paired pulse facilitation (PPF) was assessed by administering two stimulating pulses with different inter-pulse intervals (IPIs) at a stimulus amplitude that elicited a half maximal fEPSP amplitude. Two traces for each IPI were averaged together and the paired pulse ratio was calculated as the fEPSP2/fEPSP1. An input-output curve was generated and test stimuli were set to elicit responses with 40-55% maximum amplitude and 20-30% max fEPSP slope at 0.033Hz. When slices showed stable EPSPs for 20 minutes (less than 10% variability), they were stimulated with four theta burst stimulation (TBS) trains. Each train consisted of ten 100Hz bursts of 4 spikes, with a 5Hz burst frequency, each train separated by 20 seconds. Test stimulation resumed and fEPSPs were recorded for 90-100 minutes following TBS. When the recordings were complete, slices were prepared for immunostaining as described above. For analysis of fEPSP slope, four consecutive fEPSP recordings were averaged and the slope of the averaged waveform was measured using software written in Igor Pro. Graphs and statistics were generated in Graphpad Prism software. 4-15 slices for each genotype for recorded and analyzed. LTP, I/O curve and PPF in CKO animals were compared to WT using repeated measure two-way ANOVA with Bonferroni post-hoc analysis. We also analyzed the last 8 min and the first 20min of EPSP

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recordings and compared CKO animals to control animals using a two-way ANOVA analysis with Bonferroni post-hoc analysis.

Behavioral Assays

All tests were performed using 12-14 week old male mice that were handled 2 weeks prior to behavior testing. Tests were conducted in a least stressful (EPM) to most stressful (Morris water maze) sequence with a rest day between each behavioral test.

Morris Water Maze: A pool of water was made opaque using white paint. A platform was hidden under the surface in the middle of one quadrant. Visual cues were present on the walls of the room, which was illuminated by only dim floor lighting. Mice were tracked using Ethovision software. Mice were placed in a quadrant and allowed to locate the platform for 60 sec. If mice did not locate the platform, they were guided to the platform where they remained for 10 seconds. This was repeated 3 more times from a different starting quadrant. The 4-trial training block was repeated after 30minutes. Mice were trained over 4 days. On the last day, the platform was removed for the final trial of the final training block to conduct a probe trial. Mice were placed in the quadrant opposite the target quadrant and allowed to swim freely for 60 seconds while their movement was tracked. 24 hours later, mice were tested using the probe trial setup to measure memory retention. Escape latencies for each block during the training period were averaged and analyzed. Mice that exhibited more than one floating trial per block were excluded from the analysis. Data was analyzed in Graphpad prism software. Escape latencies from each trial block were averaged together and the average escape latency for each trial block was compared between control and CKO animals using a repeated measure two-way ANOVA with Bonferronni post-hoc analysis. Each genotype was compared to control animals in separate tests.

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T-tests comparing different genotypes at specific trial blocks were also performed to highlight any potential trends at a specific trial block.

Novel Object Recognition: Mice were habituated to the testing field 24hr prior to NOR test. On the day of the test, mice were placed in the testing field with two identical objects for 10 minutes. 3 hours later, mice were reintroduced to the same field, but one object was replaced with a novel object of similar size for 5 minutes of exploration. Mice were manually scored for amount of time spent interacting with the objects during the habituation and testing phase. Mice that did not explore objects during the testing phase for at least a total of 4 seconds were excluded from the analysis. Discriminate index (DI) was calculated using the equation (novel time – familiar time)/(novel time + familiar time). Data was analyzed using Graphpad software. T-tests were used to compare DI to fixed value of 0 and also to compare DI between a CKO mouse model and control animals.

Elevated plus maze: Mice were placed in a plus shaped maze with two arms enclosed by walls, two arms unenclosed and a central hub. Mice were placed in the hub and allowed to explore for 5 minutes while tracked using Ethovision. The duration spent in the closed arms, open arms and hub was measured. In addition, entrances into each arm were also scored and analyzed. All data was analyzed in Graphpad software. T-tests were performed between CKO and control animals to compare time spent in the different areas of the maze.

RESULTS

Characterization of ERK2 conditional knockout mice.

We have previously generated an ERK2 CKO mouse line using the Emx1-Cre transgene (ERK2 CKO^{Emx}). Using this model, we have shown that ERK2 is required for normal development of the cortex, but is expendable for development of the hippocampus (Pucilowska

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et al., 2012; Vithayathil et al., 2015). As a follow up to these developmental studies, we wanted to determine if developmental loss of ERK2 in the forebrain would affect post-natal maturation and function of the hippocampus. Previous studies targeting the MAPK pathway post-natally utilized the CamK2a-Cre (Kelleher et al., 2004; Chen et al., 2006), which was used in this study to generate a post-natal knockout of ERK2 (ERK2 CKO^{CamK}) to compare to the developmental knockout (ERK2 CKO^{Emx}). The mutant mice are compared to ERK2$^{flox/flox}$, which are referred to as WT mice in the rest of this study, as no difference in ERK2 expression has been observed in this or previous studies between WT and ERK2$^{flox/flox}$ mice (Samuels et al. 2008).

In order to evaluate ERK1/2 signaling we performed immunostaining of activated phosphorylated ERK1/2 in the CA1 region of the hippocampus in WT, ERK2 CKO^{Emx} and ERK2 CKO^{CamK} mice at P90 after stimulation of CA1 neurons with an LTP stimulus. Both CKO mice appear to show a significant reduction in levels of phosphorylated ERK1/2 in the hippocampus, primarily in the CA1 region (Figure 1A). ERK2 CKO^{CamK} showed residual phospho-ERK immunoreactivity in CA3 and the DG as seen in other reports of CamK-Cre expression (Kelleher et al., 2004). We validated these findings by examining phosphorylated and total ERK1/2 levels in the hippocampus of P90 mice by western analysis (Figure 1B). Both ERK2 CKO mice exhibited no changes in ERK1 protein levels (Figure 1C), but ERK1 activity, measured by phosphorylated ERK1, was significantly increased in both ERK2 CKO^{Emx} and ERK2 CKO^{CamK} mice by 58% (t-test $p<0.001$) and 27% (t-test $p<0.05$), respectively, which is consistent with previous reports (Figure 1D) (Imamura et al., 2008; Pucilowska et al., 2012).

ERK2 CKO^{CamK} expressed normal levels of ERK2 at P10 (Figure 1E), which is consistent with the Camk2a-Cre transgene being expressed around P15-P20 (Tsien et al., 1996). Importantly, at P90 both ERK2 CKO mouse models did show significant decreases in ERK2

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protein levels in the hippocampus. Interestingly, while ERK2 CKO^{Emx} mice showed almost complete loss of ERK2 in the hippocampus (t-test, $p<0.001$), ERK2 CKO^{CamK} mice exhibited an approximate 40% decrease in ERK2, which was statistically significant (t-test, $p<0.01$) (Figure 1F). The CamK2a-Cre does not have high expression in the CA3 or dentate gyrus (DG) of the hippocampus and the magnitude of knockdown is consistent with previous observations using the CamK2a-Cre (Chen et al., 2006).The remaining ERK2 protein in the ERK2 CKO^{CamK} mice was phosphorylated at a normal level indicating that there is no hyperactivation of ERK2 in cells that do not express Cre transgene (Figure1G). These data show that the different Cre transgenes effectively knocked down ERK2 in the hippocampus, although there are some differences in regional expression between the Emx1-Cre and Camk2a-Cre.

Synaptogenesis is intact in CA1 pyramidal cells in CKO mice

While we had previously observed normal neurogenesis in the hippocampus of ERK2 CKO^{Emx} mice, ERK signaling is also known to be important for synaptic development and spine formation (Hans et al., 2004; Giachello et al., 2010; Vithayathil et al., 2015). Therefore, we assessed synaptogenesis by examining dendritic spine density as well as spine length, surface area and volume to determine if synapse formation was impaired. Pyramidal neurons in the CA1 region of the P90 hippocampus were traced and spines in the apical dendrites residing in the stratum radiatum were analyzed (Figure 2A-H). Pyramidal neurons in CA1 showed no differences in spine density, surface, area, length or volume in the ERK2 CKO^{Emx} mice compared to WT mice (Figure 2A-D). We repeated this analysis in the ERK2 CKO^{CamK} mice and found that CA1 pyramidal cells in these mice showed normal spine density, length, surface area and volume as well (Figure 2E-H).

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Additionally, we performed a western analysis of synaptic proteins at excitatory synapses, since dendritic spine counts were performed on a small sample of neurons. We assayed protein levels of PSD95, the AMPA receptor subunits GRIA1 and GRIA2 as well as the NMDA receptor subunits GRIN2A and GRIN2B (Figure 2I-N). We found no significant changes in the expression of any of these synaptic proteins in the hippocampus of ERK2 CKO^{Emx} and ERK2 CKO^{CamK} mice when compared to WT mice at P90. These results, along with the synaptic spine analysis, indicate that synaptic development in CA1 is largely intact in both ERK2 CKO mouse models.

Behavioral phenotypes in the CKO^{Emx} and CKO^{CamK} adult mice.

Spatial Memory

Since depletion of ERK signaling with pharmacological blockers showed impairments in spatial memory and LTP we wanted to determine any behavioral consequences of chronic loss of ERK2 with respect to learning and memory formation as well as other behaviors linked to hippocampal function. First we assessed the spatial memory of ERK2 CKO mice using the Morris water maze (MWM). Mice were trained over the course of 4 days with two trial blocks per day, where each trial block consisted of 4 trials, with the last trial on the fourth day being the probe test. However, during the training period, we observed that a significant number of the CKO^{CamK} mice remained immobile or floated for the entire trial duration (Figure 3A). We did not observe this immobile or floating behavior in the CKO^{Emx} mice (Figure 3A). Animals that failed to search for the platform during the training period consistently were excluded from further analysis. Over the 8 trial blocks WT mice showed a decline in escape latency over the course of training. ERK2 CKO^{Emx} mice were not statistically different from WT mice when the data was analyzed by a repeated measure two-way ANOVA ($F=0.03$, $DFn=1$, $DFd=238$, $p=0.86$) with a

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post-hoc Bonferroni analysis showing no significant differences at individual trial blocks. The ERK2 CKO^{Emx} and ERK2 CKO^{CamK} mice exhibited escape latencies similar to WT mice by the last training day. However, they did show a trend toward delayed learning. ERK2 CKO^{Emx} mice showed a significant difference in escape latency on trial block 3 compared to WT mice (t-test, p=0.03), which was trended towards significance on trial block 4 (t-test, p=0.06) (Figure 3B). ERK2 CKO^{CamK} mice on the other hand exhibited a statistically significant difference compared to WT when analyzed by repeated measure two-way ANOVA (F=4.25; DFn=1, DFd=210; p=0.0481), although a Bonferroni post-hoc analysis did not show significant differences in individual training blocks. However non-significant trends were noted at trial block 3 (t-test, p=0.053) and 4 (t-test, p=0.07) (Figure 3B). These data indicate that while ERK2 CKO^{Emx} and ERK2 CKO^{CamK} showed normal training by trial block 8, these mice may exhibit slightly delayed learning. Memory retention was assessed by conducting probe trails on day four immediately following training and 24 hours later to assess training efficacy and memory retention, respectively. When analyzed by two-way ANOVA, the initial probe trial following training showed that the WT, CKO^{Emx} and CKO^{CamK} mice preferred the target quadrant when compared to the other quadrants in a Bonferroni post-hoc analysis, but no genotype effects were observed (Figure 3C). In the probe trial conducted 24 hours later (24hr Ret), we found that WT and CKO^{Emx} still preferred the target quadrant to a lesser extent, but CKO^{CamK} mice did not appear to have a quadrant preference. It is important to note that the sample size of the CKO^{CamK} during the retention trials was only 5 animals due to the fact that some of the mice would not actively search for the platform, which decreased the power of the statistical analysis. We also assessed both platform crossings and target quadrant duration as measures of spatial memory retention. A repeated measure two-way ANOVA showed no genotype effect when ERK2

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CKO^{Emx} and CKO^{CamK} mice were compared to WT mice in both platform crossings and target quadrant duration in the probe trial and retention trial (Figure 3C,D). The CKO^{CamK} mice do exhibit a trend towards decreased platform crossing during the retention trial (t-test, p=0.15). However, these findings indicate that spatial memory retention in the ERK2 CKO^{Emx} mouse models is largely intact.

Novel Object Recognition

We also assessed novel object recognition memory, which has been primarily shown to require the perirhinal cortex (Brown and Aggleton, 2001; Barker and Warburton, 2011). The ERK2 CKO^{Emx} mice exhibited no changes in novel object preference when compared to WT mice (Figure 3E). Importantly, both WT mice and ERK2 CKO^{Emx} preferred the novel object because their discrimination index showed a significant difference when compared to the hypothetical value of 0 or no object preference (WT: t-test, p=0.0003; CKO^{Emx}: t-test, p=0.03) (Figure 3E). Interestingly, ERK2 CKO^{CamK} mice exhibited a lack of novel object preference and showed a discrimination index that was significantly different from WT animals (t-test, p=0.0464), but not significantly different from 0 (Figure 3E). Thus, ERK2 CKO^{CamK} exhibit no object preference. To determine if the impaired memory performance in the CKO^{CamK} mice was secondary to changes in exploration during the habituation period we assessed object exploration during habituation. Surprisingly, we observed that both ERK2 CKO mouse models exhibited increased object exploration when compared to WT mice (ERK2 CKO^{Emx}: t-test, p=0.01; ERK2 CKO^{CamK}: t-test, p=0.066) (Figure 3F). These findings show that loss of ERK2 in both CKO models resulted in increased novel object exploration, but only the ERK2 CKO^{CamK} exhibit impaired object recognition memory.

Anxiety-like behavior

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The ERK2 CKO^{Emx} mice had previously been reported to exhibit an anxiety-like behavior that was most pronounced when tested on the elevated plus maze (Pucilowska et al., 2012). We examined the ERK2 CKO^{CamK} mice with this test to determine if they exhibited a similar anxiety-like phenotype. ERK2 CKO^{CamK} mice showed no differences in total entrances into the arms of the maze indicating that these mice showed no deficits in exploration or mobility (Figure 3G). However, ERK2 CKO^{CamK} mice did spend less time in the open arms and significantly more time in the closed arms of the maze (t-test, p=0.043) (Figure 3H,I). These data show that the ERK2 CKO^{CamK} mice exhibit a preference for the closed arms consistent with increased anxiety-like behavior.

LTP is intact in the ERK CKO mice.

Lastly, we assessed long-term potentiation in the ERK2 CKO mice. We initially compared input-output curves of CA1 pyramidal neuron, which showed no differences between ERK2 CKO^{Emx}, CKO^{CamK} and WT mice (Figure 4A).

In order to assess presynaptic function, paired pulse facilitation was tested in the different mutant animals (Figure 4B). Paired pulse rations of ERK2 CKO^{Emx} and ERK2 CKO^{CamK} were not significantly different from control animals when compared by two-way ANOVA. This finding suggests there are no gross functional presynaptic defects in the ERK2 deficient animals.

Surprisingly, we also found that the mutant animals did not show significant changes in late phase LTP in the CA3/CA1 circuit when LTP was induced by stimulation of Schaeffer collaterals with a four train TBS protocol (Figure 4C). ERK2 CKO^{Emx} and ERK2 CKO^{CamK} both exhibited LTP that was not significantly different from control animals when analyzed by a repeated measure two-way ANOVA. ERK2 CKO^{Emx} showed a significant interaction between genotype and time ($F=2.06$; $DFn=51$ $DFd=867$; $p<0.0001$), which confounds the genotype effect,

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but no significant effects were seen at any time point in a Bonferroni post-hoc analysis. A two way ANOVA analysis of the last 8 minutes also showed no significant effect of genotype on potentiation in either ERK2 CKO animal model, however there was a trend toward significance in the ERK2 CKO^{CamK} mice (p=0.055), which had a 16% reduction in LTP at 90min post-LTP (no significance by post-hoc analysis). Interestingly, a two-way ANOVA analysis of the minutes 2-20 following TBS did show a genotype effect when ERK2 CKO^{Emx} mice were compared to WT (F=4.72; DFn=1, DFd=126; p=0.0475). These data indicate that loss of ERK2 signaling, developmentally or post-natally does not significantly impact late-phase LTP in CA1 pyramidal cells, but early LTP is enhanced in the ERK2 CKO^{Emx} mice

To determine if the presence of ERK1 was sufficient for normal LTP, we generated an ERK1 and ERK2 double knockout line (ERK1/2 DKO^{CamK}), which were ERK2 CKO^{CamK} mice bred on an ERK1 null background. At P90, the DKO^{CamK} were grossly normal in appearance with normal cytoarchitecture of the hippocampus when stained with cresyl violet (Figure 5A). Immunostaining of phospho-ERK1/2 in the hippocampus of adult P90 mice showed decreased staining in CA1 layer (Figure 5B, B'). Western analysis at P90 also showed decreased ERK2 levels in the hippocampus and absence of ERK1 (Figure 5C-E).

When ERK1/2 DKO^{CamK} hippocampal slices were analyzed for electrophysiological changes at P90, they showed a left shift in I/O curves compared to WT animals (Figure 5F). Repeated measure two-way ANOVA analysis indicated a genotype effect that was statistically significant (F=10.75; DFn=1 DFd=66, p=0.0074). Bonferroni post-hoc test showed significant differences at inputs of 80μA, 90μA and 100μA. At higher stimulation inputs (>200μA) no significant differences were observed. Paired-pulse facilitation was not significantly different from control animals (Figure 5G). LTP was assessed in the DKO^{CamK} animals and when

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compared to control animals by repeated measure two-way ANOVA analysis, no genotype effect on LTP was observed. However, a two-way ANOVA analysis of minute 84-90 post-stimulation did show a statistically significant effect in the DKO^{CamK} mice (F=4.64; DFn=1 DFd=56, p=0.03) (Figure 5H). At 90min post-TBS stimulation, DKO^{CamK} was associated with a 20% reduction in LTP compared to control animals (not significant by post-hoc analysis).

DISCUSSION

Mutations in the ERK/MAPK pathway lead to variety of disorders classified as NCFC syndromes, or RASopathies (Samuels et al., 2009; Rauen et al., 2010). Dissecting the role of the different components of this pathway provides critical insight into both the overlapping and differential presentations of the various NCFC syndromes, specifically the various degrees of cognitive impairment. The ERK/MAPK signaling pathway has been previously associated with a variety of cellular functions. Importantly, MAPK signaling has been implicated as an important pathway governing synaptic plasticity. Pharmacologic and genetic strategies to inhibit MEK activity have resulted in impaired long-term potentiation (LTP) that correlates with impaired spatial memory formation (Adams and Sweatt, 2002; Kelleher et al., 2004; Sweatt, 2004). However, these strategies did not directly assess ERK1/2 signaling. In addition, the acute nature of pharmacologic studies does not accurately represent the chronic dysregulation observed in genetic disorders. We addressed this question by using genetic strategies in order to re-examine the role of ERK1/2 signaling in excitatory neurons in the hippocampus.

We had previously reported that conditional deletion of ERK2 using the Emx1-Cre resulted in loss of ERK2 beginning at E9.5 in the dorsal telencephalon, which includes the hippocampus (Pucilowska et al., 2012). However, we observed that loss of ERK2 signaling at

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this developmental time point did not impair morphogenesis of the hippocampus (Vithayathil et al., 2015). We also generated ERK2 conditional knockouts using a Camk2a-Cre, which results in deletion of ERK2 in the third post-natal week primarily in excitatory neurons of the cortex and CA1. Since this Cre line has been used in other conditional knockouts of upstream components of the MAPK pathway, it served as an ideal model which could be compared to previous studies (Kelleher et al., 2004; Chen et al., 2006). In the current study we found that loss of ERK2 during embryonic or early post-natal development does not affect maturation of the neurons in CA1 or the DG, as we observed no differences in spine density or synaptic proteins in these mice.

In the novel object recognition task, ERK2 CKO^{Emx} mice performed similar to WT mice, while the ERK2 CKO^{CamK} mice exhibited no object preference. While the behavioral differences in the developmental and post-natal knockouts is interesting, the major caveat to comparing the ERK2 CKO^{Emx} and ERK2 CKO^{CamK} lines with respect to behavior is that two different Cre lines were used to generate a knockout of ERK2. The Emx1-Cre and CamK2a-Cre, while largely overlapping, do exhibit small differences in expression patterns, which could account for the behavioral differences seen in the two ERK2 CKO models. The Emx1-Cre caused significant loss of ERK2 in the dentate gyrus DG and CA3 regions, whereas the Camk2a-Cre lines had residual expression of ERK2 in these regions.

We also noted another important difference between the ERK2 CKO^{Emx} and CKO^{CamK} mice with respect to coping strategies in the Morris Water Maze (MWM). During the training phase of the MWM test, CKO^{CamK} mice were more likely to exhibit passive coping strategies, which entailed floating until the end of the trial. On the other hand, CKO^{Emx} and WT mice reflected more active coping strategies by actively searching for the platform. Again, these findings could be the result of differences in regional expression of the Emx-Cre and Camk2a-

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Cre lines. Camk2a-Cre is also sparsely expressed in the striatum, unlike the Emx1-Cre, and loss of ERK activity in the striatum may affect coping behavior in the ERK2 CKO^{CamK} mice since ERK signaling in the striatum has been shown to affect goal-directed behaviors and coping strategies (Koolhaas et al., 1999; Cabib and Puglisi-Allegra, 2012).

It is important to acknowledge that, surprisingly, the loss of ERK2 in both mouse models in this study resulted in very modest spatial memory deficits as well as no overt changes in LTP. ERK2 is present at three times the level of ERK1 in the hippocampus and previous studies showed spatial memory deficits with less than 50% reductions in total ERK activity (Ortiz et al., 1995; Blum et al., 1999; Kelleher et al., 2004; Satoh et al., 2007). We report that ERK2 CKO^{Emx} mice exhibited a slight delay during the training phase on day 2, and ERK2 CKO^{CamK} showed trends towards delayed learning on days 2 and 3. CKO^{CamK} also showed impaired 24 hour retention, but, due to the passive coping behavior, the sample size for the retention tests were very small. The delay in spatial memory is consistent with findings observed in ERK2 hypomorph mice, however, a more severe phenotype was expected given the complete absence of ERK2 in the genetic knockouts. The likely explanation for our findings is that in mice lacking ERK2 signaling, ERK1 is able to compensate for the loss of ERK2 over time, thus permitting normal LTP and spatial memory. This is supported by the fact that ERK1 phosphorylation was elevated in the ERK2 CKO^{Emx} mice and that ERK1/2 DKO^{CamK} mice showed changes to late-phase LTP. Although the effect on LTP in the DKO^{CamK} mice is small compared to the pharmacologic inhibitor data, the changes in the input/output curve indicate potential increased excitability of the pyramidal neurons. A caveat to the LTP findings in the DKO^{CamK} mice is that the small sample size along with the mixed genders may mask gender differences. In addition,

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primarily male mice were used in the LTP studies since our behavior studies were also conducted in male mice.

Interestingly, ERK1 knockout mice, which have intact spatial memory, have been previously reported to have decreased LTP by theta burst stimulation, but intact LTP using a high frequency 100Hz stimulation protocol (Selcher et al., 2001). Furthermore, the recruitment of the ERK/MAPK pathway during various stimulation protocols is unclear, with some studies showing that the theta burst stimulation (TBS) protocol is a more robust activator of the MAPK pathway compared to the high frequency 100Hz stimulation protocol (Winder et al., 1999; Selcher et al., 2003; Zhu et al., 2015).

Alternatively, ERK1 may not be sufficient to compensate for loss of ERK2 without recruitment of other compensatory mechanisms. Previous genetic models of impaired ERK signaling used a conditional knock-in of a dominant negative MEK1 and the ERK2 hypomorph. However, these models do not appear to affect baseline ERK phosphorylation and decreased ERK activity is seen only in response to stimulation (Kelleher et al., 2004; Satoh et al., 2007). In the ERK2 CKO mice we examined, baseline levels of ERK activity have been permanently altered and could potentially induce compensatory pathways and mechanisms. Interestingly, conditional knockouts of ERK2 using a Nestin-Cre caused significant behavioral changes that were not exacerbated by further inhibition of ERK1 using MEK inhibitors which suggests that ERK1 cannot compensate for loss of ERK2 with respect to some behavioral tasks, namely fear conditioning and social interaction (Satoh et al., 2011).

The incongruence between genetic and pharmacologic findings is not without precedence. Recently, a conditional knockout of PKM-ζ using the CamK2a-Cre revealed no impaired LTP or spatial memory even though pharmacologic studies had previously identified a

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function for PKM-ζ for LTP formation (Volk et al., 2013). Similarly, knockouts of Shp2, an activator the MAPK pathway, using the CamK2a-Cre also found that LTP and spatial memory to be largely intact with minor delays in learning in the Morris Water Maze (Kusakari et al., 2015). The Shp2 CKO mice also highlighted the complexity of the regulatory mechanisms that modify MAPK/ERK activity, as ERK1/2 activity was upregulated or downregulated in the Shp2 CKO mice depending on the manner of stimulation of neural networks (Kusakari et al., 2015). MAPK/ERK signaling clearly has a role in LTP and spatial memory based on all the previous studies, however, the data in the present study provides further information regarding the role of ERK1/2 signaling in the context of diseases that impair activity in a chronic timescale.

In summary, loss of ERK2 in the forebrain of two different mouse models resulted in delayed spatial learning, but intact spatial memory retention, which was consistent with the observation that LTP is intact in both ERK2 CKO mice. Normal LTP in these mice is likely due to either other compensatory mechanisms or the presence of ERK1, as loss of both ERK1 and ERK2 resulted in decreased late phase LTP. Interestingly, the ERK1/2 DKO^{CamK} mice are the first ERK1/2 double knockout mouse line that is not lethal. This would suggest that ERK signaling is more important during development compared to its post-natal functions. While the results of this study do not obviate a role of ERK signaling for LTP and spatial memory, it does provide further insight into the region specific functions for ERK signaling that mediate specific behavioral and physiologic processes.

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FIGURE 1 – ERK2 is absent in the entire hippocampus of CKO^{Emx} mice and primarily CA1 of CKO^{CamK} mice.

(A) Immunostaining of phospho-ERK1/2(green) in the hippocampus of WT, CKO^{Emx} and CKO^{CamK} mice at P90 following theta-burst stimulation of CA1. Bottom row magnification of boxed areas showing CA1 region of the hippocampus in the WT, CKO^{Emx} and CKO^{CamK} mice. sp=stratum pyramidale, sr=stratum radiatum.

(B) Western blot of hippocampal tissue from WT, CKO^{Emx} and CKO^{CamK} mice at P90.

(C-F) Densitometric analysis of relative protein levels of (C) total ERK1, (D) ERK1 activity (phospho-ERK1 normalized to ERK1), (E,F) total ERK2 protein at P10 (E) and P90 (F) and (G) ERK2 activity (phospho-ERK2 normalized to ERK2 levels) in WT (n=16), CKO^{Emx} (n=8), and CKO^{CamK} (n=6). *p<0.05, **p<0.01, ***p<0.001 using t-test.

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FIGURE 2 – ERK2 CKO^{Emx} and CKO^{CamK} mice exhibit normal spine densities of apical dendrites in CA1.

(A-D) Representative image of apical dendrite with analysis of (A) spine density, (B) spine surface area, (C) spine length and (D) spine volume in apical dendrites of CA1 pyramidal neurons and dentate gyrus granule cells in P90 WT (n=5) and CKO^{Emx} (n=5) mice.

(F-I) Analysis of (B) spine density, (C) spine surface area, (D) spine length and (E) spine volume in apical dendrites of CA1 pyramidal neurons in P90 WT (n=4) and CKO^{CamK} (n=4) mice.

(J-N) Relative protein levels of (J) PSD95, (K) GRIA2, (L) GRIA1, (M) GRIN2A and (N) GRIN2B in the P90 hippocampus of WT (n=9), CKO^{Emx} (n=6), CKO^{CamK} (n=5) using densitometry analysis of western blots.

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FIGURE 3 – Behavioral assessment of spatial memory, object recognition memory and anxiety in ERK2 CKO mice.

(A-F) *Morris Water Maze:* (A) WT, CKO^{Emx} and CKO^{CamK} mice were assessed for the number of trials where they remained immobile for the entire trial indicative of passive coping. **p<0.01 (B) Evaluation of training during the Morris water maze by measuring escape latencies against 8 trial blocks conducted over 4 days. p-values noted are from t-tests of CKO animals compared to WT. (C-D) Time spent in each quadrant of the arena during a probe trial on day 4 (Probe) and on day 5 (24hr ret) to assess for memory retention. *p<0.05, **p<0.01, ***p<0.001 from post-hoc Bonferroni test. Probe trial on day 4 (probe) and day 5 memory retention (24hr ret) were measured by looking at (E) platform location crossings and (F) target quadrant duration.

(G-H) *Novel Object Recognition*: (G) Novel object preference was assessed in P90 WT, CKO^{Emx} and CKO^{CamK} mice. t-test, *p<0.05 (compared to a hypothetical value of 0 or no preference), #p<0.05 (compared to WT). (H) Object exploration during the habituation phase was analyzed in mice as well showing elevated object exploration in CKO mice. **p<0.01 using t-test.

(I-K) *Elevated Plus Maze*: (I) Total arm entrances made by WT and CKOCamK mice during the duration of the test. (J) Time spent in the open arms of the maze and (K) time spent in the closed arms of the maze. *p<0.05 using t-test.

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FIGURE 4 – LTP is intact in mice lacking ERK2 in the hippocampus.

(A) Input/Output curves of CA3 to CA1 connection in WT (n=8), CKO^{Emx} (n=5), and CKO^{CamK} (n=9).

(B) Paired pulse facilitation in WT (n=7), CKO^{Emx} (n=5) and CKO^{CamK} (n=5) mice at different inter-pulse intervals.

(C) CA1 long-term potentiation (LTP) following a 4 train TBS stimulation after 20 minutes of baseline recordings from WT (n=12: 6males, 6females), CKO^{Emx} (n=5: 4 males, 1female) and CKO^{CamK} (n=6: 6 males) hippocampal slices. *$p<0.05$ (Genotype effect from 2-way ANOVA).

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FIGURE 5 – LTP is impaired in mice lacking ERK1 and ERK2 in the hippocampus.

(A) Cresyl Violet staining of hippocampus in P90 WT and DKO^{CamK} mice.

(B) Immunostaining of phospho-ERK1/2(green) in the hippocampus of WT and DKO^{CamK} mice at P90 following theta-burst stimulation of CA1. Bottom row magnification of boxed areas showing CA1 region of the hippocampus in the WT and DKO^{CamK} mice. sp=stratum pyramidale, sr=stratum radiatum.

(C) Western blot of hippocampal tissue from WT and DKO^{CamK} mice at P90.

(D,E) Densitometric analysis of relative protein levels of (C) total ERK1, (D) total ERK2 protein in WT and DKO^{CamK} mice. *$p<0.05$, ***$p<0.001$ using t-test.

(F) Input/Output curves of CA3 to CA1 connection in WT (n=12) and DKO^{CamK} (n=5) mice. *$p<0.05$, ***$p<0.001$ from Bonferroni post-hoc test (significant genotype effect in two-way ANOVA).

(G) Paired pulse facilitation in WT (n=7) and DKO^{CamK} (n=7) mice at different inter-pulse intervals.

(H) CA1 long-term potentiation (LTP) following a 4 train TBS stimulation after 20 minutes of baseline recordings from WT (n=12: 6males, 6females) and DKO^{CamK} (n=5: 2males, 4females) hippocampal slices. DKO^{CamK}. *$p<0.05$ using one way ANOVA of minutes 84-90 (bar).

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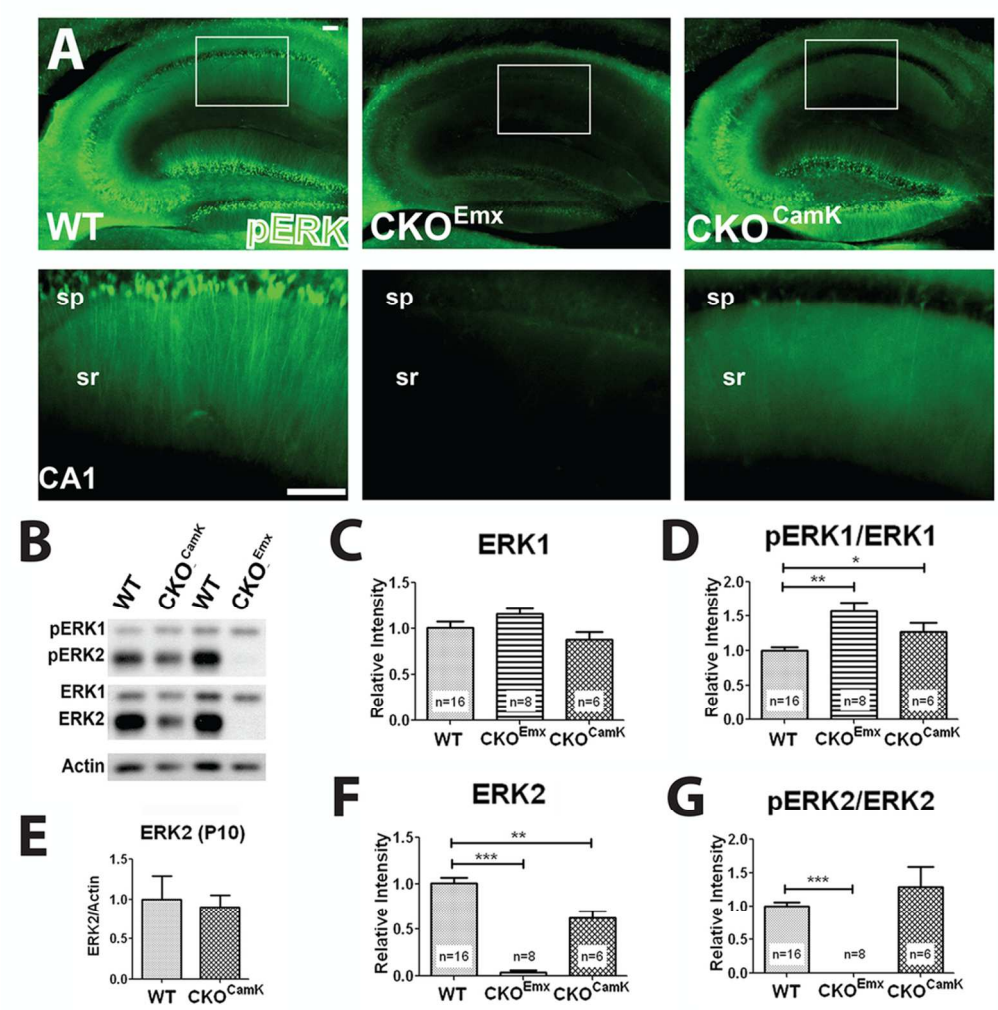

FIGURE 1 – ERK2 is absent in the entire hippocampus of CKO^{Emx} mice and primarily CA1 of CKO^{CamK} mice.

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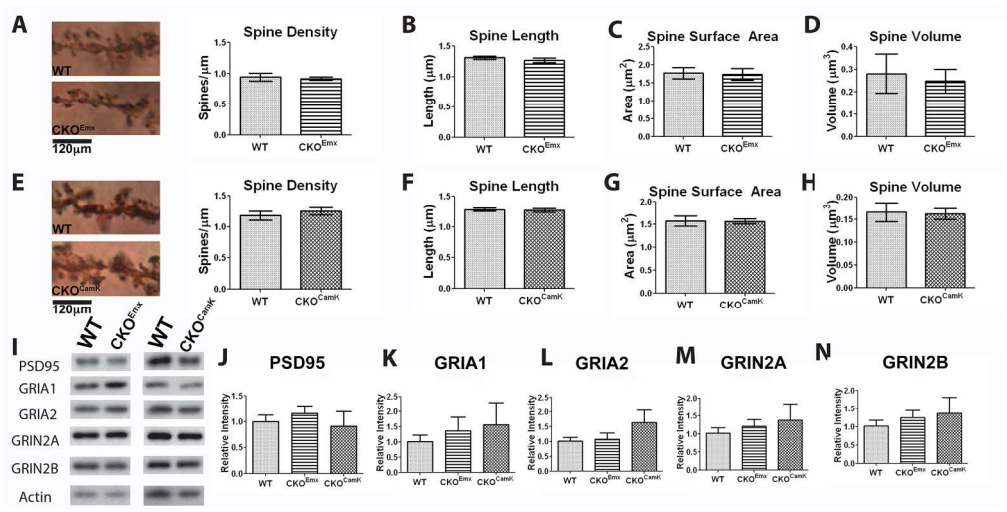

FIGURE 2 – ERK2 CKO^{Emx} and CKO^{CamK} mice exhibit normal spine densities of apical dendrites in CA1.

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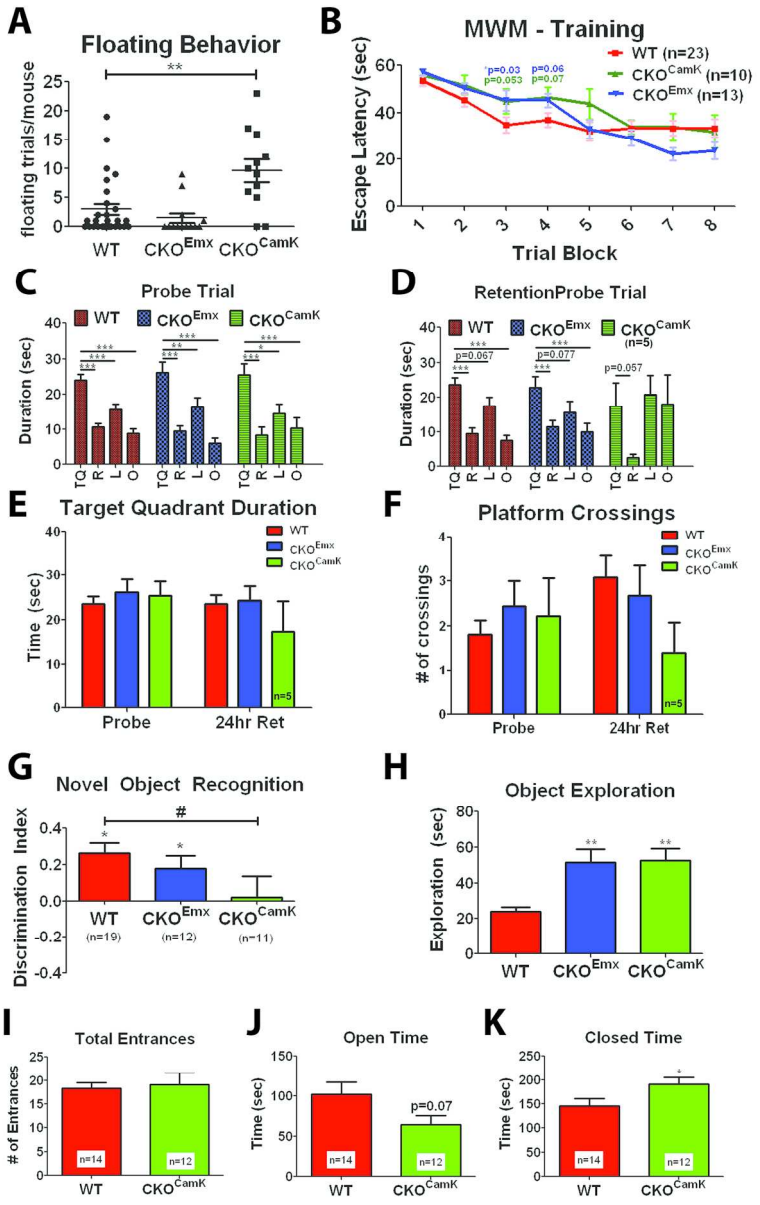

FIGURE 3 – Behavioral assessment of spatial memory, object recognition memory and anxiety in ERK2 CKO mice.

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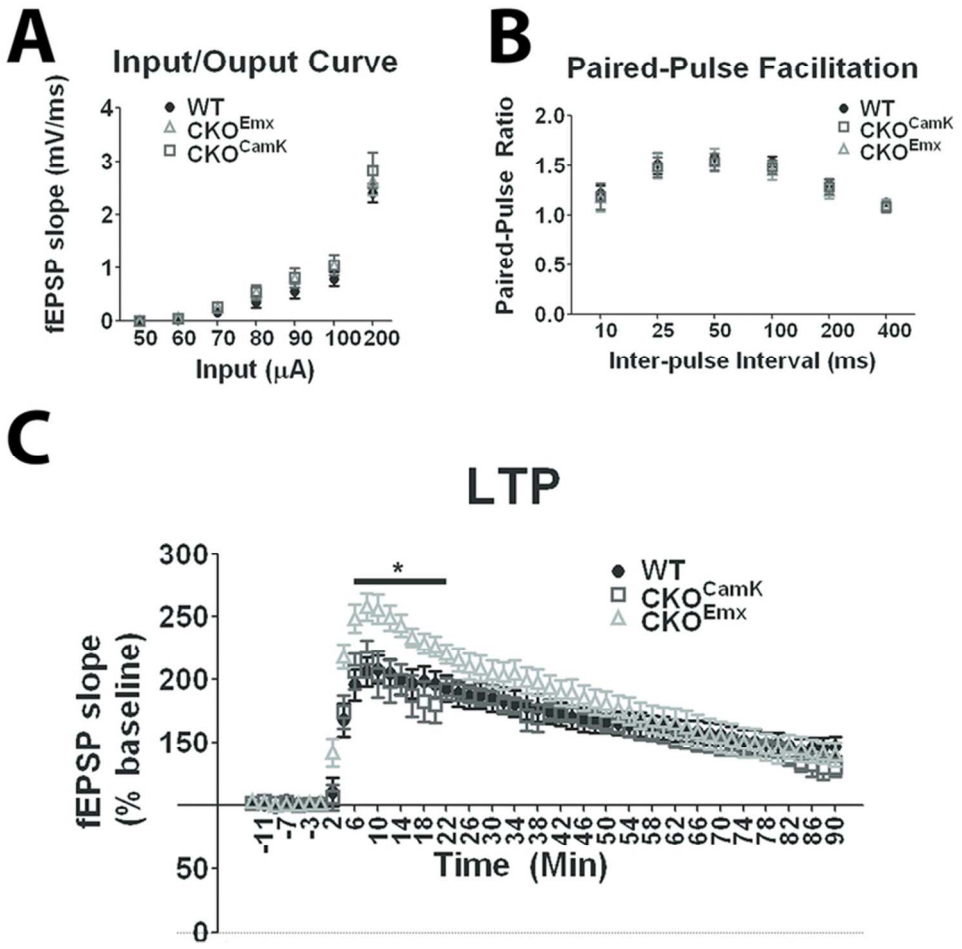

FIGURE 4 – LTP is intact in mice lacking ERK2 in the hippocampus.

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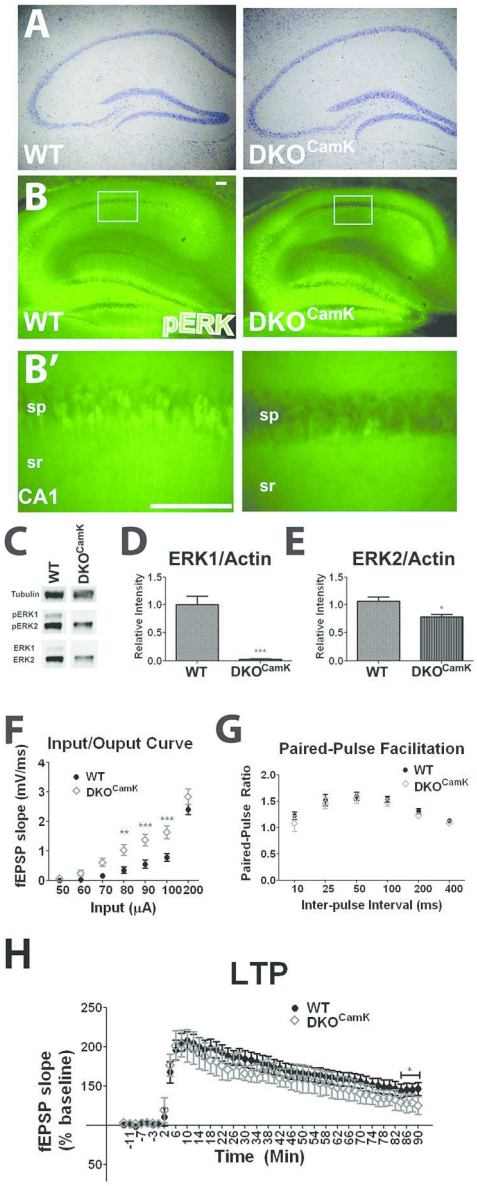

FIGURE 5 – LTP is impaired in mice lacking ERK1 and ERK2 in the hippocampus.

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