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Altered NMDA Receptor Function in Primary Cultures of Hippocampal Neurons from Mice Lacking the *Homer2* Gene

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Abstract

N-Methyl-D-Aspartate (NMDA) receptors are inhibited during acute exposure to ethanol and are involved in changes in neuronal plasticity following repeated ethanol exposure. The postsynaptic scaffolding protein Homer2 can regulate the cell surface expression of NMDA receptors *in vivo*, and mice with a null mutation of the *Homer2* gene exhibit an alcohol-avoiding and –intolerant phenotype that is accompanied by a lack of ethanol-induced glutamate sensitization. Thus, *Homer2* deletion may perturb the function or acute ethanol sensitivity of the NMDA receptor. In this study, the function and ethanol sensitivity of glutamate receptors in cultured hippocampal neurons from wild-type (WT) and *Homer2* knock-out (KO) mice were examined at 7 and 14 days *in vitro* (DIV) using standard whole-cell voltage-clamp electrophysiology. As compared to wild-type controls, NMDA receptor current density was reduced in cultured hippocampal neurons from *Homer2* KO mice at 14 DIV, but not at 7 DIV. There were no genotype-dependent changes in whole-cell capacitance or in currents evoked by kainic acid. The GluN2B-selective antagonist ifenprodil inhibited NMDA-evoked currents to a similar extent in both wild-type and *Homer2* KO neurons and inhibition was greater at 7 versus 14 DIV. NMDA receptor currents from both WT and KO mice were inhibited by ethanol (10–100 mM) and the degree of inhibition did not differ as a function of genotype. In conclusion, NMDA receptor function, but not ethanol sensitivity, is reduced in hippocampal neurons lacking the *Homer2* gene.

Abstract
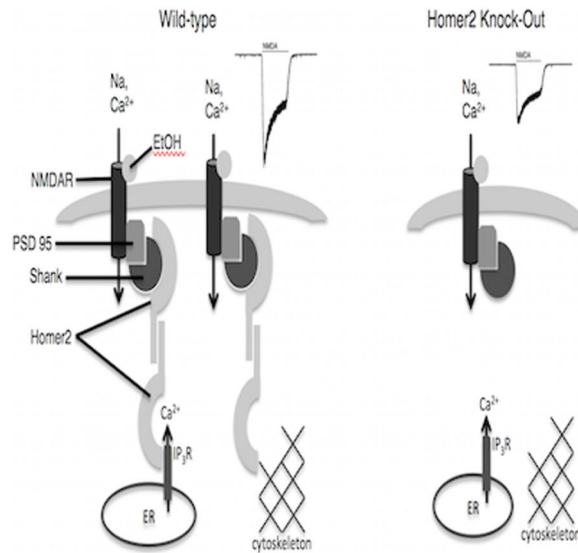
Introduction

N-methyl-D-aspartate (NMDA) receptors are ligand-gated ion channels activated by the neurotransmitter glutamate. These receptors play an important role in the neuroadaptations observed in synaptic structure and receptor distribution following repeated exposure to drugs of abuse, including alcohol (Chandler, 2003; Chandler et al., 2006). As reviewed elsewhere (Shiraishi-Yamaguchi and Furuichi, 2007), the postsynaptic scaffolding proteins known as Homer (Vesl) also appear to be critical in the reorganization of synaptic structure associated with various forms of neuronal plasticity. The Homer family of proteins are products of three different mammalian genes (*Homer1*,- *2*, and *–3*) whose expression is regulated by neuronal activity (Xiao et al., 2000). Constitutively expressed Homer proteins (e.g, Homer1b/c, Homer2a/b, and Homer3) contain a N-terminal Enabled/Vasp homology domain (EVH1) domain that allows for an interaction with Group 1 metabotropic glutamate receptors (mGluRs), inositol 1,4,5-trisphosphate receptors (IP$_3$) and other scaffolding proteins, such as Shank. These Homer proteins also express a C-terminal coiled-coil domain that mediates oligomerization (Brakeman et al., 1997; Fagni et al., 2002; Kato et al., 1998; Naisbitt et al., 1999). Homer, through concatenated interactions with Shank, guanylate kinase-associated protein (GKAP) and post-synaptic density protein (PSD-95), can cluster NMDA receptors and mGluRs with intracellular IP$_3$- or ryanodine-sensitive Ca^{2+} stores to synaptic regions of the cell (Naisbitt et al., 1999; Tu et al., 1999).

Deletion of the *Homer2* gene in mice produces a number of abnormalities in glutamatergic signaling and in the nucleus accumbens, these include reduced basal extracellular glutamate content, reduced function and total protein expression of both mGluR1 and the cystine-glutamate exchanger, as well as reduced plasma membrane expression of the NR2 subunits

of the NMDA receptor (Szumlinski et al., 2004; Szumlinski et al., 2005). In the hippocampus, Homer proteins may help regulate the trafficking and clustering of NMDA receptors within dendritic spines (Ehlers, 2002; Fagni et al., 2002; Sala et al., 2001) where various forms of synaptic plasticity including long-term potentiation (LTP) take place. Alterations in hippocampal spine dynamics, NMDA receptors (Carpenter-Hyland et al., 2004b) and glutamatergic plasticity (Zorumski et al., 2014) may underlie some of the cognitive deficits observed in alcohol-dependent subjects. Given the altered ethanol phenotype of *Homer2* KO mice, it can be speculated that some of these changes may arise from an alteration in the function or ethanol sensitivity of the NMDA receptor. In this study, we employed an electrophysiological approach to examine NMDA receptor function and the ethanol sensitivity of these receptors in primary cultures of hippocampal neurons from WT and *Homer2* KO mice.

MATERIALS AND METHODS

Subjects

Mice with null mutations of *Homer2* and their WT littermates (F10-12; C57BL/6J X 129Xi/SvJ) were generated and maintained by heterozygous mating as described previously (Szumlinski et al., 2004). Breeding pairs of WT and KO mice were housed in an AAALAC-approved animal facility in standard mouse cages (lights on: 0800 h; 25 ° C) with food and water available *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (2011).

Preparation and maintenance of hippocampal neuronal cultures

Neuronal cell cultures were prepared and maintained as previously described (Smothers et al., 1997). Briefly, hippocampi were excised from E18-19 WT or *Homer2* KO mouse fetuses, trypsinized, triturated, and the resulting dissociated cells distributed onto poly-l-lysine-coated 35 mm dishes at a density of 300 – 400 thousand/dish. The plating medium for these cells consisted of Neurobasal media supplemented with 10% fetal bovine serum, 10% heat-inactivated horse serum, and 2 mM L-glutamine. After 24 hours, the plating medium was replaced with Neurobasal media supplemented with B-27 and 2 mM L-glutamine. Cultures were maintained at 37°C in a humidified 5%/90% air mixture. The mitotic inhibitor, 5-fluoro-2-deoxyuridine + uridine (Fd-U), was added to the medium after 4–5 days in vitro (DIV) to retard the proliferation of non-neuronal cells. Culture media was replenished every 4 days. All media components were purchased from Invitrogen (Carlsbad, CA). Fd-U was obtained from Sigma Chemical Company (St. Louis, MO).

Electrophysiology

Whole-cell voltage-clamp recordings were performed on neurons 7–14 DIV. Individual culture dishes were mounted on the stage of an Olympus IX50 microscope and continuously perfused at room temperature (23°C) with an external solution of the following composition (in mM): NaCl 150, KCl 2.5, $CaCl_2$ 2.0, HEPES 10, glucose 10 and glycine 0.01. The pH was adjusted to 7.4 with NaOH and the osmolality was adjusted to 325 mOsm/kg H_2O using sucrose. Tetrodotoxin (100 nM) was added to the external solution to block action potential

firing. Patch electrodes (resistances between $4 - 7$ MΩ) were pulled from borosilicate glass capillaries (Warner Instrument LLC, Hamden, CT) and filled with an internal solution of the following composition (mM): N-methyl-D-glucamine 100, CsCl 40, HEPES 10, $MgCl_2$ 2, Na_2ATP 4, and EGTA 10, pH 7.4 Neurons were voltage-clamped at -50 mV using an Axopatch 200B amplifier (Axon Instruments, Union City, CA) in whole-cell voltage-clamp mode. Current records were filtered at 1 kHz (eight-pole Bessel filter) and digitized at 2kHz using an Apple Macintosh G4 computer (Apple Computer Inc. Cupertino, CA) and a 16-bit analog-to-digital converter (ITC-16; Instrutech Corp., Port Washington, NY). Software control of data acquisition and agonist application was performed by the Pulse Control module running under the Igor Pro program (version 4.03; WaveMetrics, Portland, OR). Leak currents were continually monitored as an indicator of seal and cell integrity and cell capacitance values were recorded.

Drug solutions were prepared in the external solution from either frozen stock or powder. Internal solutions were used for each experiment from frozen stocks. Solutions were applied to cells using a SF-77A Perfusion Fast Step apparatus (Warner Instrument LLC, Hamden, CT) which consisted of a three-barrel, linear-arrayed glass capillary tube (ID 700 μm) that was positioned within 50 μm of the cell body. Drug containing solutions were perfused from the center barrel while control solution was continuously perfused through adjacent barrels. Solution exchange was performed by computer-controlled lateral movement of the barrel assembly. Ethanol was obtained from Aaper Alcohol and Chemical Company (Shelbyville, KY). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Whole-cell voltage-clamp recordings from hippocampal neurons were carried out using a 6 second drug application. Current amplitude was measured at the end of the agonist application. The inhibition of NMDA receptor currents ($I_{Control}$) was calculated as percent ethanol inhibition $= [1-(I_{NMDA+EtOH}/I_{Control})] \times 100$, where $I_{NMDA+EtOH}$ represents the response to co-application of NMDA + ethanol, and $I_{Control}$ represents the average of two responses to NMDA, one before and one after the co-application of ethanol.

Data analysis

Statistical analysis was performed using Prism 4.0 (Graphpad Software, Inc., San Diego, CA) using either Analysis of Variance (ANOVA) followed by post-hoc testing or the Student's unpaired t-test. Statistically significant differences were those having a p value less than 0.05. Data for ethanol inhibition are expressed as percent of averaged control response (mean ± S.E.M.) unless otherwise noted and n represents the number of cells.

RESULTS

Hippocampal neurons from WT or *Homer2* KO mice were grown as a mixed neuronal/glial population in a dissociated cell culture. *Homer2* KO hippocampal neurons exhibited no gross abnormalities in morphology or density relative to WT neuronal cultures. In addition, no genotypic differences were observed in growth, differentiation or survival of the cultured neuronal population.

NMDA-induced whole-cell current in hippocampal cultures

NMDA receptor currents were examined in 14 DIV hippocampal neurons from WT and *Homer2* KO cultures using standard whole-cell voltage-clamp techniques. As illustrated in Figure 1A, application of 100 μM NMDA (in the presence of 10 μM glycine) induced inward currents in neurons from both genotypes at a holding potential of −50 mV. However, currents from *Homer2* KO mice were generally smaller compared to those obtained from WT neurons. To exclude the possibility that this resulted from differences in neuronal cell size, the concentration-response relationship for NMDA-induced currents was normalized to whole-cell capacitance (Figure 1B). The capacitance values were 15.2 ± 1.5 pF ($n = 18$) for WT neurons and 19.3 ± 1.6 pF ($n = 16$) for *Homer2* KO neurons and were not significantly different (*t*-test, p > 0.05). Despite the reduction in current density over the range of NMDA concentrations used, there was no difference in the calculated EC_{50} values for NMDA between WT (27.4 μM) and KO (32.6 μM) animals. There was also no genotype-specific difference in macroscopic desensitization of NMDA responses in the 14 DIV cultures. The steady-state to peak ratio (SS/Pk) in neurons from WT mice (0.79 ± 0.02) was not different from that obtained in neurons from *Homer2* KO mice (0.74 ± 0.04).

NMDA-induced currents in cultured hippocampal neurons are maximal at 14 DIV and do not change significantly between 14 and 21 DIV (data not shown). However, during the first 7–10 days in culture, NMDA receptors show increases in density and a change in subunit composition (Williams et al., 1993). Homer2 expression is also developmentally regulated with the highest mRNA expression at PN8 and lower levels at PN15 (Kato et al., 1998). To determine whether the reduction in NMDA receptor current density observed at 14 DIV is also present at earlier times, NMDA-induced currents were examined in WT and *Homer2* KO hippocampal neurons at 7 DIV (Figure 2A). As previously observed, the amplitude of NMDA-evoked currents at 7 DIV was less than that measured at 14 DIV (Figure 2A). However, unlike more mature cultures, there was no significant difference in the amplitude of NMDA currents between WT and *Homer2* KO mice at 7 DIV (Figure 2B). WT and *Homer2* KO neurons also did not differ in their apparent affinity for NMDA as EC_{50} values for WT and KO neurons were 38.1 μM and 23.8 μM, respectively (Figure 2B). As observed for 14 DIV cultures, no changes were observed between the values for whole-cell capacitance of WT (14.1 ± 1.1 pF; $n = 4$) and KO neurons (19.1 ± 1.9 pF; $n = 6$) or in SS/Pk ratio (WT, 0.76 ± 0.06; *Homer2*KO, 0.74 ± 0.06).

To determine if deletion of *Homer2* alters the transition in GluN2 subunit composition that occurs during development, the effect of the GluN2B-selective antagonist, ifenprodil, was examined in WT and KO cultures. Ifenprodil (10 μM) significantly inhibited NMDA-induced currents in both WT and KO neurons at 7 DIV and 14 DIV (Figure 3). Consistent with previous studies (Williams et al., 1993) the magnitude of ifenprodil inhibition decreased with time in culture. However, there were no significant differences in the magnitude of ifenprodil inhibition of NMDA-induced currents between wild-type and *Homer2* KO neurons at either time point.

Kainate-induced whole-cell current in hippocampal cultures

Additional experiments were performed to examine whether the downward shift in the NMDA dose response observed in *Homer2* KO mice was specific for NMDA receptors. As shown in Figure 4A, kainic acid (100 uM) induced large non-desensitizing currents in both WT and KO neurons at 14 DIV. However, unlike NMDA responses, the amplitude of these currents was not different between WT and KO mice (Figure 4B). As reported previously, there were also no differences in whole-cell capacitance values between WT (15.2 ± 1.5 pF; $n = 18$) and KO neurons (19.3 ± 1.6 pF; $n = 16$).

Ethanol sensitivity of NMDA receptors

NMDA receptors are inhibited during acute exposure to ethanol (Lovinger et al., 1989) and the degree of inhibition is affected by receptor subunit composition (Jin and Woodward, 2006; Lovinger, 1995; Masood et al., 1994; Mirshahi and Woodward, 1995; Smothers et al., 2001). To determine whether *Homer2* deletion influenced the ethanol sensitivity of NMDA receptors, NMDA-induced currents were measured in the absence and presence of ethanol in 14 DIV hippocampal neurons from WT and KO mice. Ethanol, at concentrations from 10–100 mM, inhibited steady-state NMDA-induced currents in both WT and KO hippocampal cultures (Figure 5A). As shown by the sample traces, this inhibition was rapid in onset and currents fully reversed upon washout. There were no differences in the extent of ethanol inhibition of NMDA currents between WT and KO mice at any ethanol concentration tested (Figure 5B). To determine whether deletion of Homer2 may affect ethanol inhibition during initial application of NMDA, we measured the peak NMDA current in the absence and presence of 100 mM ethanol. Ethanol inhibition of peak NMDA currents in wild-type neurons (percent of control; 24.20 ± 2.53, n=12) was not significantly different from that obtained with Homer2 KO neurons (percent of control 22.03 ± 3.77, n=6). There was also no genotype-specific difference in the SS/Pk ratio in the presence of ethanol (data not shown).

DISCUSSION

This study provides electrophysiological characterization of NMDA receptor function in primary cultures of neurons prepared from *Homer2* KO mice. At 14 DIV, the current density of NMDA receptors in hippocampal neurons derived from *Homer2* KO mice was approximately half that observed in WT neurons. The observed deficit in NMDA receptor current density produced by *Homer2* deletion is unlikely due to gross abnormalities in neuronal morphology as no differences were observed in whole-cell capacitance – an electrophysiological measure of cell size (Baginskas and Raastad, 2002). Furthermore, it is unlikely that the deficit is due to a non-selective disturbance of ionotropic glutamate receptor expression since currents induced by kainic acid were not affected in *Homer2* KO cultures. The reduction in NMDA receptor current density is consistent with the role of molecular scaffolding proteins such as Homer2 in development and maturation of post-synaptic spines (Shiraishi et al., 2003; Shiraishi-Yamaguchi et al., 2009). In mature neuronal cultures, over-expression of Shank and long-form Homer1 isoforms increases spine head diameter and PSD-95 staining intensity (Sala et al., 2001), while over-expression of the dominant negative Homer isoform, Homer1a, reduces spine size, levels of NR1 protein and

NMDA receptor-mediated EPSCs (Sala et al., 2003). Homer2 isoforms also co-cluster with NMDA receptors within the synapse in mature hippocampal cultures (Shiraishi et al., 2003), where their ability to interact with the activated form of the small GTPase Cdc42 regulates synapse number and the amplitude of miniature EPSPs (Shiraishi-Yamaguchi et al., 2009). In cultures of hippocampal neurons, a mutation that impairs Homer2-Cdc42 interactions reduces the number of synapses, as well as EPSP amplitudes (Shiraishi-Yamaguchi et al., 2009). The former result is in line with the results of a recent study of constitutive *Homer2* KO mice indicating that an absence of Homer2 increases the density of immature, long, thin dendritic spines, at least within the nucleus accumbens of adult mice (McGuier et al., 2015). Although dendritic morphology within the hippocampus of *Homer2* null mutant mice has yet to be determined *in situ*, the present finding of reduced NMDA receptor conductance in these animals could reflect a disproportionate number of immature, long, thin spines, which typically lack a major PSD network (Jones and Powell, 1969; Peters and Kaiserman-Abramof, 1970).

NMDA receptor density normally increases with developmental age and this is observed both *in vivo* and *in vitro* cultures of neurons (Williams et al., 1993). This increase in receptor function is accompanied by a shift in subunit expression from GluN2B- to GluN2A-containing receptors (Li et al., 1998; Williams et al., 1993), resulting in altered function and pharmacological sensitivity of the receptor (Cull-Candy et al., 2001; Kirson and Yaari, 1996; Williams et al., 1994). Although Homer2 and GluN2B-containing NMDA receptors exhibit coincident localization throughout neuronal development (Shiraishi et al., 2003), the average amplitude of NMDA currents in *Homer2* KO neurons was reduced at 14 DIV, but not 7 DIV, in the current study. This observation suggests that Homer2 may be especially important as neurons undergo a shift in NR2 subunit expression during later maturation. However, the results from the ifenprodil experiments suggest that this shift in subunit expression as measured functionally may not be affected by *Homer2* deletion as ifenprodil inhibited NMDA receptor current to a similar extent in 14 DIV cultures of WT and KO neurons. These findings suggest that, although total NR2 subunit expression may be reduced in *Homer2* KO mice (Szumlinski et al., 2005), the developmentally regulated change in subunit composition of NMDA receptors is not grossly impaired. Consistent with this interpretation, a recent report failed to detect genotypic differences in GluN2 subunit expression within the PSD fraction of homogenates from the nucleus accumbens of WT and *Homer2* mice (McGuier et al., 2015).

Although *Homer2* deletion significantly reduced whole-cell NMDA currents, the inhibition of NMDA receptor currents by ethanol was not altered in *Homer2* KO hippocampal neurons. This finding was somewhat surprising as previous behavioral studies suggested that the ethanol sensitivity of NMDA receptors might be enhanced in *Homer2* KO mice (Szumlinski et al., 2005). In that study, *Homer2* KO mice showed longer duration of the loss of righting reflex (LORR) following an acute injection of a sedative dose of ethanol, that may reflect, at least in part, inhibition of NMDA receptors. However, LORR following ethanol injection was not altered in mice expressing ethanol-resistant NMDA receptors (den Hartog et al., 2013) suggesting that other channels or processes are responsible for this effect of ethanol. In addition to differences in ethanol-induced sedation, *Homer2* KO mice

also fail to develop behavioral or neurochemical sensitization following repeated alcohol exposure (Szumlinski et al. 2005). Although other receptor systems and processes must be considered, the results of the present study appear to rule out a difference in the acute ethanol sensitivity of the NMDA receptor as mediating these effects. However, it is possible that genotypic differences in behavioral responses to ethanol may be related to the significant reduction in NMDA current density observed in the *Homer2* KO mice as this should, in theory, augment sensitivity to alcohol and other NMDA receptor antagonists. Indeed, *Homer2* KO mice exhibit increased behavioral responsiveness to both phencyclidine and MK-801 (Szumlinski et al., 2006; Szumlinski et al., 2004), as well as the competitive NMDA antagonist CPP (Szumlinski et al. 2005). The reduction in NMDA receptor function observed in *Homer2* KO neurons could also impair post-translational processes (e.g., kinase or phosphatase activity) that influence receptor trafficking that may underlie some ethanol-induced neuroadaptations (Carpenter-Hyland et al., 2004a; Chandler, 2003). While we have yet to test this hypothesis directly, Homer2 and NMDA receptor subunit expression is co-regulated with activated PI3K, PKCε, and/or ERKs within several limbic regions of ethanol-experienced animals (Cozzoli et al., 2012; Cozzoli et al., 2015; Cozzoli et al., 2014; Cozzoli et al., 2009; Goulding et al., 2011) in a manner consistent with the hypothesis that alcohol regulation of Homer2-NMDA receptor clustering may be critical for ethanol-induced neuroplasticity of relevance to the development and treatment of alcoholism. Finally, it is important to note that all experiments in the present study used primary cultures prepared from embryonic wild-type and knock-out mice. While we think it unlikely, it is possible that different results might be obtained from studies utilizing brain slices from adult animals. Future studies are needed to address this possibility.

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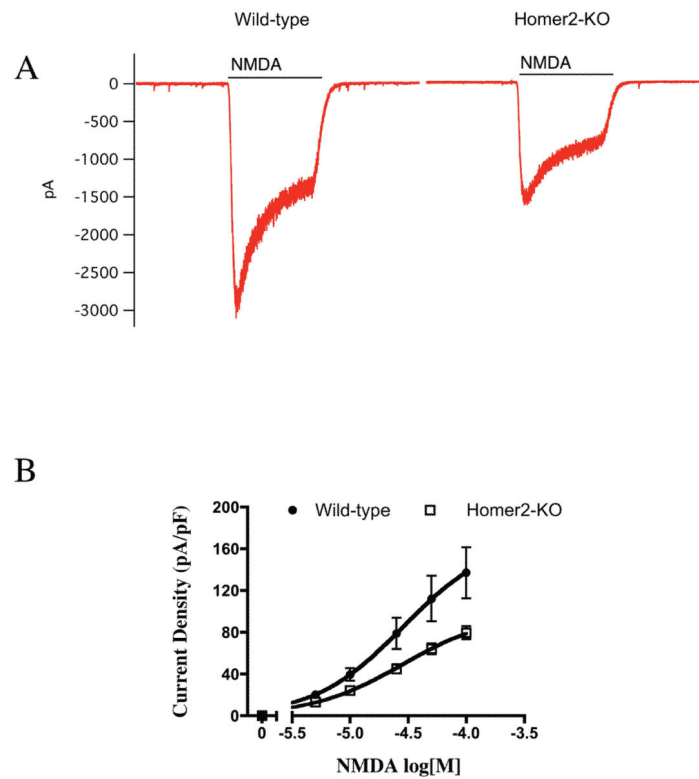
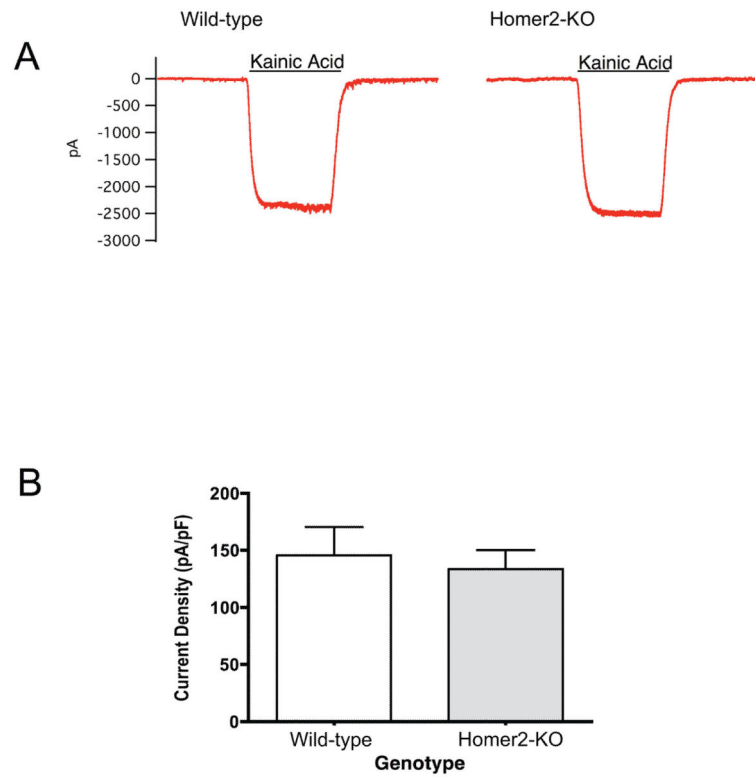**Figure 1.**
Effect of *Homer2* deletion on NMDA receptor current at 14 DIV. (A) Representative traces of whole-cell currents induced by 100 μM NMDA (10 μM glycine) from WT and *Homer2* KO mouse hippocampal neurons. Agonist was applied for 6 seconds. (B) Concentration-response graph of NMDA-induced currents from 14 DIV hippocampal neurons of WT and KO mice. Data represent steady-state currents normalized to whole-cell capacitance and are expressed as the mean (± S.E.M.) from 16 – 17 neurons per group. ANOVA indicated a significant effect of genotype (p<0.001).
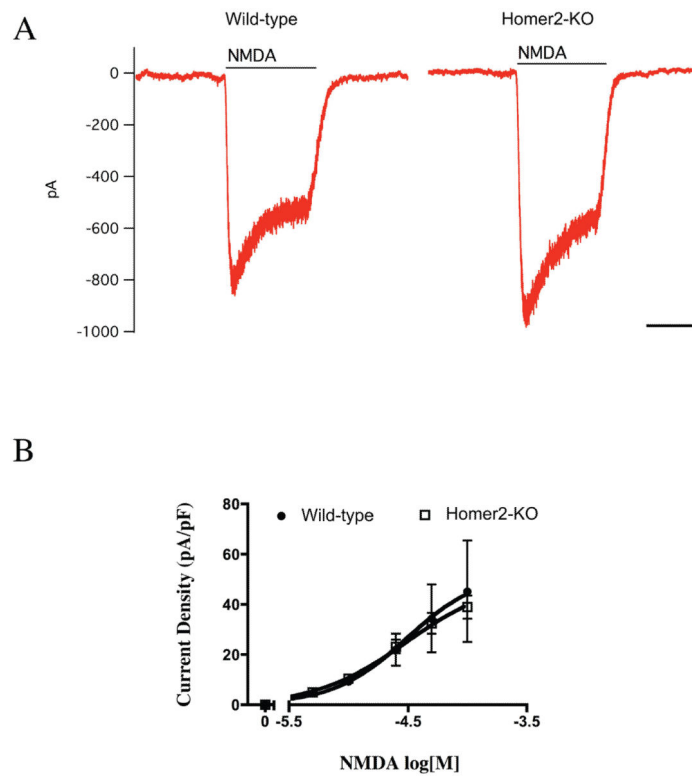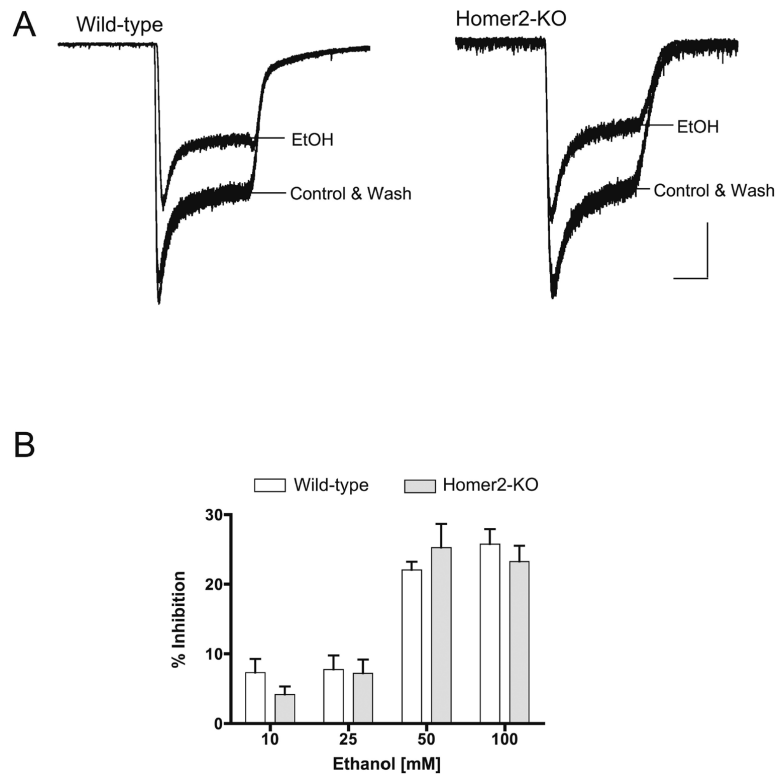
Figure 2.
Effect of *Homer2* deletion on NMDA receptor current from neurons at 7 DIV. (A)
Representative traces of whole-cell currents induced by 100 μM NMDA (10 μM glycine)
from WT and *Homer2* KO mouse hippocampal neurons. Agonist was applied for 6 seconds.
(B) Concentration-response graph of NMDA-induced currents from hippocampal neurons of
WT and KO mice. Data represent steady-state currents normalized to whole-cell capacitance
and are expressed as the mean (± S.E.M.) from 4 – 6 neurons per group. ANOVA indicated
no significant difference between groups (p > 0.05).
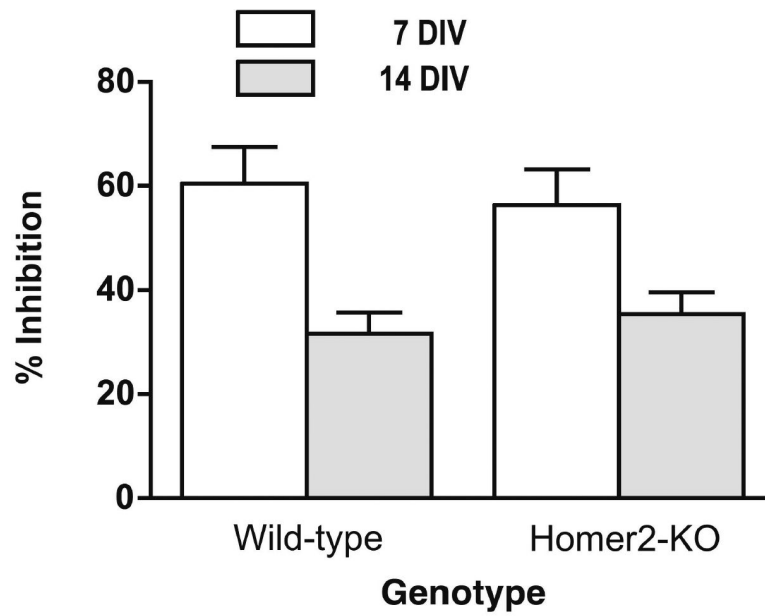
Figure 3.
Effect of age in culture on the ifenprodil sensitivity of NMDA-induced currents in WT and KO neurons. Summary graph of ifenprodil (10 μM) inhibition of NMDA (100 μM; 10 μM glycine) induced currents from WT and *Homer2* KO mouse hippocampal neurons at 7 DIV and 14 DIV. Data represent the percent inhibition by ifenprodil and are expressed as the mean (± S.E.M.) from 3 – 6 neurons per group.

Figure 4.
Effect of *Homer2* deletion on non-NMDA receptor currents at 14 DIV. (A) Representative traces of whole-cell currents from WT and *Homer2* KO mouse hippocampal neurons during a 6 second application of 100 µM kainic acid. (B) Summary graph of kainic acid-induced currents from WT and *Homer2* KO mouse neurons. Data represent steady-state currents normalized to whole-cell capacitance and are expressed as the mean (± S.E.M.) from 12 – 14 neurons per group.

Figure 5.
Inhibition of NMDA currents by ethanol. (A) Representative traces of ethanol ethanol inhibition of NMDA (100 μM; 10 μM glycine) induced whole-cell current from WT and *Homer2* KO hippocampal neurons. Currents are from single neurons in the absence (control and washout) and presence of 100 mM ethanol. Drug application time was 6 seconds. Horizontal and vertical calibration bars are 2.5 seconds and 1000 pA for WT and 500 pA for KO. (B) Summary of ethanol inhibition of NMDA-induced current. Data represents the mean (± S.E.M.) from 16–18 neurons from each group.