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Ephrin-B3 regulates glutamate receptor signaling at hippocampal synapses

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Abstract

B-ephrin - EphB receptor signaling modulates NMDA receptors by inducing tyrosine phosphorylation of NR2 subunits. Ephrins and EphB RTKs are localized to postsynaptic compartments in the CA1, and therefore potentially interact in a non-canonical *cis*-configuration. However, it is not known whether *cis*- configured receptor-ligand signaling is utilized by this class of RTKs, and whether this might influence excitatory synapses. We found that ablation of ephrin-B3 results in an enhancement of the NMDA receptor component of synaptic transmission relative to the AMPA receptor component in CA1 synapses. Synaptic AMPA receptor expression is reduced in ephrin-B3 knockout mice, and there is a marked enhancement of tyrosine phosphorylation of the NR2B receptor subunit. In a reduced system co-expression of ephrin-B3 attenuated EphB2-mediated NR2B tyrosine phosphorylation. Moreover, phosphorylation of EphB2 was elevated in the hippocampus of ephrin-B3 knockout mice, suggesting that regulation of EphB2 activity is lost in these mice. Direct activation of EphB RTKs resulted in phosphorylation of NR2B and a potential signaling partner, the non-receptor tyrosine kinase Pyk2. Our data suggests that ephrin-B3 limits EphB RTK-mediated phosphorylation of the NR2B subunit through an inhibitory *cis*- interaction which is required for the correct function of glutamatergic CA1 synapses.

Keywords

NMDA receptor; ephrin-B3; EphB2 RTK; hippocampus; CA1; excitatory synapse; In *cis* signaling; Pyk2

INTRODUCTION

NMDA receptors are among the most important proteins present in the postsynaptic density (PSD) of excitatory synapses. In addition to their well-described roles in long-term potentiation and depression (LTP and LTD), they are critical to several developmental processes including

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synaptic maturation, synaptogenesis and synapse elimination. Therefore, through diverse intracellular signaling cascades, NMDA receptors enable synaptic activity to coordinate and refine the connectivity between neurons in the brain (Cohen and Greenberg, 2008).

NMDA receptors directly associate with EphB receptor tyrosine kinases (RTKs) (Dalva et al., 2000). EphB receptors and their cognate ligands, the B-ephrins, are transmembrane bidirectional signaling molecules that orchestrate a wide array of developmental processes (Kullander and Klein, 2002). Their signaling roles at synapses are less well understood; however it has been demonstrated that B-ephrin mediated activation of EphB RTKs potentiates NMDA receptor signaling in cultured neurons (Takasu et al., 2002). B-ephrin binding to EphB RTKs initiates forward signaling which recruits the Src family of tyrosine kinases to phosphorylate key tyrosine residues on the cytoplasmic tail of NR2 receptor subunits. Thus EphB RTKs and B-ephrins are important regulators of NMDA receptors. In the CA1 region of the hippocampus, EphB RTKs and B-ephrins are localized to PSDs and potentially are co-localized to the same PSD (Grunwald et al., 2004). This raises the possibility of a non-canonical *cis*- signaling interaction between EphB receptors and B-ephrins at CA1 synapses. Such an interaction has been described for the A-class RTKs but not EphBs; thus when A-ephrins are presented in *cis*- to EphA RTKs, rather than initiating Eph RTK forward signaling, they instead inhibit signaling by the receptor (Carvalho et al., 2006).

Here we tested whether ephrin-B ligands regulate hippocampal CA1 synapse function *in vivo* by monitoring synaptic transmission in CA1 neurons from three lines of ephrin-B3 mutant mice. In ephrin-B3 hypomorphic mice (ephrin-B3^{neo/neo}), there was a marked enhancement of the ratio of NMDA receptor to AMPA receptor mediated excitatory postsynaptic currents (EPSCs). However in ephrin-B3 reverse signaling incompetent mice, there were no similar differences in the NMDA/AMPA EPSC ratio (NA ratio). mEPSC amplitudes were also reduced in ephrin-B3^{neo/neo} mice suggesting that synapses had fewer AMPA receptors. Examination of synaptic NMDA receptors uncovered no obvious alteration in NMDA receptor stoichiometry. However we observed a significant enhancement of tyrosine phosphorylation specific to the NR2B receptor subunit. Using a reduced recombinant system we found that co-transfection of NMDA receptors and EphB2 RTKs resulted in a robust enhancement of NR2B subunit tyrosine phosphorylation which importantly was reduced by co-expression of ephrin-B3. We hypothesized that loss of ephrin-B3 would be accompanied by increased activation of EphB RTKs and signaling partners important for regulating tyrosine phosphorylation of NMDA receptors. We found that the tyrosine phosphorylation of EphB2 was elevated in ephrin-B3^{neo/neo} hippocampus. Moreover, direct stimulation of EphB2 RTK activity in cultured cortical neurons led to phosphorylation of NR2B and a potential signaling partner proline-rich tyrosine kinase 2 (Pyk2), supporting the involvement of this important regulator of NMDA receptors in the signaling pathway. We suggest a model in which *cis*- interacting ephrin-B3 inhibits the activity of EphB RTKs in CA1 synapses. Loss of this regulation leads to chronic upregulation of EphB RTK signaling, enhanced NR2B phosphorylation, and reduction of the AMPA receptor content of hippocampal synapses. These results demonstrate that ephrin-B3 is an important regulator of NMDA receptors, and thus influences the maturation of glutamatergic synaptic transmission.

RESULTS

EphB RTKs and B-ephrins are localized to postsynaptic densities in CA1 synapses in the hippocampus (Grunwald et al., 2004) raising the possibility that they interact through an atypical *cis*- configuration that might affect glutamatergic synapses. Therefore we investigated whether the functional properties of hippocampal synapses were affected by the ablation of ephrin-B3. Excitatory synapses contain NMDA and AMPA receptors at variable densities, which are largely determined by the maturation state of the synapse (Crair and Malenka,

1995; Hsia et al., 1998; Barth and Malenka, 2001), therefore examination of the ratio of NMDA/AMPA EPSCs in a population of synapses is a good indicator of their developmental state. Whole-cell patch-clamp recordings were made from CA1 neurons in acute hippocampal slices from ephrin-B3^{neo/neo} mice and littermate controls. AMPA receptor-mediated EPSCs were measured by stimulating the Schaffer collateral input while holding the cell at a hyperpolarized membrane potential (−70 mV). A depolarizing step to +40 mV was used to elicit an outward EPSC, and the NMDA component was measured 60ms after the onset of the current (see Methods) (Marie et al., 2005). For each recording we calculated the ratio of the NMDA receptor-mediated EPSC to AMPA receptor mediated EPSC (NA ratio). We found that in ephrin-B3 hypomorphic mice the NA ratio was significantly higher than in littermate controls. In ephrin-B3^{wt/wt} the NA ratio was 0.32 ± 0.04 (n = 14) (Figure 1Ai & Aiii) which is similar to what has previously been reported in the hippocampus (Marie et al., 2005). In knockout animals (ephrin-B3^{neo/neo}) the NA ratio was 0.45 ± 0.04 (n = 24, p < 0.05) (Figure 1Aii & Aiii). The NA ratio in heterozygous animals (ephrin-B3^{wt/neo}) was similar to that in wildtype littermates (0.29 ± 0.06 , n = 6, p > 0.05) (Figure 1Aiii) demonstrating that complete loss of ephrin-B3 is required for this phenotype.

Ephrin-B3 ligands, upon activation by their cognate partners the EphB RTKs, can themselves signal through their cytoplasmic domains (Klein, 2009). To determine whether the altered NA ratios depended on ephrin-B3 reverse signaling, we made recordings from two mutant mice in which the ephrin-B3 protein was rendered reverse signaling incompetent. In ephrin-B3^{lacZ/lacZ} mice the intracellular signaling domain of ephrin-B3 protein is replaced by a bacterial lacZ cassette to form an ephrin-B3-β-galactosidase fusion protein (Yokoyama et al., 2001). In recordings from CA1 neurons in ephrin-B3^{lacZ/lacZ} mice, we did not observe any alteration in the NA ratio (ephrin-B3^{wt/wt}: 0.27 ± 0.05 , n = 11; ephrin-B3^{lacZ/lacZ}: 0.22 ± 0.02 , n = 9, p > 0.05) (Figure 1Bi & Bii). Expression of the bulky β-galactosidase fusion protein in these mice may potentially interfere with native aggregation processes between bound EphBs and mutant ephrin-B3, resulting in disrupted forward signaling. Therefore we examined synaptic transmission in another mutant mouse line in which the transmembrane domain and the extracellular binding region of ephrin-B3 are preserved while the cytoplasmic domain is truncated (ephrin-B3^{tr}) (see Methods). In ephrin-B3^{tr} mice we again found that the NA ratio was not perturbed, further confirming that ephrin-B3 reverse signaling is not involved in producing this synaptic phenotype (ephrin-B3^{wt/wt}: 0.17 ± 0.02 , n = 7; ephrin-B3^{tr}: 0.24 ± 0.06 , n = 8; ephrin-B3^{tr}: 0.23 ± 0.04 , n = 6, p > 0.05). Therefore ablation of ephrin-B3 results in a significant enhancement of the NA ratio, however blocking B-ephrin reverse signaling has no effect on glutamatergic synaptic transmission in CA1 synapses.

The elevated NA ratio observed in ephrin-B3^{neo/neo} mice could be a result of a change in the average density or function of either the NMDA or AMPA component of synaptic transmission at CA1 synapses. To determine the average quantal AMPA response of CA1 synapses we made recordings of mEPSCs in the presence of 50 μM D-APV and 1 μM tetrodotoxin (TTX). The mean amplitude of mEPSC events was significantly lower in ephrin-B3^{neo/neo} recordings in comparison to those from littermate ephrin-B3^{wt/wt} mice (ephrin-B3^{wt/wt}: 16.4 ± 0.2 pA, n = 4; ephrin-B3^{neo/neo}: 13.7 ± 0.2 pA, n = 5, p < 0.0001, Kolmogorov-Smirnov (KS) two-sample test) (Figure 2A & B). In contrast, the frequency of mEPSC events was not different between the two genotypes (ephrin-B3^{wt/wt}: 0.55 ± 0.11 Hz, n = 4; ephrin-B3^{neo/neo}: 0.57 ± 0.10 Hz, n = 5, p > 0.05, KS test) (Figure 2C). A decrease in the amplitude of mEPSC events suggests that the average synaptic density of AMPA receptors, and thus the synaptic weight of CA1 synapses, is reduced in ephrin-B3 knockout mice. Moreover the finding that the frequency of events is not altered argues that the number of synaptic contacts and release probability are unlikely to be substantially altered.

To further investigate this reduction in quantal amplitude, and to determine specifically if this deficit was at Schaffer collateral synapses, we recorded strontium evoked mEPSCs (Sr^{2+} minis). Replacing extracellular Ca^{2+} with Sr^{2+} desynchronizes evoked release, resulting in the ability to detect mEPSCs evoked by the activation of a specific input pathway to the recorded cell (Goda and Stevens, 1994). Consistent with our previous result, we found that the amplitude of Sr^{2+} mEPSCs recorded in ephrin-B3^{neo/neo} mice was significantly smaller than those recorded from Schaffer collateral synapses in wildtype mice (ephrin-B3^{wt/wt}: 21 ± 0.2 pA, $n = 8$; ephrin-B3^{neo/neo}: 19 ± 0.1 pA, $n = 8$, $p < 0.05$, KS test) (Figure 2D). These results demonstrate that the average strength of individual synapses in the CA1 is reduced in ephrin-B3^{neo/neo} mice. In order to directly assess whether glutamate receptor protein expression was altered in juvenile ephrin-B3^{neo/neo} mice, we performed quantitative immunoblot experiments, but in contrast to what has been reported in adult ephrin-B3^{neo/neo} mice (Rodenas-Ruano et al., 2006), found no alterations in total AMPA or NMDA receptor expression (Supplemental Figure 1 & Table 1).

We have found specific alterations in glutamate receptor signaling which suggest that ablation of ephrin-B3 results in postsynaptic changes in CA1 synapses. B-ephrins are localized presynaptically in some hippocampal synapses, and presynaptic reverse signaling is required for mossy fiber LTP (Contractor et al., 2002; Armstrong et al., 2006). In addition, ephrin-B3 co-localizes with presynaptic proteins in axons of cultured hippocampal neurons (Rodenas-Ruano et al., 2006). Therefore, it is possible that presynaptic function may also be perturbed in CA1 of ephrin-B3^{neo/neo} mice. To address this possibility, we measured a form of short-term plasticity. Pairs of synaptic stimulation were delivered to Schaffer collateral inputs at 40ms, 80ms, and 200ms. Knockout and littermate control mice displayed the same paired-pulse facilitation over these intervals ($p > 0.05$, two-way ANOVA followed by post-hoc Bonferroni tests.) (Supplemental Figure 2). We also performed paired-pulse facilitation experiments in reverse signaling incompetent mice (ephrin-B3^{lacZ/lacZ}). Again there was no difference in facilitation at any of the intervals tested when compared to recordings from littermate controls (ephrin-B3^{wt/wt}: 2.3 ± 0.14 (40 ms), 2.4 ± 0.18 (80 ms), 1.4 ± 0.09 (200 ms), $n = 6$; ephrin-B3^{lacZ/lacZ}: 2.0 ± 0.09 (40 ms), 1.9 ± 0.05 (80 ms), 1.4 ± 0.04 (200 ms), $n = 7$ $p > 0.05$ (two-way ANOVA followed by post-hoc Bonferroni test)). These findings demonstrate that short-term presynaptic plasticity is not compromised by either the complete removal of ephrin-B3 or in the presence of a reverse signaling incompetent mutant.

NMDA receptors are known to undergo developmental changes in subunit composition over the course of the first three postnatal weeks. A switch from NR2B-containing receptors to those predominantly composed of NR2A is accompanied by a quickening of the deactivation kinetics of the NMDA receptor EPSC (Barth and Malenka, 2001; Bellone and Nicoll, 2007). Native NMDA receptors are likely to be of mixed stoichiometry; both di-heteromeric receptors containing either NR2A and NR2B (with the NR1 subunit), and tri-heteromeric complexes containing both the NR2B and NR2A receptor subunits (Sheng et al., 1994; Chazot and Stephenson, 1997). The proportion of NR2B containing receptors can be determined by the use of the NR2B specific antagonist ifenprodil (Williams, 1993), which blocks both di-heteromeric and tri-heteromeric NR2B containing receptors (although the block of the tri-heteromeric complex is less complete) (Hatton and Paoletti, 2005). To address the possibility of alteration in the stoichiometry of hippocampal NMDA receptors, NMDA EPSCs were isolated in CA1 neurons in the presence of the AMPA/kainate antagonist CNQX (50 μM). Addition of ifenprodil (3 μM) blocked the NMDA component by $63 \pm 7\%$ ($n = 8$) in wildtype animals (Figure 3A & C). In interleaved recordings from ephrin-B3^{neo/neo} mice we found that the ifenprodil sensitivity was not significantly different ($59 \pm 4\%$ inhibition, $n = 17$, $p > 0.05$) (Figure 3B & C). The NR2B subunit confers slower deactivation kinetics on NMDA receptors, therefore we also measured the decay time constant (Cathala et al., 2000) of NMDA EPSCs in CA1 pyramidal neurons. The measured decay time of the NMDA EPSC did not differ

significantly between ephrin-B3 knockout and wildtype animals (ephrin-B3^{wt/wt}: 134 ± 12 ms, $n = 8$; ephrin-B3^{neo/neo}: 160 ± 17 ms, $n = 11$, $p > 0.05$) indicating similar deactivation kinetics of the NMDA EPSC in each phenotype (Figure 3D). Therefore it is unlikely that the stoichiometric arrangement of synaptic NMDA receptors is altered in ephrin-B3^{neo/neo} mice. Moreover, consistent with this, there is no alteration in the expression of NMDA receptor subunits in the hippocampus (Supplemental Figure 1 & Table 1).

EphB RTKs directly interact with NMDA receptors, but not AMPA receptors (Dalva et al., 2000). Activation of EphB RTKs results in recruitment of the Src family of tyrosine kinases and phosphorylation of key tyrosine residues on the cytoplasmic tail of the NR2 NMDA receptor subunit (Takasu et al., 2002). We hypothesized that loss of a *cis*- coupling between postsynaptic EphB RTKs and B-ephrins in ephrin-B3^{neo/neo} mice might result in altered EphB signaling, which in turn might affect tyrosine phosphorylation of NMDA receptors. Therefore, we investigated whether phosphorylation of the NR2 subunits was altered in ephrin-B3^{neo/neo} mice. Hippocampal homogenates from ephrin-B3^{neo/neo} and littermate ephrin-B3^{wt/wt} mice were immunoprecipitated with a pan phospho-tyrosine (p-Tyr) antibody to immunoprecipitate all tyrosine-phosphorylated proteins. Immunoprecipitated proteins were then probed with antibodies specific to the NR2A or NR2B NMDA receptor subunits to determine the relative amount of tyrosine phosphorylation of these two substrates (Figure 4A). We did not find any difference in the amount of NR2A protein immunoprecipitated by the pan p-Tyr antibody (ephrin-B3^{wt/wt}: 100 ± 27 %, $n = 7$; ephrin-B3^{neo/neo}: 100 ± 22 %, $n = 7$, $p > 0.05$). However, interestingly we found a significant enhancement of tyrosine phosphorylated NR2B protein in ephrin-B3^{neo/neo} mice (140 ± 13 %, $n = 11$, $p < 0.05$) (Figure 4A & B).

The NR2B receptor subunit is the most prominently tyrosine-phosphorylated protein in the PSD (Moon et al., 1994). Of the 25 tyrosine residues on the carboxyl terminal tail of the receptor, three sites have been clearly identified to be substrates for tyrosine phosphorylation by members of the Src family of kinases (Nakazawa et al., 2001). Therefore to confirm that NR2B tyrosine phosphorylation is upregulated in ephrin-B3^{neo/neo} mice, we immunoprecipitated NR2B from hippocampal homogenates and immunoblotted with phospho-specific antibodies that recognize activation of tyrosine residues Y1252, Y1336, and Y1472 (Figure 4C) (Takasu et al., 2002). We found a significant and specific upregulation of tyrosine phosphorylation at site Y1252 (150 ± 19 %, $n = 5$, $p < 0.05$), but not at sites Y1336 (105 ± 11 %, $n = 4$) or Y1472 (117 ± 16 %) (Figure 4C & D). Additionally, no alteration in Y1252 phosphorylation was found in the reverse signaling deficient mutant (ephrin-B3^{lacZ/lacZ}: 118 ± 18 , $n=5$, $p>0.05$), demonstrating that the upregulation of tyrosine phosphorylation is specific in the ephrin-B3 hypomorphic mice. Taken together these data demonstrate a clear and specific increase in tyrosine phosphorylation of the NR2B receptor subunit in ephrin-B3^{neo/neo} mice.

Given our findings that there is elevated tyrosine phosphorylation of NR2B in ephrin-B3 deficient mice, we asked whether ephrin-B3 may normally inhibit EphB RTK mediated modification of NMDARs when they are expressed in the same cellular compartment. We used a reduced recombinant expression system to directly address a potential B-ephrin-EphB *cis*-inhibitory interaction. HEK 293 cells were co-transfected with the NMDA receptor subunits NR1a and NR2B along with a tagged EphB2 construct (EphB2-FLAG). We first observed that co-expression of EphB2-FLAG, but not the dominant negative construct (DN-EphB2-FLAG), elevated phosphorylation levels of Y1252, Y1336, and Y1472 of the NR2B subunit (data not shown). This is consistent with previous findings that overexpression of EphB2 results in a dramatic increase of NR2B subunit phosphorylation in a reduced system (Takasu et al., 2002). To ascertain whether the presence of ephrin-B3 was able to interfere with EphB2-mediated NR2B subunit tyrosine phosphorylation, we co-expressed NMDA receptors and EphB RTKs in the presence and absence of ephrin-B3 (Figure 5). We found that the

phosphorylation of Y1252 and, to a lesser degree, Y1336 could be repressed by the presence of ephrin-B3. In these experiments we used two different ratios of EphB2. The percent inhibition of Y1252 phosphorylated NR2B relative to ephrin-B3 untransfected cells was $-34 \pm 10\%$, $n = 4$, $p < 0.05$ ($1.5 \times$ EphB2-FLAG) and $-39 \pm 13\%$, $n = 4$, $p < 0.05$ ($2 \times$ EphB2-FLAG) (Figure 5A & B). A smaller reduction in phosphorylation of NR2B Y1336 was observed with ephrin-B3 co-transfection but only in the experiments with $2 \times$ EphB2-Flag ($-21 \pm 8\%$, $n = 4$, $p < 0.05$) (Figure 5Aii). Ephrin-B3 overexpression did not cause a statistically significant alteration in EphB2-mediated phosphorylation of NR2B Y1472 (Percent change relative to ephrin-B3 untransfected cells, $21 \pm 21\%$, $n = 4$, $p > 0.05$ ($1.5 \times$ EphB2-FLAG) and $27 \pm 18\%$, $n = 4$, $p > 0.05$ ($2 \times$ EphB2-FLAG)). Co-expression of B-ephrins did not reduce EphB2-Flag expression ($137 \pm 23\%$ of control, $n = 12$, $p > 0.05$), therefore this could not account for the reduced phosphorylation of Y1252. When EphB2 and ephrin-Bs are co-expressed in the same cells, in addition to interacting *in cis*, there is still the possibility that there are *trans* interactions with proteins on neighboring cells. To determine whether EphB2-mediated phosphorylation of NR2B could be influenced by expression of ephrin-B3 solely on neighboring cell populations, we transfected cells separate with EphB2/NMDARs and B-ephrins and mixed the two cell populations after expression. In these experiments we found that the presence of B-ephrin expressing cells in the mixed culture had no effect on Y1252 phosphorylation (Figure 5Aii, lower panel; *trans*-). These data demonstrate that ephrin-B3 is able to interfere selectively with EphB2-mediated phosphorylation of NR2B tyrosine phosphorylation sites when co-expressed in a model system.

Our data demonstrate that tyrosine phosphorylation of the NR2B subunit is elevated and synaptic AMPA receptors are reduced in young ephrin-B3^{neo/neo} mice. An obvious question for us to address was whether LTP in the CA1 region, which is dependent upon the activation of NMDA receptors and the trafficking of AMPA receptors into synapses, is altered in ephrin-B3^{neo/neo} mice. Previous studies in adult ephrin-B3 hypomorphic mice have reported either impairment in CA1 LTP (Grunwald et al., 2004) or no deficits (Armstrong et al., 2006). To determine if CA1 LTP was expressed normally in the young animals ($< P21$) that we had used in this study, we performed LTP experiments using whole-cell recording. For these experiments we recorded excitatory postsynaptic potentials (EPSPs) in current clamp mode and utilized a naturalistic theta-burst pairing protocol to induce LTP. This consisted of presynaptic stimulation delivered at theta-frequency, paired with brief current injections to elicit back-propagating action potentials (see Methods) (Hoffman et al., 2002) (Figure 6A). In both ephrin-B3^{wt/wt} and ephrin-B3^{neo/neo} mice this induction protocol elicited a robust LTP which, when measured at 35–40 minutes post-induction, was not different in magnitude (ephrin-B3^{wt/wt}: $210 \pm 46\%$, $n = 6$; ephrin-B3^{neo/neo}: $170 \pm 30\%$, $n = 5$, $p > 0.05$) (Figure 6). These findings are similar to those we had previously reported in adult mice, in which we failed to find deficits in CA1 LTP using field potential EPSP recordings in either ephrin-B3^{neo/neo} or ephrin-B3^{lacZ/lacZ} mice (Armstrong et al., 2006).

Elevated activity of EphB2 RTKs might also be directly observed in the hippocampus when ephrin-B3 is ablated. Hippocampal homogenates were examined with a phosphorylation site-specific antibody and revealed a significant enhancement of Y-EphB2 in ephrin-B3^{neo/neo} mice (ephrin-B3^{wt/wt}: $100 \pm 14\%$, $n = 3$; ephrin-B3^{neo/neo}: $140 \pm 14\%$, $n = 3$, $p < 0.05$) (Figure 7A). Potential signaling partners that may regulate the selective tyrosine phosphorylation of NR2B might also be upregulated in these mice. However, examination of hippocampal homogenates from ephrin-B3^{neo/neo} with a general Src-family kinase antibody that recognizes multiple non-receptor tyrosine kinases at the activation site complementary to Y416 of Src revealed no differences between genotypes (ephrin-B3^{wt/wt}: $100 \pm 16\%$, $n = 3$; ephrin-B3^{neo/neo}: $105 \pm 22\%$, $n = 3$, $p > 0.05$). We therefore directly stimulated EphB RTKs with B-ephrin fusion proteins in cultured cortical neurons and examined the phospho-tyrosine reactive proteins. As expected, we found that a brief 30 min stimulation of EphB RTKs resulted in enhancement of NR2B

phosphorylation consistent with previous findings of EphB-mediated phosphorylation of NR2A (Grunwald et al., 2001) ($134 \pm 9\%$, $n = 3$, $p < 0.05$) (Figure 7B). EphB2 stimulation also caused a significant enhancement of endogenous phosphorylated Pyk2 in cortical cultures ($163 \pm 32\%$, $n = 3$, $p < 0.05$) (Figure 7B). Pyk2 is a non-receptor tyrosine kinase that is activated by calcium, highly enriched in the hippocampus, and is known to phosphorylate NR2B subunits (Lev et al., 1995; Heidinger et al., 2002). These data are the first to demonstrate that EphB2 activation can upregulate Pyk2 activity, and taken together with our other findings support the model that native ephrin-B3 regulates the activity of EphB RTK in the hippocampus. Loss of negative modulation of EphB RTK function in ephrin-B3 hypomorphic mice results in the chronic activation of signaling pathways that enhance NMDAR phosphorylation and alter glutamatergic signaling at CA1 synapses.

DISCUSSION

Regulation of glutamatergic signaling is critical for the correct development and functioning of the CNS. Here we have demonstrated that ephrin-B3 is required for the proper balance of glutamate receptors at excitatory synapses in the CA1 of the hippocampus. Loss of ephrin-B3 results in an enhancement of the NA ratio, a reduction in the synaptic weight of individual synapses (due to a decrease in synaptic expression of AMPA receptors), up-regulation of NMDA receptor NR2B subunit and EphB2 tyrosine phosphorylation. Using a reduced recombinant system we provide evidence that supports a model where postsynaptic *cis*-interactions between B-ephrins and EphB RTKs limit the tyrosine kinase activity of the receptor. Loss of this regulation results in elevated tyrosine phosphorylation of the NR2B receptor subunit possibly through Pyk2 activity, which results in a reduced incorporation of AMPA receptors into glutamatergic synapses. The conventional view has been that increased NMDA receptor signaling, (an expected consequence of enhanced tyrosine phosphorylation of the NR2B subunit (Wang and Salter, 1994)), would result in enhanced synaptic plasticity and increased recruitment of AMPA receptors into synapses. However, recently it was demonstrated that the NR2B receptor subunit limits the AMPA receptor complement of synapses during development (Hall and Ghosh, 2008). This mechanism could explain the correlation between AMPA receptor synaptic density and NR2B receptor subunit composition that is observed at many developing cortical synapses (Crair and Malenka, 1995; Barth and Malenka, 2001).

EphB RTKs and B-ephrins are emerging as key regulators of excitatory synapses. A recent study demonstrated that activation of ephrin-B2 reverse signaling reduced AMPA receptor internalization in cultured neurons (Essmann et al., 2008). Moreover, it was demonstrated that the mean mEPSC amplitude was significantly reduced in neurons cultured from ephrin-B2 knockout animals compared to those in wildtype neurons. These results are qualitatively similar to what we have found in ephrin-B3 knockout mice; however there may be several mechanistic differences between the actions of ephrin-B2 and ephrin-B3. We propose that the alteration of AMPA receptor density in ephrin-B3^{neo/neo} mice does not require B-ephrin reverse signaling, because no alterations were observed in reverse signaling incompetent mice. However, ephrin-B2 modulation of synaptic strength does require reverse signaling and the association between ephrin-B2 and the AMPA receptor interacting protein GRIP (glutamate receptor interacting protein) (Essmann et al., 2008). Our observations suggest that the intracellular signaling domain of ephrin-B3 is not required for the phenotype we observe when ephrin-B3 is ablated *in vivo*. Instead the interaction between the extracellular domain of ephrin-B3 and EphB RTKs must play a role, which will likely require forward signaling through the receptor.

We found a selective enhancement of the NR2B subunit phosphorylation at Y1252 in the ephrin-B3^{neo/neo} hippocampus that was not observed in the ephrin-B3^{lacZ/lacZ} mice. Furthermore, no changes were found in the post-translational modification of NR2B sites

Y1336 and Y1472 in ephrin-B3^{neo/neo} mice. In a reduced system, co-expression of ephrin-B3 significantly depressed the EphB2 RTK-mediated enhancement of NR2B Y1252 modification, with only a modest effect at Y1336 (in experiments with 2× EphB2-FLAG, but not with 1.5× EphB2-FLAG), and no change in Y1472, providing support for the Y1252 site playing a prominent role in EphB2-NR2B interactions. NR2B tyrosine sites 1252, 1336, and 1472 were first identified as the critical residues modified by the Src family kinases (Cheung and Gurd, 2001; Nakazawa et al., 2001). Due to the robust phosphorylation of Y1472 and the reliable responsiveness of this site to LTP induction protocols, there has been particular interest in this site (Nakazawa et al., 2001). However, each of the sites may be substrates under divergent conditions. For example, phosphorylation of Y1336 is observed during conditions of chronic NMDAR activation. In this instance, a Fyn- and calpain-directed signaling cascade coordinates the hyper-phosphorylation of Y1336 that is associated with the cleavage of the NR2B C-terminal domain and a change in the functional properties of the NMDAR. (Wu et al., 2007). Therefore site specific tyrosine modification is not unprecedented and a selective regulation of NR2B at Y1252 sensitive to interactions between ephrin-B3 and EphB2 may have a unique functional property that is critical for synapse stability.

The central role of both NMDA receptors and AMPA receptors in synaptic plasticity led us to address whether CA1 LTP was altered in ephrin-B3 knockout animals. We performed whole-cell current-clamp recording and used a theta-burst pairing protocol that provides a more naturalistic pattern of activation (Hoffman et al., 2002), compared to high frequency stimulation paradigms that are often used for LTP induction. Despite the changes in basal synaptic transmission in ephrin-B3 hypomorphic mice, we did not find any difference in LTP; a robust potentiation was observed in both wildtype and interleaved knockout experiments. Previous studies in adult mice have reported that both ephrin-B2 and ephrin-B3 knockout mice show deficits in CA1 LTP (Grunwald et al., 2004). However, we also previously measured CA1 LTP in adult ephrin-B3^{neo/neo} and ephrin-B3^{lacZ/lacZ} mice and had found no deficits (Armstrong et al., 2006). A significant decrease in the expression of NMDA receptor subunits in synaptoneurosomes from the hippocampus of adult ephrin-B3^{neo/neo} mice had been proposed to underlie the LTP deficits (Rodenias-Ruano et al., 2006). In the present study we did not find similar alteration in the expression of NR1 in juvenile ephrin-B3^{neo/neo} mice (see Supplemental Material). It is possible that the complex pleiotropic roles of B-ephrins and EphBs interacting with the genetic background of mice could account for these discrepant results; however, our study underscores that B-ephrins in the CA1 region are not necessary mediators of LTP (Malenka and Bear, 2004), but instead are modulators of plasticity through their multiple actions on glutamate receptors and synapse development.

Based upon our finding we propose a novel B-ephrin-EphB *cis* inhibitory interaction in CA1 synapses. There have been no prior reports of B class receptor-ligands interacting in *cis*, however, EphA RTKs and their cognate ligands A-ephrins have been previously demonstrated to have similar inhibitory interactions (Carvalho et al., 2006). Thus it is likely that the specific subcellular localization of Eph receptors and their binding partners will play a significant role in defining their function. This is highlighted in the distinct roles that B-ephrins play at two separate hippocampal synapses. At the main mossy fiber synapse, formed between the axons of dentate granule neuron and CA3 pyramidal neurons, EphB RTKs and B-ephrins signal trans-synaptically (Contractor et al., 2002). EphB RTKs expressed in the postsynaptic CA3 neurons interact with presynaptically localized B-ephrins, and ephrin reverse signaling into the large mossy fiber bouton is required for NMDA receptor independent mossy fiber LTP (Contractor et al., 2002; Armstrong et al., 2006). In CA1 synapses where EphB RTKs are co-localized with their cognate ligands in the same postsynaptic compartment, we provide evidence that their functional interaction results in a very different role for B-ephrins as inhibitory signaling partners of the EphB RTKs.

Glutamate receptor signaling is critical to brain function and plasticity. EphB-RTKs and their cognate ligands, B-ephrins, are emerging as key regulators, not only of developmental processes that are required for the formation of synaptic contacts, but also for ongoing modification of glutamatergic synaptic transmission. The present study demonstrates a role for ephrin-B3 in regulating synaptic NMDA and AMPA receptors which could be important for the functional maturation of synaptic transmission in the hippocampus.

EXPERIMENTAL METHODS

Animals

Animals were treated in accordance with Northwestern University Institutional Animal Care and Use Committee. Both ephrin-B3^{neo/neo} and ephrin-B3^{lacZ/lacZ} mouse strains have been previously described (Yokoyama et al., 2001). Ephrin-B3^{neo/neo} mice were created by incorporating a neomycin cassette into the forth intron disrupting the downstream gene. A truncated transcript is produced, which likely results in misfolded protein and a loss of function (Yokoyama et al., 2001). Ephrin-B3^{l/t} mice were generated by crossing ephrin-B3^{lacZ/lacZ} mice to CMV-Cre “deleter” mice, thus recombining the *loxP* sites flanking the lacZ cassette and placing an in-frame stop sequence downstream of the transmembrane region to allow expression of the truncated protein. Mice were maintained as heterozygous crosses on an isogenic CD-1 background and homozygote offspring and wildtype littermates were used in experiments with the experimenter blind to the genotype. Animals were genotyped before and after completion of the experiments with PCR of tail digests.

Slice preparation and electrophysiology

Postnatal day 14 (P14) to P21 juvenile mice were anesthetized with isoflurane and decapitated. The brain was rapidly removed into an ice-cold oxygenated sucrose artificial cerebral spinal fluid (ACSF) containing the following (in mM): 85 NaCl, 75 sucrose, 25 NaHCO₃, 25 glucose, 4 MgCl₂, 2.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, equilibrated with 95% O₂/5% CO₂ and supplemented with 0.1 kynurenic acid/0.01 (DL)-APV. Each hemisphere was mounted on a sectioning stage and 350 μm thick horizontal brain sections were made with a vibratome (Leica Microsystems, Inc). Immediately after sectioning, slices were transferred to a recovery chamber and rapidly heated to 29°C followed by a slow equilibration to room temperature, with the gradual exchange of sucrose ACSF for oxygenated sodium ACSF solution containing the following (in mM): 125 NaCl, 25 NaHCO₃, 25 glucose, 2.4 KCl, 2 MgCl₂, 1.25 NaH₂PO₄, 1 CaCl₂, gassed with 95 % O₂/5 % CO₂ and supplemented with 0.1 kynurenic acid/0.01 (DL)-APV. After at least one hour recovery, individual slices were transferred to a recording chamber and visualized with an upright microscope (Zeiss). In the recording chamber, slices were continuously perfused with oxygenated sodium ACSF containing 2mM CaCl₂ and 1mM MgCl₂. All experiments were performed at 30 °C. Whole-cell patch clamp recordings were made from visually identified pyramidal cells in the CA1 region using a 700B patch-clamp amplifier (Molecular Devices). Series resistance was continuously monitored using hyperpolarizing voltage steps generated by pClamp 9 or 10.2 software (Molecular Devices), and recordings were discarded if there was a >15% change during the course of the experiment. For voltage clamp experiments a CsF internal solution was used containing the following (in mM): 95 CsF, 25 CsCl, 10 Cs-HEPES, 10 Cs-EGTA, 10 QX-314 [N-(2,6-dimethylphenyl)carbamoyl-methyl] triethylammonium bromide, 5 4-AP, 2 NaCl and 2 Mg-ATP, pH adjusted to 7.3 with CsOH (290 mOsm). For current clamp recordings a KMeSO₄ internal solution was used containing the following (in mM): 125 KMeSO₄, 11 Na-HEPES, 10 phosphocreatine, 5 KCl, 5 NaCl, 4 Na-ATP, 1 MgCl₂, 0.3 Na-GTP, pH 7.4 (292 mOsm). EPSCs or EPSPs were stimulated with a monopolar glass electrode placed in the stratum radiatum 100–150 μm away from the recorded cell. Inhibition was blocked by the GABA_A antagonists bicuculline (10 μM) and picrotoxin (50 μM). Stimuli were controlled by pClamp

9 or 10.2 software through a Digidata 1300 or 1440 series interface (Molecular Devices) coupled to an A360 stimulation isolation unit (Warner Instruments, Hamden, CT). Data collection and filtering was performed with pClamp 10.2 software.

NMDA/AMPA ratio

AMPA EPSCs were measured as the peak of the evoked current at a holding potential of -70 mV. For NMDA EPSCs, the cell was depolarized to $+40$ mV and the mean amplitude of the EPSC was measured between $60 - 62.5$ ms after the onset of the current. At this time the AMPA component has fully decayed and the remaining current is mediated by NMDA receptors (Marie et al., 2005). 20 interleaved sweeps of the NMDA and AMPA EPSCs were measured for each cell.

NMDA EPSCs

To isolate the NMDA EPSC alone, recordings were made in the presence of $50 \mu\text{M}$ CNQX. To block NR2B-containing receptors we used $3 \mu\text{M}$ ifenprodil. The decay time constant of NMDA currents were measured by dividing the total area of the current ($\int I(t)dt$) by the peak amplitude (I_{peak}) of the NMDA receptor EPSC according to previously published methods (Cathala et al., 2000).

mEPSCs

mEPSCs were recorded in voltage-clamp in the presence of $50 \mu\text{M}$ (DL)-APV and $1 \mu\text{M}$ tetrodotoxin (TTX). At the end of each experiment, $50 \mu\text{M}$ CNQX was added to ensure that events were AMPA receptor mediated. Data was collected by pClamp 10 and analyzed with the MiniAnalysis Program (Synaptosoft). Strontium evoked mEPSC (Sr^{2+} mEPSCs) were measured by first evoking an AMPA EPSC in regular ACSF, and then switching to an external containing 6 mM Sr^{2+} and 0.5 mM Ca^{2+} . Desynchronized release was detected as Sr^{2+} mEPSCs up to 1 second after the stimulus-locked event.

LTP

EPSPs were recorded in current clamp mode from CA1 neurons. A relatively short baseline of $5 - 10$ minutes was used, so as to avoid washout of LTP which occurs in whole cell recording (Malinow and Tsien, 1990). LTP was induced using a theta burst pairing protocol (Hoffman et al., 2002) that consisted of pairing 5 coincident EPSPs and postsynaptic action potentials (activated by short somatic current injection; 2 nA for 2 ms) at a frequency of 100 Hz . Five bursts were combined at a frequency of 5 Hz to produce a theta burst train and a total of three trains was delivered at 0.1 Hz . LTP was measured as the potentiation $35 - 40$ minutes after induction compared to the baseline period.

Biochemistry

Synaptoneurosomes were prepared according to previously described methods (Villasana et al., 2006). In brief, the hippocampi were dissected from the brain and homogenized with a glass pestle into 2 ml of an ice cold HEPES-buffer containing (in mM): 150 NaCl , 50 NaF , 10 HEPES , $10 \text{ NaH}_2\text{PO}_4$, 1 EDTA , 2 EGTA , $0.5 \text{ dithiothreitol}$; this buffer was supplemented with a protease inhibitor cocktail (1 tablet/ 10 mls , Roche) and phosphatase inhibitor cocktails (Sigma, phosphatase inhibitor cocktails 1 and 2, $100 \mu\text{l}$ of each 10 mg/ml solution). Half of the whole homogenate was passed through a $100 \mu\text{m}$ filter followed by a $5 \mu\text{m}$ filter (Millipore), and the final homogenate was centrifuged at $1,000g$ for 10 min at 4°C . The supernatant was discarded and the remaining pellet containing the synaptoneurosome fraction was resuspended into $100 \mu\text{l}$ buffer. The protein content was determined using the BCA assay (Pierce) and diluted into a denaturing sample buffer containing dithiothreitol, urea, 10% sodium dodecyl sulfate, and 2% v/v β -mercaptoethanol. Equivalent protein content of each sample ($10 - 20$

µg) was resolved via SDS-PAGE with 7.5 – 10 % TRIS-HCl acrylamide gels and blotted onto 0.45 µm pore polyvinylidene fluoride membranes in a methanol/TRIS-glycine buffer.

For immunoprecipitation experiments, the hippocampus was isolated and lysed with an ultrasonic tissue disruptor in HEPES-buffer supplemented with 1 mM activated Na₃VO₄ on ice. For immunoprecipitation of proteins, a spin column-based kit utilizing immobilized protein A/G beads was used according to manufacturer's specifications in the presence of 1mM activated Na₃VO₄ throughout all steps (Catch and Release® Phosphotyrosine, clone 4G10® and v 2.0 Immunoprecipitation Kits, each from Millipore). For immunoprecipitation of phosphotyrosine-containing proteins, 600 µg of tissue was incubated in the presence of 3 µg 4G10 antibody or mouse IgG for 30 min at 37°C. For immunoprecipitation of NR2B, 1500 µg of tissue or mouse IgG was incubated in the presence of 4 µg NR2B antibody at 4°C for 18 hours. Precipitated proteins were eluted after boiling affinity beads in sample buffer containing DTT, Urea, and 5% v/v β-mercaptoethanol at 95°C for 10 min. Equivalent volumes were resolved via SDS-PAGE and immunoblotting performed.

For immunoblotting, all membranes were blocked for 1 hour in 0.05% Tween 20-Tris-buffered saline (TTBS) supplemented with an antibody specific blocking agent (5% nonfat dry milk, 0.02% purified casein protein, or sterile filtered 5% bovine serum albumin) and 1 mM Na₃VO₄ where appropriate. The following primary antibodies were used: NR1 (1:1000, Millipore), NR2A (1:1500, Millipore), NR2B (1:750, Antibodies, Inc.), GluR1 (1:1500, Millipore), GluR2 (1:1000, Antibodies, Inc.), PSD-95 (1:10,000; Affinity BioReagents, Golden, CO), Pyk2 (1:1000, Cell Signaling), β-actin (1:50,000, Sigma-Aldrich), and NR2B-specific phosphotyrosine antibodies Y-1252, Y-1336, and Y-1472 (1:2000, (Takasu et al., 2002)) Membranes were incubated in primary antibodies 2 hours at room temperature or overnight at 4 °C with gentle shaking, then rinsed in 3 changes of TTBS and incubated for 2 hours at room temperature with the appropriate mouse or rabbit HRP-conjugated secondary antibody (1:50,000–1:100,000; Jackson ImmunoResearch, West Grove, PA). After rinsing, blots were visualized with enhanced chemiluminescence (Lumi-Light, Roche) and exposed on film. The exposure time (5 s to 10 min) was adjusted to be within the linear range of each signal. Blots were stripped with 2 % SDS, 2 mM glycine, pH 2.0 for 1 hour at 55 °C, rinsed vigorously with 5 washes of TTBS, and re-probed with appropriate antibodies to correct for immunoprecipitation efficiency (anti-mouse IgG or NR2B), or protein loading error (β-actin). Images were digitized with a desktop scanner to a grayscale resolution of 300 dpi and files were analyzed with Scion Image software or Image J (NIH, Bethesda, MD). For examination of EphB2 tyrosine phosphorylation in Ephrin-B3^{neo/neo} mice, 40 µg of hippocampal homogenate from 3 pairs of littermates were assayed via Western blot for tyrosine phosphorylated EphB2 (Y-EphB2, 1:1000)(Takasu et al., 2002). These blots were stripped and re-probed for total EphB2 (1:1000) and analyzed via densitometry for the phospho/total ratio.

Transfection HEK 293 cells on the 27–32nd passage were grown to ~80% confluency over 3 days on 10 cm plates and transfected using calcium phosphate precipitation. Equivalent levels of NR1a (4µg) and NR2B (4µg) DNA were transfected in each experiment. Ephrin-B3-myc was introduced at a relatively high level (16µg) to ensure maximum effect. EphB2-FLAG was titrated between 4, 6, and 8 µg per experimental condition. Total DNA for each experimental condition was held constant by transfecting with pcDNA-GFP. DNA constructs for ephrin-B3-myc, EphB2-FLAG, and DN-EphB2-FLAG were previously characterized and described elsewhere (Takasu et al., 2002) Following transfection cells were maintained in media containing 10 µM D-APV. Transfected cells were incubated for 24 hours, before being rinsed in ice-cold PBS and harvested into 500µl modified RIPA lysis buffer supplemented with phosphatase inhibitors. Transfection efficiency was estimated to be 60–70% by visualizing equivalent transfection of pcDNA-GFP alone before harvest. The HEK 293 cell lysates were ultrasonicated then cleared for 10 min at 4°C, 14,000 rpm to remove cellular debris prior to

immunoprecipitation and SDS-PAGE. For some experiments, one batch of HEK 29 cells were transfected with NMDAR subunits and EphB and a separate set were transfected with ephrin-B3. Two days after transfection cells in each batch were detached from plates and resuspended in media at a ratio of 1:1. Cells were re-plated and allowed to grow until 95% confluent (1 day), before harvesting for analysis with Western blot.

Cortical Neuron Experiments

Rat cortical neurons were cultured onto 50 mm plates according to methods described previously from E18 embryos (Xie et al., 2005). Media from 10–14 day-old cultures was replaced with ACSF and cultures were allowed to equilibrate for 2 hours prior to treatment. ephrin-B1-Fc chimera (R&D Systems) was preclustered by adding anti-human Fc fragment-specific antibody (10:1 ratio, Jackson ImmunoResearch) and gently mixed at room temperature for 30 min. Cortical cultures were treated with 0.5 µg/ml Fc fragment alone or 0.5 µg/ml Fc-ephrinB1 cluster for 30 min. This timepoint was chosen to achieve maximal activation of downstream signaling partners (Grunwald et al., 2001). Cells were rinsed 3 times in ice-cold PBS and harvested by scraping into a modified RIPA lysis buffer supplemented with inhibitors.

Data Analysis

Statistical analysis was performed using Excel (Microsoft), Origin (Microcal) or GraphPad Prism (Graphpad Software). Two sample comparisons were made using the Student's *t*-test, nonparametric data were compared using the Kolmogorov-Smirnov test and comparison of repeated measures was made using the two-way ANOVA. Statistical significance is reported when $p < 0.05$. Where it is not explicitly stated in the text the Student's *t*-test was used for statistical comparison, in other cases the particular test applied to the data is specified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. NMDA/AMPA ratio is enhanced in CA1 pyramidal neurons from ephrin-B3^{neo/neo} mice
(Ai) Representative traces of EPSCs recorded at -70 mV and +40 mV from ephrin-B3^{wt/wt} mice. The AMPA component was measured as the peak of the current at -70 mV, and the NMDA component was measured 60–62.5 ms after the onset of the outward current at +40 mV. From this time point onwards, AMPA receptors do not contribute significantly to the response and the remaining current is mediated wholly by NMDA receptors (shaded area) (Marie et al., 2005). Calibration: 50 pA, 100 ms **(Aii)** Representative traces from ephrin-B3^{neo/neo} mice. Calibration: 25 pA, 100 ms **(Aiii)** Grouped data from all recordings from ephrin-B3^{neo/neo} mice and heterozygote and wildtype littermates. **(Bi)** Representative EPSC traces from reverse signaling incompetent ephrin-B3^{lacZ/lacZ} expressing mice. Calibration: 25 pA, 100 ms **(Bii)** Grouped data from ephrin-B3^{lacZ/lacZ} and interleaved littermate control recordings. Significance ($p < 0.05$) is denoted by an asterisk.

Figure 2. Amplitude of mEPSCs is reduced in ephrin-B3^{neo/neo} mice
 (Ai) Representative mEPSC recordings from ephrin-B3^{wt/wt} and (Aii) ephrin-B3^{neo/neo} mice. Calibration: 10 pA, 250 ms (Bi) Cumulative distribution of mEPSC amplitudes measured in wildtype (red) and hypomorphic mice (blue). (Bii) Mean amplitudes of mEPSCs in ephrin-B3^{wt/wt} (red) and ephrin-B3^{neo/neo} mice (blue). (Ci) Cumulative distribution of mEPSC frequencies in wildtype and knockout animals. (Cii) Graph of mean frequencies of mEPSC events. (Di) Cumulative distribution of Sr²⁺ mEPSC amplitudes measured in recordings from wildtype (red) and knockout (blue) animals.

Figure 3. Ifenprodil sensitivity of the NMDA EPSC is not altered in ephrin-B3^{neo/neo}
(A) Representative whole-cell voltage-clamp recordings of NMDA EPSC (+40 mV) from a wildtype mouse before and after application of the NR2B selective antagonist ifenprodil (3 μ M). **(B)** Representative NMDA EPSCs recorded from ephrin-B3^{neo/neo} mouse. Calibration: 20 pA, 100 ms. **(C)** Grouped data from all recordings demonstrating the percentage inhibition of the NMDA EPSC by ifenprodil. **(D)** The mean NMDA EPSC deactivation time constant (τ_{decay}) in wildtype and littermate ephrin-B3^{neo/neo} mice.

Figure 4. Selective upregulation of tyrosine phosphorylation of the NR2B NMDA receptor subunit in ephrin-B3^{neo/neo} mice

(A) Representative gel run with hippocampal homogenates immunoprecipitated with a pan-tyrosine phosphorylation antibody (4G10, p-Tyr). Phosphorylated proteins were immunoblotted with NR2A and NR2B antibodies. The bands observed in the immunoprecipitation are specific in comparison to negative (IgG - 5 μ g) and positive (Input - 40 μ g hippocampal homogenate) controls within each experiment. Each blot was probed with anti-mouse IgG to reveal the reduced heavy chain IgG bands. (B) Quantification of tyrosine-phosphorylated NR2 subunits. Immunoreactive bands were quantified by densitometry and normalized to the reduced heavy chain IgG to correct for immunoprecipitation efficiency. For each series of experiments, the relative immunoreactivity densitometric value was normalized to wildtype. (C) Representative blot of hippocampal homogenates immunoprecipitated with NR2B and immunoblotted with site specific phosphotyrosine antibodies (Y1252, Y1336, and Y1472). (D) Quantification of site specific tyrosine phosphorylation of NR2B. The measured densitometric value was normalized to total NR2B to determine the ratio of phosphorylated

NR2B. The values are expressed as a percent of control (wildtype) immunoreactivity. Significant values ($p < 0.05$) are indicated by an asterisk.

Figure 5. Ephrin-B3 attenuates EphB2-mediated NR2B subunit Y1252 phosphorylation
(A) HEK 293 cells were transfected with NMDA receptor (equivalent amounts of NR1a and NR2B cDNA) in the presence and absence of FLAG-tagged EphB2 and ephrin-B3 as indicated. Representative immunoblots of cell lysates confirm the presence of EphB2-FLAG and ephrin-B3 (pan-ephrin antibody). **(Aii)** Top panel, *Cis*-: Representative immunoblots from experiments where NMDAR subunits, EphB2 and ephrin-B3 were co-transfected and cell lysates that were immunoprecipitated with NR2B and immunoblotted with tyrosine site specific antibodies to Y1252, Y1336, and Y1472 of the NR2B subunit. Lower panel, *Trans*-: Representative immunoblots from mixing experiments, where NMDAR subunits and EphB2 were co-transfected, and ephrin-B3 was transfected into separate cells before mixing (see Experimental Methods). **(B)** Quantification of the percent decrease in EphB2-mediated NR2B Y1252 phosphorylation in the presence of ephrin-B3 from 4 individual experiments. For each experiment the densitometric value of phosphorylated/total NR2B subunit in the presence of ephrin-B3 (4:1 ratio respective to the NMDAR) was compared to ephrin-B3 untransfected cells in the presence of EphB2-FLAG (1.5-fold and 2-fold ratios respective to the NMDAR). Data is presented as percent reduction of the ephrin-B3 untransfected samples for each of the EphB2 cDNA concentrations. Comparisons were made using the Student's *t*-test.

Figure 6. Magnitude of theta-burst pairing-induced LTP is not different in ephrin-B3^{neo/neo} mice
(A) Theta-burst pairing protocol. Brief (2 nA, 2 ms) postsynaptic depolarization - evoked action potentials (top) were coincident with evoked EPSPs (represented below). A train consisted of five burst pairings (50 ms duration, 100 Hz), delivered at theta-frequency (200 ms inter-burst interval, 5 Hz). A total of three trains of theta-burst pairings were delivered at 0.1 Hz to induce LTP. Calibration: 50 mV, 50 ms. **(Bi)** Representative EPSP recorded in whole-cell current clamp mode from CA1 pyramidal neurons in wildtype mice before (1) and 35 – 40 min after LTP induction (2) **(Bii)** Time-course of a single LTP experiment from ephrin-B3^{wt/wt} mouse. Shaded area represents time after LTP induction. **(Ci)** Representative EPSPs recorded from ephrin-B3^{neo/neo} mice before (1) and after LTP induction (2). Calibration: 2 mV, 50 ms **(Cii)** Time-course of a single LTP experiment from ephrin-B3^{neo/neo} mouse. **(D)** Grouped data from all LTP recordings in ephrin-B3^{wt/wt} (red) and ephrin-B3^{neo/neo} mice (blue). For clarity data are parsed to show amplitudes from each group at one minute intervals. **(E)** Grouped data showing the magnitude of LTP calculated as the % potentiation between 35 – 40 min after induction compared to the pre-induction control period (shaded areas in (D)).

Figure 7. Ephrin-B3 ablation leads to activation of EphB RTKs and direct activation of Eph RTKs enhances phosphorylation of Pyk2 and NR2B subunit

(A) Whole hippocampal homogenates from wildtype and ephrin-B3^{neo/neo} mice were examined for the phosphorylation of EphB2 (Ai) Representative gel immunoblotted with a tyrosine phosphorylation site-specific antibody (Y-EphB2) and total EphB2. (Aii) Quantification of Y-EphB2 immunoreactivity. (B) EphB RTKs were activated by treating cortical neurons for 30 min with preclustered ephrin-B1-Fc (EB-Fc) or Fc fragment (Fc) alone and samples were immunoprecipitated with a pan-tyrosine antibody. (Bi) Representative blot of EphB RTK-stimulated cultures immunoprecipitated with pan-tyrosine antibody and immunoblotted with Pyk2 and NR2B (Bii) Quantification of NR2B and Pyk2. For each series

of experiments, the relative immunoreactivity densitometric value was compared to the vehicle (Fc fragment) control. The measured densitometric value was normalized to total IgG to determine the ratio of phosphorylated protein. The values are expressed as a percent of control immunoreactivity. Significant values ($p < 0.05$) are indicated by an asterisk.