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Dynamic Regulation of Homer Binding to Group I Metabotropic Glutamate Receptors by Preso1 and Converging Kinase Cascades

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ABSTRACT

In rat sympathetic neurons from the superior cervical ganglia (SCG) expressing metabotropic glutamate receptor mGluR1 or mGluR5, overexpression of scaffolding Homer proteins, which bind to a Homer ligand in their C termini, cause receptor clustering and uncoupling from ion channel modulation. In the absence of recombinant Homer protein overexpression, uncoupling of mGluRs from voltage-dependent channels can be induced by expression of Preso1, an adaptor of proline-directed kinases that phosphorylates the Homer ligand and recruits binding of endogenous Homer proteins. Here we show that in SCG neurons expressing mGluR1 and the tyrosine receptor kinase B, treatment with brain-derived neurotrophic factor (BDNF) produces a similar uncoupling of the receptors from calcium channels. We investigated the pathways that mediate this uncoupling and compared it with uncoupling observed with Preso1 expression. Both BDNF- and Preso1-induced uncoupling require residues T1151 and S1154 in the mGluR1 Homer ligand (TPPSPF). Uncoupling via Preso1 but not BDNF was prevented by expression of a dominant negative Cdk5, suggesting that endogenous Cdk5 mediates Preso1-dependent phosphorylation of mGluR1. Dominant negative Cdk5 did not block the BDNF effect but this was sensitive to inhibitors of the mitogen-activated protein kinase kinase/extracellular signal–regulated kinase cascade. Interestingly, the BDNF pathway appeared to require native Preso1 binding to mGluR, because overexpression of the Preso1 FERM domain, which mediates the Preso1–mGluR interaction, prevented BDNF-induced uncoupling. These data suggest that the BDNF/tyrosine receptor kinase B and Cdk5 pathways converge at the level of mGluR to similarly induce Homer ligand phosphorylation, recruit Homer binding, and uncouple mGluRs from channel regulation.

Introduction

Metabotropic glutamate receptors (mGluRs) are family C G protein–coupled receptors that regulate neuronal excitability, synaptic plasticity, and many other processes in nearly every region of the brain (Vaidya et al., 2013). The group I mGluRs mGluR1 and mGluR5 and their splice variants couple to $G\alpha_{q/11}$ as well as $G\alpha_{i/o}$ proteins (Niswender and Conn, 2010). The splice variants mGluR1a, mGluR5a, and mGluR5b are often closely associated with the postsynaptic density (PSD) by virtue of their association with the Homer scaffolding proteins (Brakeman et al., 1997), which bind to a proline-rich PPXXF motif on their C termini (Tu et al., 1998). Association of these mGluRs with the scaffolding Homer proteins positions them in close proximity to the PSD and, in turn, a set of effector proteins in that milieu, such as the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Kammermeier and Worley, 2007) and *N*-methyl-D-aspartic acid (NMDA) receptors (Bertaso et al., 2010), diacylglycerol (DAG) lipase (Jung et al., 2007; Roloff et al., 2010), and other proteins that can mediate synaptic plasticity, as well as inositol trisphosphate (IP_3) receptors via their direct interaction with Homer proteins (Tu et al., 1998). By contrast, association with scaffolding Homer proteins uncouples mGluR1 and mGluR5 from extrasynaptic effectors such as voltage-gated calcium and potassium channels (Kammermeier et al., 2000).

In this way, association with Homer proteins can act as a molecular switch prioritizing coupling of group I mGluRs to different sets of effectors. Cells can regulate this switch in one respect by upregulation of Homer-1a, a naturally occurring dominant negative (DN) variant of Homer-1 (Brakeman et al., 1997), which expresses as an immediate early gene after periods of strong neural activity, or downstream of other

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDNF, brain-derived neurotrophic factor; DAG, diacylglycerol; DN, dominant negative; ERK, extracellular signal–regulated kinase; ERK KM, ERK K71M; IP_3, inositol trisphosphate; MEK, mitogen-activated protein kinase kinase; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartic acid; PCR, polymerase chain reaction; PD98059, 2-(2-amino-3-methoxyphenyl)chromen-4-one; PSD, postsynaptic density; RT, reverse transcription; SCG, superior cervical ganglion; TrkB, tyrosine receptor kinase B.

signaling cascades (Sato et al., 2001; Nielsen et al., 2002). Another regulatory mechanism was uncovered recently, in which phosphorylation of the Homer binding site (TPPSPF in mGluRs) enhances the interaction of Homer proteins with mGluR1 and mGluR5 (Park et al., 2013). We recently showed that the protein Preso1 can act as a protein kinase–anchoring protein for proline-directed kinases such as Cdk5 and extracellular signal–regulated kinase (Erk) (Hu et al., 2012). Although Preso1 can bind to both group I mGluRs and Homer proteins, binding to the mGluR1 or mGluR5 C-terminal tail was both necessary and sufficient to direct phosphorylation of this site by these kinases downstream of mGluR signaling and tyrosine receptor kinase B (TrkB) receptor activation by brain-derived neurotrophic factor (BDNF) (Hu et al., 2012). In addition, investigators are beginning to describe some effects downstream of phosphorylation. For example, mGluR phosphorylation can lead to association with Pin-1 prolyl isomerase in the absence of binding by scaffolding Homer proteins, which promotes coupling of mGluR5 to NMDA receptors (Park et al., 2013). This association with Pin-1 and subsequent isomerization appears to play an important role in mGluR-NMDA plasticity and in certain modalities of addiction (Park et al., 2013), and it is regulated by Homer association and Homer ligand phosphorylation. Furthermore, by regulating Homer binding, phosphorylation of this site may play an important role in autism and cognitive dysfunction by uncoupling group I mGluRs from the postsynapse (Guo et al., 2016). Phosphorylation of the Homer site can be initiated by several different cascades including BDNF and dopamine receptor activation (Hu et al., 2012; Park et al., 2013).

Here we examine the specific signaling intermediates of two separate pathways that lead to mGluR1 Homer ligand phosphorylation and enhanced Homer scaffolding. We examined this pathway in a highly tractable, adult neuronal experimental system comprising sympathetic neurons from the rat superior cervical ganglion (SCG), in which the efficacy of coupling of heterologously expressed group I mGluRs to native calcium channels provides a sensitive measure of the degree of association of these receptors with natively expressed Homer proteins (Kammermeier et al., 2000). This is done by whole-cell patch-clamp electrophysiological experiments to isolate the native, mostly N-type calcium currents, which are strongly inhibited downstream of G protein–coupled receptor activation (Herlitze et al., 1996; Ikeda, 1996). We found that enhanced Homer binding via phosphorylation of the Homer ligand on mGluRs could be initiated either via overexpression of Preso1 or by activation of the TrkB receptor with BDNF. Although these pathways work primarily via different proline-directed kinases, they are both dependent on Preso1 scaffolding for phosphorylation of the mGluR Homer site.

Materials and Methods

Cell Isolation, cDNA Injection, and Preparation. A detailed description of the cell isolation and cDNA injection protocol is published elsewhere (Lu et al., 2009). Animal protocols were approved by the University of Rochester Committee on Animal Resources. Briefly, both SCGs were removed from adult male Wistar rats (150–300 g) after decapitation and were incubated in Earle's balanced salt solution (Life Technologies Inc., Rockville, MD) containing 0.5 mg/ml trypsin (Worthington Biochemicals, Freehold, NJ) and 1.2 mg/ml type IV collagenase (Worthington Biochemicals) for 1 hour at 35°C. Cells were then centrifuged (50*g*) twice for 6 minutes, transferred to minimum essential medium (Fisher Scientific, Pittsburgh, PA), plated on poly(L-lysine)–coated 35-mm polystyrene tissue culture dishes, and incubated (95% air and 5% CO_2; 100% humidity) at 37°C prior to DNA injection. After injection, cells were incubated overnight at 37°C and imaging or patch-clamp experiments were performed the next day.

Injection of cDNA was performed with an Eppendorf FemtoJet microinjector and the InjectMan NI2 micromanipulator (Brinkmann, Westbury, NY) 4–6 hours after cell isolation. Plasmids were stored at −20°C as a 1 $\mu g/\mu l$ stock solution in Tris/EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8). Neurons were coinjected with "enhanced" green fluorescent protein cDNA (0.02 $\mu g/\mu l$, pEGFPN1 or C1; Clontech Laboratories, Mountain View, CA) to facilitate later identification of successfully injected cells. All constructs were sequence verified prior to use in experiments. Polymerase chain reaction (PCR) products were purified with silica membrane spin columns (Qiagen, Valencia, CA) or a GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO) prior to restriction digestion and ligation. Midi- or maxipreps were prepared using Qiagen anion exchange columns.

The mGlu1 (pCDNA3.1+), mGluR5b (pRK5), Preso1, TrkB receptor, Homer-1c, Homer-2b, DN Cdk5, and ERK KM (pRK5) constructs were injected at 100–130 ng/μl, where indicated. BDNF (Sigma-Aldrich) was applied to cells in culture at 3.6 μM at least 1 hour prior to patch-clamp recording, where indicated. PD98059 [2-(2-amino-3-methoxyphenyl)-chromen-4-one] was obtained from Sigma-Aldrich.

Electrophysiology and Data Analysis. Patch-clamp recordings were made from 8250 glass (King Precision Glass, Claremont, CA). Pipette resistances were generally 1–3 MΩ, yielding uncompensated series resistances of 2–7 MΩ. Series resistance compensation of 80% was used in all recordings. Data were recorded using an EPC-7 patch-clamp amplifier (formerly HEKA Elektronic, Lambrecht, Germany; now Harvard Bioscience, Holliston, MA) or an Axon Axopatch-1D patch-clamp amplifier (formerly Axon Instruments, Sunnyvale, CA; now Molecular Devices, Sunnyvale, CA). Voltage protocol generation and data acquisition were performed using custom data acquisition routines (donated by Stephen R. Ikeda, National Institutes of Health National Institute on Alcohol Abuse and Alcoholism, Rockville, MD) within Igor Pro software (WaveMetrics, Lake Oswego, OR) on a Macintosh mini computer with an InstruTech ITC18-USB data acquisition board (HEKA Elektronik). Currents were sampled at 0.5- to 5-kHz low-pass filtered at 3 kHz using the filter in the patch-clamp amplifier, digitized, and stored on the computer for later analysis. All experiments were performed at 21–24°C (room temperature). Data analysis was performed using Igor Pro software.

For calcium current recordings, the external (bath) solution contained 145 mM tetraethylammonium methanesulfonate, 10 mM HEPES, 15 mM glucose, 10 mM $CaCl_2$, and 300 nM tetrodotoxin, pH 7.4 (osmolality, 320 mOsm/kg). The internal (pipette) solution contained 120 mM *N*-methyl-D-glucamine methanesulfonate, 20 mM tetraethylammonium, 11 mM EGTA, 10 mM HEPES, 10 mM sucrose, 1 mM $CaCl_2$, 4 mM MgATP, 0.3 mM Na_2GTP, and 14 mM Tris creatine phosphate, pH 7.2 (osmolality, 300 mOsm/kg). For M-current recordings, the external solution contained 150 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 15 mM glucose, and 300 nM tetrodotoxin, pH 7.4 (osmolality, 320 mOsm/kg). The internal solution contained 150 mM KCl, 0.1 mM K_4BAPTA, 10 mM HEPES, 4 mM MgATP, and 0.1 mM Na_2GTP, pH 7.2 (osmolality, 300 mOsm/kg). The glutamate concentration in all experiments was 100 μM. The glutamate concentration used was 100 μM, BDNF was applied at 3.6 nM, and PD98059 was applied at 3 μM.

Results

Uncoupling of Group I mGluRs from Calcium Current Modulation in SCG Neurons by Homer, Preso1, and TrkB Receptor Activation by BDNF. We previously showed that association with scaffolding Homer proteins

(Homer-1b, 1c, 2, and 3) uncouples group I mGluRs from modulation of voltage-dependent calcium channels (Kammermeier et al., 2000). These Homer proteins assemble the receptors into large clusters that are analogous to the PSD (Kammermeier, 2006; Kammermeier and Worley, 2007). Because voltage-dependent calcium and potassium channels are likely excluded from these clusters, their modulation by mGluRs within the cluster is greatly weakened. Interestingly, coupling to effectors that are coassembled (e.g., the IP_3 receptors, DAG lipases, and AMPA receptors) is strengthened by scaffolding Homer proteins (Xiao et al., 2000).

Indeed, as shown in Fig. 1, when mGluR1 is heterologously expressed in SCG neurons by intranuclear cDNA injection (Lu et al., 2009), receptor activation by 100 μM glutamate results in a strong, reversible inhibition of the native calcium currents. Coexpression of Homer-1c significantly reduced the calcium current inhibition from 44% ± 5% (n = 6) in the absence of Homer-1c (the bold open circles in Fig. 1 show percent inhibition measurements from individual cells) to 24% ± 6% (n = 6) in the presence of Homer-1c (light open circles).

Our previous work showed that activation of TrkB could lead to a Preso1-dependent phosphorylation of the mGluR Homer binding site in cortical neurons (Hu et al., 2012). Thus, to test whether this pathway would lead to Homer binding and functional uncoupling of mGluRs from calcium channels in SCG neurons, TrkB was coexpressed with mGluR1 in SCG neurons, and coupling to calcium currents was tested by applying 100 μM glutamate to untreated cells expressing mGluR1 and TrkB or to cells pretreated for at least 1 hour with 3.6 nM BDNF (Fig. 1). As with neurons expressing mGluR1 alone, untreated cells expressing both mGluR1 and TrkB exhibited strong calcium channel inhibition of 48% ± 5% (n = 15, bold triangles in Fig. 1), but coupling was significantly reduced to 30% ± 5% (n = 15, light triangles) when cells were pretreated with BDNF. These data are consistent with a model in which activation of the proline-directed kinase ERK is activated downstream of TrkB, resulting in phosphorylation of the Homer site of mGluR1 and leading to enhanced binding of endogenous Homer proteins and at least partial uncoupling from calcium channel modulation.

Next, we reproduced the experiment performed in Hu et al. (2012), in which coexpression of Preso1 with mGluR1 in SCG neurons led to uncoupling of mGluR1 from calcium channel modulation. Here, Preso1 reduced channel inhibition to 16% ± 5% (n = 6, light diamonds in Fig. 1B) from 47% ± 5% (n = 15, bold diamonds) in matched control cells expressing mGluR1 alone (Fig. 1B). Thus, uncoupling of mGluR1 from SCG calcium currents was observed after Homer-1c overexpression, Preso1 overexpression, and activation of TrkB with BDNF.

The BDNF Effect Is Lost When Homer Ligand Phosphorylation Sites on mGluR1 Are Mutated. Next, we tested the prediction that the uncoupling of mGluR1 from calcium channel modulation by TrkB activation was dependent on phosphorylation of the Homer ligand on mGluR1. As shown in Fig. 2, the mGluR1 double-mutant T1151A,S1154A (altering the Homer site from TPPSPF to APPAPF results in the mGluR1 TASA mutant) coupled to calcium currents in SCG neurons similarly to the wild-type mGluR1. This mutant remains capable of binding Homer proteins, but the affinity of

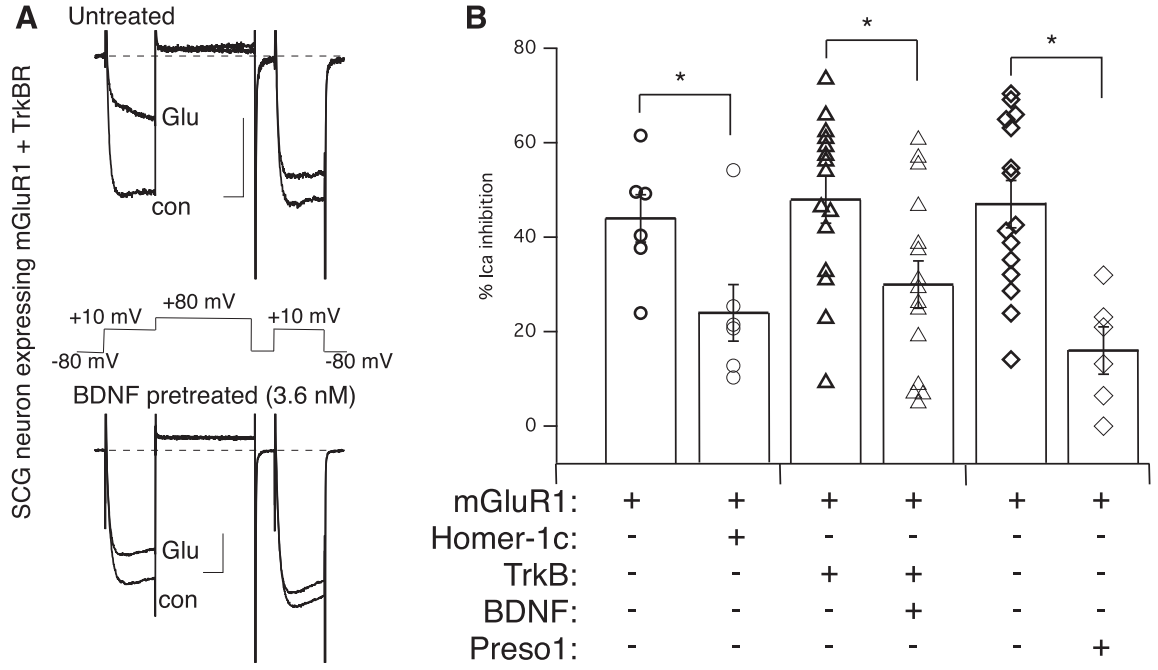

Fig. 1. Group I mGluRs in SCG neurons are uncoupled from calcium channel modulation by overexpression of Homer proteins, Preso1, or activation of TrkB with BDNF. (A) Sample current traces illustrating baseline currents (uninhibited, labeled "con") and currents inhibited by 100 μM glutamate in a cell expressing mGluR1 and TrkB, either untreated with BDNF (upper) or treated with 3.6 nM BDNF for >1 hour (lower). Currents were elicited with the "triple pulse" voltage protocol illustrated (center). This protocol consists of two test pulses to +10 mV separated by a strong depolarizing step designed to illustrate the voltage dependence of inhibition mediated by G$\beta\gamma$ (Elmslie et al., 1990). Scale bars indicate 0.2 nA and 10 milliseconds (upper) and 0.5 nA and 10 milliseconds (lower). (B) Average ± S.E.M. inhibition (bars) by 100 μM glutamate in cells with the indicated expression and drug treatment. Individual symbols indicate percent inhibition values from individual cells in each group. *$P \leq 0.05$ (t test). Glu, glutamate; ICa, voltage-gated calcium current.

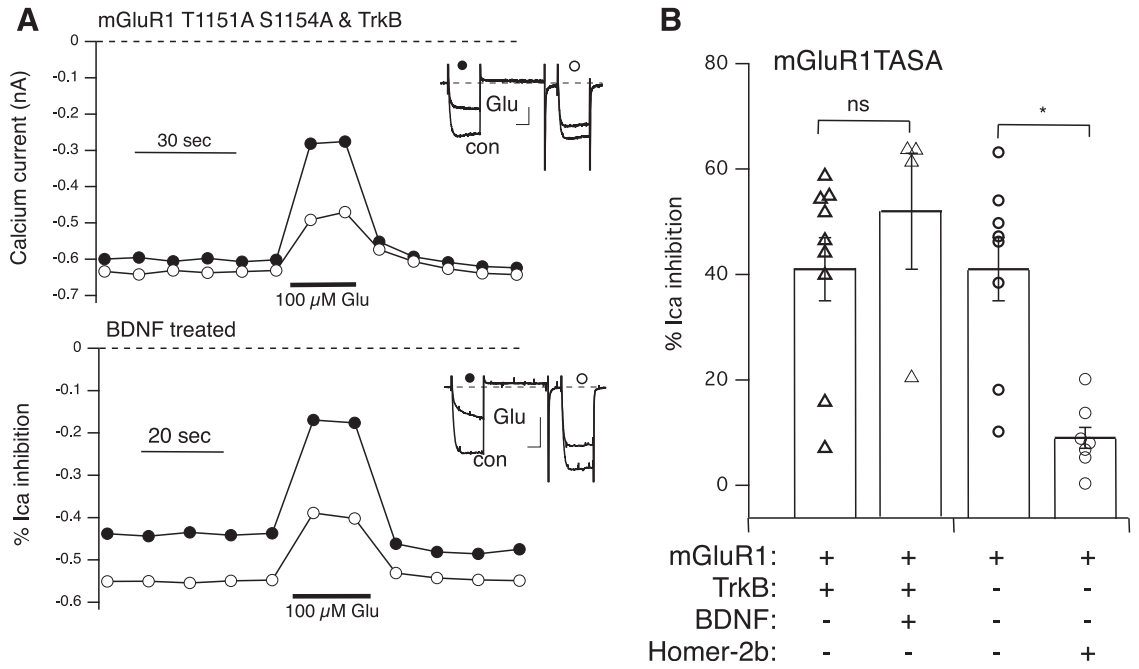

Fig. 2. TrkB activation with BDNF does not uncouple the mGluR1 TASA mutant from calcium current modulation. (A) Sample calcium current amplitudes and current traces (insets) illustrating inhibition by 100 μM glutamate in SCG neurons expressing the mGluR1 TASA mutant, which cannot be phosphorylated at the Homer binding site, with TrkB in untreated cells (upper) and cells pretreated with 3.6 nM BDNF for $\geq$1 hour (lower). Time courses illustrate amplitude measurements taken at the indicated times in the insets (filled and open circles) for each cell shown. Scale bars in each inset represent 0.2 nA and 10 milliseconds. (B) Average $\pm$ S.E.M. inhibition (bars) by 100 μM glutamate in cells with the indicated expression and drug treatment. Individual symbols indicate percent inhibition values from individual cells in each group. $^*P \leq 0.05$ (t test). con, control; Glu, glutamate; ICa, voltage-gated calcium current; ns, not significantly different.

the site for Homer proteins cannot be regulated by phosphorylation (Hu et al., 2012). When this receptor was expressed with TrkB (untreated with BDNF), activation with 100 μM glutamate produced an average of 41% $\pm$ 9% inhibition of the current (n = 9). Similarly, when cells expressing mGluR1 TASA and TrkB were pretreated with BNDF, the average inhibition of the current was 52% $\pm$ 11% (n = 4; Fig. 2). To demonstrate that the mutant receptor could still interact with Homer proteins, mGluR1 TASA was expressed alone or with Homer-2b. Under these conditions, 100 μM glutamate produced inhibitions of 41% $\pm$ 6% (n = 8) and 9% $\pm$ 2% (n = 7), suggesting that the mutant receptor can bind and be scaffolded by Homer proteins but with the phosphorylation sites mutated cannot be regulated by proline-directed kinases to enhance Homer association (Fig. 2B).

Uncoupling of mGluR1 from Calcium Channels by Preso1 Overexpression But Not TrkB Activation Requires the Proline-Directed Kinase Cdk5. To test the involvement of the proline-directed kinase Cdk5 in the pathways activated by Preso1 overexpression and TrkB activation by BDNF in SCG neurons, each pathway was induced in the absence and presence of the DN Cdk5 mutant. As shown in Fig. 3A, in paired negative control experiments replicating those in Fig. 1, expression of mGluR1 with TrkB and untreated with BDNF resulted in inhibition of 51% $\pm$ 11% (n = 12) of the calcium currents with 100 μM glutamate. In mGluR1/TrkB cells pretreated with 3.6 nM BDNF, the inhibition was significantly reduced to 35 $\pm$ 9% (n = 6). However, when DN Cdk5 was coexpressed, BDNF was not occluded from reducing the calcium current modulation (Fig. 3A). In these cells, inhibition was 25% $\pm$ 6% (n = 6), suggesting that the effect of BDNF through TrkB does not require Cdk5 activation. To verify the role of Cdk5 in the mGluR1–calcium channel uncoupling observed when Preso1 is overexpressed in SCG neurons, channel modulation was examined when mGluR1 was expressed alone and when mGluR1 was coexpressed with DN Cdk5 and with DN Cdk5 and Preso1. As shown in Fig. 3B, activation of mGluR1 with glutamate in SCG neurons resulted in a 42% $\pm$ 6% inhibition of the calcium current (n = 14), consistent with the values observed in previous figures. Coexpression of DN Cdk5 without Preso1 resulted in a similar inhibition of 41% $\pm$ 4% (n = 6), indicating that endogenous Cdk5 is negligibly active in this assay. However, when DN Cdk5 was expressed with mGluR1, coexpression of Preso1 failed to uncouple the receptor from calcium current modulation. In these cells, an inhibition of 36% $\pm$ 6% was observed (n = 9), which was not significantly different from either control value. Together, these data indicate that the regulation of mGluR1–calcium channel coupling in SCG neurons by Preso1 overexpression requires active Cdk5, but the pathway initiated by TrkB activation with BDNF does not.

The TrkB Pathway Requires ERK Activation, But the Preso1 Pathway Does Not. Next, the possibility that the Preso1 pathway may act through the proline-directed kinase ERK was tested, as was the somewhat intuitive prediction that BDNF activation of TrkB requires ERK (Fig. 4). Interestingly, the DN ERK construct ERK K71M (ERK KM) was unable to prevent uncoupling of mGluR1 from calcium current modulation. Calcium currents were inhibited by 52% $\pm$ 3% (n = 10) in SCG neurons expressing mGluR1 alone. Preso1 expression reduced the response both in the absence

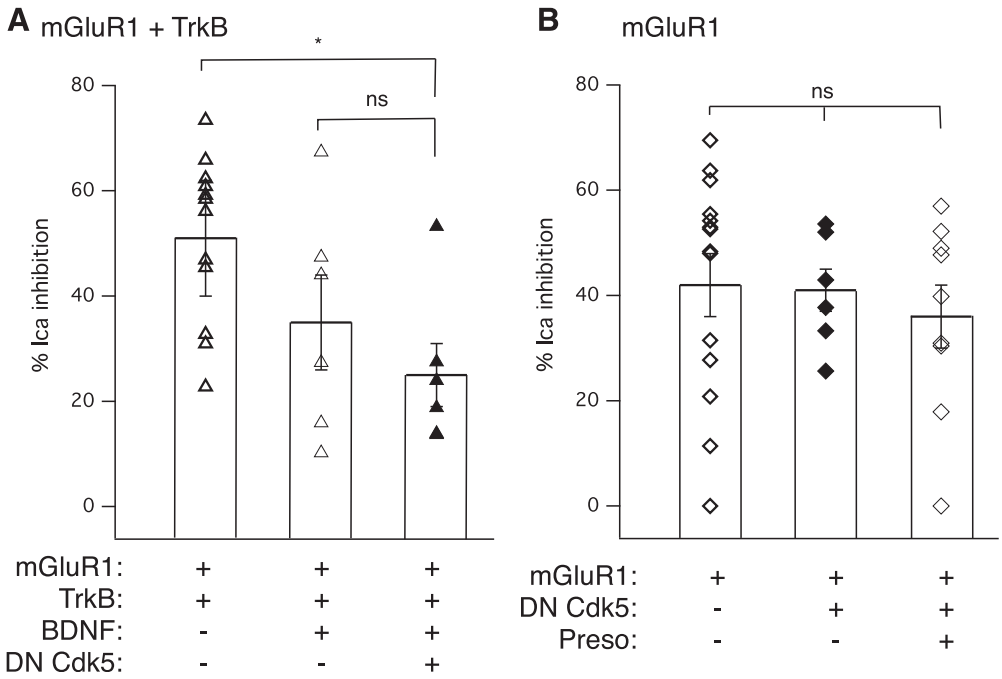

Fig. 3. mGluR1 uncoupling from calcium currents by BDNF but not Preso1 overexpression is insensitive to inhibition of Cdk5. (A and B) Average ± S.E.M. inhibition (bars) by 100 μM glutamate in cells with the indicated expression and drug treatment. Individual symbols indicate percent inhibition values from individual cells in each group. *$P \leq 0.05$ (analysis of variance). ICa, voltage-gated calcium current; ns, not significantly different.

(34% ± 4%, $n = 9$) and presence (33% ± 4%, $n = 6$) of ERK KM (Fig. 4), whereas coexpression of ERK KM alone (with mGluR1) had no effect (47% ± 5%, $n = 8$). These data indicate that while Preso1-dependent uncoupling of mGluR1 from calcium channels requires active Cdk5, it does not require activation of ERK.

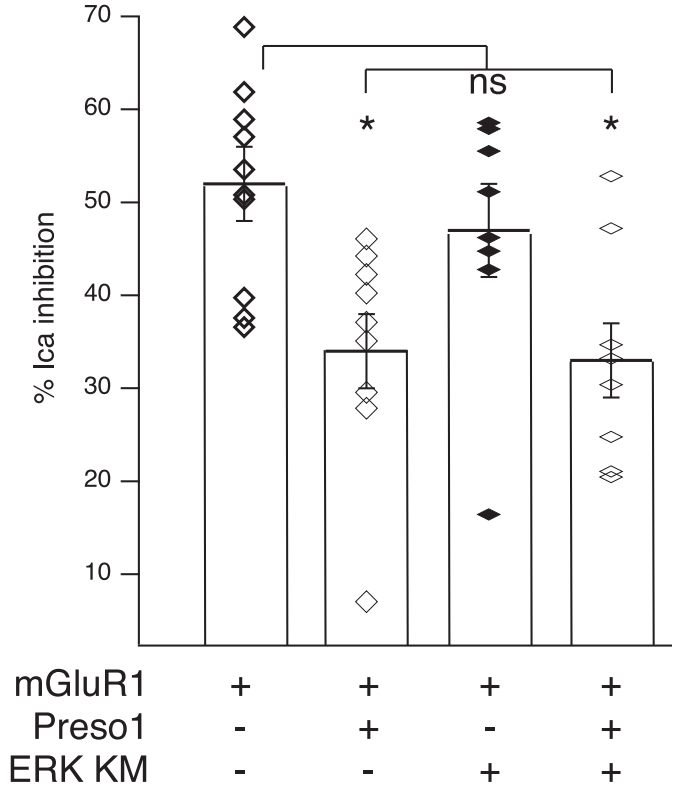

Fig. 4. TrkB activation with BDNF can still uncouple mGluR1 from calcium current modulation in the presence of the DN ERK mutant, ERK KM. (A) Average ± S.E.M. inhibition (bars) by 100 μM glutamate in cells with the indicated expression and drug treatment. Individual symbols indicate percent inhibition values from individual cells in each group. *$P \leq 0.05$ (analysis of variance). ICa, voltage-gated calcium current; ns, not significantly different from control.

The role of ERK in the TrkB pathway was also investigated (Fig. 5A). Because the effect of BDNF application via TrkB was more acute than overnight expression of Preso1, the pharmacological mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 was used to inhibit the ERK signaling cascade rather than ERK KM expression. As in the equivalent experiments described above, SCG neurons expressing mGluR1 and TrkB but untreated with BDNF produced a calcium current inhibition of 54±3 ($n = 13$). After pretreatment with BDNF, a significant reduction in modulation was seen. In these cells, inhibition was only 32% ± 5% ($n = 16$). Simultaneous pretreatment with 3.6 nM BDNF and 30 μM PD98059 restored strong coupling. In these cells, inhibition was 47% ± 6% ($n = 5$), as expected. Thus, the uncoupling of group I mGluRs from voltage-dependent calcium channels in SCG neurons requires activation of the MEK/ERK pathway, but similar uncoupling induced by Preso1 overexpression does not.

The BDNF Effect on mGluR1 Requires Binding of Endogenous Preso. Preso1 is a large protein containing several canonical protein interaction domains (Lee et al., 2008), including a FERM domain in the N-terminal half that directly binds to mGluR1, as well as a Homer ligand in the C-terminal half. Recruitment of proline-directed kinases to the mGluR C tail by Preso1 requires a direct Preso1–mGluR interaction but not a direct Preso1–Homer interaction (Hu et al., 2012). Because the TrkB pathway that leads to uncoupling of group I mGluRs from SCG calcium channels appears to work through proline-directed kinases and phosphorylation of the mGluR Homer binding site, the possibility that this pathway requires endogenous Preso1 protein was considered. In this model, TrkB activation with BDNF would lead to activated ERK. However, in the absence of Preso1, either the levels or localization of ERK are insufficient to

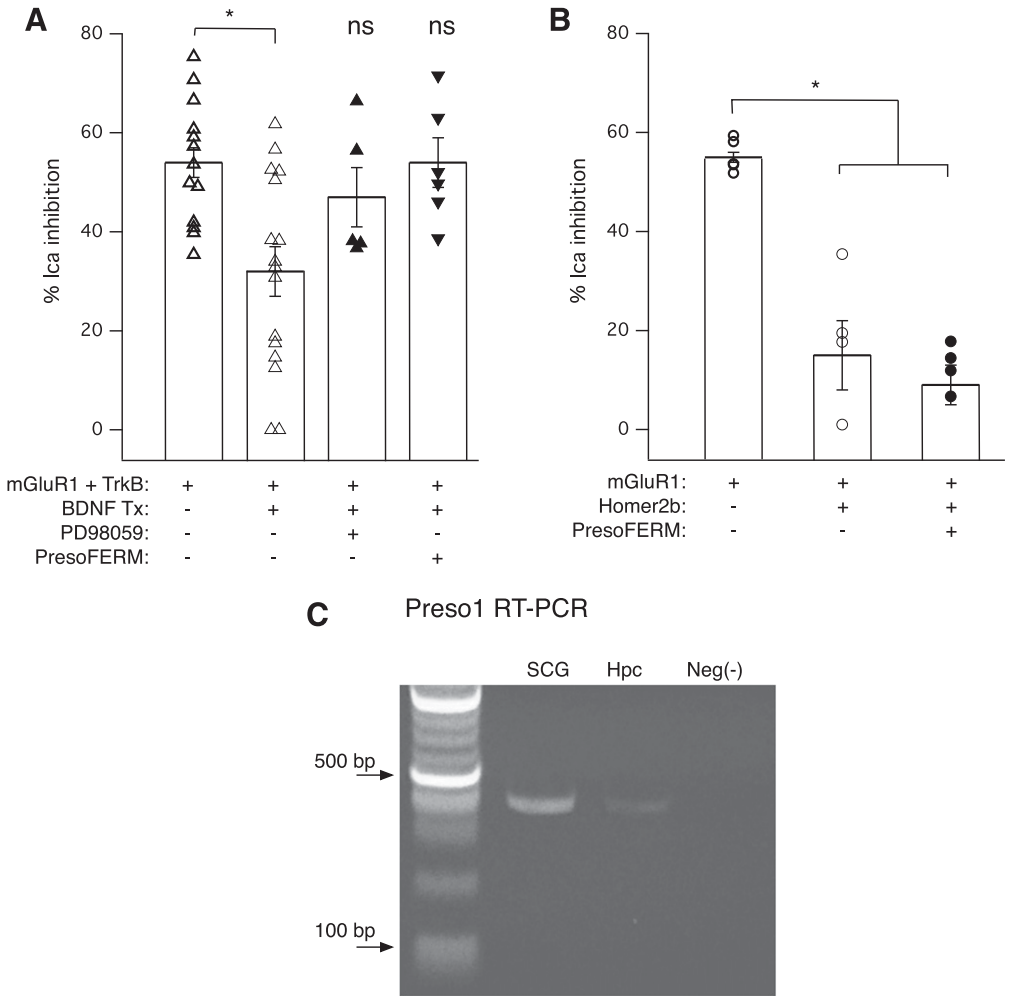

Fig. 5. mGluR1 uncoupling from calcium currents by BDNF is prevented with the MEK inhibitor PD98059. The BDNF effect requires endogenous Preso1 binding, but uncoupling mediated by Homer overexpression does not. (A and B) Average ± S.E.M. inhibition (bars) by 100 μM glutamate in cells with the indicated expression and drug treatment. Individual symbols indicate percent inhibition values from individual cells in each group. *$P \leq 0.05$ (analysis of variance). (C) SCG neurons express Preso1 mRNA. Results of an RT-PCR experiment designed to detect Preso1 message. Identical reactions were run for the experiments illustrated in each lane with the exception of the RNA used as the template. Lane 1 [SCG] used total RNA isolated from dissociated rat SCG neurons, lane 2 [Hpc] used total RNA isolated from cultured neonatal rat hippocampal neurons, and Lane 3 [Neg(−)] is the negative control (no RNA). Primers were designed to amplify a 407-bp band. The forward primer sequence was 5′-ATACGACATTGCC, and the reverse primer sequence was 5′-TGGCTGAAGTCAG in all lanes. These primers are predicted to span three exon-intron boundaries. ns, not significantly different from control. ICa, voltage-gated calcium current; ns, not significantly different.

potently phosphorylate the Homer site on expressed mGluRs. Thus, native Homer proteins are not sufficiently recruited, and uncoupling of the receptors from channel modulation does not occur. Alternatively, it is possible that activation of the MEK/ERK pathway via TrkB in SCG neurons is sufficient to phosphorylate the Homer site of group I mGluRs. To distinguish between these possibilities, a truncated mutant Preso1 protein containing only the FERM domain was coexpressed with mGluR1 and TrkB to determine whether it could prevent BDNF-induced uncoupling. The assumption is that the FERM domain would be expressed at high enough levels to prevent endogenous Preso1 from binding the receptors and recruiting active ERK to allow phosphorylation. As shown in Fig. 5A, expression of the FERM domain of Preso1 did in fact prevent BDNF-mediated uncoupling. In these cells, calcium channel inhibition averaged 54% ± 5% ($n = 6$) compared with the untreated negative controls (54% ± 3%) and BDNF-treated controls (32% ± 5%) described above. These data are consistent with the interpretation that BDNF-mediated uncoupling of mGluR1 from calcium channel modulation requires interaction of a full-length Preso1, or a similar protein, with the mGluR C tail.

To rule out the possibility that the FERM domain of Preso1 simply occludes uncoupling by preventing Homer association with the putatively phosphorylated Homer ligand, the Preso1 FERM domain was expressed with mGluR1 and Homer-2b to test whether it prevented uncoupling when Homer-2b was overexpressed. As shown in Fig. 5B, the Preso1 FERM domain did not prevent Homer-2b–mediated uncoupling. In five cells expressing mGluR1 alone, channel inhibition averaged 55% ± 1%, whereas inhibition was 15% ±7% ($n = 5$) and 9% ± 4% ($n = 5$) in cells coexpressing Homer-2b or Homer-2b with Preso1 FERM, respectively. Thus, it appears unlikely that expression of Preso1 FERM directly occludes Homer association with the C-terminal tail of mGluR1.

The data above suggest that TrkB-mediated phosphorylation of the mGluR Homer ligand requires recruitment of a proline-directed kinase, presumably ERK, to the C tail of the

receptor. Since Preso1 was not overexpressed in these experiments, one would predict that Preso1 must be natively expressed in SCG neurons. To test for the presence of Preso1 mRNA, reverse transcription (RT)-PCR primers designed to detect Preso1 message were used in RT-PCR experiments targeting RNA isolated from isolated rat SCG neurons. As a positive control, RNA was isolated from rat hippocampal neurons, which are known to express Preso1 (Lee et al., 2008). As a negative control, the reaction was run in the absence of added RNA (Fig. 5C). Both the SCG and hippocampal RNA yielded a positive band at approximately 400 bp, whereas the negative control produced no detectable bands. These data suggest that SCG neurons do in fact express Preso1, at least at the message level.

Discussion

In this study, we demonstrate that two proline-directed kinases, ERK and Cdk5, can be recruited to induce phosphorylation of the Homer binding site on group I mGluRs in adult rat sympathetic neurons from the SCG. As shown previously (Hu et al., 2012), overexpression of the neuronal adaptor protein Preso1 in SCG neurons leads to phosphorylation of residues in the Homer binding site in the C terminus of group I mGluRs. This phosphorylation results in increased binding of Homer proteins endogenously expressed in these neurons, leading to uncoupling of the receptors from modulation of voltage-dependent calcium channels. This effect is dependent on the phosphorylation sites in the Homer ligand (TPPSPF) of mGluR1/5. Furthermore, mutants of mGluR5 that cannot bind Homer are unaffected (Hu et al., 2012). Here, we show that specific inhibition of the proline-directed kinase Cdk5 using a DN mutant specifically prevents the uncoupling of mGluRs from voltage-dependent calcium channels after Preso1 overexpression. In addition, we describe another parallel pathway that can also lead to phosphorylation of group I mGluRs and consequent uncoupling of these receptors from calcium channel modulation. By expressing TrkB and activating it with BDNF treatment, we observe a similar uncoupling that also requires the Homer ligand phosphorylation sites to be intact. This pathway is, however, insensitive to DN Cdk5 expression but is disrupted by inhibitors of MEK. In addition, overexpression of the Preso1 FERM domain, which can bind the C tail of group I mGluRs, prevents the effect or TrkB/BDNF, suggesting that natively expressed Preso1 binding to the receptor may be necessary for this pathway as well. By contrast, we show that uncoupling resulting from direct overexpression of Homer-2b (Kammermeier et al., 2000) is insensitive to the presence of Preso1 FERM, suggesting that this effect is specific to phosphorylation of the Homer site and not due to simply preventing the mGluR-Homer interaction. Thus, two parallel pathways using different proline-directed kinases can both converge at the level of the receptor to produce phosphorylation, enhanced Homer binding, and thus uncoupling of mGluRs from inhibition of voltage-dependent calcium channels. These findings demonstrate the generality of the Preso1 pathway and suggest additional means of physiologic regulation of the fidelity of Homer-dependent scaffolding and coupling to downstream effectors.

The finding that BDNF/TrkB-dependent uncouples group I mGluRs from calcium channel modulation suggested that SCG neurons likely natively express Preso1. In fact, using RT-PCR to detect Preso1 mRNA, we show that Preso1 mRNA is present in rat SCG neurons. These data further illustrate the utility of the

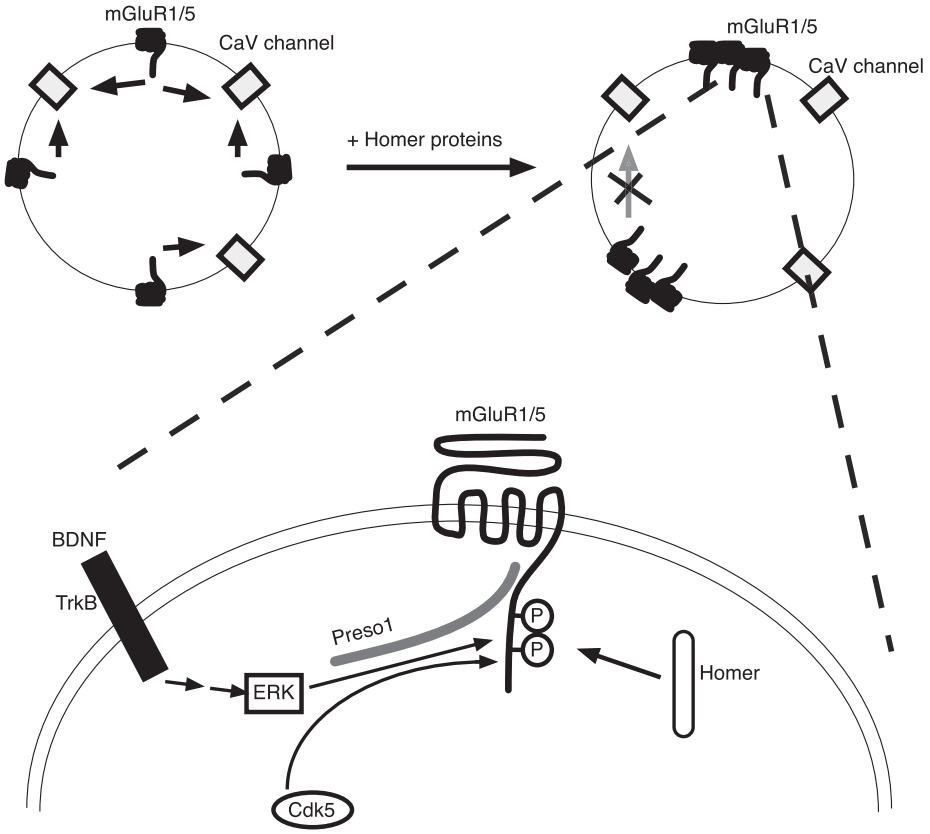

Fig. 6. Cartoon illustrating the refined model in which two converging proline-directed kinase cascades in SCG neurons can converge upon activation to produce Homer ligand phosphorylation, more efficient binding of scaffolding Homer proteins, and result in uncoupling of group I mGluRs from voltage-dependent channel modulation. CaV, voltage-gated calcium channel.

SCG preparation as a useful tool in studying Homer-dependent regulation of group I mGluR-effector coupling. Although these cells do not natively express mGluRs, they appear to natively express PSD proteins such that Homer proteins that can organize mGluRs into PSD-like clusters that retain the ability to toggle coupling from extrasynaptic effectors (e.g., voltage-dependent ion channels) in the absence of scaffolding Homer protein binding to canonical PSD effectors (e.g., DAG lipase) when bound to Homer proteins.

These data support a model for regulation of Homer binding, and thus mGluR-effector coupling, as depicted in Fig. 6. The upper portion of Fig. 6 illustrates Homer-dependent uncoupling of mGluR1 from calcium channels that results from receptor clustering induced by Homer protein association. The lower schematic focuses on phosphorylated, Homer-associated receptors (although Homer proteins are not shown to reduce clutter) and shows the group I mGluR (mGluR1/5) bound to Preso1 (in gray) at the proximal region of the receptor's intracellular C terminus. Preso1 can also bind directly to Homer proteins via a canonical, PPXXF Homer ligand, but this interaction is not necessary for its role as an adaptor for proline-directed kinases (Hu et al., 2012) and is therefore not depicted here. While bound to the tail of the receptor, Preso1 can help localize at least two proline-directed kinases, ERK or Cdk5, in close proximity to the serine and threonine residues of the mGluR Homer ligand (TPPSPF), where they can efficiently phosphorylate these residues and enhance Homer binding affinity. When more group I mGluRs are bound to Homer proteins, they will assemble into clusters akin to the PSD, from which they can more efficiently couple to PSD effectors (i.e., AMPA receptors) and other Homer binding proteins (i.e., DAG lipase, IP_3 receptors) but are uncoupled from extrasynaptic effectors such as voltage-dependent calcium and potassium channels. Under basal conditions, SCG neurons likely express both ERK and other kinases in the MAPK pathway as well as CdK5; however, without some other experimental intervention, either the expression levels or activity are too low to detectably alter the phosphorylation of the Homer ligand on expressed group I mGluRs. Upon either overexpression of heterologous Preso1 or activation for at least an hour of heterologous TrkB by BDNF, these kinases are activated and recruited to phosphorylate the Homer site and regulate its binding.

Finally, these data have significance beyond the regulation of mGluR coupling by virtue of the Homer scaffold. For example, it was recently demonstrated that group I mGluRs can be regulated by the Pin-1 isomerase, which catalyzes isomerization of the pS1126-P prolyl bond, leading to enhancement of mGluR regulation of NMDA receptor currents, which may be important in certain forms of plasticity and may play an important role in mGluR signaling that underlies addiction (Park et al., 2013). Interestingly, the association of Pin-1 with group I mGluRs is promoted by Homer-1a, is antagonized by Homer-1c (a long scaffolding Homer), and requires dual phosphorylation of the mGluR Homer ligand. Although the mGluR–calcium channel coupling assay employed is upstream of predicted effects of Pin-1 isomerization, those data strongly indicate the importance of an enhanced understanding of the pathways that lead to Homer site phosphorylation and the numerous downstream effects that result.

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Authorship Contributions

Participated in research design: Hu, Worley, Kammermeier.
Conducted experiments: Kammermeier.
Contributed new reagents or analytic tools: Hu, Worley, Kammermeier.
Performed data analysis: Kammermeier.
Wrote or contributed to the writing of the manuscript: Kammermeier.

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