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# Dynamic regulation of Homer binding to group I mGluRs by Preso1 and converging kinase cascades.

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Running Title:

**Phosphorylation of the mGluR Homer binding site by kinases.**

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**Abstract**

In rat sympathetic neurons from the superior cervical ganglia (SCG) expressing metabotropic glutamate receptors (mGluRs) 1 or 5, 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 or 5 and the receptor tyrosine kinase TrkB, treatment with 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 requires 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 endogenous Cdk5 mediates Preso1-dependent phosphorylation of mGluR. dnCDK5 did not block the BDNF effect but this was sensitive to inhibitors of the MEK-ERK kinase cascade. Interestingly, the BDNF pathway appeared to require native Preso1 binding to mGluR, as over-expression of the Preso1 FERM domain, which mediates the Preso1-mGluR interaction, prevented BDNF-induced uncoupling. These data suggest that BDNF-TrkB and CDK5 pathways converge at the level of mGluR to similarly induce Homer ligand phosphorylation, recruit Homer binding, and uncouple mGluRs from channel regulation.

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**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 5 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 5b are often closely associated with the post synaptic density 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 Homers positions them in close proximity to the post synaptic density and in turn, a set of effector proteins in that milieu such as AMPA (Kammermeier and Worley, 2007) and NMDA receptors (Bertaso *et al.*, 2010), DAG Lipase (Jung *et al.*, 2007; Roloff *et al.*, 2010) and other proteins that can mediate synaptic plasticity, and via their direct interaction with Homers (Tu *et al.*, 1998), $IP_3$Receptors. By contrast, association with scaffolding Homers uncouples mGluR1 and 5 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 up regulation of Homer-1a, a naturally occurring dominant negative variant of Homer-1 (Brakeman *et al.*, 1997), which expresses as an immediate early gene following periods of strong neural activity, or downstream of other signaling cascades (Sato *et al.*, 2001; Nielsen *et al.*, 2002). Recently, another regulatory mechanism has been uncovered in which phosphorylation of the Homer binding site (TPPSPF in mGluRs) enhances the interaction of Homers with mGluR1 and 5 (Park *et al.*, 2013). We have recently shown that the protein Preso1 can act as a protein kinase anchoring protein for proline-directed kinases such as Cdk5 and Erk (Hu *et al.*, 2012). While Preso1 can bind to both group I

mGluRs and Homer proteins, binding to the mGluR1 or 5 C-terminal tail was both necessary and sufficient to direct phosphorylation of this site by these kinases downstream of mGluR signaling and TrkB receptor activation by brain derived neurotrophic factor (BDNF) (Hu *et al.*, 2012). In addition, some effects downstream of phosphorylation have begun to be described. 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 is regulated by Homer association and Homer ligand phosphorylation. Further, 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, 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 (Ikeda, 1996; Herlitze *et al.*, 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. While these pathways work primarily via different proline directed kinases, they are both are 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 Committee on Animal Resources (UCAR). Briefly, both SCGs were removed from adult male Wistar rats (150-300 g) following decapitation, and 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 ºC. Cells were then centrifuged (50 x 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 ºC prior to DNA injection. After injection, cells were incubated overnight at 37 ºC and imaging or patch clamp experiments were performed the following day.

Injection of cDNA was performed with an Eppendorf FemtoJet microinjector and Injectman NI2 micromanipulator (Brinkmann, Westbury, NY) 4-6 hours following cell isolation. Plasmids are stored at –20 ºC as a 1 $\mu$g/$\mu$l stock solution in TE buffer (10 mM TRIS, 1 mM EDTA, pH 8). Neurons were co-injected with "enhanced" green fluorescent protein cDNA (0.02 $\mu$g/$\mu$l; pEGFPN1 or C1; Clontech Laboratories) to facilitate later identification of successfully injected cells. All constructs were sequence verified prior to use in experiments. PCR products were purified with Qiagen (Valencia, CA)

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silica membrane spin columns or Sigma GenElute Plasmid Miniprep Kit 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/$\mu$l, where indicated. BDNF (Sigma Aldrich, St. Louis, MO) was applied to cells in culture at 3.6 $\mu$M at least 1 hour prior to patch-clamp recording, where indicated. PD98059 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 (formerly HEKA Elektronik, Germany, now Harvard Biosciences) or Axon Axopatch 1D patch-clamp amplifier (formerly Axon Instruments, now Molecular Devices, Sunnyvale, CA). Voltage protocol generation and data acquisition were performed using custom data acquisition routines (donated by Stephen R. Ikeda, NIAAA, Rockville, MD) within Igor Pro (WaveMetrics, Lake Oswego, OR) on a Macintosh mini computer with an Instrutech ITC18-USB data acquisition board (HEKA). Currents were sampled at 0.5-5 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 preformed at 21-24ºC (room temperature). Data analysis was performed using Igor Pro.

For calcium current recordings, the external (bath) solution contained (in mM): 145 tetraethylammonium (TEA) methanesulfonate (MS), 10 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 glucose, 10 $CaCl_2$, and 300 nM tetrodotoxin, pH 7.4, osmolality 320 mOsm/kg. The internal (pipette) solution contained: 120 N-methyl-D-glucamine (NMG) MS, 20 TEA, 11 EGTA, 10 HEPES, 10 sucrose, 1 $CaCl_2$, 4 MgATP, 0.3 $Na_2GTP$, and 14 tris creatine

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phosphate, pH 7.2, osmolality 300 mOsm $kg^{-1}$. For M-current recordings, the external solution contained: 150 NaCl, 2.5 KCl, 10 HEPES, 1 $MgCl_2$, 2 $CaCl_2$, 15 glucose, and 300 nM tetrodotoxin, pH 7.4, osmolality 320 mOsm $kg^{-1}$. The internal solution contained: 150 KCl, 0.1 $K_4$BAPTA, 10 HEPES, 4 MgATP, and 0.1 $Na_2$GTP, pH 7.2, osmolality 300 mOsm/kg. The glutamate concentration in all experiments was 100 $\mu$M. The glutamate concentration used was 100 $\mu$M, BDNF was applied at 3.6 nM, PD98059 at 3 $\mu$M.

**Results**.

*Uncoupling of group I mGluRs from calcium current modulation in SCG neurons by Homer, Preso1, and TrkB receptor activation by BDNF*.

We have shown previously (Kammermeier *et al.*, 2000) that association with scaffolding Homer proteins (Homer-1b, 1c, 2, and 3) uncouples group I mGluRs from modulation of voltage dependent calcium channels. These Homer proteins assemble the receptors into large clusters that are analogous to the post synaptic density (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 co-assembled, such as the $IP_3$ receptors, DAG lipases, and AMPA receptors, is strengthened by scaffolding Homers (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 $\mu$M glutamate results in a strong, reversible inhibition of the native calcium currents. Co-expression of Homer-1c significantly reduced the calcium current inhibition from 44±5% (n=6) in the absence of Homer-1c (bold open

circles show % inhibition measurements from individual cells) to 24±6% (n=6) in the presence of Homer-1c (light open circles).

Our previous work had shown that activation of the receptor tyrosine kinase 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 co-expressed with mGluR1 in SCG neurons and coupling to calcium currents was tested by applying 100 $\mu$M glutamate to untreated cells expressing mGluR1 and TrkB, or cells pretreated for at least 1 hour with 3.6 nM BDNF (Fig. 1A, 1B). As with neurons expressing mGluR1 alone, cells expressing both mGluR1 and TrkB that were untreated exhibited strong calcium channel inhibition of 48±5% (n=15, bold triangles), but when pretreated with BDNF, coupling was significantly reduced to 30±5% (n=15, light triangles). These data are consistent with a model in which activation of the proline directed kinase ERK is activated downstream of TrkB, resulting phosphorylation of the Homer site of mGluR1, leading to enhanced binding of endogenous Homers and at least partial uncoupling from calcium channel modulation.

Next, we reproduced the experiment performed in Hu et al., (Hu *et al.*, 2012) in which co-expression 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) 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 following Homer-1c over-expression, Preso1 over-expression, and activation of TrkB with BDNF.

*BDNF effect is lost when Homer ligand phosphorylation sites on mGluR1 are mutated*.

Next, the prediction that the uncoupling of mGluR1 from calcium channel modulation by TrkB activation was dependent on phosphorylation of the Homer ligand on mGluR1 was tested. As shown in Fig. 2, the mGluR1 double mutant T1151A,S1154A (altering the Homer site from TPPSPF to APPAPF: "mGluR1 TASA") 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 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 $\mu$M glutamate produced an average of 41±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±11% (n=4; Fig. 2A&B). 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 $\mu$M glutamate produced inhibitions of 41±6% (n=8) and 9±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 dominant negative Cdk5 mutant (DN Cdk5). 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 the calcium currents with 100 $\mu$M glutamate of 51±11% (n=12). In mGluR1/TrkB cells pretreated with 3.6 nM BDNF, the inhibition was significantly reduced to

359% (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±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, when 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±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±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±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 dominant negative ERK construct ERK KM was unable to prevent uncoupling of mGluR1 from calcium current modulation. Calcium currents were inhibited by 52±3% (n=10) in SCG neurons expressing mGluR1 alone. Preso1 expression reduced the response both in the absence (34±4%, n=9) and presence (33±4%, n=6) of ERK KM (Fig. 4), while co-expression 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.

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 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). Following 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 $\mu$M 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, and 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 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 channel inhibition averaged 54±5% (n=6) compared to 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 5 cells expressing mGluR1 alone, channel inhibition averaged 55±1%, while in cells coexpressing Homer-2b or Homer-2b with Preso1 FERM, inhibition was 15±7% (n=5) and 9±4% (n=5), 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, 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 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 ~400 bp, while 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. Further, 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 dominant negative mutant specifically prevents the uncoupling of mGluRs from voltage dependent calcium channels following 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 the receptor tyrosine kinase TrkB

and activating it by BDNF pretreatment, we observe a similar uncoupling that also requires the Homer ligand phosphorylation sites to be intact. This pathway is however insensitive to dominant negative 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 physiological regulation of the fidelity of Homer-dependent scaffolding and coupling to downstream effectors.

The finding that BDNF/TrkB dependent uncoupling of 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 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 post-synaptic density proteins such that Homer proteins that can organize mGluRs into PSD-like clusters that retain the ability to toggle coupling from extra-synaptic effectors, such as voltage dependent ion channels, in the absence of scaffolding Homer protein binding, to canonical PSD effectors, such as DAG Lipase and AMPA channels, when bound to Homers.

These data support a model for regulation of Homer binding, and thus mGluR-effector coupling, as depicted in Fig. 6. The upper portion 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 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 it's 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 Rs) and other Homer binding proteins (i.e. DAG Lipase, $IP_3$Rs) but are uncoupled from extra-synaptic 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, although without some other experimental intervention, either the expression levels or activity is 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 plays an important role in mGluR signaling that underlies addiction (Park *et al.*, 2013). Interestingly, association of Pin-1 with group I mGluRs is promoted by Homer-1a, antagonized by Homer-1c (a long scaffolding Homer), and requires dual phosphorylation of the mGluR Homer ligand. While 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: Worley, Hu, and Kammermeier

Conducted experiments: Kammermeier

Contributed new reagents or analytic tools: Worley, Hu, and Kammermeier

Performed data analysis: Kammermeier

Wrote or contributed to the writing of the manuscript: Kammermeier

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**Footnotes**.

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**Figure Legends**.

Figure 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 $\mu$M glutamate ("Glu") 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 Gβγ (Elmslie *et al.*, 1990). Scale bars indicate 0.2 nA and 10 msec (*upper*) and 0.5 nA and 10 msec (*lower*). B, Average ± SEM inhibition (bars) by 100 $\mu$M glutamate in cells with the indicated expression and drug treatment. Individual symbols indicate % inhibition values from individual cells in each group. * indicated significant differences, $p \leq 0.05$, T-Test.

Figure 2. TrkB activation with BDNF does not uncouple the mGluR1 TASA mutant from calcium current modulation. A, Sample calcium current current amplitudes and current traces (insets) illustrating inhibition by 100 $\mu$M 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 ≥ 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 msec. B, Average ± SEM inhibition (bars) by 100 $\mu$M glutamate in cells with the indicated expression and drug treatment. Individual symbols indicate %

inhibition values from individual cells in each group. * indicated significant differences, $p \leq 0.05$, T-Test, ns = not significantly different.

Figure 3. mGluR1 uncoupling from calcium currents by BDNF but not Preso1 overexpression is insensitive to inhibition of Cdk5. A & B, Average ± SEM inhibition (bars) by 100 $\mu$M glutamate in cells with the indicated expression and drug treatment. Individual symbols indicate % inhibition values from individual cells in each group. * indicated significant differences, $p \leq 0.05$, ANOVA, ns = not significantly different.

Figure 4. TrkB activation with BDNF can still uncouple mGluR1 from calcium current modulation in the presence of the dominant negative ERK mutant, ERK KM. A, Average ± SEM inhibition (bars) by 100 $\mu$M glutamate in cells with the indicated expression and drug treatment. Individual symbols indicate % inhibition values from individual cells in each group. * indicates significant differences, $p \leq 0.05$, ANOVA, ns = not significantly different from control.

Figure 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 & B, Average ± SEM inhibition (bars) by 100 $\mu$M glutamate in cells with the indicated expression and drug treatment. Individual symbols indicate % inhibition values from individual cells in each group. * indicates significant differences, $p \leq 0.05$, ANOVA, ns = not significantly different from control. 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, the reverse primer sequence was: 5’- TGGCTGAAGTCAG in all lanes. These primers are predicted to span 3 exon-intron boundaries.

Figure 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.

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Figure 1

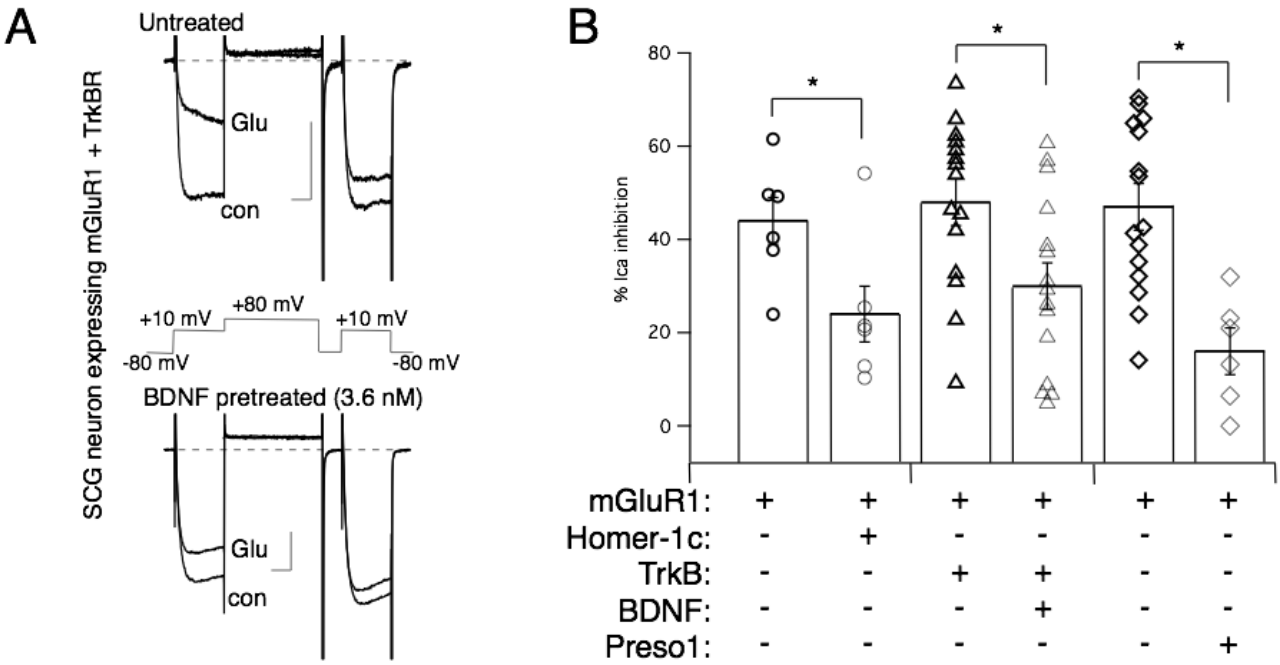

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Figure 2

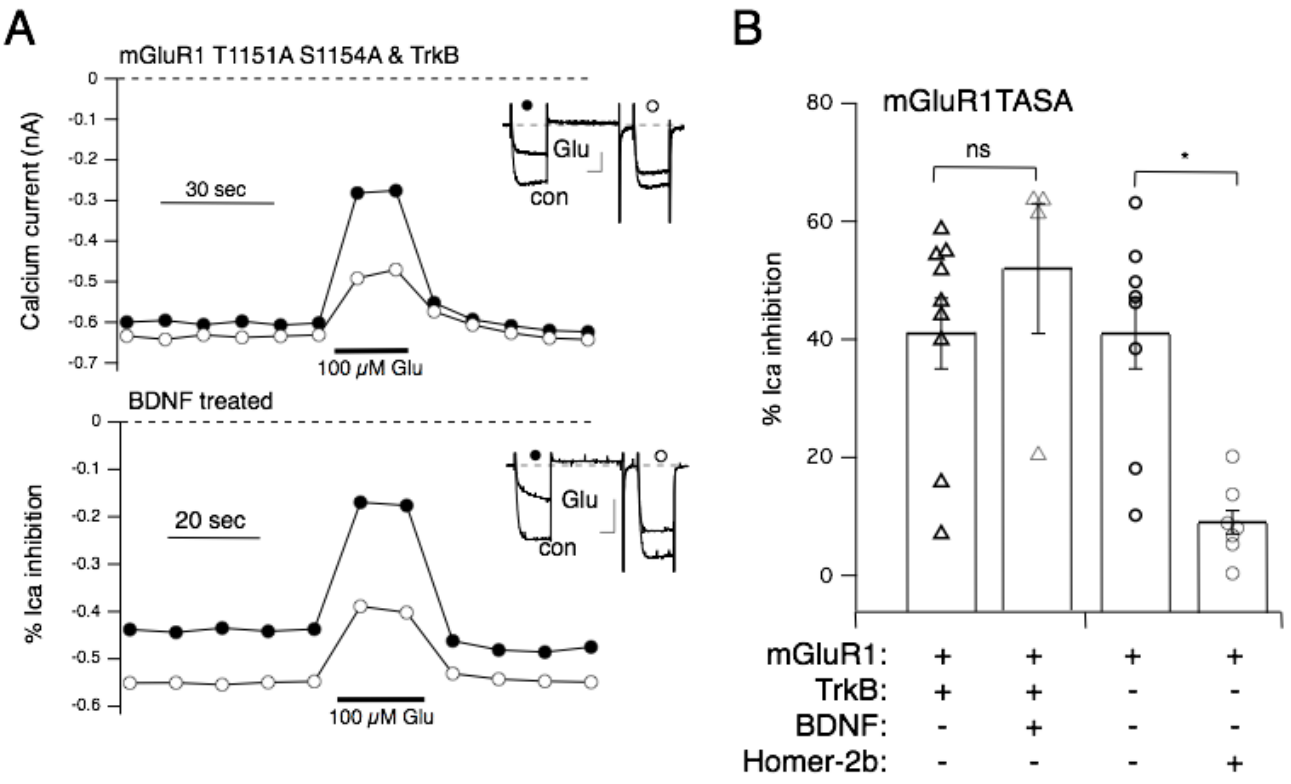

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Figure 3

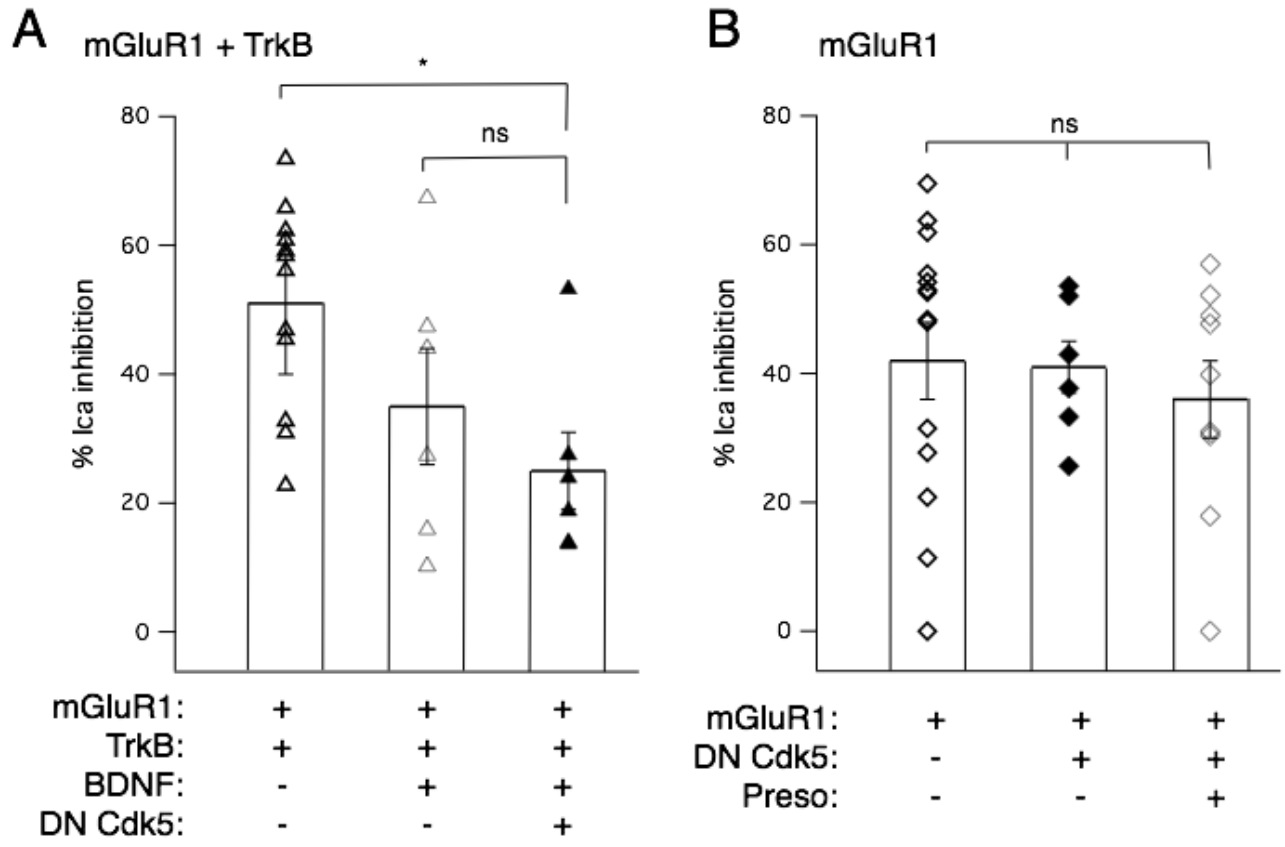

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Figure 4

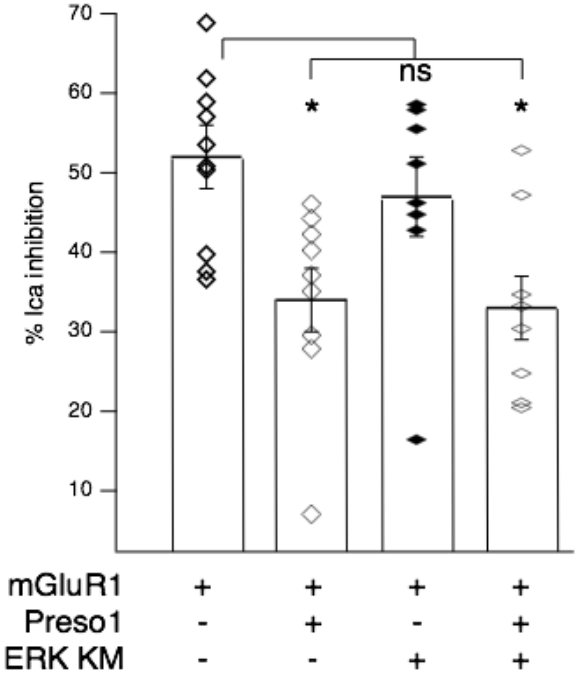

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Figure 5

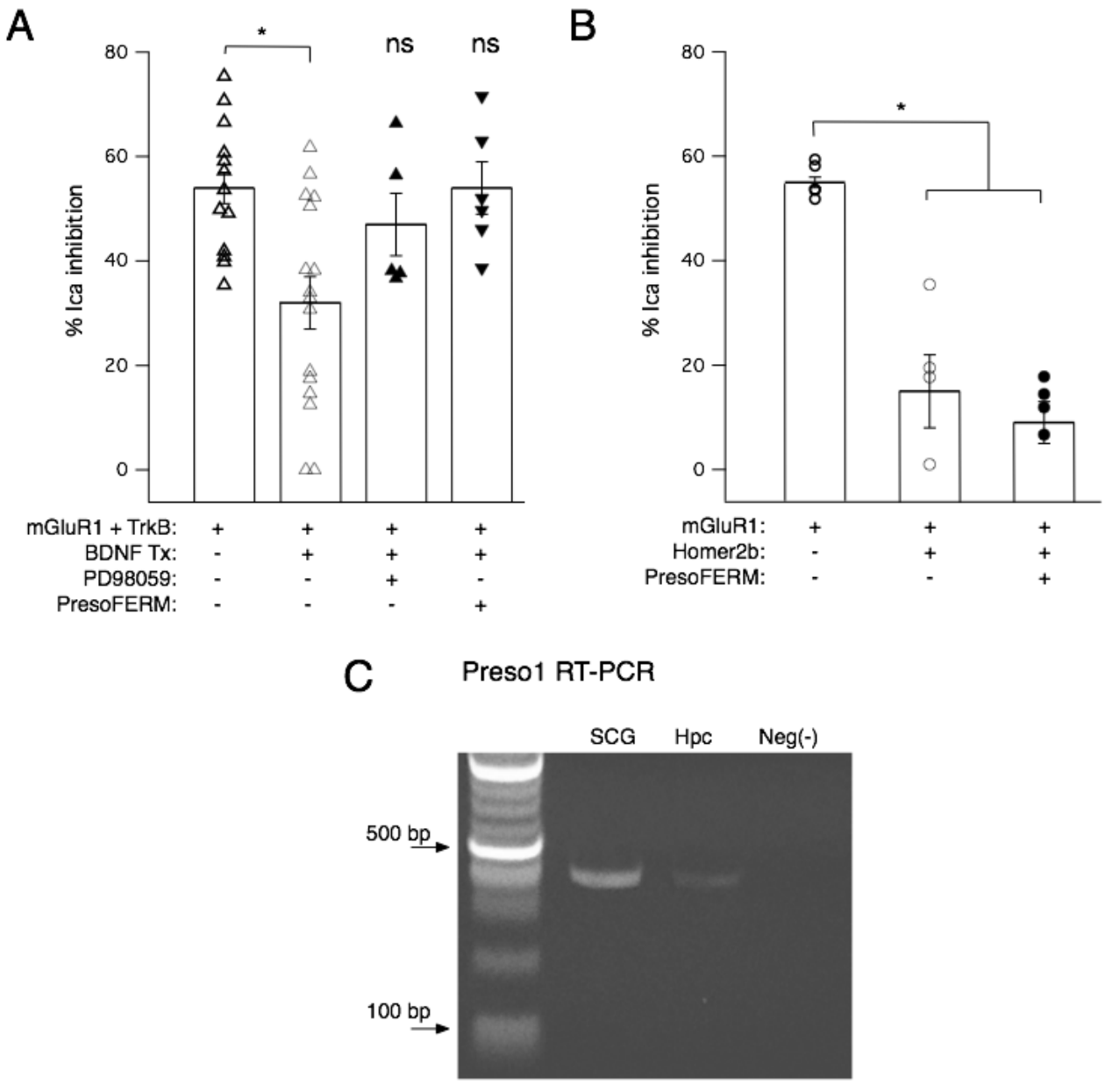

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Figure 6