The interaction between mGluR1 and the calcium channel Ca$_{V2.1}$ preserves coupling in the presence of long Homer proteins

Donald Beqollari[†] and **Paul J. Kammermeier**[*]
University of Rochester Medical Center, Department of Pharmacology and Physiology, Rochester, NY 14642

Abstract

Group I metabotropic glutamate receptors (mGluR1 and 5) are G protein coupled receptors that regulate neuronal activity in a number of ways. Some of the most well studied functions of group I mGluRs, such as initiation of multiple forms of mGluR-dependent long term depression, require receptor localization near the postsynaptic density (PSD). This localization is in turn dependent on the Homer family of scaffolding proteins which bind to a small motif on the distal C-termini of mGluR1 and 5, localize the receptors near the PSD, strengthen coupling to postsynaptic effectors and simultaneously uncouple the mGluRs from extra-synaptic effectors such as voltage dependent ion channels. Here the selectivity of this uncoupling process was examined by testing the ability of Homer-2b to uncouple mGluR1 from multiple voltage dependent calcium channels including Ca$_{V2.2}$ (N-typ e), Ca$_{V3.2}$ (T-type), and Ca$_{V2.1}$ (P/Q-type) expressed in rat sympathetic neurons from the superior cervical ganglion (SCG). Of these, only the mGluR1-Ca$_{V2.1}$ modulatory pathway was insensitive to Homer-2b expression. Uncoupling from this channel was achieved by coexpression of an mGluR1 C-terminal protein designed to disrupt a previously described direct interaction between these two proteins, suggesting that this interaction allows incorporation of Ca$_{V2.1}$ into the mGluR1/Homer signaling complex, thereby preserving modulation in the presence of scaffolding Homer proteins.

Keywords

G protein coupled receptor; calcium channel; sympathetic neuron; pharmacology

1. Introduction

1.1

Metabotropic glutamate receptors (mGluRs) are a family of G protein coupled receptors activated by the excitatory neurotransmitter glutamate. There are 8 mammalian mGluR genes with widespread expression in the central nervous system, and more limited expression peripherally (Schoepp, 2001). Two of the mGluRs (mGluR1 and 5) comprise the group I mGluR subfamily, which couple to the G$_{q/11}$ and G$_{i/o}$ proteins and commonly

[*]Correspondence to: Paul J. Kammermeier, 601 Elmwood Avenue, Box 711, Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY 14642, Voice: 585-275-5606, Fax: 585-273-2652, paul_kammermeier@urmc.rochester.edu.
[†]Present address: Brown University, Department of Neuroscience, Providence, RI 02912.

exhibit postsynaptic expression at glutamatergic synapses (Hay and Kunze, 1994; Choi and Lovinger, 1996; Kammermeier and Ikeda, 1999; Schoepp, 2001).

Group I mGluRs localize near the postsynaptic density (PSD) through their interaction with the Homer family of scaffolding proteins, which anchor not only mGluR1/5 to the PSD but also several other proteins including IP_3 receptors (IP_3Rs) (Brakeman et al., 1997; Ehrengruber et al., 2004; Duncan et al., 2005), some transient receptor potential (TRP) cation channels (Yuan et al., 2003), dynamin (Gray et al., 2003), and others (Ehrengruber et al., 2004; Shiraishi-Yamaguchi et al., 2009). Interestingly, a natural dominant negative Homer subtype has been described which interacts with Homer binding partners, but does not act as a scaffold (Brakeman et al., 1997; Xiao et al., 1998; Tu et al., 1999). These so called "short" Homers (Homer-1a and Ania-3), exhibit regulated expression, and as such are upregulated following periods of strong neuronal activity (Brakeman et al., 1997; Kato et al., 1997). Interaction with the scaffolding, or "long," Homers induces strong mGluR coupling to PSD localized effectors such as AMPA receptors (Kammermeier and Worley, 2007), and to synaptic responses such as mGluR-dependent forms of long-term plasticity (Kirschstein et al., 2007; Ronesi and Huber, 2008; Ueta et al., 2008). At the same time, the scaffolding Homers uncouple group I mGluRs from other effectors such as the plasma membrane ion channels $Ca_{V2.2}$ and KCNQ potassium channels (Kammermeier et al., 2000).

To date, long Homer protein mediated uncoupling from mGluR signaling has been demonstrated only for $Ca_{V2.2}$ channels and KCNQ channels (Kammermeier et al., 2000), but that uncoupling should occur from any effector localized outside the mGluR-Homer-PSD signaling complex. As such, any effector localized within this complex should remain coupled to mGluR1/5 in the presence of long Homers, even if these effectors are closely related to $Ca_{V2.2}$. Recently, a direct interaction between mGluR1 and $Ca_{V2.1}$ has been reported by Kitano et al. (Kitano et al., 2003), who showed that these proteins directly interact in expression systems and in cerebellar neurons. This interaction allowed us the opportunity to test the dogmatic model explaining the regulation of mGluR signaling by Homer proteins. Specifically, we hypothesized that the mGluR1-$Ca_{V2.1}$ interaction should allow incorporation of this channel into the mGluR-Homer signaling complex and thus preserve its modulation by mGluR1 when Homer proteins are expressed, in contrast to the mGluR1-$Ca_{V2.2}$ pathway (Kammermeier et al., 2000; Kammermeier, 2008). If this were the case, it would represent the first demonstration of group I mGluR mediated modulation of a voltage dependent channel that remains robust when scaffolding Homers are abundantly expressed. Thus, modulation of $Ca_{V2.1}$ currents by mGluR1, and its sensitivity to Homer protein over-expression, was examined in rat sympathetic neurons from the superior cervical ganglion (SCG), a system in which Homer-dependent regulation of mGluR signaling has been demonstrated (Kammermeier et al., 2000; Kammermeier, 2008; Won et al., 2009).

2. Materials and Methods

2.1 Electrophysiology and data analysis

Pipettes for patch-clamp experiments were generated using a Sutter (Novato, CA) P-97 horizontal puller with 8250 glass (Garner Glass, Claremont, CA) and had resistances of 1–3 MΩ. Series resistances were 1–5 MΩ prior to electronic compensation of 80%. Whole-cell patch-clamp recordings were made with an EPC-7 (Heka Elektronik, Germany) or Axon 200B (Molecular Devices, Sunnyvale, CA) patch clamp amplifier. Voltage protocol generation and data acquisition were performed using custom software (courtesy Stephen R. Ikeda, NIAAA, Rockville, MD) on a Macintosh G3 or G4 computer (Apple Computer, Cupertino, CA) with an InstruTech (Heka Elektronik) ITC-16 or ITC-18 data acquisition board. Currents were low-pass filtered at 3–5 kHz using the 4-pole Bessel filter in the patch clamp amplifiers, digitized at 2–5 kHz and stored on the computer for later analysis.

Experiments were performed at 21–24 °C (room temperature). Patch-clamp data analysis was performed using the Igor Pro software package (Wavemetrics, Lake Oswego, OR).

The external (bath) recording solution contained (in mM): 155 tris hydroxymethyl aminomethane, 20 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 glucose, 10 $CaCl_2$, and 0.0003 tetrodotoxin (TTX), pH 7.4. The internal (pipette) solution contained: 120 N-methyl-D-glucamine (NMG) methanesulfonate, 20 TEA, 11 EGTA, 10 HEPES, 10 sucrose, 1 $CaCl_2$, 4 MgATP, 0.3 Na_2GTP, and 14 tris creatine phosphate, pH 7.2. L-Glutamate (Sigma) was used as the agonist for mGluRs. SNX482 (SNX) and ω-conotoxin GVIA (CTX) were obtained from Tocris Bioscience (Ellisville, MO). L-Glutamate was obtained from Sigma-Aldrich (St. Louis, MO). All drugs and control solutions were applied to cells using a custom, gravity-driven perfusion system positioned ~100 μm from the cell, allowing rapid solution exchange (250 ms). The degree of calcium current inhibition was calculated as the maximum current inhibition in the presence of drug compared to the last current measurement prior to drug application.

2.2 Primary culture preparation, cDNA injection, antibodies, and plasmid generation

A description of cell isolation and cDNA injection is found elsewhere (Ikeda et al., 1995; Lu et al., 2009). Animal protocols were approved by the university committee on animal resources (UCAR). Briefly, SCGs were removed from adult male Wistar rats (175–225 g) after CO_2 euthanasia and decapitation, then incubated in Earle's balanced salt solution (InVitrogen, Life Technologies Carlsbad, CA) containing 0.6 mg/ml trypsin (Worthington Biochemicals, Freehold, NJ) & 0.8 mg/ml collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 60 minutes at 35 °C. Cells were transferred to minimum essential medium (InVitrogen/Gibco), plated on poly-L-lysine (Sigma Chemical Co., St. Louis, MO) coated culture dishes and incubated at 37 °C for 2–4 hours before cDNA injection. Injected cells were incubated overnight at 37 °C (95% air and 5% CO_2; 100% humidity) and patch clamp experiments were performed the next day. Anti-CaV2.1 antibody was obtained from Abcam (Cabbridge, MA). Anti-HA was obtained from Covance (Princeton, NJ). Both were used at a dilution of 1:500 for experiments shown in Fig. 1.

Injection of cDNA was performed with an Eppendorf 5247 microinjector and InjectMan NI 2 micromanipulator (Madison, WI) 3–5 hours following cell isolation. Injection electrodes were made with a Sutter P-97 horizontal electrode puller (Novato, CA) from thin-walled, borosilicate glass (World Precision Instruments, Sarasota, FL). Plasmids were stored at −20 °C as a 0.4 – 1 μg/μl stock solution in TE buffer (10 mM TRIS, 1 mM EDTA, pH 8). All mGluR constructs were injected at 100–130 ng $μl^{-1}$ (pCDNA3.1$^+$; InVitrogen). All neurons were co-injected with "enhanced" green fluorescent protein cDNA (0.02 μg/μl; pEGFPN1; BD Biosciences-Clontech, Palo Alto, CA) for identification of successfully injected cells.

Constructs were all sequence confirmed, and PCR products were purified with Qiagen (Valencia, CA) silica membrane spin columns prior to restriction digestion and ligation. Midipreps were prepared using Qiagen anion exchange columns, and amplified in either Top10 or DH5α E. coli. (InVitrogen).

3. Results

3.1 mGluR1 and Ca$_{V2.1}$ interact

To verify that an interaction between mGluR1 and Ca$_{V2.1}$ could be detected, we expressed in HEK293 cells a hemagglutinin (HA)- tagged mGluR1, Ca$_{V2.1}$ (with β2A and α2δ), or both constructs together and performed co-immunporecipitation experiments. Cell lysates were immunoprecipitated (IP) with an anti-HA antibody and blots probed using an anti Ca$_{V2.1}$ antibody. Results of these experiments are shown in Fig. 1, which also illustrates

samples from the supernatant (S) and flow-through (FT), as well as the IP for each expression condition. A prominent band just under 250 kd, the expected size of $Ca_{V2.1}$, was evident in the lysates of $Ca_{V2.1}$ expressing cells and also appeared (although less prominently) in the IP only when the channel was co-expressed with HA-mGluR1 (Fig. 1A, HA-mGluR1a + $Ca_{V2.1}$, "IP" lane). Analogous experiments were performed using myc-tagged mGluR1 with $Ca_{V2.1}$ with similar results (Fig. 1B). These data confirm the findings of Kitano et al. (Kitano et al., 2003) of an interaction between mGluR1 and $Ca_{V2.1}$.

3.2 $Ca_{V2.1}$ channels can be expressed in rat sympathetic neurons and modulated by mGluR1

The long forms of the Homer scaffolding proteins (Homer 1b, 1c, 2 and 3) bind to a proline rich motif near the C-terminus of mGluR1 and 5 (Brakeman et al., 1997; Xiao et al., 1998; Tu et al., 1999), causing these receptors to form clusters (Kammermeier et al., 2000; Kammermeier, 2006), and organize around the post-synaptic density (Ango et al., 2000). As a result of this interaction, mGluR1/5-mediated modulation of voltage dependent, N-type calcium channels ($Ca_{V2.2}$) and M-type potassium channels (K_{V7}) is disrupted (Kammermeier et al., 2000). The simplest explanation for this Homer-mediated uncoupling is that clustering of mGluR1/5 by Homers physically sequesters the receptors away from the voltage gated channels such that subsequent coupling is impaired. To date however, this model has not been directly tested. We reasoned that interaction between mGluR1 and $Ca_{V2.1}$ may preserve mGluR1-$Ca_{V2.1}$ modulation even in the presence of Homer protein over-expression (and mGluR1 clustering) since the channels may be incorporated into the mGluR1/Homer signaling domains that putatively exclude other voltage dependent channels, such as $Ca_{V2.2}$ and M-channels. To test this, we examined mGluR1 modulation of calcium currents in rat sympathetic neurons from the superior cervical ganglion (SCG), a system in which Homer-mediated uncoupling of mGluR1 from calcium current modulation has been demonstrated (Kammermeier et al., 2000; Kammermeier, 2008). Since these neurons do not express $Ca_{V2.1}$ channels natively, it was necessary to express them heterologously.

Calcium currents in SCG neurons expressing mGluR1 alone were strongly inhibited by the cocktail of 1 μM ω-conotixin GVIA, a selective $Ca_{V2.2}$ blocker (Reynolds et al., 1986), and 500 nM SNX482, a selective $Ca_{V2.3}$ blocker (Newcomb et al., 1998) (Fig. 2A). The toxin cocktail reduced calcium current density in 13 cells from 26 ± 3 pA/pF to 6 ± 2 pA/pF, a reduction of about 80% (Fig. 2B). In neurons co-expressing the α subunit of $Ca_{V2.1}$, the toxin insensitive fraction of the current was greatly enhanced. In these cells, the toxin cocktail reduced the current from 66 ± 13 pA/pF to 37 ± 9 pA/pF in 6 cells, an inhibition of only 44% (Fig. 2B). Surprisingly, it was not necessary to co-express calcium current accessory subunits to induce $Ca_{V2.1}$ expression. Nevertheless, these data confirm that $Ca_{V2.1}$ channels can be effectively expressed in SCG neurons.

Next, the ability of mGluR1 activation to modulate $Ca_{V2.1}$ currents was examined. As shown in the current density time courses in Fig. 2A, both the total current (before toxin application under both expression conditions) and the more isolated "$Ca_{V2.1}$ current" (in the presence of toxins in $Ca_{V2.1}$ expressing cells) was rapidly and reversibly inhibited upon application of 100 μM glutamate, demonstrating that $Ca_{V2.1}$ currents are modulated by mGluR1 in SCG neurons, which do not natively express any mGluRs (Kammermeier and Ikeda, 1999; Kammermeier and Yun, 2005). Indeed, in 5 uninjected control SCG neurons, application of 100 μM glutamate failed to inhibit the current, producing $1.2 \pm 1.6\%$ inhibition. These data confirm that SCG neurons represent a null mGluR background.

3.3 Ca$_{V2.1}$ current modulation is insensitive to Homer protein expression

To test whether Ca$_{V2.1}$ currents are, by virtue of their interaction with mGluR1, insensitive to uncoupling by Homer protein expression, we examined their modulation via mGluR1 in the absence and presence of over-expressed Homer-2b, which strongly uncouples mGluR1 from modulating the native SCG calcium currents (Kammermeier et al., 2000; Won et al., 2009). Indeed, in control cells expressing mGluR1 and Ca$_{V2.1}$, the isolated Ca$_{V2.1}$ currents (in the presence of toxins) were inhibited by $31 \pm 3\%$ (n=15), and by $29 \pm 4\%$ (n=10) in Homer-2b co-expressing cells (Fig. 3A, B). In the same cells, the magnitude of the total calcium current inhibition was also examined, which includes Ca$_{V2.1}$ currents as well as the native Ca$_{V2.2}$ and Ca$_{V2.3}$ currents (Zhu and Ikeda, 1994), as an internal control for Homer-2b expression. Inhibition of the total current was significantly reduced by Homer-2b, from $32 \pm 4\%$ in the 15 control cells to $19 \pm 2\%$ in the 10 Homer-2b expressing cells (Fig. 3A, B). These data demonstrate that modulation of Ca$_{V2.1}$ currents in SCG neurons by mGluR1 is insensitive to Homer protein expression.

3.4 Over-expression of other voltage dependent calcium channels in SCG neurons does not result in Homer insensitivity

To ascertain whether the modulation of Ca$_{V2.1}$ currents by mGluR1 in the presence of Homer-2b was the result of channel over-expression, modulation of two other over-expressed voltage dependent calcium channels in SCG neurons was examined in the absence and presence of Homer-2b (Fig. 4). First, the expression and mGluR1 modulation of the T-type calcium channel Ca$_{V3.2}$ (Perez-Reyes et al., 1998) was examined. As expected, co-expression of Ca$_{V3.2}$ in SCG neurons resulted in a prominent, low-voltage activated, rapidly inactivating component of the calcium current (Fig. 4A). These currents were elicited and separated from the native calcium currents using a two step voltage protocol shown in Fig. 4A (*upper*). From a holding potential of −80 mV, 100 msec voltage steps were applied to −40 mV to elicit and inactivate the low-voltage activated Ca$_{V3.2}$ current ("LVA"), then to +10 mV to elicit the native, high-voltage activated currents ("HVA"). A sample current trace from an uninjected control cell (Fig. 4A) shows that the LVA step did not elicit any current. However, when Ca$_{V3.2}$ channels were expressed a prominent, rapidly inactivating current was evident during the LVA step. The amplitude of this current varied widely from less than 10 to about 55 pA/pF, but was detectable in each Ca$_{V3.2}$ expressing cell examined, and never seen in uninjected cells. By the end of the 100 msec LVA step, nearly all of the Ca$_{V3.2}$ current was inactivated, and therefore the current elicited during the HVA step was made up of almost entirely natively expressed calcium currents. Note that the driving force for calcium current is considerably smaller at +10 mV than at −40 mV, so the contribution of Ca$_{V3.2}$ channels to the HVA current is much smaller than appears from a cursory glance at the current traces in Fig. 4A.

Next, the ability of Ca$_{V3.2}$ currents to be modulated by mGluR1 was examined by applying 100 μM glutamate to SCG neurons co-expressing mGluR1 and Ca$_{V3.2}$. Indeed, a rapid and reversible inhibition of the current was observed, averaging $20 \pm 3\%$ (n=21). Although there are a plethora of T-type calcium modulatory pathways described in the literature, most of the Ca$_{V3.2}$ inhibitory mechanisms described are G protein mediated (Chemin et al., 2006), and thus appropriate for comparison to the Ca$_{V2}$ modulation studied here. In the same cells, the native HVA currents were inhibited by $30 \pm 4\%$. Interestingly, in neurons in which Homer-2b was co-expressed, modulation of both the LVA and HVA components was significantly reduced to $9 \pm 2\%$ and $9 \pm 3\%$, respectively (n=17). These data confirm that mGluR1-mediated modulation of an over-expressed calcium channel in SCG neurons can be effectively uncoupled by Homer protein expression.

To compare the Homer protein sensitivity of mGluR1 modulation of a more closely related, over-expressed calcium channel, experiments were performed in SCG neurons over-expressing $Ca_{V2.2}$ (beyond native expression levels). Interestingly, co-injection of just the $Ca_{V2.2}$ subunit cDNA was not sufficient to increase the current density in SCG neurons (not shown). Therefore, it was necessary to co-inject small amounts of cDNA for the calcium channel accessory subunits β2A and α2δ (Campbell et al., 1988; Ellis et al., 1988) in each of the $Ca_{V2.2}$-expressing cells. In these cells, the average calcium current density was increased to 118 ± 14 pA/pF (n=16) from the typical value of around 30 pA/pF for control cells measured under identical conditions. As expected, the over-expressed $Ca_{V2.2}$ currents appeared to be modulated to a similar degree as the native currents, and were significantly uncoupled upon Homer-2b expression. 100 μM glutamate applied to cells expressing mGluR1 and $Ca_{V2.2}$ in the absence and presence of Homer-2b were inhibited by $31 \pm 9\%$ (n=5) and $11 \pm 3\%$ (n=11), respectively. These data provide further evidence that the observed insensitivity to Homer-mediated uncoupling from mGluR1 modulation of $Ca_{V2.1}$ is not the result of channel over-expression.

3.5 Expression of an mGluR1 C-terminal construct imparts Homer sensitivity to $Ca_{V2.1}$ modulation

To determine whether the interaction between mGluR1 and $Ca_{V2.1}$ was responsible for preserving coupling in the presence of Homer-2b, an mGluR1 C-terminal protein was co-expressed to disrupt the interaction. Kitano et al. (Kitano et al., 2003) demonstrated that the interaction between the two proteins was localized to their C-termini, so over-expression of the cytoplasmic C-tail of mGluR1 should compete for binding to the $Ca_{V2.1}$ tail and prevent or reduce binding of the full length mGluR1. Thus a construct (mG1-CT) consisting of the C-tail of mGluR1, truncated just before the canonical Homer binding site (amino acids 842–1150 of mGluR1a) and fused to CFP to verify expression at its N-terminus, was made and expressed in SCG neurons with mGluR1 or mGluR1 + Homer-2b (Fig. 5). Deletion of the Homer binding site from the CT construct was necessary to insure that it did not act to sequester expressed Homer-2b. When mG1-CT was co-expressed with mGluR1 and similar experiments as those reported in Fig. 3 (above) were performed, both the total calcium current and the isolated $Ca_{V2.1}$ currents (in the presence of the toxin cocktail) were similar to control conditions. 100 μM glutamate produced an average inhibition in these cells of $33 \pm 7\%$ and $19 \pm 5\%$, respectively (n=6; Fig. 5A, B). However, when Homer-2b was co-expressed with mGluR1 and mG1-CT, glutamate-mediated inhibition of both the total current and the isolated $Ca_{V2.1}$ current was significantly reduced to $14 \pm 4\%$ and $7 \pm 1\%$ (n=7), respectively (Fig. 5A, B). These data support the hypothesis that modulation of $Ca_{V2.1}$ by mGluR1 is protected from Homer-mediated uncoupling by virtue of its interaction with the C-tail of mGluR1. Further, since $Ca_{V2.1}$ modulation by mGluR1 was not significantly reduced upon expression of mG1-CT (in the absence of Homer-2b), it may be concluded that modulation *per se* does not require direct association between receptor and channel, but association seems to be required for modulation only when mGluR1 is organized into clusters by Homer-2b. Finally, because Homer-2b expression can uncouple the native currents from modulation by mGluR1 in the presence of $Ca_{V2.1}$, it can be inferred indirectly that association with the channel does not prevent Homer-induced clustering of mGluR1. That is, it appears unlikely that the mGluR1-$Ca_{V2.1}$ interaction prevents Homer binding to the mGluR1 C-tail.

To independently verify that the mG1-CT construct could disrupt the interaction between mGluR1 and $Ca_{V2.1}$ in SCG neurons, a clustering assay was employed using a fluorescently tagged channel (Venus-$Ca_{V2.1}$). We hypothesized that since Homer proteins organize mGluR1 into clusters when co-expressed in SCG neurons (Kammermeier, 2006), clustering of Venus-$Ca_{V2.1}$ should be apparent when co-expressed with Homer-2b and mGluR1, but

not with Homer-2b alone. Further, if mG1-CT prevents mGluR1-Ca$_{V2.1}$ association, it should also disrupt Venus-Ca$_{V2.1}$ clustering. For this experiment, a non-fluorescent version of mG1-CT was used. This construct was similar to mG1-CT but was tagged to the non-fluorescent FP variant Amber. The results of these experiments are shown in Fig. 6. Fig. 6A shows three sample total internal reflection fluorescence (TIRF) images of SCG neurons expressing Venus-Ca$_{V2.1}$ with Homer-2b (*left*), Homer-2b and mGluR1 (*center*), and with Homer-2b, mGluR1 and mG1-CT-Amber (*right*). In nearly all cells expressing Venus-Ca$_{V2.1}$ with Homer-2b and mGluR1, a punctate expression pattern was evident that was largely absent in cells from the other expression groups. However, perhaps predictably this clustering was less complete and less consistent than the clustering of mGluR1-GFP in the presence of Homer proteins reported previously (Kammermeier, 2006). Thus to quantify the observed clustering, a cumulative histogram of cluster number vs. size was generated from all of the TIRF images in each group. These data are shown in Fig. 6B. A detailed description of the quantification procedure is provided elsewhere (Kammermeier, 2006). A significant reduction in the number of clusters per cell was evident in neurons expressing mGluR1-CT-Amber from this analysis, of clusters ranging from 25 to 50 pixels[2]. Similarly sized clusters of mGluR1-GFP were evident in SCG neurons co-expressing Homer proteins in our previous report (Kammermeier, 2006). These data support our prediction that expression of mG1-CT can effectively disrupt the interaction between mGluR1 and Ca$_{V2.1}$ in SCG neurons, and support the interpretation that the loss of mGluR1-Ca$_{V2.1}$ coupling when Homer-2b and mG1-CT are co-expressed is due to this disruption.

4. Discussion

4.1

The dogmatic model to explain how Homer proteins regulate mGluR-effector coupling suggests that long, scaffolding Homers (Homer-1b, 1b, 2 and 3) bind to mGluR1 and 5 and organize the receptors into clusters (Kammermeier, 2006) in neuronal expression systems, or near the postsynaptic density (PSD) in native neurons (Ango et al., 2000). Clustered mGluRs represent a signaling mode in which coupling to effectors outside the clusters, specifically the voltage dependent ion channels Ca$_{V2.2}$ and KCNQ channels (Kammermeier et al., 2000), is disrupted while coupling to IP$_3$ receptors (which are also Homer binding proteins) or other effectors within the PSD is strengthened. The second mode is represented by mGluR1 or 5 unbound to Homer proteins or bound to the natural dominant negative splice variants Homer-1a or Ania-3. Such receptors are unclustered and localized apart from the PSD. As such, they couple strongly to plasma membrane ion channels and are uncoupled from PSD localized effectors, such as AMPA receptors (Kammermeier and Worley, 2007), and functions, such as induction of multiple forms of mGluR-dependent plasticity (Ronesi and Huber, 2008; Ueta et al., 2008). The data in the present study further the model by illuminating a mechanism by which one voltage dependent calcium channel, Ca$_{V2.1}$, is selectively rendered exempt from mGluR1 uncoupling by Home protein expression by virtue of a direct interaction between the receptor and the channel (Fig. 7). In addition, these data show for the first time a Homer-dependent uncoupling of mGluR1 from the T-type calcium channel Ca$_{V3.2}$ (Fig. 7). This uncoupling is predicted by the dogmatic model but had not been previously demonstrated.

We have not addressed the possibility of an analogous interaction with mGluR5, nor did the Nakanishi group who first described the mGluR1-Ca$_{V2.1}$ interaction (Kitano et al., 2003), but these receptors share fairly high homology in the proximal C-tail believed to participate in the interaction (55% sequence identity in the first 94 amino acids of the respective C-tails). Thus, one may predict that a similar interaction could occur with mGluR5, the only other member of the group I mGluR family and only other Homer interacting mGluR (Brakeman et al., 1997).

It is interesting to speculate about the implications of these findings, particularly in the context of cerebellar Purkinje neurons, which express both mGluR1 and $Ca_{V2.1}$ at high levels (McDonough et al., 1997; Knöpfel and Grandes, 2002). $Ca_{V2.x}$ channels are widely known as presynaptic calcium channels that mediate calcium entry into synaptic terminals during depolarization and thus trigger neurotransmitter release (Catterall, 2011). Indeed G protein mediated inhibition of the $Ca_{V2.x}$ channels is a principally important pathway for presynaptic inhibition of neurotransmitter release. In cerebellar Purkinje neurons however, $Ca_{V2.1}$ channel expression is clearly not limited to presynaptic terminals. At least one study describes an apparent postsynaptic localization of $Ca_{V2.1}$ in Purkinje neurons (Kulik et al., 2004), in addition to its expression elsewhere in these cells. It is possible that the direct interaction between mGluR1 and $Ca_{V2.1}$ may be necessary for localization near the PSD. If so, one may extrapolate that this localization is thus indirectly dependent on Homer protein scaffolding, and that the principal site of importance of the findings in the present study would be at this site where we predict that strong voltage dependent calcium channel modulation via mGluR1 would be maintained. This would be in contrast to other neuronal subtypes, few of which express both mGluR1 and $Ca_{V2.1}$ at such high levels.

It is difficult to predict the precise physiological role of postsynaptic mGluR1-$Ca_{V2.1}$ coupling in Purkinje cells or why it is necessary to maintain with this specific receptor-channel pair, but it is possible that $Ca_{V2.1}$ channels strongly influence dendritic electrical properties and that this negative feedback pathway is necessary for proper dendritic signal integration. Alternatively, calcium levels near the PSD may require fairly tight regulation in Purkinje cells, as calcium entry through $Ca_{V2.1}$ has been linked to calcium-calmodulin dependent protein kinase II (CaMKII) activation. Further, this Purkinje cell $Ca_{V2.1}$-CaMKII pathway has been shown to regulate Homer phosphorylation which in turn can alter the apparent affinity for scaffolding Homer proteins for mGluRs (Mizutani et al., 2008). Thus it is possible that both $Ca_{V2.1}$ localization near the PSD in Purkinje cells as well as the negative feedback provided by mGluR1-mediated $Ca_{V2.1}$ inhibition postsynaptically may be an important feature of Purkinje cell physiology.

Acknowledgments

Abbreviations

mGluR	metabotropic glutamate receptor
SCG	superior cervical ganglion
Glu	L-glutamate

References

Ango F, Pin JP, Tu JC, Xiao B, Worley PF, Bockaert J, Fagni L. Dendritic and axonal targeting of type 5 metabotropic glutamate receptor is regulated by homer1 proteins and neuronal excitation. Journal of Neuroscience. 2000; 20:8710–8716. [PubMed: 11102477]

Brakeman PR, Lanahan AA, O'Brien R, Roche K, Barnes CA, Huganir RL. Worley P.F. Homer: a protein that selectively binds metabotropic glutamate receptors. Nature. 1997; 386:284–288. [PubMed: 9069287]

Campbell KP, Leung AT, Sharp AH. The biochemistry and molecular biology of the dihydropyridine-sensitive calcium channel. Trends in Neurosciences. 1988; 11:425–430. [PubMed: 2469159]

Catterall WA. Voltage-gated calcium channels. Cold Spring Harb Perspect Biol. 2011; 3:a003947. [PubMed: 21746798]

Chemin J, Traboulsie A, Lory P. Molecular pathways underlying the modulation of T-type calcium channels by neurotransmitters and hormones. Cell Calcium. 2006; 40:121–134. [PubMed: 16797700]

Choi S, Lovinger DM. Metabotropic glutamate receptor modulation of voltage-gated Ca2+ channels involves multiple receptor subtypes in cortical neurons. J Neurosci. 1996; 16:36–45. [PubMed: 8613803]

Duncan RS, Hwang S-Y, Koulen P. Effects of Vesl/Homer proteins on intracellular signaling. Exp Biol Med (Maywood). 2005; 230:527–535. [PubMed: 16118402]

Ehrengruber MU, Kato A, Inokuchi K, Hennou S. Homer/Vesl proteins and their roles in CNS neurons. Mol Neurobiol. 2004; 29:213–227. [PubMed: 15181235]

Ellis SB, Williams ME, Ways NR, Brenner R, Sharp AH, Leung AT, Campbell KP, McKenna E, Koch WJ, Hui A. Sequence and expression of mRNAs encoding the alpha 1 and alpha 2 subunits of a DHP-sensitive calcium channel. Science. 1988; 241:1661–1664. [PubMed: 2458626]

Gray NW, Fourgeaud L, Huang B, Chen J, Cao H, Oswald BJ, Hémar A, McNiven MA. Dynamin 3 is a component of the postsynapse, where it interacts with mGluR5 and Homer. Curr Biol. 2003; 13:510–515. [PubMed: 12646135]

Hay M, Kunze DL. Glutamate metabotropic receptor inhibition of voltage-gated calcium currents in visceral sensory neurons. J Neurophysiol. 1994; 72:421–430. [PubMed: 7965024]

Ikeda SR, Lovinger DM, McCool BA, Lewis DL. Heterologous expression of metabotropic glutamate receptors in adult rat sympathetic neurons: subtype-specific coupling to ion channels. Neuron. 1995; 14:1029–1038. [PubMed: 7538309]

Kammermeier PJ, Ikeda SR. Expression of RGS2 alters the coupling of metabotropic glutamate receptor 1a to M-type K+ and N-type Ca2+ channels. Neuron. 1999; 22:819–829. [PubMed: 10230801]

Kammermeier PJ, Worley PF. Homer 1a uncouples metabotropic glutamate receptor 5 from postsynaptic effectors. Proc Natl Acad Sci USA. 2007; 104:6055–6060. [PubMed: 17389377]

Kammermeier PJ, Xiao B, Tu JC, Worley PF, Ikeda SR. Homer proteins regulate coupling of group I metabotropic glutamate receptors to N-type calcium and M-type potassium channels. Journal of Neuroscience. 2000; 20:7238–7245. [PubMed: 11007880]

Kammermeier PJ, Yun J. Activation of metabotropic glutamate receptor 1 dimers requires glutamate binding in both subunits. J Pharmacol Exp Ther. 2005; 312:502–508. [PubMed: 15466247]

Kammermeier PJ. Surface clustering of metabotropic glutamate receptor 1 induced by long Homer proteins. BMC Neurosci. 2006; 7:1. [PubMed: 16393337]

Kammermeier PJ. Endogenous homer proteins regulate metabotropic glutamate receptor signaling in neurons. Journal of Neuroscience. 2008; 28:8560–8567. [PubMed: 18716215]

Kato A, Ozawa F, Saitoh Y, Hirai K, Inokuchi K. vesl, a gene encoding VASP/Ena family related protein, is upregulated during seizure, long-term potentiation and synaptogenesis. FEBS Letters. 1997; 412:183–189. [PubMed: 9257717]

Kirschstein T, Bauer M, Muller L, Ruschenschmidt C, Reitze M, Becker AJ, Schoch S, Beck H. Loss of Metabotropic Glutamate Receptor-Dependent Long-Term Depression via Downregulation of mGluR5 after Status Epilepticus. Journal of Neuroscience. 2007; 27:7696–7704. [PubMed: 17634364]

Kitano J, Nishida M, Itsukaichi Y, Minami I, Ogawa M, Hirano T, Mori Y, Nakanishi S. Direct interaction and functional coupling between metabotropic glutamate receptor subtype 1 and voltage-sensitive Cav2.1 Ca2+ channel. J Biol Chem. 2003; 278:25101–25108. [PubMed: 12704197]

Knöpfel T, Grandes P. Metabotropic glutamate receptors in the cerebellum with a focus on their function in Purkinje cells. Cerebellum (London, England). 2002; 1:19–26.

Kulik A, Nakadate K, Hagiwara A, Fukazawa Y, Luján R, Saito H, Suzuki N, Futatsugi A, Mikoshiba K, Frotscher M, Shigemoto R. Immunocytochemical localization of the alpha 1A subunit of the P/Q-type calcium channel in the rat cerebellum. Eur J Neurosci. 2004; 19:2169–2178. [PubMed: 15090043]

Lu VB, Williams DJ, Won Y-J, Ikeda SR. Intranuclear microinjection of DNA into dissociated adult mammalian neurons. Journal of Visualized Experiments: JoVE. 2009

McDonough SI, Mintz IM, Bean BP. Alteration of P-type calcium channel gating by the spider toxin omega-Aga-IVA. Biophysical Journal. 1997; 72:2117–2128. [PubMed: 9129813]

Mizutani A, Kuroda Y, Futatsugi A, Furuichi T, Mikoshiba K. Phosphorylation of Homer3 by Calcium/Calmodulin-Dependent Kinase II Regulates a Coupling State of Its Target Molecules in Purkinje Cells. Journal of Neuroscience. 2008; 28:5369–5382. [PubMed: 18480293]

Newcomb R, Szoke B, Palma A, Wang G, Chen XH, Hopkins W, Cong R, Miller J, Urge L, Tarczy-Hornoch K, Loo JA, Dooley DJ, Nadasdi L, Tsien RW, Lemos J, Miljanich G. Selective peptide antagonist of the class E calcium channel from the venom of the tarantula Hysterocrates gigas. Biochemistry. 1998; 37:15353–15362. [PubMed: 9799496]

Perez-Reyes E, Cribbs LL, Daud A, Lacerda AE, Barclay J, Williamson MP, Fox M, Rees M, Lee JH. Molecular characterization of a neuronal low-voltage-activated T-type calcium channel. Nature. 1998; 391:896–900. [PubMed: 9495342]

Reynolds IJ, Wagner JA, Snyder SH, Thayer SA, Olivera BM, Miller RJ. Brain voltage-sensitive calcium channel subtypes differentiated by omega-conotoxin fraction GVIA. Proc Natl Acad Sci USA. 1986; 83:8804–8807. [PubMed: 2430302]

Ronesi JA, Huber KM. Homer Interactions Are Necessary for Metabotropic Glutamate Receptor-Induced Long-Term Depression and Translational Activation. Journal of Neuroscience. 2008; 28:543–547. [PubMed: 18184796]

Schoepp DD. Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system. J Pharmacol Exp Ther. 2001; 299:12–20. [PubMed: 11561058]

Shiraishi-Yamaguchi Y, Sato Y, Sakai R, Mizutani A, Knöpfel T, Mori N, Mikoshiba K, Furuichi T. Interaction of Cupidin/Homer2 with two actin cytoskeletal regulators, Cdc42 small GTPase and Drebrin, in dendritic spines. BMC Neurosci. 2009; 10:25. [PubMed: 19309525]

Tu JC, Xiao B, Naisbitt S, Yuan JP, Petralia RS, Brakeman P, Doan A, Aakalu VK, Lanahan AA, Sheng M, Worley PF. Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. Neuron. 1999; 23:583–592. [PubMed: 10433269]

Ueta Y, Yamamoto R, Sugiura S, Inokuchi K, Kato N. Homer 1a suppresses neocortex long-term depression in a cortical layer-specific manner. J Neurophysiol. 2008; 99:950–957. [PubMed: 18077661]

Won Y-J, Puhl HL, Ikeda SR. Molecular reconstruction of mGluR5a-mediated endocannabinoid signaling cascade in single rat sympathetic neurons. Journal of Neuroscience. 2009; 29:13603–13612. [PubMed: 19864572]

Xiao B, Tu JC, Petralia RS, Yuan JP, Doan A, Breder CD, Ruggiero A, Lanahan AA, Wenthold RJ, Worley PF. Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. Neuron. 1998; 21:707–716. [PubMed: 9808458]

Yuan JP, Kiselyov K, Shin DM, Chen J, Shcheynikov N, Kang SH, Dehoff MH, Schwarz MK, Seeburg PH, Muallem S, Worley PF. Homer binds TRPC family channels and is required for gating of TRPC1 by IP3 receptors. Cell. 2003; 114:777–789. [PubMed: 14505576]

Zhu Y, Ikeda SR. Modulation of Ca(2+)-channel currents by protein kinase C in adult rat sympathetic neurons. J Neurophysiol. 1994; 72:1549–1560. [PubMed: 7823085]

Highlights

- Modulation of CaV2.1 by mGluR1 is insensitive to Homer-2b expression.

- Other calcium channels are uncoupled from mGluR1 by Homer-2b.

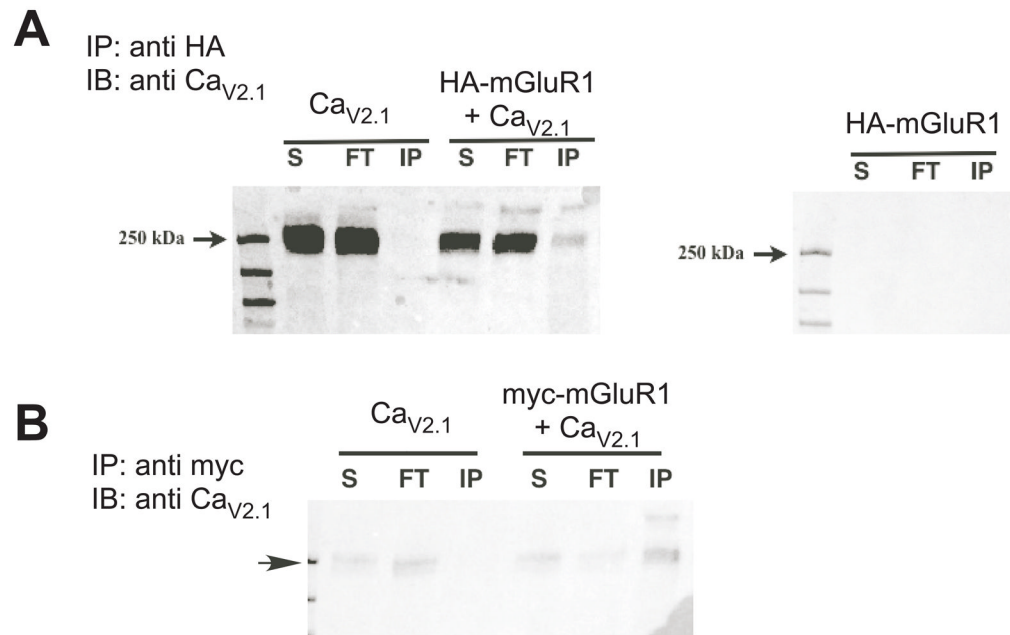- Preventing the interaction between mGluR1 and CaV2.1 permits Homer-uncoupling.

Fig. 1.
Ca$_{V2.1}$ co-immunoprecipitates with mGluR1 in HEK293 cells. A, Anti-Ca$_{V2.1}$ (1:500) immunoblot showing protein from supernatant (S), flow-through (FT) and from the anti-HA immunoprecipitation (1:500) experiment (IP), with expression conditions indicated above: Ca$_{V2.1}$ alone and HA-mGluR1 + Ca$_{V2.1}$ (*left*) and HA-mGluR1 alone (*right*). B, Similar to A, but using an anti-myc antibody paired with myc-mGluR1 for the IP. Arrow indicates 250 kd marker.
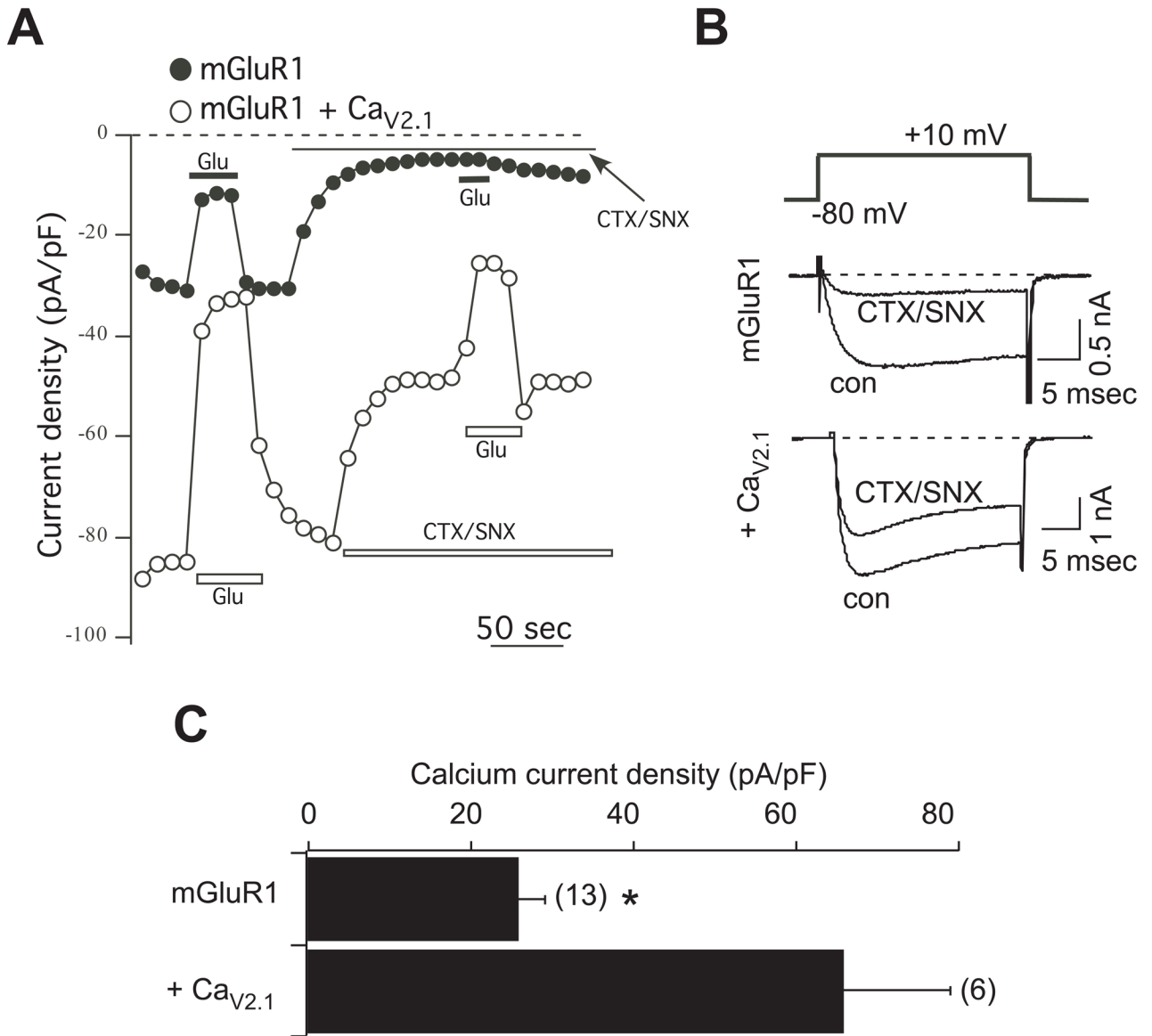
A

B

C

Fig. 2.

$Ca_{V2.1}$ expression in SCG neurons. A, Sample current density time courses from cells expressing mGluR1 alone (filled circles), or mGluR1 plus $Ca_{V2.1}$ (open circles). Current inhibition by 100 μM glutamate (Glu) is evident under both conditions. CTX/SNX indicates application of 1 μM ω-conotoxin GVIA and 500 nM SNX482. Current measurements were obtained 10 msec into a test pulse to +10 mV. B, Sample current traces from the same cells as in A. The voltage protocol is shown above, and consisted of a 25 msec step from a −80 mV holding potential to + 10 mV. Control (uninhibited) currents are shown (con) as are currents maximally inhibited by the CTX-SNX toxin combination. C, Average (± SEM) current densities for SCG neurons expressing mGluR1 alone or mGluR1 plus $Ca_{V2.1}$. Asterisk indicates that current densities are significantly different (T-test, p < 0.05).
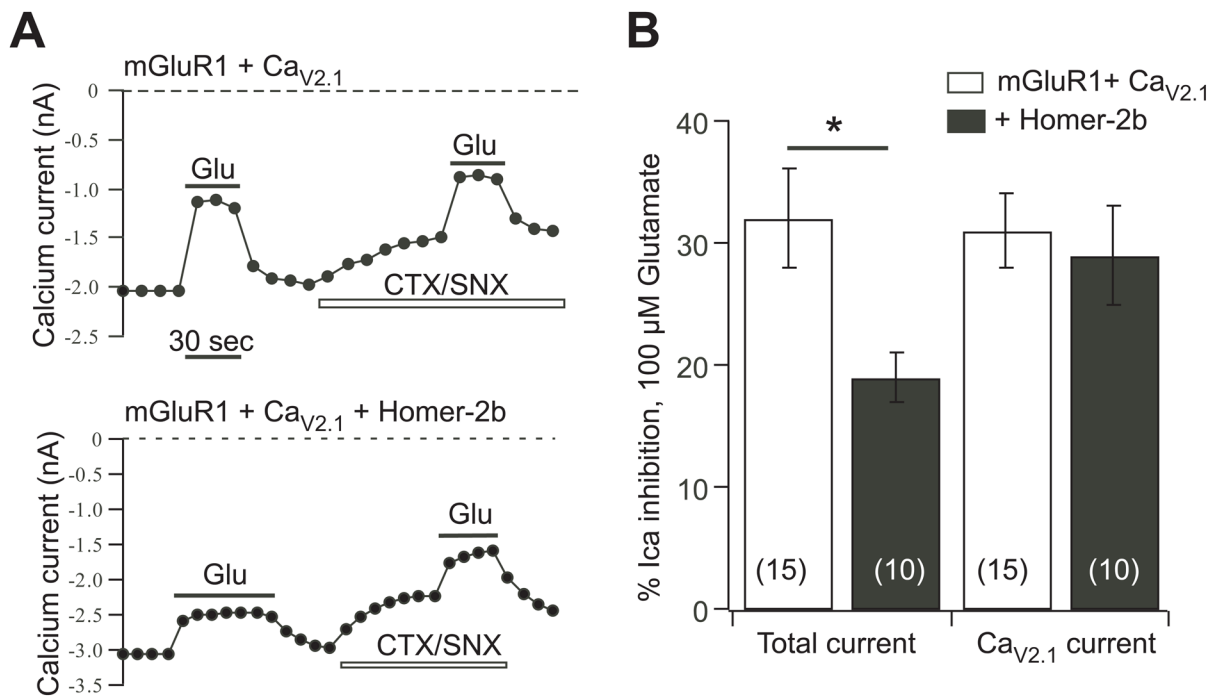
A

B

Fig. 3.
Ca$_{V2.1}$ current modulation by mGluR1 is insensitive to Homer protein expression. A, current amplitude time courses showing modulation by glutamate (Glu) of the total current and the isolated Ca$_{V2.1}$ current (in the presence of CTS/SNX) for SCG neurons expressing mGluR1 and Ca$_{V2.1}$ (upper) or mGluR1, Ca$_{V2.1}$, and Homer-2b (lower). B, average (± SEM) calcium current inhibition by 100 μM glutamate of the total current (left) and isolated Ca$_{V2.1}$ current (right) for neurons with the indicated expression. Number of cells for each condition shown in parentheses.

A

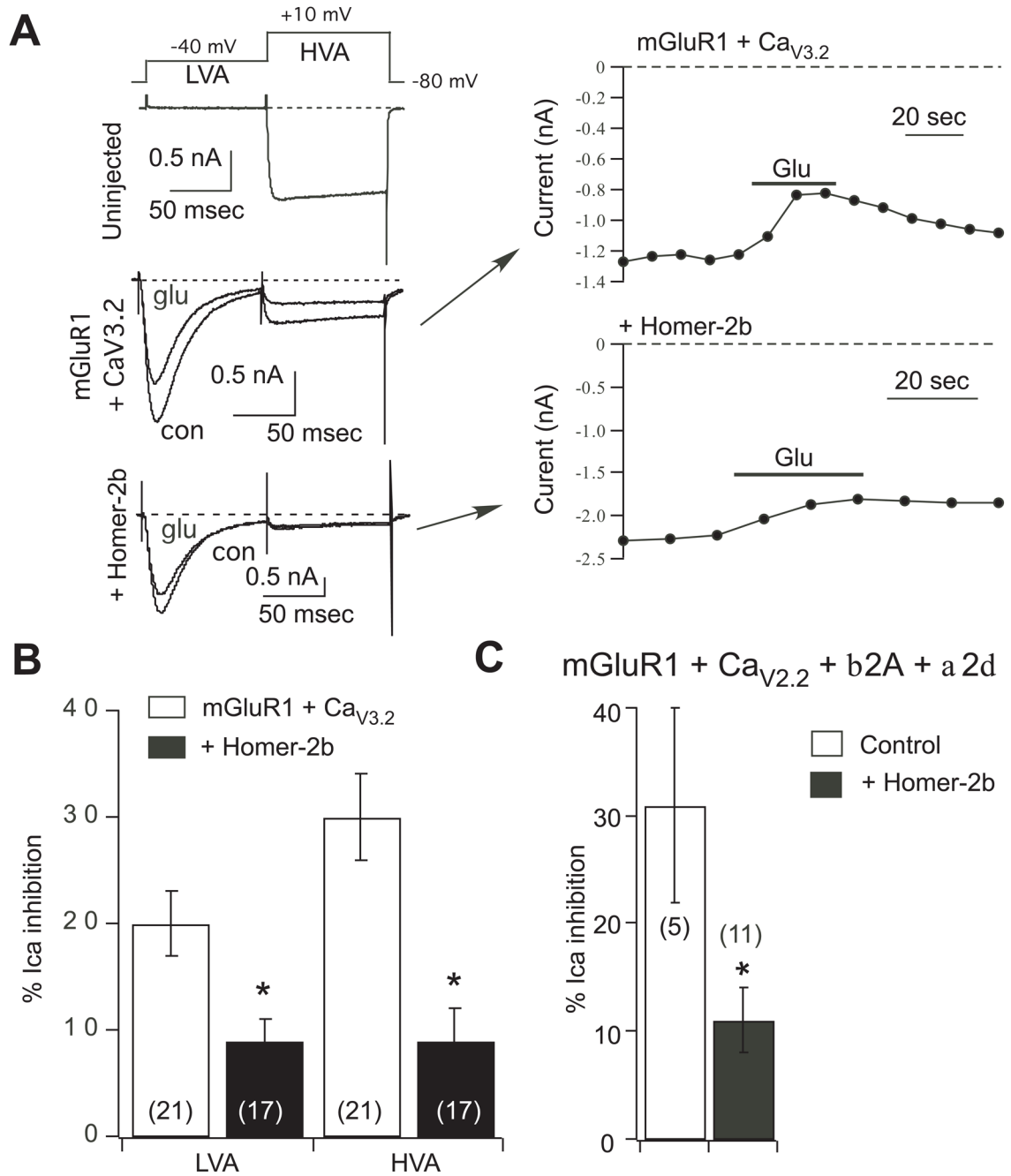**B**

C

mGluR1 + Ca$_{V2.2}$ + b2A + a2d

Fig. 4.
Overexpression of other voltage dependent calcium channels in SCG neurons does not preserve coupling to mGluR1 in the presence of Homer-2b. A, sample current traces (*left*) and time courses (*right*) illustrating the two-step voltage protocol (*top*) used to separate low voltage activated (Ca$_{V3.2}$) currents (LVA) from the native high voltage activated currents (HVA), and inhibition by 100 μM glutamate. Uninjected SCG neurons (*upper left*) showed no LVA currents. Neurons injected with mGluR1 and Ca$_{V3.2}$ (*middle left and upper right*) had prominent LVA and HVA currents, both of which were modulated by 100 μM glutamate (Glu). Additional co-expression of Homer-2b (*lower left and lower right*) uncoupled both the LVA and HVA currents from glutamate modulation. B, Average (±

SEM) LVA and HVA calcium current inhibition by 100 μM glutamate in cells with the indicated expression. Number of cells is shown in parentheses. C, Average (± SEM) calcium current inhibition by 100 μM glutamate in control cells (expressing mGluR1, Ca$_{V2.2}$, and accessory proteins) and in cells co-expressing Homer-2b. Asterisk indicates a statistically significant difference from paired control (without Homer-2b; p 0.05). Number of cells is shown in parentheses.
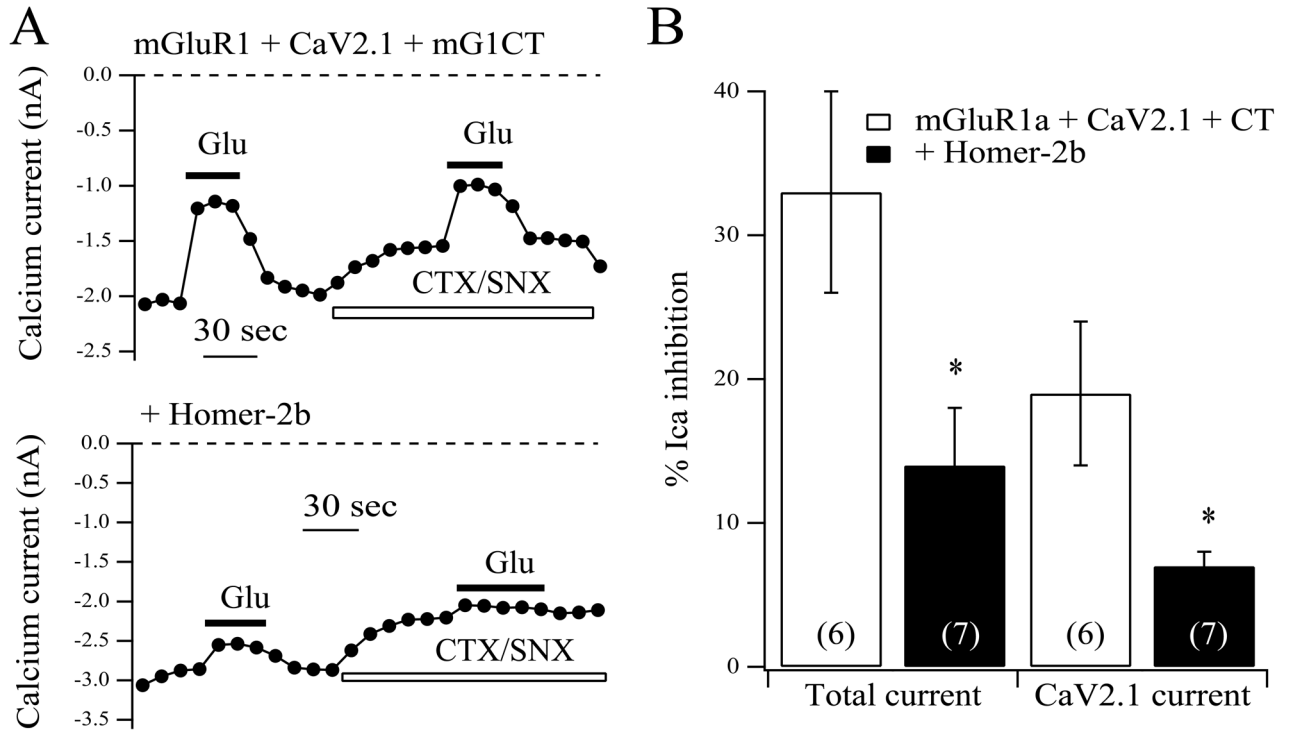
Fig. 5.

Ca$_{V2.1}$ current modulation by mGluR1 is rendered sensitive to Homer protein expression when an mGluR1-CT construct is co-expressed to prevent the receptor-channel interaction. A, Current amplitude time courses showing modulation by glutamate (Glu) of the total current and the isolated Ca$_{V2.1}$ current (in the presence of CTS/SNX) for SCG neurons expressing mGluR1, mG1-CT and Ca$_{V2.1}$ (upper) or mGluR1,, mG1-CT, Ca$_{V2.1}$, and Homer-2b (lower). B, average (± SEM) calcium current inhibition by 100 μM glutamate of the total current (left) and isolated Ca$_{V2.1}$ current (right) for neurons with the indicated expression. Number of cells for each condition shown in parentheses. Asterisk indicates a statistically significant difference from paired control (without Homer-2b; p 0.05).
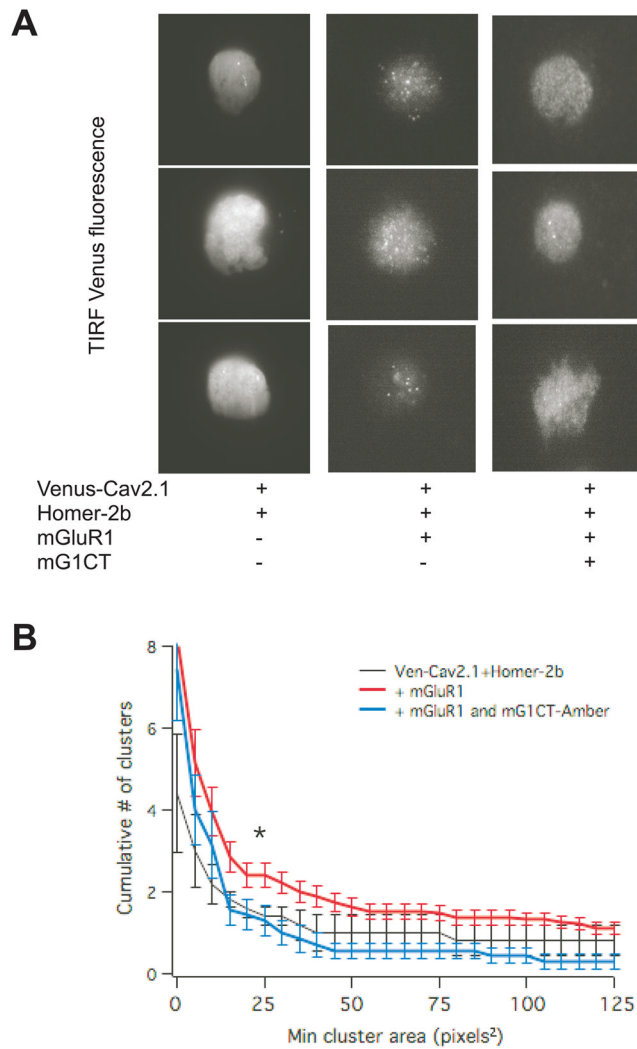
Fig. 6.
Coexpression of mGluR1 and Homer-2b induces Venus-Ca$_{V2.1}$ clustering, which is reduced by expression of an untagged mGluR1-CT construct. A, TIRF fluorescence images showing three sample images each of SCG neurons with the indicated expression. B, Clusters were quantified with a cumulative histogram of cluster number *vs.* size (in pixels^2). * indicates statistically significant difference between the number of clusters in cells expressing Venus-Ca$_{V2.1}$, mGluR1 and Homer-2b (red; n=19) compared to cells also expressing mGluR1-CT-Amber (blue; n=7). Cluster histogram for neurons expressing Ven-Ca$_{V2.1}$ + Homer-2b (n=5) are shown in black. Lines represent average clusters ± SEM at 5 pixels^2 bins.
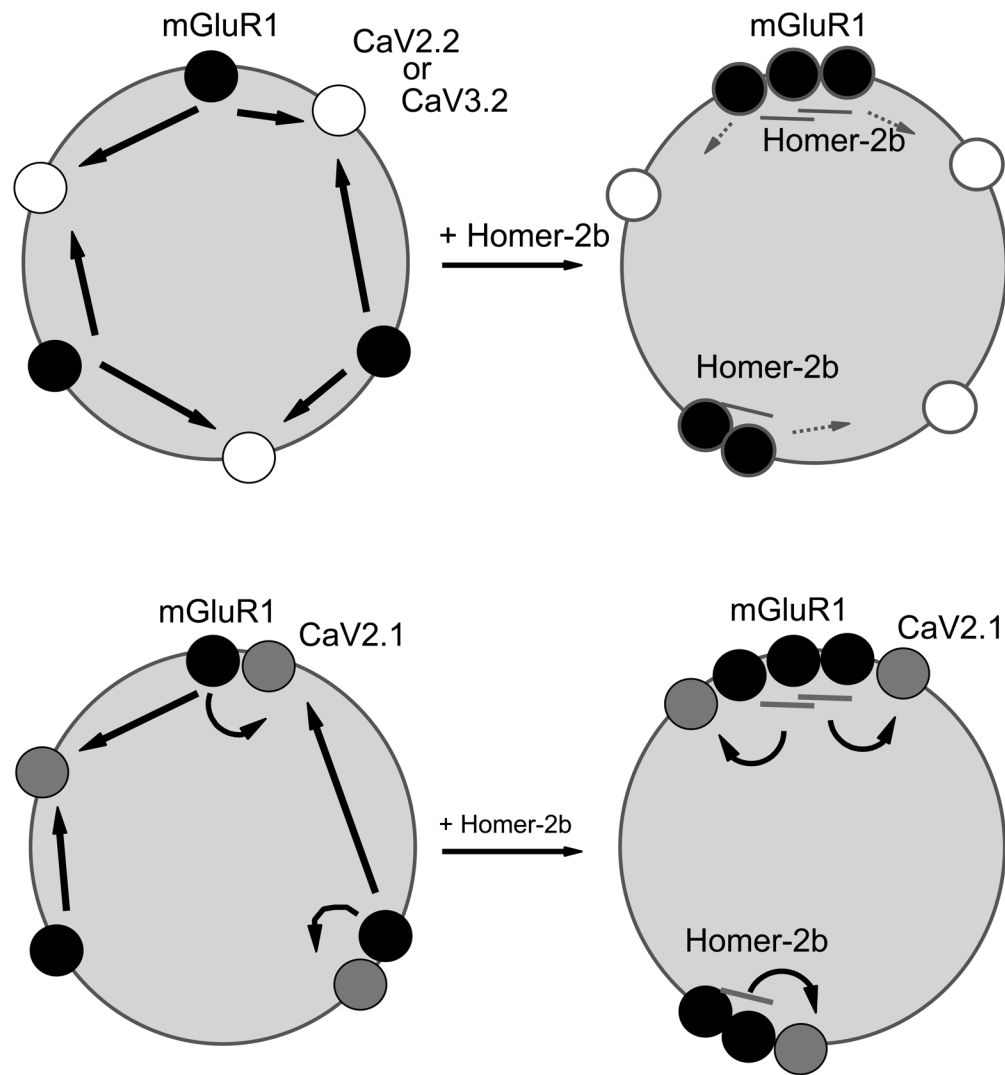
Fig. 7.
Revised model describing the regulation of mGluR signaling by Homer proteins. *Upper* model shows the traditional model in which unclustered, Homer-free mGluR1 couples strongly to voltage dependent calcium channels ($Ca_{V2.2}$ and $Ca_{V3.2}$). Interaction with scaffolding Homer proteins (Homer-2b) results in mGluR1 clustering and uncoupling from channel modulation. *Lower* model shows revised scenario in which interaction with and coupling to $Ca_{V2.1}$ is preserved in the presence and absence of clustering and Homer expression.