Nerve injury-induced changes in Homer/glutamate receptor signaling contribute to the development and maintenance of neuropathic pain.

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Abstract

While group1 metabotropic glutamate receptors (mGluRs) and ionotropic NMDA receptors regulate nociception, the precise molecular mechanism(s) contributing to glutamate signaling in chronic pain remain unclear. Here we not only confirmed the key involvement of Homer proteins in neuropathic pain, but also distinguished between the functional roles for different Homer family members and isoforms. Chronic constriction injury (CCI) of the sciatic nerve induced long-lasting, time-dependent increases in the postsynaptic density expression of the constitutively expressed (CC) isoforms Homer1b/c and/or Homer2a/b in the spinal dorsal horn and supraspinal structures involved in nociception (prefrontal cortex, thalamus), that co-occurred with increases in their associated mGluRs, NR2 subunits of the NMDA receptor and the activation of downstream kinases. Virus-mediated over-expression of Homer1c and Homer2b after spinal (intrathecal) virus injection exacerbated CCI-induced mechanical and cold hypersensitivity however *Homer1* and *Homer2* gene knock-out (KO) mice displayed no changes in their neuropathic phenotype. In contrast, over-expression of the immediate early gene (IEG) Homer1a isoform reduced, while KO of *Homer1a* gene potentiated neuropathic pain hypersensitivity. Thus, nerve injury-induced increases in CC-Homers expression promote pain in pathological states, but IEG-Homer induction protects against both the development and maintenance of neuropathy. Additionally, exacerbated pain hypersensitivity in transgenic mice with reduced Homer binding to mGluR5 supports also an inhibitory role for Homer interactions with mGluR5 in mediating neuropathy. Such data indicate that nerve injury-induced changes in glutamate receptor/Homer signaling contribute in dynamic but distinct ways to neuropathic pain processing, which has relevance for the etiology of chronic pain symptoms and its treatment.

Key words: Homer Proteins, Group1 Metabotropic Glutamate Receptors, NMDA Receptors, Spinal Cord, Neuropathic Pain

1. Introduction

Peripheral nerve injury leads to the development of neuropathic pain as a consequence of injury-induced central sensitization. This involves increases in excitatory neuronal firing, glutamate release within peripheral and central nervous systems, and subsequent reorganization of the nociceptive network [e.g. 9,31,32]. Glutamate transmission, particularly through the Gq/o-coupled Group1 metabotropic glutamate receptors (mGluR1/5), as well as (or in conjunction with) ionotropic NMDA glutamate receptors, has been highly implicated in nociception [e.g. 7,16,45,72]. Thus, a likely molecular candidate contributing to changes in spinal and supraspinal glutamate signaling in chronic pain is the Homer family of proteins, which constitutes a part of the signaling scaffold regulating the trafficking, clustering and function of both Group1 mGluRs and NMDA receptors [e.g. 13,14,53].

Homer proteins are encoded by 3 genes (*Homer1,2,3*) that give rise to constitutively expressed, coiled-coiled (CC; Homer1b/c/d/g/h, Homer2a/b and Homer3; a.k.a. CC-Homers) and immediate early gene (IEG) products (from *Homer1*, Homer1a and ania-3) [13,14]. In contrast to CC-Homers, IEG Homers lack the CC-domain and are incapable of multimerization or multi-protein interactions. Thus, their induction upon synaptic activity destabilizes CC-Homer interactions (including those with glutamate receptors), enabling synaptic rearrangement [6,26]. While the role for Homer proteins in regulating both pre- and postsynaptic aspects of glutamate signaling is well-characterized [e.g. 53] and tissue/nerve injury-induced adaptations within glutamate signaling were shown to be critically involved in the development and maintenance of chronic pain [7,16,45,72], there is recently a growing line of evidence implicating Homer proteins in the regulation of nociception. Homer proteins are expressed in brain regions conveying nociceptive information [e.g. 33,53,56], as well as in the spinal dorsal horn neurons that receive sensory input [36,40,57,71]. Both IEG-Homer1a and CC-Homer1b/c are selectively up-regulated in the spinal dorsal horn neurons in model of chronic inflammatory pain and the manipulation of their protein levels alters inflammation-induced mechanical and thermal hyperalgesia [57,71]. While *Homer1a* induction and increased Homer1b/c exhibit distinct temporal profiles within the spinal dorsal horn following chronic constriction injury (CCI) of the

sciatic nerve [36,37,40] or chronic compression of dorsal root ganglion [35], the functional relevance of changes in Homers expression has been examined to date only in the early stage (4 h after CCI) of the development of neuropathic pain [36].

Thus, in the present study, we used a combination of genetics and immunoblotting approaches to further confirm the important role for Homer proteins in regulating pain sensitivity and more importantly to delineate the relative functional roles played by different Homer family members and isoforms in the development and maintenance of neuropathic pain in mice. We first determined the profile of changes in Homer proteins expression after CCI together with injury-induced alterations in the protein level/activation of Homers-associated mGluRs (mGluR1a/5) and downstream kinases (PKCε, PI3K and ERK1/2) within structures involved in nociception. Next, we examined the effects of Homers overexpression after spinal (intrathecal) delivery and the global Homers deficiency on both the development and maintenance states of CCI-induced neuropathic pain. Finally, we investigated whether the mGluR5-Homer proteins interaction affects neuropathic pain hypersensitivity.

2. Material and Methods

2.1. *Subjects*

C57BL/6J mice. The expression of the CC-Homer isoform Homer1b/c is up-regulated within spinal cord dorsal horn following sciatic nerve ligation [36,37]. Thus, immunoblotting experiments extended these earlier data to another CC-Homer isoform, Homer2a/b, and related changes in CC-Homer expression to those of their associated Group1 mGluRs (mGluR1a/5) and the NR2a/b subunits of the NMDA receptor, as well as to the activational state of downstream kinases (ERK1/2, PKCε and PI3K) to determine whether or not chronic constriction injury (CCI) of the sciatic nerve induces changes in the spinal and supraspinal expression/activation of Group1 mGluR/Homer signaling pathways. Follow-up behavioral experiments then assayed for the functional relevance of observed changes in Homer protein expression for neuropathic pain hypersensitivity. All of these studies employed adult male C57BL/6J (B6) mice (8 weeks of age; 25–30 g; the Jackson Laboratories, Bar Harbor, ME). B6 mice were allowed to acclimate to the colony room for at least 7 days after arrival and were housed in polyethylene cages (4-5 per cage), in a room controlled for temperature (25°C) and humidity (71%) under a regular 12-h day/night cycle (lights on at 7:00 A.M.; lights off at 7:00 P.M.). Standard laboratory rodent chow and water were available *ad libitum*. Animals were habituated to testing procedures for at least 3-4 days before experiments. The handling and testing of the animals were conducted during the light phase, between 9:00 a.m. and 2:00 p.m. Every effort was made to minimize the number of animals used in the study. Experimental protocols were approved by the Institutional Animal Care and Use Committee of our respective institutions and were consistent with the guidelines provided by the National Institute of Health (NIH) *Guide for Care and Use of Laboratory Animals* (NIH publication number 80-23, revised 1996) and the guidelines of the Committee for Research and Ethical Issues of IASP published in [75].

Homer Knock-out (KO) mice. Our immunoblotting data revealed time-dependent increases in Homer1b/c and Homer2a/b expression following CCI (Fig. 1) and earlier studies indicated an important role for Homer1a in regulating inflammatory pain [50]. Thus, we employed a knock-out (KO) strategy to confirm a

necessary role for IEG and CC-Homer isoforms (Homer1a, Homer1, Homer2) in the development and maintenance of neuropathic pain following CCI. For this, the behavior of *Homer1a*, *Homer1*, and *Homer2* gene KO mice was compared to their respective wild-type (WT) and heterozygous (HET) mutant mice. WT, HET and KO littermates from all 3 KO lines were bred in-house at the University of California Santa Barbara Biology II vivarium from mating of heterozygous breeder pairs (B6 X 129Xi/SvJ background). Details of the generation of these mice are provided in Hu et al. [21] for *Homer1a* KO, Yuan et al. [73] for *Homer1* KO and Shin et al. [49] for *Homer2* KO. WT, HET and KO littermate pups from a minimum of 4 different litters for each line were used for each replicate of the behavioral studies to avoid litter confounds. Experimental mice were transferred to the Psychology vivarium at approximately 5 weeks of age and allowed to acclimate to the housing conditions for 2-3 weeks prior to surgery and behavioral testing. All experimental procedures involving WT, HET and KO mice were conducted on male littermate mice, 7-8 weeks of age, housed and handled under conditions described for the B6 mice above.

mGluR5 transgenic (Tg) mice. Our immunoblotting data indicated the co-regulation of CC-Homer expression and mGluR5 by CCI (Fig. 1). Thus, we examined the functional relevance of mGluR5-Homer interactions in neuropathic pain by assaying the pain hypersensitivity of a transgenic (Tg) mouse with a phenylalanine (F) →arginine (R) point mutation within the Homer binding domain on mGluR5 at amino acid position 1128 ($mGluR5^{F1128R}$) that impairs the capacity of Homer proteins to physically interact with the receptor [12,61], but does not affect the total protein expression of either mGluR5 or Homers [12]. The generation of Tg littermates and all experiments procedures involving WT, HET and Tg mice were conducted as described for B6 and KO mice above.

2.2. *Induction and assessment of neuropathic pain*

Sciatic nerve injury. B6 mice, homozygous and heterozygous KO/Tg mice, as well as their WT counterparts, were subjected to peripheral neuropathy induced by chronic constriction injury (CCI) of the sciatic nerve as described by Bennett and Xie [4], with slight modifications for mice [42,45]. The sciatic nerve injury was

performed under isoflurane anesthesia delivered *via* a nose cone (2% isoflurane with oxygen as the carrier gas). The skin was shaved and an incision was made just below the right hipbone, parallel to the sciatic nerve. The biceps femoris and the gluteus superficialis were separated, and the right sciatic nerve was exposed. Proximal to the sciatic trifurcation, the injury was produced by three loose ligations around the sciatic nerve. The ligatures (4/0 silk) were tied loosely around the nerve with 1 mm spacing, until they elicit a brief twitch in the respective hindlimb, which prevented from applying a too strong ligation. The total length of nerve affected was 3-4 mm. Mechanical and cold sensitivity was assessed before sciatic nerve ligation (as basal pain threshold) and then every 2nd day for 2 weeks following the injury using the procedures outlined below. Mechanical sensitivity was also confirmed in all neuropathic B6 mice employed in the immunoblotting studies. In all experiments, the observer was not aware of the treatment.

Mechanical sensitivity. Mechanical sensitivity was assessed by measuring the withdrawal threshold of the paw ipsilateral to the site of ligation in response to mechanical stimuli using von Frey filaments (Stoelting, Wood Dale, IL, USA). Animals were placed in a plastic cage with a wire net floor and were allowed to habituate 20 min before the testing began. Animals were also habituated over a period of 2-3 consecutive days by recording a series of baseline measurements. The filaments were applied in ascending order, each three times at an interval of 3-5 seconds to the midplantar surface of the hindpaw as described previously [42,45] and the smallest filament eliciting a foot withdrawal response was considered the threshold stimulus. Each animal was tested 2-3 times at the interval of 10 min to determine the mean threshold at the respective time points. The strength of the von Frey stimuli ranged from 0.07 to 6.0g.

Cold sensitivity. For assessment of cold sensitivity, the acetone test was used as described previously [42]. Mice were tested for paw withdrawal response to a cold stimulus using a 50 µl drop of acetone applied with a syringe fitted with a blunted needle at the centre of the plantar surface of a hindpaw ipsilateral to the site of ligation. Acetone was applied alternatively twice with 5 min between each application and the responses scored categorically. Responses were monitored for 1 min after application and were graded to the following

4-point scale: 0, no response; 1, quick withdrawal, foot lifted and/or light shakes; 2, prolonged withdrawal and/or prolonged shake of the paw; 3, repeated flicking of the paw with licking directed at the ventral side of the paw.

Basal pain threshold. Basal pain threshold was assessed by measuring response latencies to mechanical stimuli as described above, as well as to a thermal stimulus using the hot water (54°C) tail-immersion assay, as described by Stone et al. [52]. Briefly, mice were gently wrapped in a soft cloth such that their tails were exposed, and three-quarters of the length of the tail was dipped into the hot water. The response was defined as the removal of the tail from the hot water in sec.

2.3. *Modification of pain sensitivity by intrathecal microinjection of AAVs carrying Homer cDNA*

The procedure for generating recombinant AAV vectors carrying the hrGFP cDNA or the hemagglutinin (HA) tag fused to the coding region of rat *Homer1a*, *Homer1c* and *Homer2b* have been described in detail elsewhere [e.g., 29] and were the same as those used in previously published studies [e.g.17,29,33,53,54,56,57]. The procedure for infusing AAVs into the spinal was very similar to [59]. In the present experiments, mice were anesthetized with isoflurane delivered *via* a nose cone (2% isoflurane with oxygen as the carrier gas). For more precise AAV application, a caudal cutaneous incision (1 cm) of the shaved skin above the spinal cord was made with a scalpel and a 27-gauge needle, connected to PE tubing and a microprocessor-controlled minipump (World Precision Instruments), secured in place using a manipulator arm and the AAV vectors were infused at a rate of 0.05 μl/min for 5 min (total volume = 0.25 μl). Following the 5-min infusion period, the needle was left in place for an additional 5 min and then removed very slowly. Then, the incision site was sutured shut and animals were left undisturbed for 3 weeks when AAV-mediated transgene expression peaks remained at stable levels [e.g. 29,30]. Following behavioral testing, animals were deeply anesthesized, transcardially perfused with phosphate-buffered saline, followed by paraformaldehyde. 80 μm-thick sections of the spinal cord (L2-L6; vibratome) were

examined for assessment of transduction efficiency and vector spread based on hrGFP-epifluorescence or immunohistochemical detection of the (HA)-tag.

2.4. *Evaluation of the subcellular distribution of protein expression/activational state of members of mGluR-Homer-kinase signaling pathways*

Subcellular fractionation procedures. Evidence indicates that sciatic nerve ligation produces a shift in the relative PSD expression of Homer1a versus Homer1b/c within the ipsilateral spinal cord at 4 h post-injury [36,37,40]. Thus, we conducted an immunoblotting study upon both spinal and supraspinal tissue to determine whether or not injury-induced shifts in the subcellular distribution of *Homer1* gene products: (1) extend to Homer2a/b - another CC-Homer isoform critical for the regulation of glutamate transmission [e.g. 54,56]; (2) are accompanied by changes in their associated glutamate receptor expression and/or the activation of downstream kinases [e.g. 12,17,43,47]; (3) persist for 2 weeks following injury indicating their role in the maintenance of neuropathic pain; and (4) occur in brain regions involved in nociception (thalamus and prefrontal cortex; e.g. [63]).

The procedures for preparing spinal and supraspinal tissue for subsequent analysis of subcellular distribution of proteins was identical to those described previously [15,56,58]. At 1 day, 1 week and 2 weeks following CCI, mice were decapitated and the L4-L6 segment of the spinal cord was dissected out over ice and subdivided into the ipsi- and contralateral sides of the dorsal horn. Additionally, brains were sectioned (1.0 mm thick) along the coronal plane and the entire prefrontal cortex and thalamus were dissected out over ice. Tissue was placed into 10 volumes of ice-cold homogenization buffer containing: 0.32 mM sucrose, 10 mM HEPES at pH=7.4, 5 mM NaF, 1 mM EDTA, with protease inhibitors. The tissue was homogenized using 3-5 strokes of a glass-teflon homogenizer and spun at 1 000 g for 10 minutes to obtain pellet of nuclei and large debris (the P1 nuclear fruction). This pellet (P1) was homogenized in a medium consisting of 0.32 M sucrose, 2 mM EDTA, 1% w/v sodium dodecyl sulfate, 50 μM phenyl methyl sulfonyl fluoride, 1 μg/ml leupeptin (pH=7.2) and protease inhibitors, and then subjected to low-speed centrifugation at 12 000 g for 20 min. The remaining supernatants of the P1 fraction (S1) was spun again at 10 000 g for 15 min to obtain

a crude synaptosome fraction (P2), and subsequently were lysed hypo-osmticaly in HEPES lysis buffer containing 4 mM HEPES, 1 mM EDTA and protease inhibitors and centrifugated at 25 000 g for 20 min to pellet synaptosomal membrane fraction (the LP1 PSD fraction). Protein determinations of both P1 and LP1 fractions were performed using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) and homogenates were stored at -80^{0}C until immunblotting.

Immunoblotting. The immunoblotting procedure quantified P1 and LP1 Homer levels and examined for the co-regulation of Homer expression with the Group1 mGluR subtypes mGluR1a and mGluR5, the NR2a and NR2b subunits of the NMDA receptor (that link to Homer through a Shank-containing multi-protein scaffold; [41,60]), as well as the total and phosphorylated levels of ERK1/2, PI3K and PKCε - 3 kinases regulated by glutamate receptor activation/Homer expression [e.g. 12,43,47]. Protein samples (10-20 μg) were subjected to a SDS-polyacrylamide gel electrophoresis. Bis-Tris gradient gels (4-12%) (Invitrogen, Carlsbad, CA) were used for separation of Homers, PI3K, and the p(Tyr)p85α PI3K binding motif, the latter of which was employed to index PI3K activity. Tris-Acetate gradient gels (3-8%) (Invitrogen, Carlsbad, CA) were used for separation of the glutamate receptor proteins. Proteins were transferred to PVDF membranes, preblocked with phosphate-buffered saline containing 0.1% (v/v) Tween-20 and either 5% (w/v) bovine serum albumin (for p-ERK1/2, pPKCε and for p(Tyr)p85α PI3K binding motif) or 5% (w/v) nonfat dried milk powder (for all other proteins) for at least 1 h before overnight incubation with primary antibodies. The following rabbit polyclonal antibodies were used: anti-Homer 2a/b and anti-Homer 1b/c (Dr. Paul F Worley, Johns Hopkins University School of Medicine; 1:1000 dilution), anti-mGluR5 (Upstate Cell Signaling Solutions, Lake Placid, NY; 1:1000 dilution), anti-NR2a and anti-NR2b (Calbiochem, San Diego, CA; 1:1000 dilution), anti-PI3K antibody (Upstate, Lake Placid, NY; 1:1000 dilution), and anti-p-(Tyr) PI3K p85α binding motif (Cell Signaling Technology, Beverly, MA; 1:250 dilution), anti-ERK1/2 and anti-pERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution), anti-PKCε and anti-pPKCε (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000 dilution). An anti-mGluR1a mouse polyclonal antibody (Upstate, Lake Placid, NY; 1:1000 dilution). Even loading and transfer were confirmed by probing for

calnexin using a rabbit monoclonal antibody (Stressgen, Victoria BC; 1:1000 dilution). Membranes were washed and incubated with horseradish-peroxidase-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories Inc., West Grove PA; 1:10,000-20,000 dilution) or anti-mouse secondary anti-body (Jackson Immuno Research Laboratories, West Grove, PA; 1:10,000-1:20,000) for 90 min, and immunoreactive bands were detected by enhanced chemiluminescence (ECL Plus; Amersham Biosciences Inc., Piscataway, NJ). Immunoreactive levels for all proteins were quantified by integrating band density X area using computer-assisted densitometry (Image J; NIH, Betheseda, MD). The density X area measurements for all bands were normalized to that of its appropriate calnexin signal to provide a protein/calnexin ratio. The data for experimental animals were expressed as a percent change from the mean control signal (n=3-5/membrane) for each individual membrane. The optical density of the p-ERK1/2, and p-PKCε were divided by that of the corresponding total protein band to yield a phospho-protein/total protein ratio prior to expressing the data as a percent change from controls.

2.5. *Statistical Analysis*

Data analysis and statistical comparisons were performed using GraphPad Prism™, version 4.00 for Windows, GraphPad Software, San Diego California USA. Both behavioral and biochemical results are presented in the graphs as mean ± SEM. Each group included 8–12 animals. Statistical differences between groups in immunoblotting studies were determined by one-way analyses of variance (ANOVA) with Tukey's multiple comparison post-hoc tests. Behavioral results were analyzed by two-way analyses of variance (ANOVA) with Bonferroni's multiple comparison post-hoc tests. Differences within groups subjected to the estimation of basal pain threshold were evaluated using unpaired Student's *t* test. A value of $p < 0.05$ vs. respective control group was considered to be statistically significant.

3. Results

3.1. *Sciatic nerve injury up-regulates PSD expression of specific CC-Homer and associated glutamate receptor proteins in spinal and supraspinal structures involved in nociception*

Behavior: As expected [42,45], chronic constriction injury (CCI) to the sciatic nerve in B6 mice produced a long-lasting (2-week) reduction in pain threshold to mechanical stimuli as demonstrated by the von Frey test (2.68 ± 0.09 g for naïve vs. day 2 post-injury: 0.51 ± 0.08 g, day 7 post-injury: 0.53 ± 0.10 g, day 14 post-injury: 0.57 ± 0.04 g; $F_{(6,63)} = 38.60$, $p<0.001$).

Spinal Dorsal Horn: Protein changes induced by CCI were observed within the spinal dorsal horn ipsilateral, but not contralateral, to the injury (Fig. 1 vs. Table 1).

Homers. The increase in pain sensitivity following nerve injury was accompanied by a time-dependent shift in the PSD localization of Homer1b/c within the spinal cord ipsilateral to the injury [Time effect: $F_{(3,50)}=16.57$, $p<0.0001$; Fig. 1A], but no change in Homer1b/c localization within the contralateral dorsal horn (Table 1). At 1 day after ligation, Homer1b/c levels were decreased in the PSD fraction from ipsilateral dorsal horn, while protein levels were increased by 2-2.5-fold at 1 and 2 weeks post-injury (Fig. 1A). Correspondingly, an opposite pattern of change was observed for Homer1b/c expression within the P1 fraction (Fig. 1B) [*t*-test between naïve control and 1 week after CCI: $t_{(34)}=1.95$, $p=0.03$], supporting that sciatic nerve injury elicits a time-dependent increase in Homer1b/c localization within the PSD within dorsal horn and this shift in protein localization persists for at least 2 weeks post-injury. In contrast to Homer1b/c, Homer2a/b levels within the PSD fraction from ipsilateral dorsal horn exhibited a non-significant (37-46%) increase at the later time-points in injured animals (Fig. 1A) and exhibited no pattern of change within the P1 fraction (Fig. 1B).

Glutamate Receptors. While the PSD levels of the glutamate receptor proteins failed to exhibit any significant changes early post-injury (at 1 day), mGluR1a, mGluR5 and NR2b expression was elevated at 1 week post-injury and this effect persisted for NR2b for 2 weeks post-ligation (Fig. 1A) [mGluR1a: $F_{(3,45)}=3.25$, $p=0.03$; mGluR5: $F_{(3,53)}=2.93$, $p=0.04$; NR2b: $F_{(3,52)}=5.83$, $p=0.002$]. No changes in the PSD

expression of the NR2a subunit were observed [$F_{(3,45)}$=2.04, p=0.12]. Also, neither mGluR1a/5 nor NR2a/b exhibited changes within the P1 fraction at any time (Fig. 1B). These data show for the first time that time-dependent increases in the localization of Homer1b/c in the PSD within the ipsilateral spinal dorsal horn after injury are accompanied by increases in PSD glutamate receptor expression and implicate enduring PSD over-expression of Group1 mGluR/NR2b-containing NMDA receptor/Homer1b/c complexes in mediating the increased pain sensitivity following sciatic nerve ligation.

Thalamus. The thalamus plays a key role in the transfer of nociceptive information since this structure encodes details concerning the type, temporal pattern, intensity and topographic localization of pain [18]. Much evidence also suggests that glutamate and glutamate receptors within thalamus are involved in the neuronal transmission of pain [19,51]. Thus, we also examined for changes in thalamic expression of Homers and their associated glutamate receptors.

Homers. Thalamic PSD levels of Homer1b/c did not change early post-injury, but were significantly elevated 2 weeks post-CCI (Fig. 1C) [$F_{(3,46)}$=5.07, p=0.01]. The latent increase in LP1 Homer1/c expression within thalamus was accompanied by a tendency for reduced protein expression within the P1 fraction [compare 1C vs. D; *t*-test between naïve control and 1 week after CCI: $t_{(33)}$=2.42, p=0.01]. The injury-induced changes in thalamic Homer1b/c expression were paralleled by changes in Homer2a/b expression with respect to both the increase within the LP1 fraction (Fig. 1C) [$F_{(3,35)}$=9.92, p<0.0001] and the tendency towards a reduction within the P1 fraction [Fig. 1D; *t*- test between naïve control and 1 week after injury: $t_{(33)}$=1.76, p=0.04; between naïve control and 2 weeks after injury: $t_{(33)}$=1.86, p=0.04]. These data indicate for the first time that the PSD expression of Homer2a/b is also responsive to sciatic nerve injury and that injury-induced regulation of the PSD expression of CC-Homer isoforms may be regionally selective within central nociceptive pathways.

Glutamate Receptors. Injury-induced changes in thalamic PSD Homer expression were accompanied by increases in the LP1 expression of both Group1 mGluR subtypes, and both the NR2a and NR2b subunits of the NMDA receptor (Fig. 1C) [mGluR1a: $F_{(3,43)}$=5.65, p=0.002; mGluR5: $F_{(3,42)}$=5.66, p=0.002; NR2a:

$F_{(3,39)}$=3.14, p=0.03; NR2b: $F_{(3,41)}$=8.46, p=0.0002]. However, only the elevated LP1 levels of NR2b persisted for 2 weeks post-injury (Fig. 1C). The tendency towards reduced CC-Homer1/2 expression within the P1 fraction from thalamus were accompanied by increases in both mGluR1a and mGluR5 levels, with the rise in mGluR5 persisting until the 2-week time-point [mGluR1a: $F_{(3,50)}$=4.02, p=0.01; mGluR5: $F_{(3,56)}$=9.80, p<0.0001; Fig. 1D]. No changes in P1 levels of either NR2 subunits were observed. While the precise subthalamic localization of protein changes cannot be discerned from the present study, these immunoblotting data provide novel evidence to support a robust increase in the expression of glutamate receptor-Homer complexes within thalamus, which is likely to impinge upon glutamatergic signaling within this structure critical for relaying nociceptive information to cortical structures.

Prefrontal cortex. The prefrontal cortex (PFC) is implicated in chronic pain [3,38], in particular in the modulation of the emotional and cognitive aspects of nociceptive processing [67]. Also, different Homer isoforms play distinct roles in regulating various aspects of glutamate transmission within this forebrain structure [33,54].

Homers. LP1 levels of Homer1b/c were significantly reduced relative to controls at 1 day post-injury and this was followed by an elevation in protein expression at the 2 week time-point (Fig. 1E) [$F_{(3,49)}$=9.01, p<0.0001]. While the data presented in Fig. 1F suggested that peripheral nerve injury elicited a transient reduction in PFC P1 levels of Homer1b/c, this trend was not significant [$F_{(3,55)}$=2.68, p=0.06]. In contrast to the robust changes in thalamus (Fig. 1C,D), nerve injury failed to alter PFC Homer2a/b levels within either the LP1 or the P1 fractions (Fig. 1E,F).

Glutamate Receptors. The only significant change in PSD glutamate receptor expression was an increase in mGluR1a 2 weeks after CCI [$F_{(3,41)}$=3.50, p=0.02] and this was accompanied by an increase in P1 levels of this receptor at the same time-point [$F_{(3,41)}$=3.50, p=0.02] (Fig. 1 E,F). While no changes were observed for the LP1 levels of other glutamate receptor proteins, the P1 levels for mGluR5 and NR2b were reduced at the 1 day time-point within PFC [mGluR5: $F_{(3,56)}$=5.69, p=0.002; NR2b: $F_{(3,51)}$=4.22, p=0.009; Fig. 1F]. These data indicate that sciatic nerve ligation does elicit time-dependent and enduring shifts in the subcellular

localization of Homer1b/c and its associated glutamate receptors within PFC that is likely to enhance glutamatergic signaling within this region [33] in relation to nociception.

3.2. *Sciatic nerve injury up-regulates the activity of kinases involved in CC-Homer signal transduction within spinal and supraspinal structures involved in nociception*

Stimulation of Group1 mGluRs and NMDA receptors instigates both calcium-dependent and –independent intracellular signaling cascades that involve, but are not limited to, activation of PKCs, PI3K and MAP kinase pathways [13,14,54]. Moreover, activation of all 3 kinases (or isozymes thereof) under different persistent pain states results in the induction and maintenance of pain hypersensitivity [23,66,69]. Thus, we immunoblotted further our P1 and LP1 fractions from the spinal dorsal horn, thalamus and PFC for total versus phosphorylated PKCε, and ERK1/2, as well as total PI3K and phosphorylated p85α binding motif to relate our time-dependent changes in glutamate receptor/Homer protein expression (Fig. 1) to the expression and activational state of these kinases.

Spinal Dorsal Horn: Injury-induced changes in the levels of total and/or phosphorylated kinases were observed within spinal dorsal horn ipsilateral (Fig. 2A and B), but not contralateral, to the injury (Table 1). While injury did not alter the expression or phosphorylation state of PKCε within the LP1 fraction (Fig. 2A), the levels of phospho-PKCε and the ratio of phosphorylated vs. total PKCε were significantly elevated within the nuclear fraction at the 2-week time-point (Fig. 2B) [phospho-PKCε: $F_{(3,56)}$=4.95, p=0.004; PKCε ratio: $F_{(3,51)}$=13.06, p<0.0001]. Elevations in the PSD levels of PI3K and of phospho-(Tyr)p85α PI3K binding motif (an index of PI3K activation; [74]) were observed at 1 week post-injury (Fig. 2A) [PI3K: $F_{(3,51)}$=3.93, p=0.01; p(Tyr)p85αPI3K binding motif: $F_{(3,48)}$=3.11, p=0.03]. In contrast to the LP1 fraction, no significant changes in PI3K expression or activity were observed within the P1 fraction (Fig. 2B). Finally, nerve ligation elevated significantly PSD levels of total, as well as of phospho-ERK1/2 1 week after injury, but did not change their relative expression (Fig. 2A) [ERK1/2: $F_{(3,506)}$=3.57, p=0.02; phospho-ERK1/2: $F_{(3,48)}$=3.73, p=0.02; ERK1/2 ratio: p>0.05]. Together, the data presented in Fig. 2A and B indicate that

sciatic nerve injury elicits enduring (1 week) changes in the subcellular distribution and the activational state of several kinases within the spinal dorsal horn ipsilateral to the injury.

We observed an increase in PI3K and ERK1/2 activity at the PSD (LP1 fraction), which co-occurs with increases in glutamate receptors and Homer1b/c expression – a finding consistent with a role for Homer proteins in regulating glutamate receptor-mediated stimulation of PI3K/MAPK activity [e.g., 12,47]. We also observed increases in indices of PKCε activation within the nuclear fraction (P1 fraction), which are most apparent at times when the levels of glutamate receptors and Homers are normalized within this subcellular compartment. These data suggest that time-dependent increases in the PSD expression of glutamate receptors and Homer1b/c following nerve injury (Fig. 1A) may facilitate the activation and subsequent nuclear translocation of PKCε within the spinal dorsal horn, which in turn would be predicted to promote the transcription of genes implicated in nociception.

Thalamus. No changes in PSD PI3K expression or activity were observed in thalamus (Fig. 2C), while the P1 levels of total PI3K were increased at the 1 and 2-week time-points [PI3K: $F_{(3,54)}=9.89$, $p<0.0001$] without any change in P1 indices of PI3K activity (Fig. 2D). Total LP1 levels of ERK1/2 and the P1 levels of the total and activated ERK1/2 were unchanged by CCI within thalamus (respectively, Fig. 2C,D). However, indices of activated ERK1/2 within the PSD fraction of thalamus exhibited a significant reduction at 1 day post-injury, followed by increases at the later post-injury time-points [phospho-ERK1/2: $F_{(3,54)}=3.49$, $p=0.02$; ERK1/2 ratio: $F_{(3,45)}=3.09$, $p=0.04$]. The levels of both total and phosphorylated PKCε were elevated within the LP1 fraction of thalamus at the 1 and/or 2 week time-point (Fig. 2C) [PKCε: $F_{(3,46)}=4.15$, $p=0.01$; phospho-PKCε: $F_{(3,45)}=6.64$, $p=0.0008$], but the ratio was unchanged. Despite these regional differences, we did observe an effect of nerve injury in common between the thalamus and the spinal dorsal horn related to nuclear (P1) indices of PKCε activation; the levels of phospho-PKCε and the ratio of phosphorylated vs. total kinase tended to be low at 1 day post-injury, followed by a large increase in expression at the 2-week post-injury time-point (Fig. 2D) [phospho-PKCε: $F_{(3,50)}=5.23$, $p=0.003$; phospho-PKCε ratio: $F_{(3,46)}=4.59$, $p=0.007$]. These data indicate that the development of neuropathic pain

hypersensitivity correlates with the activation and nuclear translocation of PKCε at both the spinal and thalamus levels, as well as increases in ERK1/2 and PI3K signaling that are distinct between these two sites of nociception.

Prefrontal cortex: The patterns of change in kinase activity within the PSD fraction from PFC included time-dependent increases in the levels of p(Tyr)p85α PI3K binding motif [$F_{(3,49)}$=4.19, p=0.001], total ERK1/2 [$F_{(3,49)}$=4.51, p=0.007] and phospho-ERK1/2 [$F_{(3,40)}$=6.18, p=0.001] (Fig. 2E). However, indices of PKCε activation were reduced within the LP1 fraction at 1 day post-injury (Fig. 2E) [phospho-PKCε ratio: $F_{(3,43)}$=3.06, p=0.04]. Within the P1 fraction indices of PI3K activity were significantly elevated at 2 weeks following nerve ligation (Fig. 2F) [$F_{(3,50)}$=4.24, p=0.009]. The pattern of changes observed for indices of PKCε activation within the P1 fraction of PFC was completely consistent with those observed for both the spinal dorsal horn and the thalamus; the ratio of phosphorylated vs. total PKCε exhibited a significant reduction at 1 day post-injury followed by a persistent increase (Fig. 2F) [$F_{(3,54)}$=13.75, p<0.0001]. These data for PFC support a potential role for time-dependent changes in the activational state of PI3K, ERK1/2, as well as PKCε (including its nuclear translocation) in the development and maintenance of neuropathic pain following sciatic nerve ligation.

3.3. *AAV-mediated over-expression of CC-Homer isoforms exacerbates neuropathic pain*

The proteomic data illustrated in Fig. 1 and 2 implicate a time-dependent up-regulation in endogenous glutamate receptor signaling through PKCε, PI3K and ERK1/2 at both at the spinal and supraspinal levels of the nociceptive pathway following sciatic nerve ligation. The question remains as to the functional significance of the injury-induced increases in CC-Homer expression for the development and maintenance of neuropathic pain and alterations in basal pain threshold. To address these questions, AAV-Homer1c or AAV-Homer2b were intrathecally infused to elevate protein expression within spinal cord (i.e., to mimic the effect of peripheral nerve injury on CC-Homer expression; Fig. 1). As illustrated in Fig. 3A and 5A and consistent with our earlier reports for AAV-mediated transduction within supraspinal structures [e.g. 12,

17,29,33,53,54,56], the local infusion of AAV vectors at the level of L4-L6 produced HA staining. The pattern of transduction throughout the dorsal horn of the spinal cord when assessed at 7-8 weeks postinfusion indicates for AAVs localization within axons/fibers rather than cell bodies, thus suggesting the possibility of further examination of dorsal root ganglia (Fig. 3A). The pattern was very similar to that reported previously by Tappe et al. [59] and was not apparent in animals infused with our hrGFP-control vector that lacked the HA tag (AAV-control in Fig. 3A and 5A). Thus, we confirmed selective transduction of the spinal cord by our AAV infusion procedures that persisted beyond the duration of our behavioral studies.

To mimic the effects of sciatic nerve injury on CC-Homer expression in the spinal cord (Fig. 1A), the first behavioral experiment examined if and how AAV-mediated CC-Homer over-expression after spinal delivery affects the development and maintenance of neuropathic pain. As expected, control mice exhibited a time-dependent and persistent increase in pain hypersensitivity across the 14 days of behavioral testing following CCI as measured by the von Frey test (Fig. 3B,D) and by the acetone test (Fig. 3C,E). However, both Homer1c and Homer2b over-expressing mice exhibited exacerbated pain hypersensitivity following CCI when compared to control animals as: (1) the pain threshold to mechanical stimuli (von Frey filaments) was decreased in Homer1c and Homer2b over-expressing mice vs. controls (Fig. 3B,D); (2) for both Homer1c and Homer2b over-expressing mice mechanical hypersensitivity increases with the passage of time as mechanical pain hypersensitivity in CC-Homer over-expressing mice continued to worsen across the 2 weeks of testing, while those of control animals stabilized by Day 2 post-injury [AAV-Homer1c: $F_{(1,98)}$=12.33, p=0.0007; AAV-Homer2b: $F_{(1,98)}$=13.66, p=0.0004]; and (3) both Homer1c and Homer2b over-expressing mice exhibited increased sensitivity to acetone, although the magnitude of the differences between over-expressing and control AAV mice did not change with the passage of time post-injury [AAV-Homer1c: $F_{(1,98)}$=34.07, p<0.0001; AAV-Homer2b: $F_{(1,98)}$=73.12, p<0.0001]. Thus, the overall pattern of change in mechanical and cold hypersensitivity observed following CCI matches, in large part, the profile of CCI-induced changes in CC-Homer protein expression within the spinal cord in non-transfected (B6) animals (Fig. 3D-E vs. Fig. 1A).

Localized transduction after intrathecal delivery of AAV-Homer1c and AAV-Homer2b infusions did not alter the basal pain threshold to mechanical stimuli before nerve injury (Fig. 3B,D – Day 0) nor did it alter the latency of a separate group of non-injured mice to tail withdrawal (Table 2). Thus, over-expression of both CC-Homer1 and CC–Homer2 isoforms increases sensitivity to both mechanical and cold stimuli for at least 2 weeks following sciatic nerve ligation – effects that do not reflect changes in basal pain threshold. Such data provide the first evidence that CC-Homer isoforms potentiate the development and maintenance of neuropathic pain in mice following CCI and pose CCI-induced increases in the spinal cord CC-Homers expression as an important mediator of neuropathic pain hypersensitivity.

3.4. *Homer1* and *Homer2* deletion does not affect neuropathic pain hypersensitivity

As both CC-Homer1 and CC–Homer2 over-expression after intrathecal delivery potentiated neuropathic pain hypersensitivity (Fig. 3) and siRNA-mediated knock-down of Homer1 within the spinal cord was shown recently to reduce pain behavior early (4 h) following sciatic nerve ligation [36], we next compared mechanical and cold hypersensitivity between injured mice homozygous (KO) and heterozygous (HET) for null mutations of *Homer1* and *Homer2* to their respective wild-type (WT) littermates. The pain behavior of WT mice from both lines on a mixed B6-129 background was comparable to that exhibited by inbred B6 mice in the AAV study (compare Fig. 3 vs. 4). However, no differences were observed between *Homer1* WT, HET and KO genotypes in mechanical (Fig. 4A) and cold sensitivity (Fig. 4B) following nerve injury across the 2 weeks of testing. Similarly, no differences were observed between *Homer2* WT, HET and KO genotypes (Fig. 4C,D). Moreover, no genotypic differences were observed for either KO line regarding their basal pain threshold to mechanical stimuli before nerve injury (Fig. 4A, C – Day 0) or tail withdrawal latency (Table 2). These data indicate that single deletion of neither *Homer1* nor *Homer2* alters the development and maintenance of pain hypersensitivity following peripheral nerve injury nor does it affect basal pain threshold. These data contrast with recent data indicating that intrathecal injection of *Homer1* siRNA alleviates signs of neuropathic pain early post-injury [36]. As *Homer2* deletion does not affect CC-Homer1 expression, at least in supraspinal structures [54], a possible explanation to account for the failure of

Homer1 or *Homer2* single gene deletions to block neuropathic pain hypersensitivity might relate to compensation in scaffolding/signaling by injury-induced increases in the alternate Homer isoform within the spinal dorsal horn or supraspinal structures involved in nociception (Fig. 1). Alternatively, developmental compensations in glutamatergic signaling within nociceptive pathways, secondary to *Homer1* or *Homer2* gene deletion, may mitigate or mask effects of *Homer* deletion upon pain behavior.

3.5. *Transgenic disruption of mGluR5-Homer interactions potentiates neuropathic pain*

Homer proteins physically interact through an EVH1 domain with a proline-rich motif located on the C-terminus of Group1 mGluRs and this interaction is critical for receptor trafficking and signaling [e.g. 2,5,6,25-27,61]. Sciatic nerve ligation up-regulates mGluR5 and CC-Homer1/2 expression (Fig. 1), which likely increases their probability of physical interaction. The mGluR5 receptor is highly implicated in nociception [e.g. 7,16,45,72] and the results of our AAV studies indicate an important role for the spinal cord CC-Homer1/2 over-expression in mediating neuropathic pain (Fig. 3). In an attempt to simultaneously address the issues of potential Homer1/Homer2 redundancy, as well as potential developmental compensations in mGluR5 signaling, for pain processing in our *Homer* single gene KO mice (Fig. 4), we examined the functional relevance of mGluR5-Homer interactions for the development and maintenance of neuropathic pain following sciatic nerve ligation by assaying the mechanical and cold sensitivity in a transgenic (Tg) mouse with an F1128R point mutation in mGluR5 ($mGluR5^{F1128R}$) reducing the binding of Homer proteins [2,12,61]. For this experiment, we compared mice homozygous (Tg) or heterozygous (HET) for the transgene to their WT littermates.

WT, HET and Tg mice from the $mGluR5^{F1128R}$ transgenic line (Fig. 5B,C) exhibited a time-dependent increase in sensitivity to both mechanical and cold stimuli, but this was augmented by the mutant transgene. Tg mice demonstrated significant and persistent exacerbation of sensitivity to von Frey filaments, while the phenotype of HET mice was intermediate that of Tg animals and WT controls (Fig. 5B) [Genotype X Time, $F_{(12,168)}=2.59$, $p=0.003$]. Tg mice also demonstrated exacerbated sensitivity to acetone, but this phenotype

was not gene dose-dependent (Fig. 5C) [Genotype effect, $F_{(2,168)}$=30.59, p<0.0001]. This mutation did not reflect changes in basal pain threshold as genotypic differences were not observed for either transgenic line regarding basal pain sensitivity to mechanical stimuli before nerve injury (Fig. 5B, D – Day 0) or tail withdrawal latency (Table 2). These findings for mGluR5 transgenic mice were quite surprising as they indicated that the physical interaction between mGluR5 and CC-Homers normally inhibits or protects against the development and maintenance of neuropathic pain following peripheral nerve injury.

3.6. *Manipulations of the IEG Homer1 isoform Homer1a alter neuropathic pain*

In addition to the constitutively expressed isoforms Homer1b-g, the *Homer1* gene also encodes the IEG transcriptional variants Homer1a and ania-3 [e.g. 50,53]. The mRNA and protein levels of Homer1a are significantly elevated within the spinal dorsal horn of a model of inflammatory pain [57], as well as during the early stage of peripheral nerve injury-induced neuropathic pain, at a time when CC-Homer1 isoforms are reduced (Fig. 1; [36,37]). Moreover, functional studies selectively targeting Homer1a indicate that spinal cord over-expression of Homer1a protects against, while *Homer1a* knock-down with shRNA exacerbates, symptoms of inflammatory pain [57]. Thus, we tested the hypothesis that *Homer1a* deletion would also exacerbate the development and maintenance of neuropathic pain following peripheral nerve injury.

We first employed a *Homer1a* KO strategy [21] to determine whether or not preventing the induction of Homer1a post-sciatic nerve injury [36,37] would exacerbate the development and maintenance of neuropathic pain. In support of our hypothesis, a comparison of *Homer1a* WT, HET and KO mice indicated a significant potentiation of sciatic nerve injury-induced pain hypersensitivity in mutant animals (Fig. 5D,E). Persistent genotypic differences were observed across the 2 weeks of testing with both mechanical (Fig. 5D) [Genotype effect: $F_{(2,168)}$=39.89, p<0.0001] and cold hypersensitivity increased in KO mice, relative to WT and HET animals (Fig. 5E) [Genotype effect: $F_{(2,168)}$=25.38, p<0.0001]. The exacerbated pain hypersensitivity observed in *Homer1a* KO animals following injury did not reflect changes in basal pain threshold in the responses to mechanical stimuli before CCI (Fig. 5D – Day 0) or in tail withdrawal latency

(Table 2). Thus, Homer1a induction is not necessary for basal pain threshold, but inhibits the development and maintenance of neuropathic pain following peripheral nerve injury, in a manner consistent with the notion that Homer1a induction is anti-nociceptive [57].

Next, we assessed the effects of spinal cord Homer1a over-expression via intrathecal infusions of an AAV carrying *Homer1a* cDNA [30,57] to determine whether or not Homer1a over-expression protected against the development and maintenance of pain hypersensitivity following CCI. AAV-mediated Homer1a over-expression after intrathecal delivery (Fig. 5A) completely blocked the development and maintenance of mechanical hypersensitivity post-injury examined by von Frey testing (Fig. 5F) [AAV X Time: $F_{(6,126)}$=15.23, $p<0.0001$]. Homer1a over-expression also significantly reduced the enhanced cold hypersensitivity in the acetone test (Fig. 5F) [AAV X Time: $F_{(6,126)}$=21.51, $p<0.0001$], but did not affect basal pain threshold when assessed in the tail flick test (Table 2) or upon mechanical stimulation before injury (Fig. 5F – Day 0). These data provide the first evidence that the induction of Homer1a within the spinal cord following peripheral nerve injury inhibits the development and maintenance of neuropathic pain. These observations extend earlier findings obtained for inflammatory pain [58] and further support the hypothesis put forth by Tappe and colleagues [58] that Homer1a induction may serve as a negative regulator of excitatory signaling underlying the development and maintenance of pain following peripheral injury.

4. Discussion

The present report provides *in vivo* insight into the functional involvement of different Homer isoforms, and their interaction with mGluR5, in the development and maintenance of neuropathic pain, thereby extending previous indications of an important role for *Homer1* gene products in regulating pain sensitivity [36,37,40,57,71].

4.1. *Nerve injury co-regulates CC-Homers and associated receptors/kinases*

Nerve injury-induced increases in glutamate receptors [10,24,48,65], the activity of PKCε, PI3K/MAP kinases [66,69], and Homer1 expression [36,37,40] putatively contribute to the development of neuroplastic changes associated with chronic pain. Consistent with this, Homer1b/c, as well as its associated glutamate receptors (mGluR1a/5) and downstream kinases (PKCε, PI3K, ERK1/2), were time-dependently up-regulated within both spinal and supraspinal nociceptive structures after sciatic nerve ligation (Fig. 1 and 2), in a manner temporally correlated with the expression of fully developed mechanical and cold hypersensitivity (e.g., Fig. 3). However, injury-induced increases in PSD Homer2a/b expression were observed only within thalamus (Fig. 1C). This novel finding indicates that Homer2a/b levels, at least within thalamus, are responsive to nerve injury and suggests that glutamate sensitization within thalamus, specifically involving Homer2a/b, may be additionally implicated in mediating neuropathic pain. While the functional relevance of supraspinal changes in CC-Homer1/2 expression requires further investigation, thalamic co-regulation of both CC-isoforms implicates a shared role in pain perception. Thalamic and spinal nociceptive neurons sensitize to mechanical and cold stimuli during peripheral neuropathy [18,19,63], posing an exclusive and non-exclusive role for Homer1b/c in the regulation of injury-induced glutamate sensitization within, respectively, spinal dorsal horn and thalamus. This being proposed, Homer2b over-expression exacerbated neuropathic pain symptoms in a manner qualitatively and quantitatively similar to Homer1c over-expression (Fig. 3). Thus, despite their differential regional regulation following injury, CC-Homer1 and 2 isoforms appear to be interchangeable with respect to pain perception at least at the spinal cord level.

Whether or not the injury-induced increased activation of PKCε, PI3K and/or ERK1/2 throughout the nociceptive network requires intact interactions between Homers and glutamate receptors and whether or not neuropathy-induced elevations in kinase activity might contribute to injury-induced changes in receptor/Homer expression remains to be determined. Supporting these possibilities, the co-activation of Group1 mGluRs and NMDARs stimulates ERK1/2 activation in a Homer-dependent manner [70] and the capacity of Group1 mGluRs and NMDARs to increase intracellular calcium and to activate MAP kinase pathways are both prevented by Homer1a over-expression [57]. Moreover, blocking NMDARs or ERK1/2 activity prevents the transient rise and drop, respectively, in the spinal dorsal horn Homer1a and/or Homer1b/c levels early post tissue or nerve injury [36,40,57]. Proline-directed kinases, such as ERK1/2, phosphorylate mGluR1/5 within the Homer binding domain [44] (Park JM, Hu JH, Milshteyn A, Zhang PW, Moore CG, Park S, Datko MC, Domingo RC, Reyes CM, Wang XJ, Etzkorn FA, Xiao B, Szumlinski KK, Kern D, Linden DJ, Worley PF. A prolyl-isomerase mediates dopamine-dependent plasticity and cocaine motor sensitization. Cell (submitted for publication)) and this increases the avidity of the receptor for Homer [44,47]. Moreover, CC-Homer multimerization and binding to Group1 mGluRs occludes their respective PEST (proline, glutamate, serine, threonine) sequences and reduces their ubiquitin-mediated degradation [11,20,39]. Thus, we suggest that nerve injury-induced stimulation of PKCε and/or PI3K/MAPK signaling pathways (likely secondary to glutamate receptor stimulation) may induce transcription of *Homer* genes, instigating an abhorrent feed-forward process of enhanced protein production and protein-protein interactions, which enhances synaptic efficacy at all levels of the nociceptive network and exaggerates pain hypersensitivity following nerve injury.

4.2. *Distinct functional roles for CC-Homer and IEG-Homer proteins in neuropathic pain*

Indeed, mGluR/NMDAR antagonists [e.g. 1,8,10,34,45,48,60], spinal cord *Homer1* knock-down [36,71] and spinal cord Homer1a over-expression [57], all inhibit pain sensitivity in various pain models. Consistent with these observations, spinal cord CC-Homer over-expression exacerbated (Fig. 3), while Homer1a over-expression inhibited, nerve injury-induced pain hypersensitivity (Fig. 5D-G) and none of our manipulations

influenced basal pain threshold when assessed in the tail withdrawal test (Table 2). Thus, CC-Homer and IEG-Homer isoforms display divergent functional roles in the regulation of tissue or nerve injury-induced hypersensitivity.

The observation that mGluR5 Tg mice exhibited heightened neuropathic pain symptoms akin to those produced by *Homer1a* deletion (Fig. 5) is intriguing in light of recent evidence that these two mouse lines exhibit opposite inflammatory pain phenotypes, with the mGluR5 Tg mice exhibiting an exacerbation of symptoms consistent with *Homer2/Homer3* double-KO mice [22]. Such findings, coupled with the evidence herein that none of our manipulations influenced basal pain threshold, argue against a simple model of Homer-mGluR5 in pain perception. Further supporting this argument, neither *Homer1* nor *Homer2* deletion influenced pain hypersensitivity in neuropathic pain (Fig. 4), despite siRNA evidence that *Homer1* gene products within the spinal cord are required for the development of neuropathic pain hypersensitivity early post-injury [36]. Given our AAV-cDNA results (Fig. 3) and the fact that *Homer2/Homer3* double-KO mice exhibit an inflammatory pain phenotype [22], it seems that developmental compensation secondary to single *Homer* gene deletion or redundancy in Homer function may have masked or altered the neuropathic pain phenotype of the KO mice in this study. Alternatively, the lack of an effect of single *Homer* gene deletion in *Homer1* and *Homer2* KO mice might also relate to their differences in: (1) encoding of IEG isoforms [c.f. 50]; (2) affinity for Group1 mGluRs [25]; (3) effects upon NMDAR subunit expression and function [c.f. 55]; and/or (4) the ability to regulate extracellular glutamate levels within spinal cord, PFC [25,54,56], and perhaps within other nociception-related CNS structures. Regardless, our data for *Homer1a* KO mice, together with our collection of AAV-cDNA results, speak to distinct roles for different *Homer* gene products in regulating neuropathic pain and indicate that nerve injury-induced increases in CC-Homer expression are important, but not critical, for the development and maintenance of neuropathic pain.

The available *in vitro* literature indicates comparable effects of mGluR5^{F1128R} mutation and *Homer1a* over-expression upon receptor signaling [e.g. 2,21]. While constitutive mGluR5 activity, which is CC-Homer-independent [2], could contribute to the exaggerated neuropathic pain response in the mGluR5^{F1128R}

mutants, it is not is not likely to mediate pain hyper-sensitivity in *Homer1a* KO mice as Homer1a induction stimulates constitutive receptor activity [2], but is consistently anti-nociceptive [57; Fig. 5]. The similar behavioral phenotype of mGluR5 Tg and *Homer1a* KO mice (Fig. 5) may be explained in several ways. First, interactions between mGluR5/Homer1a rather than mGluR5/CC-Homers within spinal cord may critically mediate neuroplastic changes related to pain hypersensitivity. This further suggests that nerve injury-induced induction of IEG-Homer1a and its consequent binding to mGluR5 protects against and plays dominant roles in the development and maintenance of neuropathic pain. However, *Homer1* deletion prevents the induction of IEG *Homer1* gene products, yet *Homer1* KO mice (that lack both IEG and CC-Homer1 isoforms) exhibited no obvious neuropathic pain phenotype following nerve injury (Figure 4). Alternatively, given the wealth of evidence for NMDA receptors [e.g. 10,31,38,64] and mGluR1 [e.g. 1,8,34] in mediating pain, intact signaling through either of these receptors, or perhaps some other signaling pathway(s), may be sufficient to maintain nociception in the mGluR^{F1128R} mutants and could potentially be enhanced by virtue of the fact that CC-Homers are unable to bind to mGluR5 in the Tg mice. Third, other proteins with PDZ binding domains (e.g. NHERF-2 or Tamalin) [28,46] may be able to scaffold and maintain signaling through mGluR5, in the absence of Homer binding, and thus, mediate the pain phenotype of the Tg mice.

4.3. *Hypothetical mechanisms underlying Homer's role in chronic pain*

The mechanisms through which nerve injury-induced changes in CC-Homer expression exacerbate neuropathic pain are putatively related to CC-Homer's ability to regulate the signaling pathways activated upon Group1 mGluR and NMDA stimulation [e.g. 25-27,47,70]. CC-Homers link Group 1 mGluRs to intracellular calcium stores via scaffolding with the IP3 receptor and facilitate the integration of calcium signals elicited by Group1 mGluR and NMDA receptor stimulation by virtue of binding to the NMDA-associated scaffolding protein Shank [c.f. 65]. In contrast, the transient and rapid induction of IEG-Homer1a uncouples CC-Homers from NMDA receptor/Shank and Group1 mGluRs and facilitates mGluR interactions with ion channels [e.g. 26,27]. Nerve or tissue injury-induced Homer1a induction in the spinal cord appears

to influence NMDAR-dependent calcium entry and reduces the efficiency of Gαq signaling (including MAPK activation), as well as the number of synaptic contacts on spinal cord neurons that process pain input [36,40,57]. Moreover, CC-Homers, in particular Homer2 isoforms, normally occlude Group1 mGluR inhibition of certain voltage-gated calcium channels [e.g., 26] and Homer1a over-expression, presumably such as that induced by injury/pain [35-37,40], can remove this occlusion, facilitate mGluR-calcium channel interactions and inhibition of calcium entry/neuronal transduction [5,25,27,62]. Either, or both, of these mechanisms may be proposed to account for the bi-directional effect of manipulating Homer1a expression upon nociception. However, in the WT animal, the accumulation of Homer1a in the PSD of spinal cord following nerve injury is transient [36,40]. While an increase in Homer1a expression clearly protects against the behavioral signs of neuropathic pain (Fig. 5C), it may also permit enhanced PSD trafficking of both Group1 mGluRs and NMDA receptors, as well as CC-Homers and ultimately augment the cross-talk between these receptors within the PSD [68]. While we did not examine for injury-induced changes in the PSD localization of glutamate receptors and Homers within the nociception pathway in mGluR5^{F1128R} mutant animals, the exacerbation of pain produced by the F1128R mutation of mGluR5 (Fig. 5A) would suggest increased glutamate receptor cross-talk, which is a finding in line with recent electrophysiological evidence indicating that the mGluR5^{F1128R} mutation augments brain-derived neurotrophic factor-mediated activation of NMDA receptor currents in the absence of Homer1a induction (Park JM, Hu JH, Milshteyn A, Zhang PW, Moore CG, Park S, Datko MC, Domingo RC, Reyes CM, Wang XJ, Etzkorn FA, Xiao B, Szumlinski KK, Kern D, Linden DJ, Worley PF. A prolyl-isomerase mediates dopamine-dependent plasticity and cocaine motor sensitization. Cell (submitted for publication)). Thus, while not assayed directly in the present study, potential long-term Homer1a-mediated synaptic remodeling changes within PSD may paradoxically heighten signaling through glutamate receptor/CC-Homer complexes and eventually contribute to the development of neuropathic pain hypersensitivity.

In conclusion, here we showed that the dynamic but temporally distinct regulation of both IEG and CC-Homer isoforms contribute to long-lasting increases excitatory synaptic efficacy that underlies the

development and maintenance of neuropathic pain. Moreover, the induction of the IEG-Homer1a isoform may serve acutely to inhibit neuropathic pain hypersensitivity but facilitate synaptic rearrangement that promotes pain sensitivity in the long-term. Thus, time-dependent and selective targeting of Homer expression within the spinal cord may underlie the successful therapeutic intervention in chronic long-lasting pain.

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Figure legends:

Fig. 1 Chronic constriction injury (CCI) of the sciatic nerve produces time-dependent changes in CC-Homers and glutamate receptors expression mainly within the PSD (LP1; **A, C, E**), than within the P1 (nuclear and large debris) fraction (**B, D, F**) of the ipsilateral spinal dorsal horn (**A**-**B**), thalamus (**C**-**D**) and prefrontal cortex (**E**-**F**). Each graph represents summary of the change in protein expression obtained 1, 7 and 14 days after the CCI and expressed as a percentage of the average protein expression of the naïve controls. Data are presented as mean ± SEM, *n*=10-12 of B6 mice per each experimental group. The intensity of the bands for each antibody was normalized with the intensity of its appropriate calnexin signal. The asterisk (*) denotes significance vs. naïve controls; $^{*}p<0.05$ (one-way ANOVA, followed by Tukey's comparison post-hoc test). Representative immunoblots correspond to the total protein levels of Homer1b/c, Homer2a/b, mGluR1a, mGluR5, NR2a and NR2b.

Fig. 2 Chronic constriction injury (CCI) of the sciatic nerve elevates protein expression of the members of the PI3K/MAPK signaling pathway mainly 1 and/or 2 weeks after the injury within the PSD (LP1; **A, C, E**) and the P1 fraction (**B, D, F**) of the ipsilateral spinal dorsal horn (**A**-**B**), thalamus (**C**-**D**) and prefrontal cortex (**E**-**F**). Each graph represents summary of the change in protein expression obtained 1, 7 and 14 days after the CCI and expressed as a percentage of the average protein expression of the naïve controls. Data are presented as mean ± SEM, *n*=10-12 of B6 mice per each experimental group. The intensity of the bands for each antibody was normalized with the intensity of its appropriate calnexin signal. The asterisk (*) denotes significance vs. naïve controls; $^{*}p<0.05$ (one-way ANOVA, followed by Tukey's comparison post-hoc test). Representative immunoblots correspond to the total protein levels of PKCε, phospho- PKCε, PI3K, phospho(Tyr)p85α PI3K binding motif [p(Tyr)p85α], ERK1/2 and phospho-ERK1/2.

Fig. 3 AAV-mediated over-expression of CC-Homer isoforms potentiates neuropathic pain hypersensitivity. (**A**) Representative images of green fluorescent protein (hrGFP) epifluorescence (AAV-control vector) or immunostaining for the HA tag (AAV-Homer1c and –Homer2b cDNA vectors) using a specific antibody on

L4-L6 spinal dorsal horn sections (80 μm) at 7-8 weeks after AAV infusion. HA staining was conducted also on spinal cord tissue from AAV-control animals as a control for non-specific staining. Scale bar = 200 μm. (**B-E**) AAV-mediated overexpression of two CC-Homer isoforms, Homer1c (**B-C**) and Homer2b (D-E), within the spinal cord exacerbates the injury-induced mechanical (**B, D**) and cold (**C, E**) hypersensitivity in neuropathic pain. AAV-Homer1c and AAV-Homer2b were injected intrathecally in B6 mice. Mechanical sensitivity was assessed using von Frey filaments; cold sensitivity was determined by the acetone test. The measurements were assessed before chronic constriction injury (CCI) of the sciatic nerve and then every 2nd day for 2 weeks following the injury. The same control group was used in B and C, as well as in C and E. Data are presented as means ± S.E.M, n = 8-10 in each group. The asterisk (*) denotes significance vs. control; $^{*}p<0.05$ (two-way ANOVA, followed by Bonferroni's comparison post-hoc test).

Fig. 4 Deletion of *Homer1* and *Homer2* does not affect the development and maintenance of neuropathic pain. Each graph demonstrates mechanical (**A, C**) or cold (**B, D**) hypersensitivity induced by chronic constriction injury (CCI) of the sciatic nerve in *Homer1* and *Homer2* gene knock-out mice (KO), heterozygous (HET) and wildtype controls (WT). Mechanical sensitivity was assessed using von Frey filaments; cold sensitivity was determined by the acetone test. The measurements were assessed before CCI and then every 2nd day for 2 weeks following the injury. Data are presented as means ± S.E.M, n = 10-12 in each group. The asterisk (*) denotes significance vs. WT; $^{*}p<0.05$ (two-way ANOVA, followed by Bonferroni's comparison post-hoc test).

Fig. 5 Homer1a protects against mechanical and cold hypersensitivity following chronic constriction injury (CCI) of the sciatic nerve. (**A**) Representative images of immunostaining for the HA tag (AAV-Homer1a cDNA vectors) using a specific antibody on L4-L6 spinal dorsal horn sections (80 μm) at 7-8 weeks after AAV infusion. HA staining was conducted also on spinal cord tissue from AAV-control animals as a control for non-specific staining. Scale bar = 200 μm. (**B-G**) Each graph demonstrates mechanical and cold hypersensitivity induced by CCI in transgenic mice with disrupted binding of CC-Homer to mGluR5,

mGluR5^{F1128R} (**B-C**), in *Homer1a* gene knock-out mice (KO), heterozygous (HET) and their respective wildtype controls (WT) (**D-E**), and in B6 mice after intrathecal injection of AAVs carrying Homer1a (**F-G**). Mechanical sensitivity was assessed using von Frey filaments; cold sensitivity was determined by the acetone test. The measurements were assessed before CCI and then every 2nd day for 2 weeks following the injury. Data are presented as means ± S.E.M, n = 10-12 in each group. The asterisk (*) denotes significance vs. WT/control; $^{*}p<0.05$ (two-way ANOVA, followed by Bonferroni's comparison post-hoc test).

Table 1

Summary of the lack of statistically significant changes in the protein expression of members of the Homers-glutamate receptors-PI3K/MAPK kinases signalling pathway induced by chronic constriction injury of the sciatic nerve evaluated through immunoblotting within membrane (LP1) and nuclear (P1) fractions of the contralateral spinal dorsal horn 1 day, 1 week and 2 weeks following nerve injury. P(Tyr)p85α is abbreviation for phospho-(Tyr)p85αPI3K binding motif. The values were determined by one-way ANOVA followed by Tukey's comparison post-hoc test. Each group included 8–12 animals.

	LP1		P1	
Homer1b/c	$F_{(3,44)}=1.13$	P=0.34	$F_{(3,39)}=0.07$	P=0.98
Homer2a/b	$F_{(3,36)}=1.49$	P=0.23	$F_{(3,52)}=0.21$	P=0.89
mGluR1a	$F_{(3,46)}=0.62$	P=0.61	$F_{(3,50)}=0.57$	P=0.64
mGluR5	$F_{(3,48)}=0.34$	P=0.79	$F_{(3,56)}=0.89$	P=0.45
NR2a	$F_{(3,45)}=0.31$	P=0.82	$F_{(3,56)}=0.77$	P=0.51
NR2b	$F_{(3,41)}=0.18$	P=0.91	$F_{(3,50)}=0.54$	P=0.66
PKCε	$F_{(3,47)}=2.76$	P=0.06	$F_{(3,55)}=1.56$	P=0.21
pPKCε	$F_{(3,39)}=0.06$	P=0.98	$F_{(3,56)}=1.01$	P=0.39
Ratio pPKCε	$F_{(3,37)}=0.19$	P=0.89	$F_{(3,56)}=1.03$	P=0.39
PI3K	$F_{(3,49)}=0.45$	P=0.71	$F_{(3,50)}=0.75$	P=0.53
P(Tyr)p85α	$F_{(3,35)}=0.52$	P=0.67	$F_{(3,50)}=0.05$	P=0.98
ERK1/2	$F_{(3,51)}=0.46$	P=0.71	$F_{(3,51)}=0.99$	P=0.40
pERK1/2	$F_{(3,43)}=0.41$	P=0.75	$F_{(3,51)}=1.44$	P=0.24
Ratio pERK1/2	$F_{(3,44)}=0.33$	P=0.80	$F_{(3,51)}=0.90$	P=0.45

Table 2

Summary of the mean ± SEM of the basal pain threshold for control and AAV-treated mice as well as for wildtypes (WT), heterozygous (HET) and *Homer1*, *Homer2*, *Homer1a* gene knock-out (KO) mice and mGluR5^{F1128R} transgenic mice (Tg) with disrupted Homer-mGluR5 binding. The basal pain threshold was measured in the hot (54°C) water tail-immersion assay. Each group included 10-12 animals. The last column shows no statistically significant changes within groups as determined by one-way ANOVA followed by Bonferroni's comparison post-hoc test.

	control/WT	HET	AAV/KO/Tg	
AAV-Homer1c	2.63 ± 0.10	-	2.62 ± 0.19	$t_{(14)}$=0.06 P=0.95
AAV-Homer2b	2.63 ± 0.10	-	2.47 ± 0.10	$t_{(14)}$=1.10 P=0.29
AAV-Homer1a	2.63 ± 0.10	-	2.69 ± 0.23	$t_{(14)}$=0.23 P=0.82
Homer1	2.81 ± 0.14	2.80 ± 0.09	2.84 ± 0.06	$F_{(2,35)}$=0.03 P=0.97
Homer2	3.03 ± 0.12	3.24 ± 0.13	3.01 ± 0.08	$F_{(2,31)}$=1.16 P=0.33
mGluR5^{F1128R}	2.72 ± 0.11	2.76 ± 0.12	2.96 ± 0.17	$F_{(2,34)}$=0.87 P=0.43
Homer1a	2.90 ± 0.10	2.82 ± 0.12	2.84 ± 0.10	$F_{(2,26)}$=0.17 P=0.84

Figure 1

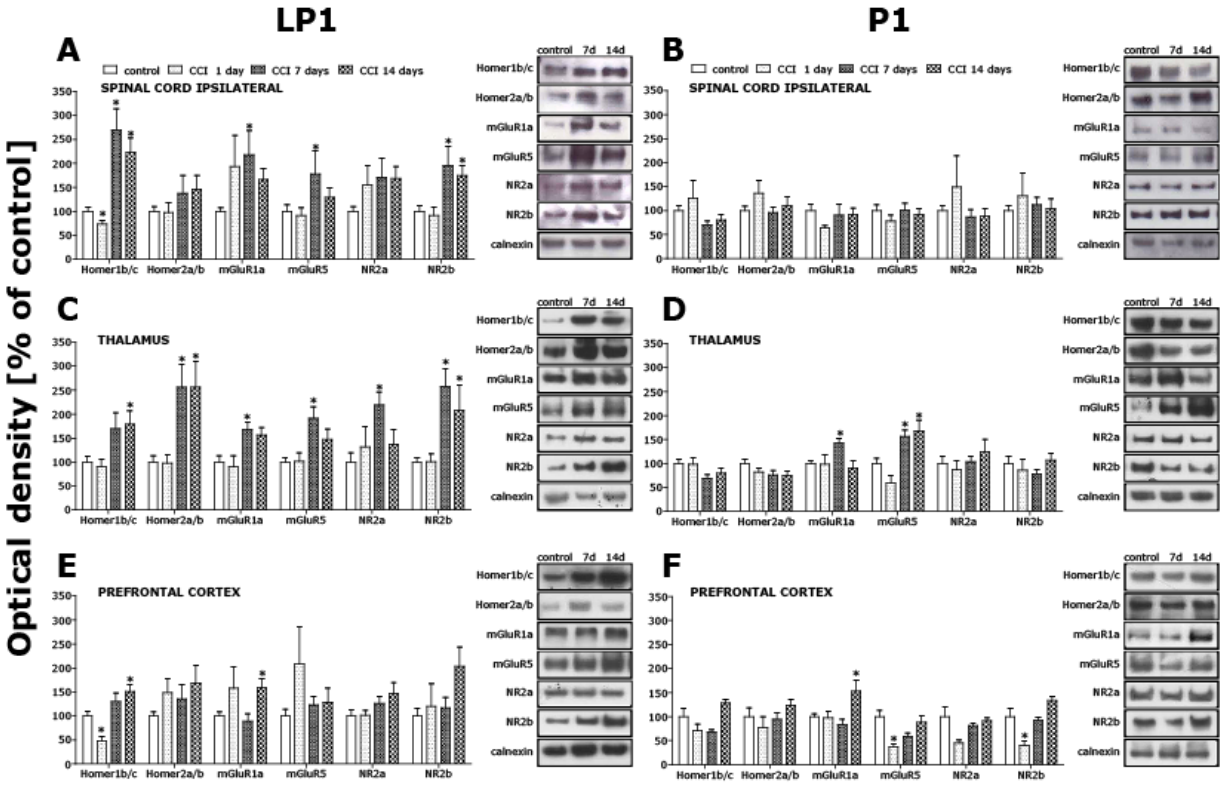

LP1
P1
A
B
C
D
E
F
control
CCI 1 day
CCI 7 days
CCI 14 days
SPINAL CORD IPSILATERAL
THALAMUS
PREFRONTAL CORTEX
Optical density [% of control]
Homer1b/c
Homer2a/b
mGluR1a
mGluR5
NR2a
NR2b
control 7d 14d
calnexin

Figure 2

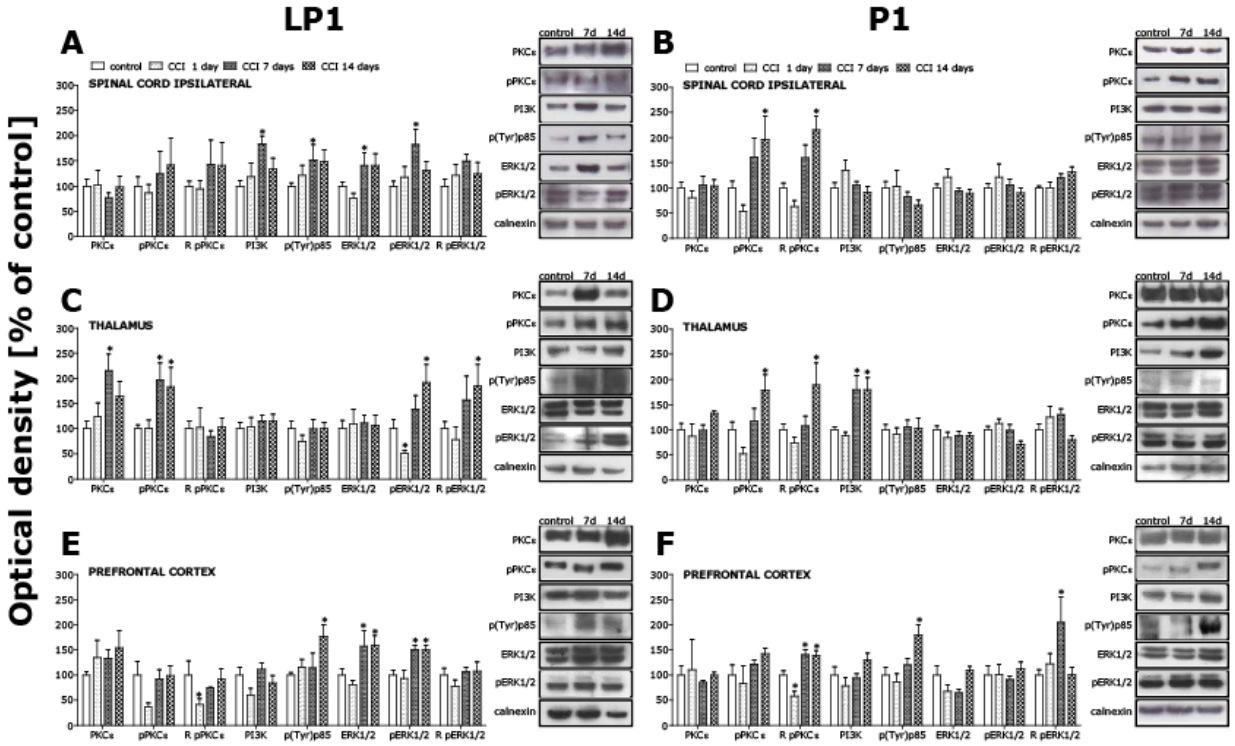

LP1
P1
A
B
C
D
E
F
control
CCI 1 day
CCI 7 days
CCI 14 days
SPINAL CORD IPSILATERAL
THALAMUS
PREFRONTAL CORTEX
Optical density [% of control]
PKCε
pPKCε
PI3K
p(Tyr)p85
ERK1/2
pERK1/2
calnexin
control 7d 14d
R pPKCε
R pERK1/2

Figure 3

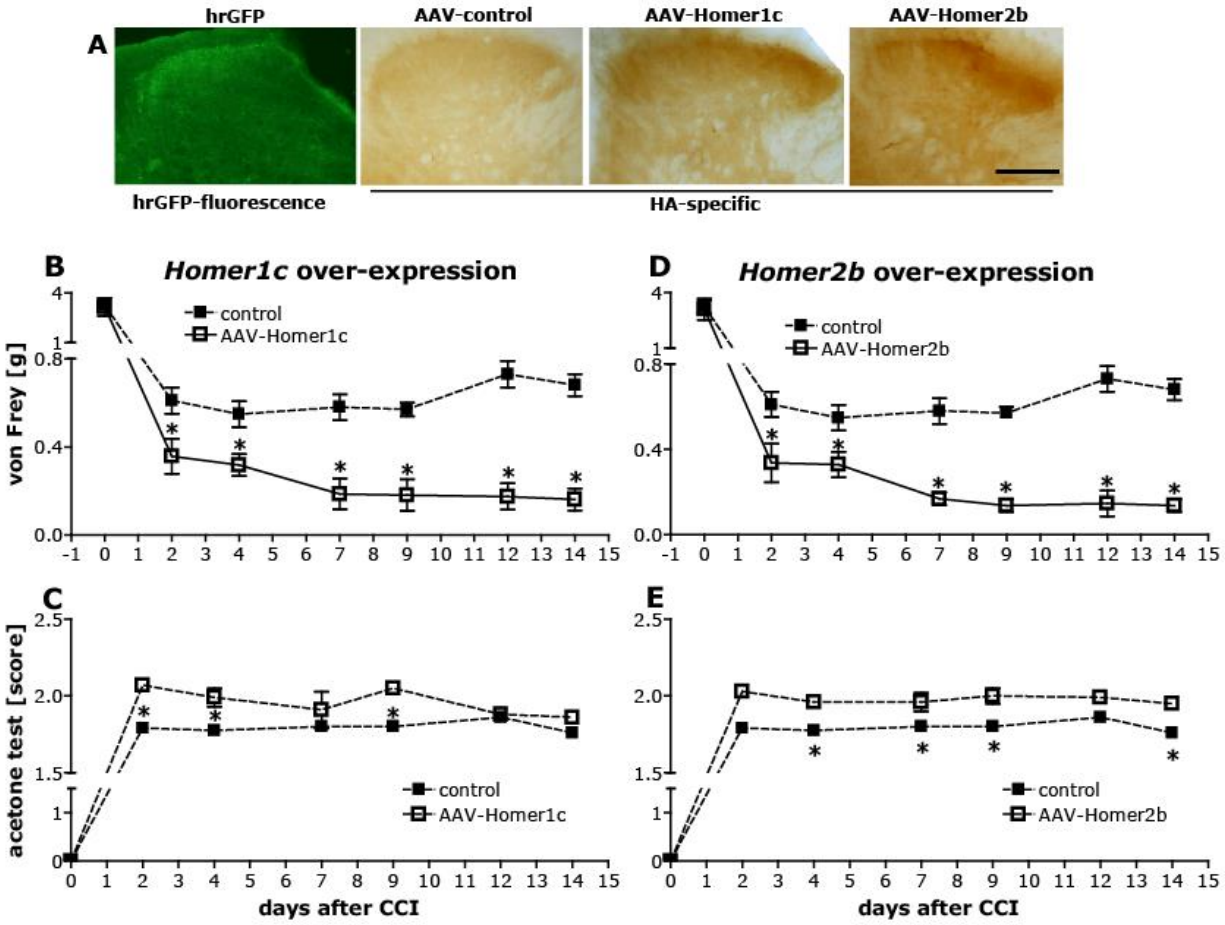

A
hrGFP
AAV-control
AAV-Homer1c
AAV-Homer2b
hrGFP-fluorescence
HA-specific
B
Homer1c over-expression
control
AAV-Homer1c
von Frey [g]
D
Homer2b over-expression
control
AAV-Homer2b
C
acetone test [score]
control
AAV-Homer1c
days after CCI
E
control
AAV-Homer2b
days after CCI

Figure 4

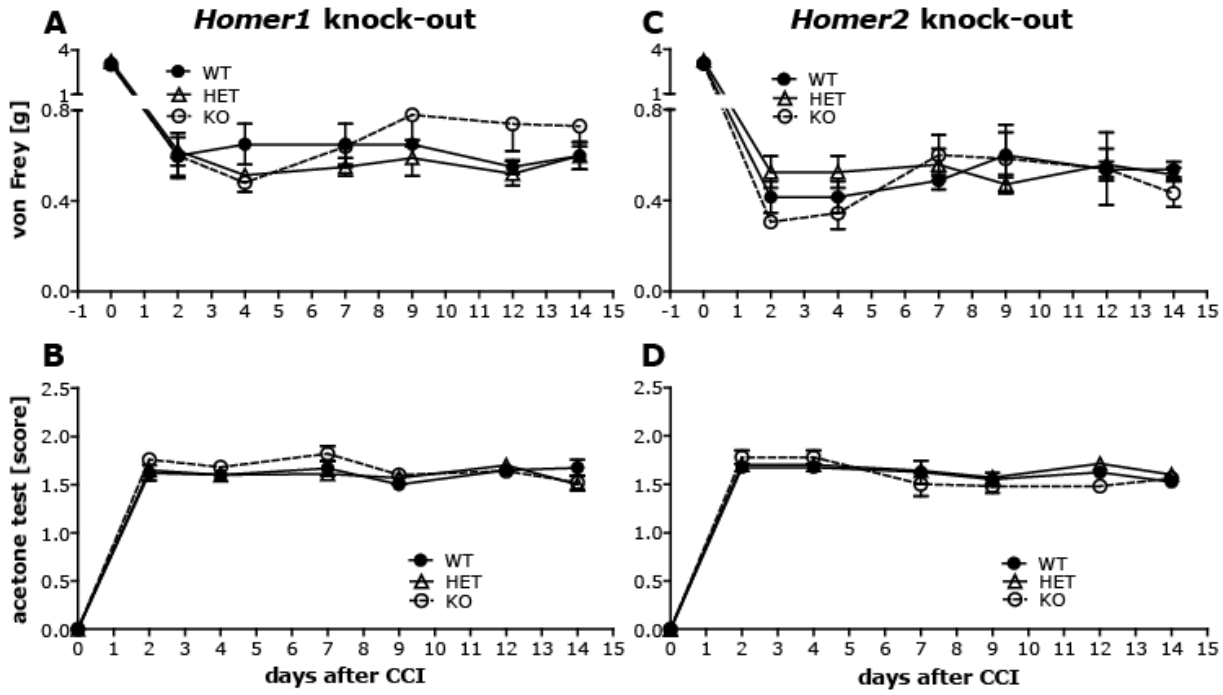
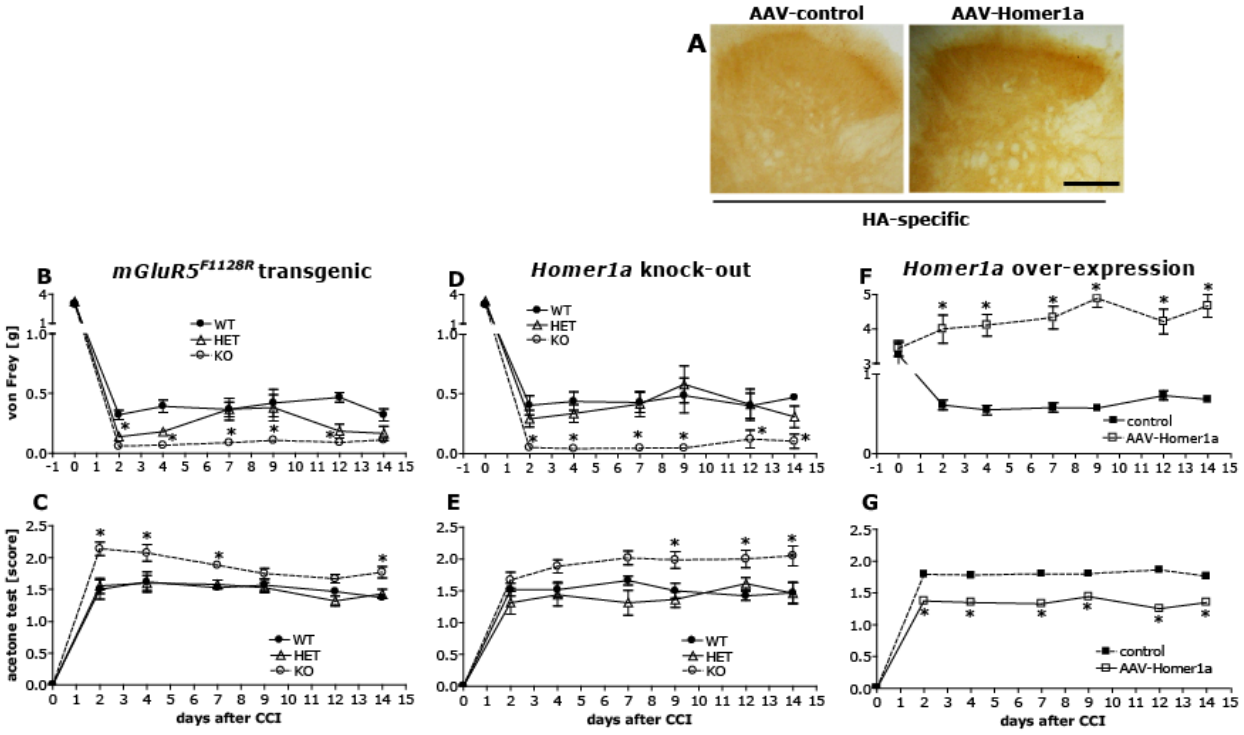

A
Homer1 knock-out
C
Homer2 knock-out
WT
HET
KO
von Frey [g]
B
D
acetone test [score]
days after CCI

Figure 5


A
AAV-control
AAV-Homer1a
HA-specific
B
mGluR5F1128R transgenic
D
Homer1a knock-out
F
Homer1a over-expression
WT
HET
KO
control
AAV-Homer1a
von Frey [g]
C
E
G
acetone test [score]
days after CCI