Homers at the interface between reward and pain

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INTRODUCTION

Comorbidity exists between chronic pain and motivational disturbances (e.g., Doth et al., 2010; Ohayon and Schatzberg, 2010; Jarcho et al., 2012; Oluigbo et al., 2012), and a cause-effect relationship between chronic pain and a blunted motivational state is apparent also in animal studies (c.f., Niikura et al., 2010). The pain processing pathway interacts at multiple levels with brain structures embedded within mesocorticolimbic subcircuits underpinning subjective responses to, as well as the incentive value of, stimuli (both appetitive or noxious), including subregions of the prefrontal cortex (PFC), nuclei of the amygdala (AMY), the ventral tegmental area (VTA), and subregions of the nucleus accumbens (NAC) (c.f., Leknes and Tracey, 2008; Becker et al., 2012). While the neurocircuity underpinning pain perception and the subjective pain response is known to involve activation within several fronto-cortical substrates and thalamus (c.f., Leknes and Tracey, 2008; Oluigbo et al., 2012), the precise neurocircuitry involved in pain-induced alterations in motivation are less well understood (Becker et al., 2012).

Patients’ hypersensitivity to pain stimuli correlates with increases in PFC-NAC connectivity in recent neuroimaging studies and, importantly, heightened connectivity is predictive of affective pain, as well as pain severity in humans (e.g., Baliki et al., 2010, 2012). In animal and human studies, noxious stimuli, including chronic constriction injury (CCI) of the sciatic nerve, alters pain-induced alterations in motivation are less well understood (Becker et al., 2012).

Keywords: Homer proteins, Group1 metabotropic glutamate receptors, NMDA receptors, neuropathic pain, heroin, nucleus accumbens, conditioned place-preference, conditioned place-aversion

Pain alters opioid reinforcement, presumably via neuroadaptations within ascending pain pathways interacting with the limbic system. Nerve injury increases expression of glutamate receptors and their associated Homer scaffolding proteins throughout the pain processing pathway. Homer proteins, and their associated glutamate receptors, regulate behavioral sensitivity to various addictive drugs. Thus, we investigated a potential role for Homers in the interactions between pain and drug reward in mice. Chronic constriction injury (CCI) of the sciatic nerve elevated Homer1b/c and/or Homer2a/b expression within all mesolimbic structures examined and for the most part, the Homer increases coincided with elevated mGluR5, GluN2A/B, and the activational state of various down-stream kinases. Behaviorally, CCI mice showed pain hypersensitivity and a conditioned place-aversion (CPA) at a low heroin dose that supported conditioned place-preference (CPP) in naïve controls. Null mutations of Homer1a, Homer1, and Homer2, as well as transgenic disruption of mGluR5-Homer interactions, either attenuated or completely blocked low-dose heroin CPP, and none of the CCI mutant strains exhibited heroin-induced CPA. However, heroin CPP did not depend upon full Homer1c expression within the nucleus accumbens (NAC), as CPP occurred in controls infused locally with small hairpin RNA-Homer1c, although intrathecal cDNA-Homer1c, -Homer1a, and -Homer2b infusions (to best mimic CCI’s effects) were sufficient to blunt heroin CPP in uninjured mice. However, arguing against a simple role for CCI-induced increases in either spinal or NAC Homer expression for heroin CPA, cDNA infusion of our various cDNA constructs either did not affect (intrathecal) or attenuated (NAC) heroin CPA. Together, these data implicate increases in glutamate receptor/Homer/kinase activity within limbic structures, perhaps outside the NAC, as possibly critical for switching the incentive motivational properties of heroin following nerve injury, which has relevance for opioid psychopharmacology in individuals suffering from neuropathic pain.
often observed in individuals suffering from chronic somatic pain (c.f., Leknes and Tracey, 2008; Becker et al., 2012; Oluigbo et al., 2012). In support of an interaction between a chronic pain state and drug reinforcement/reward, there is an absence of both opioid drug- and psychomotor stimulant-induced conditioned place-preference (CPP) in animal models of inflammatory or neuropathic pain (c.f., Niikura et al., 2010), which is consistent with very little evidence for the clinical diagnosis of addiction in individuals undergoing pharmacotherapy for chronic pain symptoms (e.g., Niikura et al., 2010; Minozzi et al., 2013). However, pain symptoms augment opioid drug consumption under operant procedures in animal models, which is theorized to reflect a compensation for a depressed mesocorticolimbic circuit (Colpaert et al., 1982, 2001; Dib and Duclaux, 1982; Lyness et al., 1989; Martin et al., 2007, 2011), fitting with extant CPP data indicating blunted drug-conditioned reward following nerve injury (c.f., Niikura et al., 2010).

Glutamate neuroadaptations within the mesocorticolimbic system are theorized to contribute significantly to drug reward/reinforcement in various addiction-related animal models (e.g., Szumlinski et al., 2008; Kalivas, 2009; Olive et al., 2012). As noxious, painful stimuli augment glutamatergic neurotransmission both at the spinal and supraspinal levels and glutamatergic hyperactivity is considered an active mediator of pain symptomatology (c.f., Chiechio and Nicoletti, 2012; Harris and Clauw, 2012; Wozniak et al., 2012; Osikowicz et al., 2013), the present study employed a combination of immunoblotting and behavioral genetic approaches to test the hypothesis that injury-induced increases in mesocorticolimbic glutamate transmission contribute to a blunted motivational state within the confines of a heroin CPP model of drug reward.

MATERIALS AND METHODS

SUBJECTS

Subjects included adult male C57BL/6J (B6) mice (8 weeks of age; 25–30 g; the Jackson Laboratories, Bar Harbor, ME, USA), as well as several strains of constitutive gene knock-out (KO) mice that were available at the time of study, including Homer1a KO (Hu et al., 2012), Homer1 KO (Yuan et al., 2003), and Homer2 KO (Shin et al., 2003) mice. Knock-in (KI) mice expressing mutant mGluR5 with a phenylalanine (F) to arginine (R) switch at position 1128 that were bred in-house at UCSB from mating of heterozygous breeder pairs (B6 × 129SvEv) background and male wild-type (WT), heterozygous (HET), and homozygous KO/KI littermate pups were employed in all studies. For the KO/KI strains bred in-house, mice were selected from a minimum of four different litters within each replicate and testing began at 7–8 weeks of age. Experimental protocols were approved by the IACUCs of our respective institutions and were consistent with the guidelines provided by NIH and the Committee for Research and Ethical Issues of IASP.

NEUROPATHIC PAIN, INFLAMMATORY PAIN, AND PAIN THRESHOLD ASSESSMENT

The procedures for inducing peripheral neuropathy by CCI of the sciatic nerve were identical to those described recently by our group (Obara et al., 2013). The total length of nerve affected was 3–4 mm. Mechanical and cold hypersensitivity at the plantar surface of the hind paw ipsilateral to the injury was assessed, respectively, using von Frey filaments (0.07–6 g; Stoelting, Wood Dale, IL, USA) and the acetone test (50 µl) before nerve injury (as one index of basal pain threshold), and on days 3, 7, and/or 14 post-CCI (e.g., Obara et al., 2003, 2013; Osikowicz et al., 2008).

IMMUNOBLOTTING

At 1 or 2 weeks after nerve injury, the entire NAC, the VTA, the entire AMY, and the PFC (anterior cingulate, prelimbic, and infralimbic cortices) were dissected from B6 mice (n = 6–8/group/time-point) over ice, homogenized in a buffer containing both protease and phosphatase inhibitors and subjected to conventional immunoblotting procedures (20 µg protein/lane) as described previously by our group (e.g., Goulding et al., 2011; Obara et al., 2013). The details regarding the antibodies employed to detect protein levels of Homer1b/c, Homer2a/b, mGluR1, mGluR5, GluN2A, GluN2B, PI3K, p(Tyr)PI3K p85α binding motif, ERK1/2, p(Thr202)/pErk1/2, PKCε, p(Ser729)PKCε, and calnexin (loading and transfer control) are provided in the legend for Figure 2. The data for neuropathic animals at the different time-points post-injury were expressed as a percent change from the mean signal of the uninjured controls for each individual membrane (n = 3–4/membrane) as published previously (e.g., Obara et al., 2013).

HEROIN-INDUCED PLACE-CONDITIONING

Mice were assayed for the development of heroin place-conditioning, starting at 14 days post-nerve injury. The apparatus and procedures for heroin place-conditioning were similar to those employed in our previous studies of drug-conditioned reward in mice (e.g., Penzner et al., 2008) and proceeded in the following four sequential phases: habituation, preconditioning test (Pre-Test), conditioning, postconditioning test (Post-test). All sessions were 15 min in duration and animals received no injections during the habituation, Pre-Test, or Post-Test sessions when they had free-access to both compartments of the apparatus. For conditioning, mice received four alternating pairings of distinct compartments with either intraperitoneal heroin (0.01–3 mg/kg; vol = 0.01 ml/kg) or an equivalent volume of saline in an unbiased fashion. Locomotor activity was monitored during all free-access sessions, as well as on the first and fourth saline/heroin conditioning session to index spontaneous and heroin-induced changes in ambulation, respectively. An increase in heroin-induced locomotion from injections 1–4 indicated the presence of locomotor sensitization. The time spent in the drug-paired vs. unpaired compartment on the Post-Test served to index place-conditioning. The dose-response study of B6 mice employed 8–9 mice/group/dose, while the sample sizes employed in the single-dose study of mutant mice were: 11–15 mice/group/genotype for Homer1a KO, 11–3 mice/group/genotype for Homer1 KO, 8–15 mice/group/genotype for Homer2 KO, and 12–18 mice/group/genotype for Grm5R/R mutant.

SURGICAL PROCEDURES AND AAV INFUSION

The procedure for generating neurotropic chimeric AAV1/2 vectors carrying the renilla green fluorescent protein (hrGFP) cDNA
or the hemagglutinin (HA) tag fused to the coding region of rat Homer1c and Homer2b have been described in detail elsewhere (e.g., Klugmann et al., 2005) and the AAV-cDNA constructs were identical to those employed previously (e.g., Klugmann et al., 2005; Tappe et al., 2006; Cozzoli et al., 2009; Goulding et al., 2011; Ary et al., 2013). The design of the AAV constructs for expression of small hairpin RNAs (shRNA) against Homer1c were described in detail in Klugmann and Szumilinski (2008). Briefly, we used a bicistronic expression cassette entailing the human U6 promoter to drive the shRNA, followed by the hrGFP reporter under the control of the chicken-beta actin (CBA) promoter for identification of transduced neurons. The shRNA-Homer1c construct was the same as that used in a recently published report, in which we demonstrated approximately 50% protein knock-down within the brain at 3 weeks post-infusion (Ary et al., 2013). AAV-shEGFP-CBA-hrGFP was used as a generic control (GFP) in our AAV studies. The surgical procedures for intra-NAC AAV infusion (0.5 µl/side) were identical to those used in previous studies (e.g., Cozzoli et al., 2009) and resulted in placement of microinjectors within the boundaries of the NAC (see Figure 5A). Studies examining behavioral response in heroin-induced place-preference test after intrathecal AAV infusion employed mice whose neuropathic pain symptoms and AAV transduction patterns within spinal cord were described before (Obara et al., 2013). Following either intracranial or intrathecal infusion, animals were left undisturbed for 3 weeks when AAV-mediated transgene expression peaks to remain at maximally stable levels prior to behavioral testing (e.g., Klugmann et al., 2005; Klugmann and Szumilinski, 2008). Sample sizes employed in the statistical analyses of the data ranged from 8 to 11 mice/group/AAV for both the NAC and spinal cord study.

**STANDARD ANALYSIS**

Behavioral and biochemical results are presented as means ± SEM (n = 8–12/group). Immunoblotting data were analyzed by one-way analyses of variance (ANOVA) with Tukey’s multiple comparison post hoc tests and these results are presented in Table 1. Behavioral results were analyzed by two-way ANOVA and significant interactions were followed up by an analysis for simple effects and Bonferroni’s multiple comparison post hoc tests, when appropriate. To confirm significant place-conditioning, a priori dependent-sample t-tests were conducted for the time spent in the heroin-paired vs. -unpaired compartment, separately for each treatment group/genotype. α = 0.05 for all analyses and the results of the statistical analyses for the behavioral assays are presented in their corresponding figure legends.

**RESULTS**

**CICI ELEVATES MESOCORTICOLIMBIC PROTEIN EXPRESSION AND ABOLISHES HEROIN CPP**

Chronic constriction injury of the sciatic nerve increased mechanical and cold hypersensitivity in B6 mice (Figures 1A,B). This hypersensitivity was associated with increased expression of the majority of our proteins of interest within all four mesocorticolimbic structures investigated (as indicated in Figure 1C), with regional distinctions in the magnitude and/or time-course of the observed protein changes (Figure 2; see Table 1). In the PFC (Figure 2A), CICI increased Homer1b/c, Homer2a/b, GluN2A, and p(Tyr)p85α at both time-points post-injury, while those for mGluR1a, GluN2B, and pPKCε were time-dependent. In the NAC (Figure 2B), CICI increased Homer1b/c, GluN2A, and pPKCε at both time-points, while kinase activation increased time-dependently and the rise in mGluR5 was transient. In the AMY (Figure 2C), the rise in mGluR5 was also transient; however, CICI increased Homer1b/c, GluN2B, PKCs, pPKCε, and p(Tyr)p85α both time-points and ERK levels increased time-dependently. Unfortunately, we could not detect a reliable signal for mGluR1α within our AMY samples. Finally, in the VTA (Figure 2D), CICI increased Homer2a/b, and GluN2A at both time-points, the rise in GluN2B, pPKCε, and the pPKCε:PKCε ratio increased time-dependently and the rise in p(Tyr)p85α and ERK were transient.

We next assayed for CICI-induced changes in heroin-conditioned reward in B6 mice as an index of motivation. All but the lowest heroin dose elicited a significant CPP in injury-naive B6 controls (Figure 3). In contrast, no heroin dose elicited CPP in injured B6 mice and the 0.1-mg/kg dose elicited a significant conditioned place-aversion (CPA). The injury-induced abolishment of CPP did not reflect impairments in motor activity as group differences were not observed regarding: (1) spontaneous locomotor activity (data not shown; total distance traveled during Habituation, Pre-Test, or Post-Test; t-tests, p.csv 0.05); (2) saline- or heroin-induced locomotor activity on injection 1 or 4; or (3) the expression of heroin-induced motor sensitization, which was observed only at the 3-mg/kg dose [data not shown; Heroin effect: F (2,48) = 25.76, p = 0.001; Heroin × Injection: F (2,48) = 2.87, p = 0.07].

**GENOTYPE × PAIN INTERACTIONS IN HEROIN CPP**

Given the CICI-induced rise in Homer expression throughout the mesocorticolimbic system, we next assayed for low-dose heroin-induced place-conditioning in naïve and CICI Homer1a, Homer1, and Homer2 null mutant mice, as well as in transgenic mice with a disrupted mGluR5-Homer interaction (Grm5R/Homer). The 0.1-mg/kg heroin dose elicited a significant CPP in injury-naive WT mice from all strains and this CPP was absent in all homozygous mutant littermate animals (Figure 4, left). Consistent with the above data from B6 mice, the 0.1-mg/kg heroin dose elicited a significant CPA in all CICI WT mice, but this too was attenuated or prevented in all homozygous mutant mouse lines (Figure 4, right). Such data pose a necessary role for Homer1a induction, as well as scaffolding by constitutively expressed (coiled-coil) CC-Homer proteins and their interaction with mGluR5 as critical for both heroin-related appetitive and aversive learning.

**AAV-MEDIATED HOMER GENE TRANSFER AND INJURY-INDUCED CPA**

The pattern of AAV-mediated neuronal transduction within the NAC was consistent with that reported previously by our group (e.g., Cozzoli et al., 2009; Goulding et al., 2011), with little spread beyond the infusion site (Figures 5A,A’). Intra-NAC cDNA-Homer1c and shRNA-Homer1c infusion potentiated and inhibited, respectively, both mechanical and cold hypersensitivity following CICI, but the effect was more pronounced in the von Frey test (Figure 5B). Neither Homer manipulation influenced basal
Table 1 | Statistical results of the one-way ANOVAs conducted on the immunoblotting data \((a = 0.05)\) and follow-up Tukey's multiple comparison post hoc tests, where appropriate.

| Region | Protein          | ANOVA        | Post hoc                      |
|--------|------------------|--------------|-------------------------------|
|        |                  | \(F_{(2,23)}\) | \(p\)                         |
| PFC    | mGluR1a          | 10.53, \(p = 0.0007\) | CCI 2 weeks > CNT = CCI 1 week |
|        | mGluR5           | 3.80, \(p = 0.04\)     |                               |
|        | GluN2A           | 6.47, \(p = 0.0007\)   | CCI 1 week = CCI 2 weeks > CNT |
|        | GluN2B           | 10.39, \(p = 0.0007\)  | CCI 2 weeks > CNT = CCI 1 week |
|        | Homer1b/c        | 13.67, \(p = 0.0002\)  | CCI 1 week = CCI 2 weeks > CNT |
|        | Homer2a/b        | 12.51, \(p = 0.0005\)  | CCI 1 week = CCI 2 weeks > CNT |
|        | pPKC             | 0.32, \(p = 0.73\)     |                               |
|        | pPKC:pPKC ratio  | 2.06, \(p = 0.16\)     |                               |
|        | PI3K             | 0.37, \(p = 0.69\)     |                               |
|        | pERK             | 0.06, \(p = 0.94\)     |                               |
|        | pERK:pERK ratio  | 0.01, \(p = 0.98\)     |                               |
| NAC    | mGluR1a          | 1.45, \(p = 0.26\)     |                               |
|        | mGluR5           | 6.97, \(p = 0.0007\)   | CCI 1 week = CCI 2 weeks      |
|        | GluN2A           | 10.52, \(p = 0.001\)   | CCI 1 week = CCI 2 weeks      |
|        | GluN2B           | 1.62, \(p = 0.23\)     |                               |
|        | Homer1b/c        | 7.96, \(p = 0.004\)    | CCI 1 week = CCI 2 weeks > CNT|
|        | Homer2a/b        | 0.24, \(p = 0.78\)     |                               |
|        | pPKC             | 1.71, \(p = 0.21\)     |                               |
|        | pPKC:pPKC ratio  | 7.28, \(p = 0.006\)    | CCI 1 week = CCI 2 weeks > CNT|
|        | PI3K             | 0.29, \(p = 0.74\)     | CCI 1 week = CCI 2 weeks > CNT|
|        | pERK             | 0.42, \(p = 0.67\)     |                               |
|        | pERK:pERK ratio  | 7.68, \(p = 0.005\)    | CCI 2 weeks > CNT = CCI 1 week|
| AMY    | mGluR1a          | –             |                               |
|        | mGluR5           | 12.59, \(p = 0.0006\)  | CCI 1 week = CCI 2 weeks      |
|        | GluN2A           | 1.26, \(p = 0.31\)     |                               |
|        | GluN2B           | 13.16, \(p = 0.0005\)  | CCI 1 week = CCI 2 weeks > CNT|
|        | Homer1b/c        | 14.41, \(p = 0.0003\)  | CCI 1 week = CCI 2 weeks > CNT|
|        | Homer2a/b        | 0.15, \(p = 0.86\)     |                               |
|        | PKC              | 12.65, \(p = 0.0006\)  | CCI 1 week = CCI 2 weeks > CNT|
|        | pPKC             | 16.43, \(p = 0.0002\)  | CCI 1 week = CCI 2 weeks > CNT|
|        | pPKC:pPKC ratio  | 0.38, \(p = 0.68\)     |                               |
|        | PI3K             | 0.45, \(p = 0.64\)     |                               |
|        | pERK             | 0.65, \(p = 0.54\)     |                               |
|        | pERK:pERK ratio  | 1.69, \(p = 0.21\)     |                               |
| VTA    | mGluR1a          | 3.63, \(p = 0.05\)     | CCI 1 week = CCI 2 weeks > CNT|
|        | mGluR5           | 0.42, \(p = 0.66\)     | CCI 1 week = CCI 2 weeks > CNT|
|        | GluN2A           | 5.08, \(p = 0.02\)     | CCI 1 week = CCI 2 weeks > CNT|
|        | GluN2B           | 6.19, \(p = 0.01\)     | CCI 2 weeks > CNT = CCI 1 week|
|        | Homer1b/c        | 1.68, \(p = 0.22\)     |                               |
|        | Homer2a/b        | 6.99, \(p = 0.007\)    |                               |
|        | PKC              | 0.17, \(p = 0.84\)     |                               |

(Continued)
The data are summarized in Figure 2 and sample sizes ranged from 6 to 8 mice/group.

Figure 1: Chronic constriction injury of the sciatic nerve results in mechanical and cold hypersensitivity in B6 mice. When assessed at 3, 7, and 14 days post-injury, B6 mice exhibited mechanical hypersensitivity as assessed in the von Frey test (A) [CCI × Time: \(F_{(3,56)} = 9.07, p < 0.0001\)] and cold hypersensitivity as measured in the acetone test (B) [CCI × Time: \(F_{(3,56)} = 10.77, p < 0.0001\)]. The data represent the mean ± SEM of 6–8 mice/group. *p < 0.05 vs. naïve (control) mice (Bonferroni’s post hoc tests).

Figure 5: Pain threshold to mechanical and cold stimuli (Figure 5B) nor did they alter simple spinal pain reflex assessed in the tail-flick test (Figure 5C).

While intra-NAC shRNA-Homer1c did not influence heroin CPP in injury-naïve animals, it prevented injury-induced heroin CPA (Figure 5D, left). In contrast to shRNA-Homer1c infusion, intra-NAC cDNA-Homer1c infusion prevented heroin-induced place-conditioning in both naïve and injured groups (Figure 5D, right).

Intrathecal infusion of cDNA-Homer1c and -Homer2b potentiates, while that of cDNA-Homer1a attenuates, CCI-induced pain hypersensitivity (Obara et al., 2013). Thus, we determined whether or not spinal Homer expression might also regulate heroin place-conditioning. Intrathecal infusion of all three AAV-cDNAs blunted heroin CPP in injury-naïve mice (Figure 6, left). In this study, the heroin CPA exhibited by GFP-infused CCI mice was not as robust as that observed in the experiments above; nevertheless, none of the AAV-cDNAs influenced the extent or direction of behavior exhibited by CCI animals (Figure 6, right).

**DISCUSSION**

Pain-associated affective and motivational blunting is hypothesized to involve injury-induced changes in mesocorticolimbic...
FIGURE 2 | Chronic constriction injury of the sciatic nerve augments glutamate-related protein expression throughout the mesocorticolimbic circuit. Summary of the changes in protein expression observed within the PFC (A), NAC (B), AMY (C), and VTA (D) in sciatic nerve-ligated mice (CCI) sacrificed at 1 or 2 weeks following injury, as well as in naïve controls. 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, Lake Placid, NY, USA; 1:1000 dilution), anti-GluN2A and anti-GluN2A (Calbiochem, San Diego, CA, USA; 1:1000 dilution), anti-PI3K antibody (Upstate; 1:1000 dilution), and anti-p-(Tyr) PI3K p85α binding motif (Cell Signaling Technology, Beverly, MA, USA; 1:250 dilution), anti-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500 dilution), anti-PKCε and anti-p(Ser729)PKCε (Santa Cruz Biotechnology; 1:1000 dilution), Anti-mGluR1a (Upstate; 1:1000 dilution) and anti-p(Tyr204)ERK1/2 (Santa Cruz Biotechnology; 1:1000 dilution) mouse polyclonal antibodies were also used. A rabbit anti-calnexin monoclonal antibody (Stressgen, Victoria, BC, Canada; 1:1000 dilution) was used as a loading and transfer control. Immunoreactive bands were detected using enhanced chemiluminescence and immunoreactivity quantified using Image J (NIH, Bethesda, MD, USA). The data for neuropathic animals at the different time-points post-CCI were expressed as a percent change from the mean signal of the uninjured controls for each individual membrane (n = 3–4/membrane). The data represent the mean ± SEM of 6–8 mice/group and detailed results of the statistical analyses of these data are presented in Table 1. *p < 0.05 vs. naïve (control) mice (see Table 1; one-way ANOVA followed by Tukey’s post hoc tests).

function (c.f., Leknes and Tracey, 2008; Becker et al., 2012; Oluigbo et al., 2012). Thus, the present study characterized CCI-induced changes glutamate receptor expression/signaling within four major components of the mesocorticolimbic system and then assayed the functional relevance of mGluR5 interactions with its scaffolding molecule Homer (Shiraishi-Yamaguchi and Furushichi, 2007) for pain-elicited changes in heroin’s incentive motivational properties.
NEUROPATHY AUGMENTS INDICES OF MESOCORTICOLIMBIC GLUTAMATE TRANSMISSION

Chronic constriction injury-induced hypersensitivity was associated with up-regulated mesocorticolimbic glutamate receptor and CC–Homer expression, as well as increased indices of ERK, PKCα, and/or PKCs activity. The present PFC replicate our earlier study (Obara et al., 2013), indicating that injury up-regulates glutamate receptor signaling within a forebrain region important for volitional control over behavior, cognition, and emotion (c.f., Arnsten and Rubia, 2012; Depue, 2012). CCI-induced increases in protein expression were observed also within VTA, NAC, and AMY, with some regional differences that are not to be unexpected. However, CCI elevated Homer1b/c levels and PI3K activation in all mesocorticolimbic regions examined. Homer proteins are involved in the recruitment of PI3K-enhancer to Group1 mGluRs to induce PI3K activity (Rong et al., 2003). PI3K induction, at least within spinal cord, contributes to the neuroprotective pain hypersensitivity (Xu et al., 2011). As an intra-NAC infusion of cDNA-Homer1c was sufficient to promote CCI-induced pain hypersensitivity, injury-induced increases in mesocorticolimbic Homer-dependent PI3K activity may contribute significantly to somatic and affective pain chronification following peripheral nerve injury. Indeed, certain AMY subregions receive direct and indirect nociceptive input from spinal cord, brainstem, thalamus, and cortex (c.f., Leknes and Tracey, 2008; Becker et al., 2012). Moreover, central sensitization, via signaling pathways involving ERK, PKCs, and PI3K, occurs within this structure in various models of chronic pain (c.f., Neugebauer et al., 2004; Neugebauer, 2006; Fu et al., 2008; Tappe-Theodor et al., 2011). Our observation of up-regulated protein expression within AMY could reflect a central sensitization of mesocorticolimbic activity that would be predicted to elicit negative emotional disturbances characteristic of chronic pain sufferers.

While we failed to detect a significant reduction in VTA ERK activity following CCI, previous studies indicated reduced VTA ERK activation and c-fos expression following injury, which was interpreted to reflect blunted VTA responsiveness and theorized to contribute to pain-induced amotivational states (e.g., Narita et al., 2003, 2004; Ozaki et al., 2004). However, CCI elevated our other indices of signaling within VTA, most notably GluN2 subunits, Homer2a/b, activated PKCs, and PI3K, which would be predicted to elevate, rather than depress, basal activity of mesolimbic dopamine neurons to heighten the saliency of both conditioned and unconditioned pain cues (Berridge, 2007; Bromberg-Martin et al., 2010). Indeed, these present immunoblotting results are consistent with human neuroimaging data indicating correlations between heightened PFC-NAC connectivity and pain chronification (Baliki et al., 2010, 2012). Thus, injury-induced plasticity within corticofugal glutamatergic and mesocorticolimbic dopaminergic projections might heighten PFC-NAC connectivity predictive of somatic and affective pain chronification. In support of this notion, NAC Homer1c expression bi-directionally altered CCI-induced pain symptoms, with increased Homer1c promoting noceception in CCI mice (see below).

HEROIN CPP AND HOMER-mGluR5 INTERACTIONS

In all experiments, repeated low-dose (0.1 mg/kg) heroin consistently supported CPP in injury-naive WT mice. Remarkably, this low-dose heroin CPP was attenuated or absent in injury-naive mice from all four mutant strains. Opioids and their withdrawal alter Homer1 gene products within the PFC and AMY (Ammon et al., 2003; Kuntz et al., 2008) and recently, polymorphisms in Homer1, as well as changes in striatal and AMY Homer1 mRNA expression, were reported in post-mortem studies of heroin addicts (Okvist et al., 2011; Jacobs et al., 2012). While constitutive Homer2 deletion does not impact heroin-induced locomotor activity (Szymlinski et al., 2004), to the best of our knowledge, these data are the first to describe the heroin reward phenotype produced by constitutive deletion of different Homer genes or transgenic disruption of mGluR5-Homer interactions. That null mutations of Homer1a and Homer1 (the latter of which eliminates both inducible and CC Homer1 isoforms; see Yuan et al., 2003) produced a more pronounced effect upon conditioning than Homer2 deletion argues a more critical role for Homer1 gene products, particularly Homer1a, in this form of heroin-related learning. Moreover, the fact that Grm5gR1 mice not only failed to exhibit heroin CPP, but tended toward CPA, argues further that the interaction between Homer1 gene products and mGluR5 is fundamental to the motivational valence of low-dose heroin, which is worthy...
Summary of the difference in the time spent in the heroin-paired and -unpaired compartments (CPP) following conditioning with 0.1 mg/kg heroin in mice with constitutive deletion of Homer1a, Homer1, or Homer2 and in mice expressing the Grm5<sup>R/R</sup> transgene. Analysis of the data from all of the mutant animals revealed significant Genotype × CCI interactions [Homer1a: F<sub>G×C</sub> = 3.50, p = 0.04; Homer1: F<sub>G×C</sub> = 6.10, p = 0.004; Homer2: F<sub>G×C</sub> = 4.14, p = 0.02; Grm5<sup>R/R</sup>: F<sub>G×C</sub> = 6.71, p = 0.002]. (A) In uninjured mice from the Homer1a study, a priori t-tests (time on paired vs. unpaired side) confirmed significant CPP in Homer1a-WT mice [t<sub>10</sub> = 6.43, p < 0.0001; n = 11], but no place-conditioning was evident in their HET or KO counterparts (t-tests, p's > 0.50; n = 13–15). In CCI mice from the Homer1a study, CPP was apparent in WT controls (t<sub>12</sub> = 2.81, p = 0.02; n = 11), but again no conditioning was apparent in their HET or KO counterparts (t-tests, p > 0.65; n = 8–12). (B) As observed in the Homer1a study, heroin elicited CPP and CPA, respectively, in uninjured and CCI Homer1 WT mice [naïve: t<sub>10</sub> = 6.12, p < 0.0001; CCI: t<sub>8</sub> = 2.34, p = 0.04], while no significant place-conditioning was apparent under either condition in HET or KO mice (n = 11–13; t-tests, p > 0.12). (C) Homer1a deleted CPP and CPA, respectively, in uninjured and CCI Homer2 WT mice [naïve: t<sub>11</sub> = 3.18, p = 0.01; CCI: t<sub>10</sub> = 2.70, p = 0.03]. No place-conditioning was apparent in HET mice under either condition (n = 15; t-tests, p > 0.90). While uninjured Homer2 KO mice did not exhibit CPP (n = 11; t-test, p = 0.15), CPP not CPA, was apparent in their CCI counterparts [t<sub>5</sub> = 3.27, p = 0.01]. (D) Heroin elicited also CPP and CPA, respectively, in naïve and CCI mice Grm5<sup>R/R</sup> (i.e., WT) [naïve: t<sub>7</sub> = 4.50, p < 0.0001; CCI: t<sub>10</sub> = 3.00, p = 0.08], while no significant place-conditioning was apparent in Grm5<sup>R/R</sup> or Grm5<sup>R/K</sup> mutants (n = 12–18; t-tests, p > 0.20). *p < 0.05 Paired vs. unpaired (i.e., conditioning; t-tests); +p < 0.05 vs. WT control (Tukey's post hoc tests).

Interestingly, Homer1a deletion abolished low-dose heroin CPP, while intra-NAC shRNA-Homer1c infusion had absolutely no effect. These data indicate either that: (1) the neural locus mediating the CPP effect of Homer1 deletion resides outside the NAC or (2) the CPP effect of Homer1 deletion reflect an absence of inducible, rather than constitutively expressed, Homer1 gene products. As the effects of Homer1a deletion mirrored those of Homer1 deletion argues in favor of the latter possibility. However, based on suggestions of regional differences in heroin-induced changes in Homer1 mRNA within PFC, AMY, and dorsal striatum (Kuntsz et al., 2008; Okvist et al., 2011; Jacobs et al., 2012), Homer1 gene products in these other addiction-relevant brain regions may contribute more so to the conditioned incentive motivational properties of low-dose heroin. It is interesting to note, however, that intra-NAC cDNA-Homer1c, as well as intrathecal cDNA-Homer1a, -Homer1c, and -Homer2b infusion, in injury-naïve mice was sufficient to block heroin CPP. The result for the NAC may be counterintuitive based on the findings from the KO studies, but, as argued below, may reflect a facilitation of low-dose heroin hyperalgesia that renders the heroin experience more aversive.

INJURY-INDUCED HEROIN CPA ALSO REQUIRES INTACT mGluRs-HOMER INTERACTIONS

Most notable and distinct from the results of earlier CPP studies in injured animals (c.f., Niikura et al., 2010), neuropathic B6 mice exhibited CPA in response to 0.1 mg/kg heroin – a dose of heroin that supported CPP in uninjured animals. In WT mice, CCI clearly augmented pain symptoms prior to heroin conditioning (see also Obara et al., 2013), supporting a causal
Homer1c in the NAC bi-directionally influences neuropathic pain symptoms and heroin CPA. (A) Half coronal section of the mouse brain at the level of the NAC targeted in the AAV infusion studies. (A’) Micrograph (4×) of GFP staining within the NAC shell produced by the shRNA-Homer1c construct [see box in (A) for orientation]. (A”) Micrograph (20×) of immunostaining for the HA-tagged cDNA-Homer1c construct within NAC shell illustrating both cell body and process staining in the tissue surrounding the microinjector tip (bracket). At 3 weeks following intra-NAC infusion, mice were subjected to CCI procedures, followed by behavioral testing. (B) Relative to GFP vector controls (GFP), altering NAC Homer1c expression bi-directionally altered both mechanical hypersensitivity assessed in the von Frey test [AAV × Time ANOVA: F(2,130) = 150.6, p < 0.0001] and cold hypersensitivity assessed in the acetone test [AAV × Time ANOVA: F(2,130) = 93.27, p < 0.0001]. *p < 0.05 vs. GFP.

(C) No changes in the tail-flick test were observed following intra-NAC AAV infusion (one-way ANOVA, p = 0.38). (D) An AAV × CCI interaction was observed for heroin-induced place-conditioning [F(2,52) = 3.79, p = 0.03]. In GFP controls, cDNA-Homer1c over-expression prevented heroin CPP, while shRNA-Homer1c was without effect [F(2,28) = 3.36, p = 0.04; Tukey’s post hoc tests]. T-tests confirmed the presence of a significant CPP in GFP controls [t(9) = 4.20, p = 0.002] and shRNA-infused control animals [t(9) = 3.49, p = 0.007]. In contrast, both Homer1c manipulations attenuated heroin CPA in CCI mice [F(2,24) = 7.37, p = 0.003; Tukey’s post hoc tests]. T-tests confirmed a significant CPA in scrambled controls [t(9) = 3.49, p = 0.007], but no conditioning in Homer1c-manipulated animals (t-tests, p > 0.05). *p < 0.05 Paired vs. unpaired (conditioning; t-tests); #p < 0.05 vs. scrambled AAV. The data represent the mean ± SEM of 8–11 mice/AAV/condition.

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relation between pain symptomatology and low-dose heroin aversion. In further support of a direct cause-effect relation between pain and heroin aversion, cDNA-Homer1 infusion into either the NAC or spinal cord augments pain hypersensitivity and abolishes heroin CPP in injury-naive animals. Furthermore, intra-NAC shRNA-Homer1c infusion, a manipulation that reduced pain hypersensitivity following CCI, prevented subsequent heroin CPA. However, neither intra-NAC nor intrathecal cDNA-Homer1c infusion potentiated heroin CPA in CCI animals. In fact, NAC cDNA-Homer1c transduction in CCI mice attenuated heroin CPA,
although the magnitude of place-conditioning was not statistically different from GFP-infused CCI controls. These data, coupled with the lack of any significant cDNA effect in our spinal cord study (where weak CPA was observed in CCI mice) argue against a ceiling effect limiting the expression of CCI-induced CPA. Arguably, however, the fact that the effects of cDNA-Homer infusion upon heroin place-conditioning were not additive with those produced by CCI alone might be interpreted to reflect mechanistic interdependency, an interpretation that would be consistent with the notion that CCI-induced increases in glutamate receptor/Homer expression are neuroadaptations that promote dysphoric states.

Indeed, the present data from the studies of transgenic mice support this possibility as no evidence for CCI-induced heroin CPA was apparent in any mutant strain; in fact, both support this possibility as no evidence for CCI-induced heroin place-conditioning were not additive with those produced by CCI alone might be interpreted to reflect mechanistic interdependency, an interpretation that would be consistent with the notion that CCI-induced increases in glutamate receptor/Homer expression are neuroadaptations that promote dysphoric states.

| Gene manipulation | CCI pain symptoms | Heroin CPP | CCI-induced heroin CPA |
|-------------------|------------------|------------|------------------------|
| CONSTITUTIVE GENE MUTATION | | | |
| Homer1a KO | ↑ | ↓ | ↓ |
| Homer1 KO | No effect | ↓ | ↓ |
| Homer2 KO | No effect | ↓ | ↓ (Full reversal) |
| Gmr5R/R | ↑ | ↓ | ↓ |
| AAV-MEDIATED GENE TRANSFER | | | |
| NAC cDNA-Homer1c | ↑ | ↓ | ↓ |
| NAC shRNA-Homer1c | ↓ | No effect | ↓ |
| IT cDNA-Homer1a | ↓ | No effect | ↓ |
| IT cDNA-Homer1c | ↑ | ↓ | No effect |
| IT cDNA-Homer2b | ↑ | ↓ | No effect |

CCI, chronic constriction injury of the sciatic nerve; CPA, conditioned place-aversion; CPP, conditioned place-preference; IT, intrathecal; NAC, intra-nucleus accumbens.

of the temporal dynamics of the interplay between inducible and CC-Homer protein expression at glutamate receptors, and likely other Homer-interacting molecules, while not always sufficient to prevent neuroplasticity within pain pathways, appears to be sufficient to prevent whatever mesocorticolimbic neuroplasticity mediating CCI-induced deficits in heroin-conditioned reward. Given the present data, it becomes important to characterize more systematically: (1) how heroin dose interacts with a chronic pain state to influence drug reward/reinforcement and to relate these interactions to the expression of different Homer isoforms, as well as their major interacting partners throughout the central nervous system; (2) to extrapolate findings for heroin to prescription opioid drugs employed in pain management, and importantly; (3) to examine the relevance of injury-induced changes in glutamate receptor/Homer expression for the incentive motivational properties of opioid and other non-opioid analgesic drugs (e.g., cannabinoids). Arguably, such lines of investigation will enable a better understanding of the molecular and cellular processes mediating pain-induced dysphoria, which has relevance not only for therapeutic intervention of pain-induced negative affective states, but also individual vulnerability to develop abuse or addiction during pain management with opioid or non-opioid drugs with high abuse potential.

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