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Spinal mediators that may contribute selectively to antinociceptive tolerance but not other effects of morphine as revealed by deletion of GluR5

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Abstract

Several groups maintain that morphine tolerance and dependence correlate with increased activity of protein kinases ERK1/2 and P38 MAPK and PKC as well as elevated levels of the neuropeptides dynorphin (DYN), substance P (sP) and calcitonin gene-related peptide (CGRP) in spinal cord dorsal horn (SCDH). They demonstrate that tolerance and dependence can be prevented, and sometimes reversed, by constitutive genetic deletion or pharmacological inhibition of these factors. Recently, we showed that mice with a constitutive deletion of the GluR5 subunit of kainate receptors (GluR5 KO) are not different from wildtype (WT) littermates with respect to baseline nociceptive thresholds as well as acute morphine antinociception, morphine physical dependence and conditioned place preference. However, unlike WT, GluR5 KO mice do not develop antinociceptive tolerance following systemic morphine administration. In this report, we examined levels of these mediators in SCDH of WT and GluR5 KO mice following subcutaneous implantation of placebo or morphine pellets. Surprisingly, spinal DYN and CGRP, along with phosphorylated ERK1/2 (pERK1/2), P38 (pP38) and PKCgamma (pPKCγ) are elevated by deletion of GluR5. Additionally, chronic systemic morphine administration increased spinal pERK1/2, pP38 and pPKCγ levels in both tolerant WT and non-tolerant GluR5 KO mice. In contrast, while morphine increased spinal DYN and CGRP in WT mice, DYN remained unchanged and CGRP was reduced in GluR5 KO mice. These observations suggest that spinal ERK1/2, P38 and PKCγ are likely involved in multiple adaptive responses following systemic morphine administration, whereas DYN and CGRP may contribute selectively to the development of antinociceptive tolerance.

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Keywords

morphine tolerance; kainate; knockout mouse; ionotropic glutamate receptor; calcitonin gene-related peptide; dynorphin

While agonists of μ opioid receptors (MOP) such as morphine are favored for management of moderate to severe pain, the clinical utility of morphine can be limited significantly by the development of antinociceptive tolerance, or a reduction in analgesic potency, which occurs in many patients receiving long-term therapy (McQuay, 1999, Inturrisi, 2002).

One common feature of several mechanistic models of morphine tolerance is the sustained activation of protein kinases, particularly Protein Kinase C γ (PKC γ) and the Mitogen-Activated Protein Kinases (MAPK) ERK1/2 and P38 α/β in spinal cord dorsal horn (SCDH). For example, PKC γ expression in rat SCDH is augmented after chronic intrathecal (i.t.) administration of morphine (Narita et al., 2004, Lim et al., 2005). Inhibition of PKC by i.t. coinfusion of the PKC antagonist chelerythrine blocks the increase in spinal PKC γ expression and the development of tolerance following i.t. morphine in rats (Granados-Soto et al., 2000). Systemic morphine tolerance in mice is attenuated by intracerebroventricular (i.c.v.) co-administration of a selective inhibitor of the receptor for activated C-kinase (RACK) binding PKC γ (Smith et al., 2007). PKC γ KO mice exhibit diminished antinociceptive tolerance following chronic systemic (Zeitz et al., 2001) or repeated i.t. (Narita et al., 2001) morphine. MOP-induced MAPK activation is suggested by the finding that recurrent daily exposure to morphine increases immunoreactivity of phosphorylated forms of ERK1/2 and P38 α/β MAPK in rat dorsal root ganglion (DRG) cultures (Ma et al., 2001). *In vivo*, repeated i.t. morphine treatment results in elevated levels of pERK1/2 and pP38 α/β (Cao et al., 2005, Cao et al., 2006, Cui et al., 2006, Wang et al., 2009) in rat SCDH in association with the development of analgesic tolerance. Both tolerance and the increased phosphorylation of MAPK are attenuated by daily i.t. co-administration with inhibitors of MAPK kinase (MEK) or P38 α/β (Cui et al., 2006, Wang et al., 2009).

Another prevailing hypothesis purports that increased release of neuropeptides such as dynorphin (DYN), substance P (sP) and calcitonin gene-related peptide (CGRP) in SCDH contributes to the development of tolerance following chronic morphine administration (Ossipov et al., 2005, Trang et al., 2005). Continuous systemic infusion of morphine via s.c. pellet implantation significantly increases levels of prodynorphin mRNA in mouse SCDH (Liang et al., 2003) as well as the post-translational product of the prodynorphin peptide, DYN A-(1–17), in rat SCDH (Gardell et al., 2002). The development of tolerance following systemic morphine infusion or repeated i.t. injection of the MOP agonist DAMGO is blocked by deletion of prodynorphin (Gardell et al., 2001) or by i.t. administration of DYN antiserum in rats (Vanderah et al., 2000, Vanderah et al., 2001). Elevated levels of sP and CGRP in DRG and in SCDH (Menard et al., 1995, Ma et al., 2001, Gardell et al., 2002, King et al., 2005, Liang et al., 2007, Wang et al., 2009) are correlated with the development of tolerance following repeated administration of i.t. or s.c. morphine for 5–7 days. Co-administration of CGRP receptor antagonists have been shown to attenuate and reverse tolerance following i.t. boluses or continuous infusion of morphine (Menard et al., 1996, Powell et al., 2000, Powell et al., 2003, Wang et al., 2009). DYN antiserum blocks the increase in CGRP and the development of tolerance, indicating that these processes are DYN-dependent (Gardell et al., 2002). Inhibition of sP/Neurokinin 1 (NK1) receptors by co-administration of SR140333 attenuates and reverses established tolerance following sustained i.t. morphine treatment in rats (Powell et al., 2003). Furthermore, ablation of spinal NK1 receptor-containing cells by i.t. sP-saporin prevents i.t. morphine tolerance and the

upregulation of spinal DYN content on day 7 of s.c. morphine infusion in rats (Vera-Portocarrero et al., 2007).

Nevertheless, since tolerance and dependence develop concurrently *in vivo*, these phenomena cannot be separated mechanistically from one another unless withdrawal is induced via the cessation of morphine treatment or the systemic administration of naloxone (Way et al., 1969). Thus, it is not possible using the aforementioned animal models to determine which of these mediators contribute selectively to tolerance and not to other adaptive responses to sustained morphine treatment in the absence of a withdrawal state. However, as GluR5 knockout (KO) mice do not develop tolerance following chronic systemic morphine administration but are otherwise indistinguishable from their WT littermates (Bogulavsky et al., 2009), we reasoned that factors involved selectively in tolerance, but not for other neuroadaptive responses, would be differentially regulated by morphine in SCDH from these mice. To investigate this possibility, we measured spinal levels of these kinases and neuropeptides in WT and GluR5 KO mice after chronic systemic morphine treatment.

EXPERIMENTAL PROCEDURES

Animals

GluR5 KO mice were produced on a 129/SvEv background at the Salk Institute in the laboratory of Dr. Stephen F. Heinemann (Mulle et al., 2000). Both GluR5 KO and their WT littermates were backcrossed with C57BL/6 mice in parallel for over 10 generations to produce congenic C57BL/6 WT and GluR5 KO lines as described previously (Bogulavsky et al., 2009). Male WT and GluR5 KO mice (8-12 weeks old, 25-30g) were maintained under climate-controlled conditions on a 12 h light/dark cycle with free access to food and water. Mice were housed individually after implantation with placebo or morphine pellets as outlined below. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Morphine tolerance paradigm

Morphine sulfate and pellet formulations of pharmacologically inert placebo (containing cellulose) and morphine (containing 25 mg morphine base), were obtained from the Research Triangle Institute (Research Triangle Park, NC) through the National Institute on Drug Abuse (Rockville, MD). On day 1, one placebo or one 25 mg morphine pellet wrapped in nylon mesh was implanted s.c. on the dorsal surface of each mouse under general isoflurane and local s.c. bupivacaine anesthesia. This paradigm results in the development of antinociceptive tolerance by day 4 after morphine pellet implantation in WT but not in GluR5 KO mice (Bogulavsky et al., 2009).

Immunoblot

On day 4 after implantation (with placebo or morphine pellets still in place), lumbar L4/L5 SCDH samples from WT and GluR5 KO mice (n = 6 animals/group) were rapidly dissected and homogenized in ice-cold Tris-EDTA (TE) buffer (50 mM Tris-HCl pH 7.4, 2 mM EDTA) or modified Radioimmunoprecipitation Assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing protease inhibitor cocktail and phosphatase inhibitor cocktails I and II (Sigma, St. Louis, MO) supplemented with 2 mM PMSF and 5 mM NaF. Lysates were flash frozen in liquid nitrogen, stored briefly at -80°C until dissections were completed, then sonicated on ice. Following the determination of protein concentrations of TE or RIPA total homogenates using DC Assay kit (BioRad), samples were diluted in Laemmli sample buffer and boiled for 5 minutes. Equal amounts of total protein (50 µg) were subjected to SDS-PAGE using 10% Tris-HCl precast gels

(BioRad) and then transferred to PVDF membranes (Millipore, Bedford, MA), which were subsequently blocked in 5% blotting grade milk (BioRad) overnight at 4°C. Blots were incubated in primary antibodies generated in rabbit for phosphoP38 α / β MAPK Thr180/Tyr182 at 1:250, phosphoERK1/2 MAPK Thr202/Tyr204 at 1:1000, phosphoPKC(pan) γ Thr514 at 1:1000, P38 α / β at 1:500, ERK1/2 at 1:5000 (Cell Signaling Technology, Beverly, MA), PKC γ at 1:20000 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, then incubated either with HRP-conjugated anti-rabbit secondary antibodies at 1:20000 (Pierce, Rockford, IL) for 1 hour at room temperature. As a loading control, blots were probed for β -actin using a mouse monoclonal antibody 1:100000 – 1:200000 (Sigma) followed by HRP-conjugated anti-mouse secondary antibodies 1:100000 (Pierce), both for 1 hour at room temperature. Membranes were developed using SuperSignal West Pico ECL kit (Pierce) and exposed to film (Kodak, Rochester, NY). Exposures yielding signal intensity in the linear range without saturation were used for densitometric analysis with Fluorchem 9900 (Alpha Innotech, San Leandro, CA). Ratios of intensity of the protein of interest to β -actin (loading control) were calculated, normalized to experimental control (WT Placebo), and averaged for animals within each group. Pilot studies comparing untreated and placebo pellet-implanted WT and GluR5 KO mice revealed that presence of the placebo pellet did not alter expression of these proteins in SCDH. Thus, the changes we report in KO mice are derived from the constitutive absence of GluR5.

Immunofluorescence

On day 4, mice were perfused intracardially under deep pentobarbital-induced anesthesia with saline followed by 4% paraformaldehyde (PFA) in Phosphate-Buffered Saline (PBS). Spinal cords were dissected, post-fixed in 4% PFA in PBS for 1 hour, cryoprotected in 30% sucrose for 3-5 days, then mounted and frozen in Tissue-Tek OCT medium (Electron Microscopy Sciences, Hatfield, PA). Transverse sections (20 μ m) from the L4/L5 lumbar region were cut on a cryostat (Leica Microsystems, Wetzlar, Germany) and adhered to glass slides. Slides were selected by systematic random sampling (Coggeshall and Lekan, 1996) for a total of 6 slides per animal, spaced 200 μ m apart. Sections were incubated overnight at room temperature with primary antibodies generated in guinea pig for calcitonin gene-related peptide α (CGRP) at 1:200 or in rabbit for substance P (sP) at 1:500, dynorphin A(1-17) (DYN) at 1:200 (Bachem, Torrance, CA). Alexa Fluor goat anti-rabbit 546 or goat anti-guinea pig 647 secondary antibody (Molecular Probes, Carlsbad, CA) was added at 1:200 for 1 hour, and coverslips were attached using Gel/Mount (Biomed, Foster City, CA). Negative control sections were processed under identical conditions, except that primary antibodies were omitted.

Images were captured in the same session using identical settings with a TRITC (for 546 secondary) or Cy5 (for 647 secondary) filter under 10X magnification on a Zeiss Axiovert 200 epifluorescence microscope equipped with a MicroMax interline black-and-white CCD camera (Zeiss Microimaging, Inc., Oberkochen, Germany), then processed using Metamorph software (MDS Analytical Technologies, Toronto, Canada). The investigator was blinded to the identity of each sample. After thresholds were set equally for the treatment groups on each slide, integrated intensity values above threshold (3X background) were counted in Laminae I-III of lumbar L4/L5 SCDH according to previous delineation (Molander et al., 1984), and then divided by the area measured for each section. Mean integrated intensity per unit area (μ M) of immunoreactivity in Laminae I-III of lumbar L4/L5 SCDH was calculated by averaging normalized values from $n = 3$ mice per group in each of two experiments for a total of $n = 6$ per group. Following analysis, representative images of PKC γ , sP and CGRP were pseudocolored red while those for pPKC γ and DYN were pseudocolored green for display purposes only.

Primers

Primers for amplification of cDNAs are listed in Table 2. Primers for *Prkcc* (PKC γ , source accession number NM_011102) targeted exon 21 and were designed using the RT-PCR design wizard in CloneManager (Sci-Ed Software, Cary, NC). Primers for amplification of *Tac1* (sP, source accession number NM_009311), *Calca* (CGRP, source accession number NM_007587); *prodynorphin* (proDYN, source accession number NM_018863) and β -*actin* (source accession number NM_007393) were synthesized based on primer sequences listed in PrimerBank (Wang and Seed, 2003) as follows: *Tac1*, PrimerBank ID 6678207a1; *Calca*, PrimerBank ID 33468845a1; *prodynorphin*, PrimerBank ID 9256622a3; β -*actin*, PrimerBank ID 6671509a2. Formation of single PCR amplicons (without primer dimers) was verified with endpoint PCR using Platinum Taq Supermix on a Biorad iCycler (Biorad, Hercules, CA) as directed (Invitrogen, Carlsbad, CA) followed by agarose gel electrophoresis.

Quantitative real-time RT-PCR

Lumbar SCDH samples from mice ($n = 6$ animals/group) were rapidly dissected under RNase-free conditions, flash frozen in liquid nitrogen, and then stored briefly at -80°C . Total RNA was extracted from SCDH of individual mice using Qiagen RNeasy Lipid Tissue Mini Kit and isolated using RNase-free DNase kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration and purity of RNA were assessed with an Eppendorf Biophotometer 6.31 spectrophotometer (Eppendorf, Westbury, NY). Complementary DNA (cDNA) templates were synthesized for all mice in the same session from equal amounts of total RNA using iScript Kit (BioRad).

SCDH samples from individual mice were prepared for real-time quantitative PCR with Power SYBR Green master kit (PE Applied Biosystems, Foster City, CA) as directed. To ensure that cDNA amplification efficiencies of SCDH from control and treated mice were equivalent, standard curves were generated from serial dilutions to determine the optimal dilution of cDNA for each primer set. Samples were analyzed in triplicate by quantitative PCR on an ABI Prism 7700 (PE Applied Biosystems) using the following parameters: 95°C for 10 min, then [95°C -15 sec; 60°C -30 sec; 72°C -30 sec] for 40 cycles, then 72°C for 10 min as described previously (Giordano et al., 2006). The specificity of PCR products was verified further by the detection of a single peak at the predicted melting temperature (T_m) using a dissociation curve. The cycle threshold (C_T) value for each amplified PCR product was calculated by the ABI prism SDS software as the intensity of SYBR green fluorescence that exceeded 10 times the standard deviation of the baseline fluorescence. The comparative C_T method was used for Relative Quantification (Livak and Schmittgen, 2001), in which the target C_T for each sample was subtracted from that of β -actin and then normalized to control (WT Placebo) to obtain a $\Delta\Delta C_T$ value. The $2^{-\Delta\Delta C_T}$ values were calculated and averaged for animals within each group.

Statistical analysis

Data were expressed as the mean percent of the control group (WT Placebo) $\pm$ SEM. p values were determined using SPSS statistics software (SPSS Inc, Chicago, IL) as follows: unpaired two-tailed t-test for two group analysis (WT Placebo v. GluR5 KO Placebo) or two-way ANOVA followed by Tukey post-test for multiple group analysis according to genotype (WT, GluR5 KO) and treatment (Placebo, Morphine). A value of $p < 0.05$ was considered significant.

RESULTS

Previously, we observed that WT and GluR5 KO mice do not differ with respect to their baseline thermal tail withdrawal (TTW) latencies on day 4 following s.c. pellet implantation with Placebo or Morphine. After a challenge dose of 16 mg/kg s.c. morphine, WT Placebo and GluR5 KO Placebo both exhibit a significant increase in TTW that does not differ in magnitude, suggesting that deletion of GluR5 does not affect acute morphine antinociception. However, WT Morphine post-challenge demonstrate no change over baseline latency, indicating the development of antinociceptive tolerance. In contrast, GluR5 KO Morphine post-challenge display an increase in TTW latency that is significantly greater than WT Morphine post-challenge yet does not differ from GluR5 KO Placebo post-challenge. In addition, cumulative dose response studies using repeated s.c. injection of morphine reveal that morphine ED_{50} is increased 4-fold in WT mice but is unchanged in GluR5 KO mice on day 4. The lack of tolerance phenotype is distinctive as GluR5 KO mice are not different from WT littermates with respect to acute morphine antinociception, central nervous system distribution of morphine and expression of morphine physical dependence, conditioned place preference and locomotor sensitization (Bogulavsky et al., 2009).

Morphine-induced activation of spinal MAPK in WT and GluR5 KO mice

Initially, we measured the expression of total and phosphorylated ERK and P38 MAPK proteins by immunoblot in samples of SCDH from WT and GluR5 KO mice on day 4 following s.c. implantation with placebo or morphine pellets. As the increased expression of pERK1/2 protein appeared to be attributable to pERK2 only, we measured phospho and total ERK2 as well as ERK1/2 combined. Optical density values were determined for ERK2 and of P38 (α/β) and depicted in Figure 1 (b,d). Two-way ANOVA analysis of phosphorylated ERK2 (pERK2) protein levels revealed a main effect of genotype ($F_{1,14} = 26.934$; $p < 0.001$) and treatment ($F_{1,14} = 27.306$; $p < 0.001$) but no interaction of genotype and treatment ($F_{1,14} = 1.119$; $p > 0.05$). Post-hoc comparisons using Tukey post-test showed that pERK2 was elevated in SCDH from morphine-tolerant WT mice ($226 \pm 11\%$, $p < 0.01$) as compared with WT placebo control mice ($100 \pm 17\%$; Figure 1 a, b). Surprisingly, expression of pERK2 also was increased significantly in SCDH from placebo-treated GluR5 KO ($226 \pm 17\%$, $p < 0.01$) versus WT mice. Spinal levels of pERK2 were augmented further in GluR5 KO mice after chronic morphine ($313 \pm 29\%$, $p < 0.05$ versus GluR5 KO placebo, $p < 0.05$ versus WT morphine). Two-way ANOVA analysis of phosphorylated p38 (pP38 α/β) protein levels revealed a main effect of genotype ($F_{1,23} = 8.817$; $p < 0.05$) and treatment ($F_{1,23} = 79.929$; $p < 0.001$) as well as an interaction of genotype and treatment ($F_{1,14} = 6.129$; $p < 0.05$). Post-hoc comparisons using Tukey post-test showed that as observed with pERK2, phosphoP38 α/β MAPK (pP38 α/β) was increased in SCDH in morphine-treated WT mice ($251 \pm 21\%$, $p < 0.001$) compared with WT placebo control mice ($100 \pm 10\%$) concurrent with the development of tolerance (Figure 1 c, d). Likewise, expression of pP38 α/β was elevated in SCDH from GluR5 KO ($171 \pm 13\%$, $p < 0.01$) relative to WT mice implanted with placebo pellets. In addition, spinal pP38 α/β was increased in GluR5 KO mice after sustained morphine administration ($256 \pm 14\%$, $p < 0.01$). However, total levels of ERK2 and P38 α/β proteins in SCDH were unchanged both by deletion of GluR5 and by s.c. morphine administration (Table 1). We did not measure expression of ERK2 or P38 α/β mRNAs as they were unaltered at the protein level. These data indicate that the systemic morphine-induced activation of spinal MAPK is not prevented in GluR5 KO mice and that the regulation of ERK2 and P38 α/β proteins in SCDH by GluR5 or by morphine likely occurs by post-translational mechanisms.

Morphine-induced upregulation and activation of spinal PKC γ in WT and GluR5 KO mice

Next, we examined the expression of total and phosphorylated forms of PKC γ in SCDH from WT and GluR5 KO mice. The phosphoPKC(pan) γ Thr514 antibody has been used previously to detect the phosphorylated form of PKC γ in mouse brain and spinal cord tissue by western blot (Shukla et al., 2006), but to our knowledge has not yet been reported for immunohistochemical analysis. Fluorescent immunohistochemistry revealed that labeling of pPKC γ (pPKC γ -ir) was detected at low levels in lamina II inner of SCDH from WT mice, a distribution which is consistent with published accounts of total PKC γ immunoreactivity (Polgar et al., 1999). Analysis of pPKC γ densitometry in WT and GluR5 KO SCDH by t-test revealed that pPKC γ -ir was increased in placebo-treated GluR5 KO ($F_{1,10} = 36.012$; $581 \pm 35\%$, $p < 0.0001$) compared with WT mice ($100 \pm 6\%$, Figure 2 a, b). Two-way ANOVA analysis of pPKC γ protein levels as measured by immunoblot revealed a main effect of genotype ($F_{1,15} = 106.272$; $p < 0.001$) and treatment ($F_{1,15} = 501.752$; $p < 0.001$) as well as an interaction of genotype and treatment ($F_{1,15} = 18.775$; $p < 0.01$). Post-hoc comparisons using Tukey post-test showed that levels of pPKC γ were elevated in SCDH from GluR5 KO ($539 \pm 38\%$, $p < 0.01$) relative to WT mice after placebo pellet implantation ($100 \pm 9\%$, Figure 2 c, d). Morphine treatment increased spinal pPKC γ in both WT ($1428 \pm 107\%$, $p < 0.001$ v. WT placebo) and GluR5 KO mice ($2504 \pm 93\%$, $p < 0.001$ v. GluR5 KO placebo, $p < 0.001$ v. WT morphine). An additional band migrating above the single ~ 75 kDa band was detected in SCDH samples from placebo-treated GluR5 KO and from morphine-treated WT and GluR5 KO mice. This band most likely corresponds to another isoform of PKC phosphorylated at a residue homologous to threonine 514 of PKC γ . Based on its higher molecular weight, possible candidates may be PKC ϵ or PKC η , both ~ 82 kDa. Therefore, the densitometry values presented in Figure 2d represent the normalized ratio of immunoreactivity from the lower ~ 75 kDa band only.

Analysis of total PKC γ densitometry in WT and GluR5 KO SCDH by t-test revealed that PKC γ -ir was increased in placebo-treated GluR5 KO ($F_{1,10} = 6.215$; $244 \pm 19\%$, $p < 0.001$) compared with WT mice ($100 \pm 9\%$, Figure 3 a, b). Two-way ANOVA analysis of PKC γ protein levels as measured by immunoblot revealed a main effect of genotype ($F_{1,15} = 21.840$; $p < 0.01$) and treatment ($F_{1,15} = 74.186$; $p < 0.001$) but no interaction of genotype and treatment ($F_{1,15} = 4.132$; $p > 0.05$). Post-hoc comparisons using Tukey post-test showed that as with phosphorylated PKC γ , total PKC γ protein as measured by immunoblot was elevated in SCDH from GluR5 KO ($140 \pm 9\%$, $p < 0.01$) as compared with WT mice ($100 \pm 4\%$) after s.c. placebo pellet implantation (Figure 3 c, d). PKC γ expression also was increased in both morphine-treated WT ($163 \pm 8\%$, $p < 0.001$) and GluR5 KO ($179 \pm 8\%$, $p < 0.01$) mice relative to WT or GluR5 KO placebo control mice, respectively. In contrast, relative steady-state levels of PKC γ mRNA, as measured by real-time quantitative RT-PCR using primers listed in Table 2, were unaltered relative to control mice by the constitutive knockout of GluR5 or after morphine administration (Table 3). Two-way ANOVA analysis of PKC γ mRNA levels revealed no main effect of genotype ($F_{1,22} = 1.113$; $p > 0.05$), treatment ($F_{1,22} = 3.795$; $p > 0.05$) or interaction of genotype and treatment ($F_{1,22} = 3.594$; $p > 0.05$). Together, these results suggest that GluR5- and morphine-mediated regulation of PKC γ expression in SCDH likely takes place at a post-transcriptional level.

Morphine-induced increase in spinal DYN and CGRP in WT but not GluR5 KO mice

Immunolabeling of the neuropeptides dynorphin (DYN), calcitonin gene-related peptide α (CGRP) and substance P (sP) were examined in spinal cord from WT and GluR5 KO mice on day 4 after pellet implantation. In accordance with previous reports in rat SCDH (Vanderah et al., 2000), immunoreactivity for DYN A-(1–17), (DYN-ir), was observed primarily in laminae I, II and V (Figure 4). Two-way ANOVA analysis of DYN-ir revealed a main effect of genotype ($F_{1,23} = 20.932$; $p < 0.001$) and treatment ($F_{1,23} = 9.846$; $p < 0.01$)

as well as an interaction of genotype and treatment ($F_{1,23} = 5.087$; $p > 0.05$). Post-hoc comparisons using Tukey post-test showed that DYN-ir was elevated in the superficial laminae of SCDH from GluR5 KO placebo-treated mice ($237 \pm 20\%$, $p < 0.01$) as compared with WT control mice ($100 \pm 17\%$). Sustained morphine treatment produced a significant increase of DYN labeling in SCDH of WT mice ($208 \pm 18\%$, $p < 0.01$ v. WT placebo). In contrast, there was no change in the integrated intensity of spinal DYN-ir in morphine-treated GluR5 KO mice ($255 \pm 24\%$, $p > 0.05$ v. GluR5 KO placebo). The relative amount of *prodynorphin* (proDYN) mRNA, as determined by qPCR, was unchanged by either deletion of GluR5 or by morphine (Table 3). Two-way ANOVA analysis of proDYN mRNA levels revealed no main effect of genotype ($F_{1,21} = 0.021$; $p > 0.05$), treatment ($F_{1,21} = 1.307$; $p > 0.05$) or interaction of genotype and treatment ($F_{1,21} = 0.989$; $p > 0.05$). These results indicate that the regulation of DYN in SCDH by GluR5 and by systemic morphine likely occurs at a post-transcriptional level.

As described previously (Gardell et al., 2002), labeling of CGRP α (CGRP-ir) was detected in laminae I and IIo (II outer) of spinal cord (Figure 5). Two-way ANOVA analysis of CGRP-ir revealed a main effect of genotype ($F_{1,23} = 4.745$; $p < 0.05$) and an interaction of genotype and treatment ($F_{1,23} = 29.918$; $p < 0.001$) but no main effect of treatment alone ($F_{1,23} = 0.218$; $p > 0.05$). Post-hoc comparisons using Tukey post-test showed that CGRP-ir was increased significantly in superficial laminae of SCDH from GluR5 KO placebo-implanted mice ($197 \pm 18\%$, $p < 0.001$ v. WT placebo) and morphine-treated WT mice ($163 \pm 7\%$, $p < 0.05$ v. WT placebo) as compared with WT control mice ($100 \pm 14\%$). Surprisingly, CGRP-ir was reduced in SCDH from GluR5 KO mice receiving morphine ($122 \pm 8\%$, $p < 0.01$ v. GluR5 KO placebo), such that the levels were not different ($p > 0.05$) from WT placebo control mice. As spinal CGRP is expressed in sensory primary afferent terminals (Rethelyi et al., 1989), the majority of CGRP mRNA would be expected to reside in DRG cell bodies, as demonstrated previously (Liang et al., 2007). We attempted to quantify *Calca* mRNA levels in SCDH; however, the amplification efficiencies for spinal samples obtained from individual mice were below the limit of quantitation ($< 85\%$), so we did not pursue this approach.

In line with previous studies (King et al., 2005), immunoreactivity of substance P (sP-ir) was localized to superficial laminae I and IIo of SCDH (Figure 6). Two-way ANOVA analysis of sP-ir revealed no main effect of genotype ($F_{1,23} = 2.745$; $p > 0.05$), treatment ($F_{1,23} = 1.590$; $p > 0.05$) or interaction of genotype and treatment ($F_{1,23} = 0.553$; $p > 0.05$). Post-hoc comparisons using Tukey post-test showed that sP labeling in SCDH was unchanged by the deletion of GluR5 ($120 \pm 7\%$, $p > 0.05$) relative to WT placebo-implanted control mice ($100 \pm 12\%$). Moreover, spinal sP-ir was unaltered on day 4 of s.c. morphine administration both in WT ($95 \pm 5\%$, $p > 0.05$ v. WT placebo) and in GluR5 KO mice ($103 \pm 8\%$, $p > 0.05$ v. GluR5 KO placebo). Two-way ANOVA analysis of sP mRNA levels revealed a main effect of treatment ($F_{1,22} = 6.138$; $p < 0.05$), but no main effect of genotype ($F_{1,22} = 0.292$; $p > 0.05$) or interaction of genotype and treatment ($F_{1,22} = 0.011$; $p > 0.05$). Post-hoc comparisons using Tukey test showed that mRNA levels of the sP precursor *preprotachykinin A* were unchanged (Table 3). Taken together, these data suggest that expression of sP is not affected by GluR5 genotype or by three days of continuous systemic morphine infusion in mice.

DISCUSSION

The distinctive behavioral phenotype of GluR5 KO mice with respect to their selective lack of antinociceptive tolerance following chronic systemic morphine treatment (Bogulavsky et al., 2009) affords the ability to examine mechanisms of tolerance separately from other morphine-mediated effects. We reasoned that factors required for the development of

antinociceptive tolerance, but not for other neuroadaptive responses after sustained morphine administration, would be differentially regulated in GluR5 KO mice in regions that mediate opioid analgesia. While GluR5 is not expressed in many areas classically associated with morphine analgesia, including the medial thalamus, rostroventral medulla, periaqueductal gray or SCDH intrinsic neurons (Bettler et al., 1990, Tolle et al., 1993, Hwang et al., 2001, Kerchner et al., 2002), it is prominent in small-diameter, unmyelinated Isolectin B4 (IB4)-positive nociceptive C fibers terminating in lamina III of the SCDH (Lee et al., 2001, Youn and Randic, 2004, Bourane et al., 2007). In addition, the TTW test we used to assess the development of tolerance (Bogulavsky et al., 2009) is predominantly a spinally-mediated phenomenon (Irwin et al., 1951). For these reasons, we measured changes in spinal mediators and our observations regarding the effects of GluR5 deletion and of chronic morphine treatment are summarized in Table 4.

Spinal mediators under tonic control by GluR5: PKC γ , ERK2, P38, DYN, CGRP

About half of the nociceptors expressing the Nerve Growth Factor (NGF) receptor TrkA are destined to become the IB4 subpopulation, undergoing a phenotypic switch during early postnatal development (P0 to P21) from NGF to Glial cell-line Derived Neurotrophic Factor (GDNF) dependence (Molliver et al., 1997). Previous studies have alluded to the potential involvement of glutamate receptors (including GluR5 KAR) in nociceptor development (Kalb and Fox, 1997, Lee et al., 2001, Stegenga and Kalb, 2001). Expression of GluR5 appears early in embryonic development in a subset of DRG cells (Bettler et al., 1990) and in the SCDH is confined to lamina II at P10 (Stegenga and Kalb, 2001). However, while it continues to be expressed in adult DRG neurons (Bettler et al., 1990), GluR5 is no longer detectable in the spinal cord by P22 (Stegenga and Kalb, 2001).

In this study, we observed that deletion of GluR5 results in a significant upregulation of basal levels of total and phosphoPKC γ , phosphoERK2, phosphoP38 α/β , DYN and CGRP in SCDH. Interestingly, mRNA expression of *Grik1* (the gene encoding GluR5) in DRG is ablated by the deletion of TrkA, indicating that the population of nociceptors that undergoes the NGF to GDNF phenotypic switch contains GluR5 (Bourane et al., 2007).

Phosphorylated forms of ERK1/2 and p38 are modulated by NGF (Averill et al., 2001, Ji et al., 2002). In the adult, GluR5 is present in the IB4-positive population of nociceptors (Bourane et al., 2007), which may make synaptic contacts with many PKC γ -containing interneurons in SCDH (Malmberg et al., 1997). DYN and CGRP are colocalized in some small diameter DRG neurons (Marvizon et al., 2009) and CGRP-positive terminals end on cellular elements containing DYN in SCDH (Carlton and Hayes, 1989). These findings suggest the potential for crosstalk between NGF and GluR5 to regulate expression of MAPK, PKC γ , DYN and CGRP, which may be altered by the constitutive absence of GluR5 in development. It has been proposed that GluR5 may be a target of Runx1 (Bourane et al., 2007), which controls the neurochemical fate of sensory neurons as peptidergic or non-peptidergic (Chen et al., 2006). Similar to Runx1 deletion mutants, GluR5 KO mice also display derepression of CGRP.

Nonetheless, the increased basal expression of ERK2, P38 α/β , PKC γ , DYN and CGRP in GluR5 mutant mice did not affect either baseline nociceptive thermal and mechanical thresholds or acute morphine antinociception (Bogulavsky et al., 2009). These findings corroborate previous reports that MAPK and PKC γ are not involved in the regulation of acute nociception (Malmberg et al., 1997, Svensson et al., 2005, Xu et al., 2008). In addition, thermal response latencies are unchanged in mice deficient in CGRP (Zhang et al., 2001) or reduced in prodynorphin knockout mice (Wang et al., 2001). Interestingly, differential expression of CGRP mediates variable sensitivity to noxious heat, such that mouse strains expressing higher basal levels of CGRP exhibit less thermal hyperalgesia in response to exogenously administered CGRP (Mogil et al., 2005).

Spinal mediators that may contribute selectively to morphine tolerance: DYN, CGRP

In agreement with published literature (Vanderah et al., 2000, Gardell et al., 2002), WT mice exhibit morphine-induced increases in SCDH immunoreactivity of DYN. We found no change in *prodynorphin* mRNA following morphine infusion, which could result either from differences in morphine administration paradigms or from species-specific differences between mice and rats, as described above. In support of previous studies (Powell et al., 2000, Gardell et al., 2002), WT mice exhibit a morphine-induced increase in spinal CGRP-ir in conjunction with the development of tolerance. Surprisingly, CGRP-ir was reduced in SCDH from GluR5 KO mice following sustained morphine versus placebo treatment, particularly in lamina II where GluR5-containing fibers terminate, suggesting possible contacts between these neuronal populations and direct modulation of CGRP release by GluR5. This reduction was of a significant magnitude such that total levels of CGRP-ir were not different from WT placebo control mice. While the mechanism remains to be defined, these data suggest that spinal DYN and CGRP may contribute selectively to the development of antinociceptive tolerance after chronic morphine administration.

Increased DYN-dependent release of CGRP from primary afferents following sustained morphine infusion (Gardell et al., 2002) may amplify NMDAR activity in SCDH neurons (Murase et al., 1989a, Vanderah et al., 2000) and may contribute to tolerance in WT mice. CGRP increases the release of glutamate (Kangrga and Randic, 1990, Takhshid et al., 2007), and enhances voltage-dependent Ca^{2+} currents in spinal cord slice preparations (Ryu et al., 1988, Murase et al., 1989b). These processes may be absent or altered in GluR5 KO mice and could underlie their failure to develop analgesic tolerance. Expression of DYN and CGRP may approach a maximal level in SCDH from GluR5 KO mice, such that these factors are less sensitive or even refractory to further modulation by systemic morphine treatment in the absence of GluR5. Alternatively, one or more pathways that regulate the spinal expression of these mediators may be missing or disrupted in GluR5 KO mice. Interestingly, deletion of the p75NTR (which binds neurotrophins including NGF) results in the absence of tolerance without effect on physical dependence following systemic morphine (Trang et al., 2009), similar to the behavioral phenotype of GluR5 KO mice. Perhaps during development prior to the NGF to GDNF phenotypic switch, GluR5 regulates expression of DYN and CGRP in sensory neurons in a manner that modulates the adult response to chronic morphine.

Spinal mediators lacking selective contribution to morphine tolerance: PKC γ , ERK2, P38

Previous studies have shown that μ opioid-mediated stimulation leads to phosphorylation of the NMDAR, potentiating its activity via removal of the Mg^{2+} block (Chen and Huang, 1991). Phosphorylation of ERK1/2 and P38 α/β depends on local increases in intracellular Ca^{2+} levels, potentially derived from influx via NMDARs (Hardingham et al., 2001, Waxman and Lynch, 2005). Collectively, these processes can contribute to the development of tolerance (Inturrisi, 1997). In this study, we found that pERK2, pP38 α/β , pPKC γ and total PKC γ are increased by systemic morphine both in tolerant WT and non-tolerant GluR5 KO mice, indicating their likely participation in other adaptive processes following sustained exposure to morphine. It should be noted that these observations do not discount the involvement of these mediators in the development of tolerance, but rather suggest that these kinases likely serve multiple roles in response to chronic morphine.

The expression of PKC γ in excitatory interneurons of the ventral aspect of SCDH lamina II inner layer (IIi) is almost completely segregated from that of μ opioid receptors (MOP) (Polgar et al., 1999, Zeitz et al., 2001). Nevertheless, it is plausible that activation of PKC γ in the subpopulation of SCDH cells that coexpress MOP could contribute to tolerance. Alternatively, other isoforms such as PKC α and PKC ϵ may be involved (Hua et al., 2002,

Smith et al., 2007). PKC γ may play a role in some aspects of morphine dependence as well, since i.c.v. inhibition (Smith et al., 2007) or global deletion (Zeitz et al., 2001) of PKC γ also affects naloxone-precipitated withdrawal behaviors in mutant mice (Zeitz et al., 2001).

Likewise, spinal MAPK activation also has been linked to morphine dependence. For example, SCDH pERK1/2 expression also is augmented during naloxone-precipitated withdrawal (Cao et al., 2005). The withdrawal-induced increase in spinal pERK1/2 levels, mechanical allodynia and scores of stereotypic abstinence behaviors were reduced by i.t. administration of the MEK inhibitor U0126 or of ERK1/2 antisense oligonucleotides. Likewise, increased phosphorylation of P38 α/β has been associated with analgesic tolerance, yet physical dependence was not assessed directly (Cui et al., 2006).

Spinal mediators unchanged by deletion of GluR5 or by systemic morphine: sP

As expected, neither steady-state levels of *Tac1* mRNA nor sP-ir in SCDH were affected by the deletion of GluR5 or by sustained morphine treatment. GluR5 is rarely coexpressed with sP in DRG neurons (Lee et al., 2001) and there is minimal overlap of IB4 and sP immunoreactivity in SCDH (Dodd and Jessell, 1985) so direct functional interactions are unlikely in this region. The finding that systemic morphine infusion did not alter spinal sP-ir on day 4 either in WT or GluR5 KO mice differs from other accounts (King et al., 2005, Liang et al., 2007), which document increased sP expression in SCDH. These investigators induced morphine tolerance using paradigms that differ from the current study: i.e., repeated s.c. injection in rats (4 days) or s.c. pellet implantation in mice (6 days).

It is possible that differences in species employed (mice v. rats) or in morphine dosing parameters may account for the disparate effects on sP expression. For example, s.c. implantation of one 25 mg morphine pellet in C57BL/6 mice for 3 days resulted in more stable blood levels of morphine and a higher degree of physical dependence when compared with repeated s.c. injection for 2-10 days (Fischer et al., 2008). Thus, pharmacokinetic parameters may impact the morphine-mediated effects on sP, as suggested previously (Kondo et al., 2005). Our findings do not preclude the possibility that spinal sP expression may be altered by prolonged systemic or intrathecal morphine.

These data suggest that activity-dependent plasticity of GluR5-dependent signaling networks mediates the morphine-induced increase in spinal DYN and CGRP levels and contribute selectively to the development of antinociceptive tolerance in WT mice, as they are not observed in GluR5 KO mice following sustained morphine treatment. While further studies are needed to elucidate mechanisms by which CGRP and DYN contribute to morphine tolerance, the GluR5 KO mouse model can serve as a valuable resource in this endeavor.

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LIST OF ABBREVIATIONS

WT	Wildtype
GluR5 KO	GluR5 knockout
SCDH	spinal cord dorsal horn
DRG	dorsal root ganglion

i.t.	intrathecal
s.c.	subcutaneous
i.c.v.	intracerebroventricular
MAPK	Mitogen Activated Protein Kinase
MEK	MAPK kinase
ERK	Extracellular Signal-Regulated Kinase
P38	P38 MAPK
pERK	phospho ERK
pP38	phospho P38
PKCγ	Protein Kinase C γ
DYN	dynorphin A-(1-17)
proDYN	prodynorphin
CGRP	calcitonin gene-related peptide α
sP	substance P
KAR	Kainate receptor
NK1	Neurokinin 1 receptor
NMDAR	N-methyl-D-aspartate receptor
MOP	mu opioid receptor
IB4	Isolectin B4
NGF	Nerve Growth Factor
GDNF	Glial cell-line Derived Neurotrophic Factor
TrkA	neurotrophin tyrosine kinase receptor A
p75^{NTR}	p75 neurotrophin receptor

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Figure 1. Levels of phosphorylated ERK2 and P38α/β MAP Kinases in spinal cord dorsal horn are increased by the deletion of GluR5 and by repeated morphine administration

(a) Immunoblot of mouse spinal cord dorsal horn (SCDH) detects bands corresponding to phosphorylated ERK1 and ERK2 (pERK1/2) at ~44 and ~42 kDa, respectively. (b) Densitometry reveals that spinal pERK2 levels are increased significantly in placebo-implanted GluR5 Knockout (GluR5 KO) mice and in morphine-treated Wildtype (WT) and GluR5 KO mice on day 4 as compared with WT placebo control mice. (c) Immunoblot of mouse spinal cord dorsal horn (SCDH) detects a band corresponding to phosphorylated P38α/β (pP38α/β) at ~38 kDa. (d) Densitometry reveals that spinal pP38α/β levels are increased significantly in placebo-implanted GluR5 KO and in morphine-treated Wildtype (WT) and GluR5 KO mice on day 4 as compared with WT placebo. (PLAC = Placebo, MOR = Morphine). Normalized ratios of (pERK2)/β-actin and (pP38α/β)/β-actin (mean ± SEM) are expressed as percent of the WT Placebo control. **, $p < 0.01$ v. WT Placebo; +, $p < 0.05$ v. GluR5 KO Placebo; #, $p < 0.05$ v. WT Morphine.

Figure 2. Levels of phosphorylated Protein Kinase Cγ in spinal cord dorsal horn are increased by the deletion of GluR5 and by repeated morphine administration

(a) Immunofluorescence labeling of phosphorylated Protein Kinase Cγ (pPKCγ-ir) in mouse spinal cord dorsal horn (SCDH). (b) Densitometry reveals that spinal pPKCγ-ir is increased significantly in placebo-implanted GluR5 Knockout (GluR5 KO) mice. (c) Immunoblot of mouse spinal cord dorsal horn (SCDH) detects a band corresponding to phosphorylated Protein Kinase Cγ (pPKCγ) at ~75 kDa. (d) Densitometry reveals that spinal pPKCγ levels are increased significantly in placebo-implanted GluR5 Knockout (GluR5 KO) and in morphine-treated Wildtype (WT) and GluR5 KO mice on day 4 as compared with WT placebo. (PLAC = Placebo, MOR = Morphine). Normalized ratios of pPKCγ/β-actin (mean ± SEM) are expressed as percent of the WT Placebo control. **, p < 0.01 v. WT Placebo; ***, p < 0.001 v. WT Placebo; +++, p < 0.001 v. GluR5 KO Placebo; ###, p < 0.001 v. WT Morphine. Scale bar, 100 μm.

Figure 3. Levels of total Protein Kinase C γ in spinal cord dorsal horn are increased by the deletion of GluR5 and by repeated morphine administration

(a) Immunofluorescence labeling of total Protein Kinase C γ (PKC γ -ir) in mouse spinal cord dorsal horn (SCDH). (b) Densitometry reveals that spinal PKC γ -ir is increased significantly in placebo-implanted GluR5 Knockout (GluR5 KO) mice. (c) Immunoblot of mouse spinal cord dorsal horn (SCDH) detects a band corresponding to total Protein Kinase C γ (PKC γ) at ~80 kDa. (d) Densitometry reveals that spinal PKC γ levels are increased significantly in placebo-implanted GluR5 KO mice and in morphine-treated Wildtype (WT) and GluR5 KO mice on day 4 as compared with WT placebo. (PLAC = Placebo, MOR = Morphine). Normalized ratios of total PKC γ / β -actin (mean $\pm$ SEM) are expressed as percent of the WT Placebo control. **, $p < 0.01$ v. WT Placebo; ***, $p < 0.001$ v. WT Placebo; ++, $p < 0.01$ v. GluR5 KO Placebo. Scale bar, 100 μ m.

Figure 4. Immunoreactivity of dynorphin A in spinal cord dorsal horn is increased by the deletion of GluR5 and by repeated morphine administration in Wildtype but not in GluR5 Knockout mice

(a) Immunofluorescence labeling of dynorphin (DYN-ir) in mouse spinal cord dorsal horn (SCDH). (b) Densitometry reveals that DYN-ir is increased significantly in placebo-implanted GluR5 Knockout (GluR5 KO) and morphine-treated Wildtype (WT) but not GluR5 KO SCDH on day 4 as compared with WT placebo. (PLAC = Placebo, MOR = Morphine). Normalized values of DYN-ir/ μm (mean $\pm$ SEM) measured in laminae I-III are expressed as percent of the WT Placebo control. **, $p < 0.01$ v. WT Placebo. Scale bar, 100 μm .

Figure 5. Immunoreactivity of calcitonin gene-related peptide in spinal cord dorsal horn is increased in Wildtype but is decreased in GluR5 Knockout mice by repeated morphine administration

(a) Immunofluorescence labeling of calcitonin gene-related peptide α (CGRP-ir) in mouse spinal cord dorsal horn (SCDH). (b) Densitometry reveals that CGRP-ir is increased significantly in placebo-implanted GluR5 Knockout (GluR5 KO) and morphine-treated Wildtype (WT) but is reduced in GluR5 KO SCDH on day 4 as compared with WT placebo. (PLAC = Placebo, MOR = Morphine). Normalized values of CGRP-ir/ μm (mean $\pm$ SEM) measured in laminae I-III are expressed as percent of the WT Placebo control. *, $p < 0.05$ v. WT Placebo; ***, $p < 0.001$ v. WT Placebo; ++, $p < 0.01$ v. GluR5 KO Placebo. Scale bar, 100 μm .

Figure 6. Immunoreactivity of substance P in spinal cord dorsal horn is unchanged by the deletion of GluR5 or by repeated morphine administration

(a) Immunofluorescence labeling of substance P (sP-ir) in mouse spinal cord dorsal horn (SCDH). (b) Densitometry reveals that sP-ir is unaltered in placebo-implanted GluR5 Knockout (GluR5 KO) and morphine-treated Wildtype (WT) and GluR5 KO SCDH on day 4 as compared with WT placebo. (PLAC = Placebo, MOR = Morphine). Normalized values of sP-ir/ μm (mean $\pm$ SEM) measured in laminae I-III are expressed as percent of the WT Placebo control. Scale bar, 100 μm .

Table 1
Total protein levels of ERK2 and P38 α / β MAP Kinases are unchanged in spinal cord dorsal horn by the deletion of GluR5 or by repeated morphine administration

Immunoblot analysis of spinal cord dorsal horn (SCDH) samples from Wildtype (WT) and GluR5 Knockout (GluR5 KO) mice (n = 6) on day 4 following s.c. implantation with placebo or 25 mg morphine pellets. Ratios of each protein to β -actin were calculated and normalized to the control group (WT Placebo). Data are expressed as a fold of control $\pm$ S.E.M.

Protein	WT Placebo	GluR5 KO Placebo	WT Morphine	GluR5 KO Morphine
ERK2	1.00 $\pm$ 0.12	1.12 $\pm$ 0.06	1.15 $\pm$ 0.11	1.03 $\pm$ 0.15
P38 α / β	1.00 $\pm$ 0.04	0.99 $\pm$ 0.03	0.99 $\pm$ 0.02	0.91 $\pm$ 0.05

Table 2
List of forward and reverse primer sets used for quantitative PCR in mouse spinal cord dorsal horn

Genes encoded are as follows: *Prkcc*, PKC γ ; *Tac1*, preprotachykinin A (precursor for sP); *Calca*, CGRP α ; *prodyn*, prodynorphin (precursor for DYN); *β -actin*, beta-actin.

Gene	Forward Primer (5' $\rightarrow$ 3')	Reverse Primer (5' $\rightarrow$ 3')	Amplicon length (bp)
<i>Prkcc</i>	CCTATCCCAAGTCACTTTCC	GAGGTGCAATTTCCAGTCTC	171
<i>Tac1</i>	AAGCGGGATGCTGATTCCTC	TCTTTCGTAGTTCTGCATTGCG	174
<i>Calca</i>	GAGGCTCTAGCTTGGACAG	AAGGTGTGAACTTGTTGAGGT	101
<i>prodyn</i>	GAGGTTGCTTTGGAAGAAGGC	TTTCCTCTGGGACGCTGGTAA	103
<i>β-actin</i>	GAAATCGTGCCTGACATCAAAG	TGTAGTTTCATGGATGCCACAG	216

Table 3
Steady-state levels of mRNAs encoding Protein Kinase C γ , Preprotachykinin A and Prodynorphin are unchanged in spinal cord dorsal horn by the deletion of GluR5 or by repeated morphine administration

Total RNA was extracted from spinal cord dorsal horn (SCDH) of Wildtype (WT) and GluR5 Knockout (GluR5 KO) mice (n = 6) on day 4 following s.c. implantation with placebo or 25 mg morphine pellets. Complementary DNAs (cDNAs) were synthesized from equal amounts of RNA and then subjected to qPCR using primers listed in Table 4.2 for *Prkcc* (PKC γ), *Tac1* (Preprotachykinin A/substance P) and *prodyn* (Prodynorphin). The target C_T for each sample was subtracted from that of β -actin and then normalized to control (WT Placebo) to obtain a $\Delta\Delta C_T$ value. $2^{-\Delta\Delta C_T}$ values were calculated and averaged for animals within each group, then expressed as a fold of control $\pm$ S.E.M.

Gene	WT Placebo	GluR5 KO Placebo	WT Morphine	GluR5 KO Morphine
<i>Prkcc</i>	1.03 $\pm$ 0.17	1.03 $\pm$ 0.05	0.94 $\pm$ 0.08	1.38 $\pm$ 0.21
<i>Tac1</i>	1.01 $\pm$ 0.19	1.13 $\pm$ 0.13	1.48 $\pm$ 0.21	1.55 $\pm$ 0.21
<i>prodyn</i>	1.03 $\pm$ 0.11	0.95 $\pm$ 0.12	1.04 $\pm$ 0.03	1.15 $\pm$ 0.09

Table 4
Summary of changes in expression of spinal cord dorsal horn proteins produced by deletion of GluR5 and/or morphine treatment

Symbols indicate the following directional differences: ↑, increase; ↓, decrease; =, no change. *, proteins with potentially selective involvement in morphine tolerance.

Protein	(-) GluR5 (v. WT PLAC)	(+) Morphine (v. WT PLAC)	(-) GluR5/(+) Morphine (v. GluR5 KO PLAC)
pERK2	↑	↑	↑
pP38α/β	↑	↑	↑
pPKCγ	↑	↑	↑
ERK2	=	=	=
P38α/β	=	=	=
PKCγ	↑	↑	↑
sP	=	=	=
DYN*	↑	↑	=
CGRP*	↑	↑	↓