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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 wild type (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 ERK2 (pERK2), P38 (pP38) and PKCgamma (pPKCγ) are elevated by deletion of GluR5. Additionally, chronic systemic morphine administration increased spinal pERK2, 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 ERK2, 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.

Key words: 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 Cγ (PKCγ) and the mitogen-activated protein kinases (MAPK) ERK1/2 and P38α/β in spinal cord dorsal horn (SCDH). For example, PKCγ 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 PKCγ 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 i.c.v. co-administration of a selective inhibitor of the receptor for activated C-kinase (RACK) binding PKCγ (Smith et al., 2007). PKCγ 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 P38α/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 pP38α/β (Cao et al., 2005, 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 P38α/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)

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Abbreviations: CGRP, calcitonin gene-related peptide α; DRG, dorsal root ganglion; DYN, dynorphin A-(1–17); ERK, extracellular signal-regulated kinase; GDNF, glial cell-line derived neurotrophic factor; GluR5 KO, GluR5 knockout; IB4, isolectin B4; i.t., intrathecal; KAR, kainate receptor; MAPK, mitogen activated protein kinase; MEK, MAPK kinase; MOP, mu opioid receptor; NGF, nerve growth factor; NK1, Neurokinin 1 receptor; NMDAR, N-methyl-D-aspartate receptor; pERK, phospho ERK; PKCγ, protein kinase Cγ; pP38, phospho P38; proDYN, prodynorphin; P38, P38 MAPK; p75NTR, p75 neurotrophin receptor; SCDH, spinal cord dorsal horn; sP, substance P; TrkA, neurotrophin tyrosine kinase receptor A; WT, wild type.

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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, 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, 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, 25–30 g) 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, USA) through the National Institute on Drug Abuse (Rockville, MD, USA). 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, USA) 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 min. 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, USA), which were subsequently blocked in 5% blotting grade milk (BioRad) overnight at 4 °C. Blots were incubated in primary antibodies generated in rabbit for phosphoP38α/β MAPK Thr180/Tyr182 at 1:250, phosphoERK1/2 MAPK Thr202/Tyr204 at 1:1000, phosphoPKC(pan)γThr514 at 1:1000, P38α/β at 1:500, ERK1/2 at 1:5000 (Cell Signaling Technology, Beverly, MA, USA), PKCγ at 1:20000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C, then incubated either with HRP-conjugated anti-rabbit secondary antibodies at 1:20000 (Pierce, Rockford, IL, USA) for 1 h 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 h at room temperature. Membranes were developed using SuperSignal West Pico ECL kit (Pierce) and exposed to film (Kodak, Rochester, NY, USA). Exposures yielding signal intensity in the linear range without saturation were used for densitometric analysis with Fluorchem 9900 (Alpha Innotech, San Leandro, CA, USA). 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.