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Prohormone convertase 2 (PC2) null mice have increased mu opioid receptor levels accompanied by altered morphine-induced antinociception, tolerance and dependence

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Abstract

Chronic morphine treatment increases the levels of prohormone convertase 2 (PC2) in brain regions involved in nociception, tolerance and dependence. Thus, we tested if PC2 null mice exhibit altered morphine-induced antinociception, tolerance and dependence. PC2 null mice and their wild-type controls were tested for baseline hot plate latency, injected with morphine (1.25 – 10 mg/kg) and tested for antinociception 30 min later. For tolerance studies, mice were tested in the hot plate test before and 30 min following morphine (5 mg/kg) on day 1. Mice then received an additional dose so that the final dose of morphine was 10 mg/kg on this day. On days 2–4, mice received additional doses of morphine (20, 40 and 80 mg/kg on days 1, 2, 3, and 4, respectively). On day 5, mice were tested in the hot plate test before and 30 min following morphine (5 mg/kg). For withdrawal studies, mice were treated with the escalating doses of morphine (10, 20, 40 and 80 mg/kg) for 4 days, implanted with a morphine pellet on day 5 and 3 days later with naloxone (1 mg/kg) and signs of withdrawal were recorded. Morphine dose- dependently induced antinociception and the magnitude of this response was greater in PC2 null mice. Tolerance to morphine was observed in wild-type mice and this phenomenon was blunted in PC2 null mice. Withdrawal signs were also reduced in PC2 null mice. Immunohistochemical studies showed up-regulation of the mu opioid receptor (MOP) protein expression in the periaqueductal grey area, ventral tegmental area, lateral hypothalamus, medial hypothalamus, nucleus accumbens, and somatosensory cortex in PC2 null mice. Likewise, naloxone specific binding was increased in the brains of these mice compared to their wild-type controls. The results suggest that the PC2-derived

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Conflict of Interest

The authors declare no conflict of interest.

List of Author contribution

TCF and KL contributed to the design of the experiments as well as in writing the manuscript. KL also analyzed and interpreted the data. TCF obtained funding for the project. DP conducted the analgesia assays; DLL and MGF conducted the immunohistochemical studies; AH did the breeding and genotyping. YL supervised and contributed to some of these experiments.

peptides may play a functional role in morphine-induced antinociception, tolerance and dependence. Alternatively, the lack of opioid peptides led to up- regulation of the MOP and altered morphine-induced antinociception, tolerance and dependence.

More than two decades ago, a new family of prohormone processing enzymes, termed prohormone convertases (PCs), was identified (Seidah et al., 1990, Smeekens and Steiner, 1990, Seidah et al., 1991, Smeekens et al., 1991). Two members of this family, PC1/3 and PC2, were found in neural and endocrine cells equipped with a regulatory secretory pathway (Seidah et al., 1990, Smeekens and Steiner, 1990, Smeekens et al., 1991). In the brain, both PC1/3 and PC2 are present, while in the pituitary of rodents, PC1/3 appears to be present in both the anterior and neurointermediate lobes, PC2 is found predominantly in the neurointermediate lobe (Birch et al., 1991, Hakes et al., 1991, Seidah et al., 1991). In general, PC1/3 makes the initial cleavage of prohormones followed by peptide cleavage by PC2 to generate smaller peptide intermediates (Johanning et al., 1996, Johanning et al., 1998). After this step, peptide intermediates with basic C-terminal residues are further processed by carboxypeptidase E and at times need to undergo other post-translational modifications to generate biological active peptides (Fricker, 1988). In other words, PC2 is a key enzyme in the biosynthesis of endogenous opioid peptides such as β-endorphin, dynorphins, enkephalins and the related peptide, orphanin FQ/nociceptin (OFQ/N) (Dupuy et al., 1994, Day et al., 1998, Allen et al., 2001).

We have previously shown that acute morphine treatment down-regulated; whereas, chronic morphine treatment up-regulated the expression of PC1/3 and PC2 in the hypothalamus and other brain regions (Espinosa et al., 2008). This is because expression of PC2 and PC1/3 enzymes is dependent on the presence of a cyclic adenosine monophosphate (AMP) response element (CRE) in their promoter region (Jansen et al., 1995, Lamas et al., 1997) and opioids are known to regulate phosphorylation of cyclic-AMP dependent regulatory element binding protein (P-CREB) level with acute opioids down-regulating P-CREB (Duman et al., 1988, Guitart et al., 1992) and chronic opioid administration increasing the levels of P-CREB (Lane-Ladd et al., 1997, Nestler and Aghajanian, 1997) in brain regions thought to be important in opioid addiction. Given the relationships between opioids and PC2 expression and potentially PC2- derived peptides, we hypothesized that the altered biosynthesis of endogenous opioids in PC2 null mice would lead to alterations in some of the responses to morphine administration. PC2 null mice (Furuta et al., 1997) represent an ideal model to test this hypothesis as these mice have reduced levels of mature Met-enk-Arg-Phe, Leu-enk and Met-enk and elevated levels of enkephalin precursors (Johanning et al., 1998). PC2 null mice also have a complete absence of Dyn A-8 and a substantial reduction of Dyn B-13 (Berman et al., 2000). Allen and colleagues (Allen et al., 2001) reported increased hypothalamic levels of β-endorphin$^{1–31}$ in PC2 null mice. They explained these surprising results as being due to the ability of PC1/3 to generate β-endorphin$^{1–31}$ with decreased PC2-mediated endoproteolytic cleavage following Lys^{29} of this peptide. The amount of unprocessed proopiomelanocortin (POMC) in the hypothalamus was unchanged (Allen et al., 2001), although it was increased in the pituitary of PC2 null mice (Allen et al., 1999). However, in their neurointermediate lobe of the pituitary, there was greatly reduced β-endorphin and α-MSH formation (Allen et al., 1999). In contrast, Laurent and colleagues

have reported almost no β-endorphin in the pituitary of these mice using both RIA and immunoprecipitation techniques (Laurent et al., 2004). Orphanin FQ/Nociceptin (OFQ/N) production has also been found to be significantly lower in PC2 deficient mice (Allen et al., 2001). Interestingly, OFQ/N has been implicated in tolerance induced by repeated microinjections of morphine into ventrolateral periaqueductal gray in rats (Ge et al., 2007). Furthermore, endogenous enkephalins have been linked to analgesic tolerance to morphine (Nitsche et al., 2002, Marquez et al., 2006). PC2 null mice were significantly less responsive to nociceptive stimuli particularly in the hot plate test following a short forced swim in warm water than wild-type mice (Croissandeau et al., 2006), suggesting that opioid-mediated stress-induced antinociception was enhanced in PC2 null mice compared to their wild-type controls. We hypothesized that antinociception induced by morphine would be enhanced in mice lacking PC2 due to lower levels of pronociceptive peptides (Cesselin, 1995, Mogil et al., 1996b). We also proposed that reduced levels of endogenous opioids in PC2 null mice would lead to increased expression of mu opioid receptor (MOP), leading to greater morphine-induced antinociception. Thus, using PC2 null mice and their wild-type littermates/controls, we examined whether morphine-induced antinociception, tolerance, dependence as well as specific binding of [^{3}H]-naloxone in the whole brain homogenate and expression of MOP in key brain regions related to these processes would be altered in mice lacking PC2 compared to their wild-type controls.

Experimental Procedures

Animals

Male (2–6 months old) PC2 null mice and their wild-type littermates were housed 2 to 4 per cage with free access to food and water in a temperature and humidity controlled room on a 12-h light/12-h dark cycle. Mice were habituated to the room for 1 h prior to any testing. All experiments were conducted in accordance with the ethical guidelines of the National Institute of Health and approved by the Institutional Animal Care and Use Committee at Western University of Health Sciences (Pomona, CA, USA) and Charles R. Drew University of Medicine and Science (Los Angeles, CA, USA). All observations were made during the light cycle.

Breeding and Screening of PC2 knockout mice

The original breeding pairs on a mixed C57BL/6 and 129SV background were generously supplied by Dr. Donald Steiner of the University of Chicago. These mice were successfully backcrossed (3–7 generations) onto a C57BL/6 background. Pups obtained from mating male and female heterozygous breeding pairs were screened by PCR using a disrupted PC2 gene as a marker (Furuta et al., 1997) on DNA prepared from an ear piece biopsy and utilized two sets of primers. One primer set generated a 117-bp product indicative of the PC2 wild-type allele and the other primer set generated a 180-bp mutant product that includes exon 3 and the neomycin resistant cassette (Furuta et al., 1997) indicative of PC2 KO mice.

The role of PC2-derived peptides in morphine-induced antinociception

To determine the role of PC2-derived peptides in morphine-induced antinociception, PC2 null mice and their wild-type littermates were tested for hot plate latency using the Socrel Model DS37 hot plate machine (Ugo Basile, Italy). On the test day, the machine reached the desired temperature (52°C) and allowed to stabilize for 5 min. Each mouse was placed in the middle of a Plexiglas cylinder (7.6 cm height) placed on the top of the hot plate (52°C) and the amount of time that elapsed for each mouse to vigorously shake its hindpaw was recorded. Mice were then injected subcutaneously (s.c.) with morphine (1.25, 2.5, 5.0 or 10 mg/kg; n = 6–20 mice per dose per genotype) and tested again on the hot plate 30 min later. A cutoff time of 40 sec was used to prevent tissue damage.

The role of PC2-derived peptides in antinociceptive tolerance to morphine

PC2 null mice and their wild-type littermates (n = 9–13 mice per genotype) were tested for antinociception following a single dose of morphine (5 mg/kg, s.c.) on day 1, as described above. The choice of the dose was based on the results of Experiment 1. Following the hot plate measurement, mice received an additional dose of morphine (5 mg/kg) so that the sum of the two doses was 10 mg/kg for each mouse on this day (day 1). Mice were then injected twice daily (morning and evening) with 10, 20 and 40 mg/kg doses of morphine on days 2, 3 and 4, respectively. Thus, the total dose of morphine was 10, 20, 40 and 80 mg/kg on days 1, 2, 3, and 4. On day 5, mice were tested for antinociception induced by the same dose of morphine (5 mg/kg, s.c.) used on day 1.

The role of PC2-derived peptides in naloxone-precipitated withdrawal

Mice (n = 5–7 mice per genotype) were treated with escalating doses of morphine for 4 days, as described in Experiment 2. On day 5, mice were implanted with a 25-mg morphine pellet in the nape of the neck and left in place for three days. Mice were then tested for signs of naloxone- precipitated withdrawal. Mice were brought to the room and habituated for 1 h. Mice were then placed in clear 2.8 L plastic beakers and allowed to habituate to the test chamber for 30 min. Mice were then weighed, injected with naloxone (1 mg/kg) and the number of animals having diarrhea, showing piloerection and/ or jumping was recorded. The number of jumps and the latency to jump was also determined.

The role of PC2-derived peptides on the level of mu opioid receptors

PC2 null and wild-type littermates/age-matched controls (n = 4 mice per genotype) were anesthetized with pentobarbital (100 mg/kg, i.p.; Sigma/Aldrich, St. Louis, MO, USA) and then transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Brains were carefully removed and post-fixed in 4% paraformaldehyde overnight at 4°C, immersed in 25% sucrose until the tissues sunk and then frozen on dry ice. Sections (20 μM thick) of the periaqueductal gray, nucleus accumbens, ventral tegmental area, lateral hypothalamus, and somatosensory cortex, brain areas implicated in antinociception, tolerance and dependence, were cut and processed for immunohistochemical studies. Sections were washed in PBS, quenched in 3% hydrogen peroxide for endogenous peroxidase activity, washed in PBS, blocked in PBS containing 10% normal goat serum and 0.15% Triton X-100, and incubated with the MOP primary

polyclonal antibody (1:500) (Neuromics, Edina, MN) in a humidification chamber (4°C) for overnight. The specificity of the antibody has been described by Chen and Pan (2006) who omitted the primary antibody (which we also did in the present study) and performed pre-absorption of the antibody by the blocking peptide (P10104) and found no immunostained neurons in the dorsal root ganglia or spinal cord. Sections were then incubated for 40 min with a biotinylated goat anti-rabbit secondary antibody (1:200) (Vector Laboratories, Burlingame, CA, USA). After washes in PBS, samples were incubated for 30 min with ABC peroxidase complex (Vector Laboratories) followed by 3,3′-diaminobenzidine/H_2O_2 as a chromogen. Finally, sections were dehydrated, coverslipped and viewed under the microscope (Olympus, Japan). Negative controls were processed in an identical manner except the primary antibody was substituted with the rabbit IgG. Each slide analyzed contained a negative control. Slides were visualized at 64X using a bright field microscope (Olympus, Japan) and analyzed by Image Pro® imaging software by counting the number of MOP-immunopositive cells per field in each of the 6 anatomical brain regions analyzed.

Binding assay

The binding assay was a modification of our earlier report (Lutfy and Yoburn, 1991). Briefly, mice lacking PC2 and their wild-type littermates/controls (n = 3 mice per genotype) were deeply anesthetized with isoflurane and euthanized. The whole brain excluding the cerebellum was removed and homogenized in Tris buffer (50 mM, pH = 7.4). The homogenates were then centrifuged at 18,000g for 15 min. This procedure was repeated twice. Following the second spin, the supernatant was discarded, the pellet resuspended in 40 volumes of the buffer, incubated at 25°C for 30 min and then spun for 15 min. The supernatant was discarded, the pellet resuspended in 40 volumes of buffer and assayed in triplicates for [^{3}H]-naloxone (1nM) binding in the presence and absence of non-labeled naloxone (1000 nM) to define the non-specific and total binding, respectively. Specific binding was defined as total minus non-specific binding.

Data Analysis

Data represent mean (±SEM) of the hot plate latency or percent maximum possible effect (% MPE) calculated as (Test latency – baseline latency) / (Cut-off – baseline latency) X 100. A two- factor analysis of variance (ANOVA) or repeated measure ANOVA was used to analyze the data. The *post-hoc* Bonferroni test was used to reveal significant differences between various groups. Comparison of naloxone-precipitated signs of withdrawal (number of jumps, latency to jump and percent of animals showing piloerection or diarrhea), MOP-immunopositive cells and specific [^{3}H]-naloxone binding between the wild-type and PC2 null mice were made using the unpaired Student’s t test. $p<0.05$ was considered statistically significant.

Results

The role of PC2-derived peptide in morphine-induced antinociception

Figure 1A illustrates percent maximum possible effect (% MPE) as a function of morphine’s dose (1.25, 2.5, 5.0 or 10.0 mg/kg, s.c.) in PC2 null mice [PC2 (−/−)] and their wild-type littermates/age-matched controls [PC2 (+/+)]. A two-way ANOVA revealed a significant

effect of morphine dose ($F_{3,73}$ = 22.9; p<0.001) and a significant effect of genotype ($F_{1,73}$ = 14.0; p<0.0001), but not a significant interaction between the two factors ($F_{3,73}$ = 0.14; p>0.05), showing that morphine dose-dependently induced antinociception in both wild-type and PC2 null mice. Post-hoc testing indicated that the magnitude of this response was greater in null mice compared to their wild-type littermates/age-matched controls, in particular at low doses of morphine (p<0.05).

The role of PC2-derived peptide in morphine tolerance

Figure 1B shows the antinociceptive effect of morphine (5 mg/kg, s.c.) in PC2 null mice and their wild-type littermates/controls on day 1 (D1) and day 5 (D5). A two-way repeated measures ANOVA revealed a significant effect of test day ($F_{1,40}$ = 22.8; p<0.0001), a significant effect of genotype ($F_{1,40}$ = 22.2; p<0.001) and a significant interaction between the two factors ($F_{1,40}$ = 5.71; p<0.01). Post-hoc testing showed that morphine-induced antinociception was reduced in wild-type mice, i.e., the %MPE was significantly lower on day 5 compared to day 1 (p<0.05), showing that tolerance developed to the antinociceptive effect of morphine. On the other hand, the %MPE was comparable on days 1 and 5 in PC2 null mice, suggesting that tolerance was blunted in these mice (Fig. 1B).

The role of PC2-derived peptide in naloxone-precipitated withdrawal

A challenge dose of naloxone (10 mg/kg) induced signs of withdrawal in mice of both genotypes pretreated with morphine, showing that both groups became dependent on morphine (Fig. 2). However, the magnitude of withdrawal signs was reduced in PC2 null mice, as evidenced by a longer latency to jump (Fig. 2A), decreased number of jumps (Fig. 2B), as well as reduced percentage of animals having diarrhea (Fig. 2C) or exhibiting piloerection (Fig. 2D) in null mice compared to their wild-type littermates/age-matched controls (p<0.05).

The role of PC2-derived peptides on the level of mu opioid receptors

Immunohistochemical studies revealed MOP-immunopositive cells in all the brain regions tested in mice lacking PC2 and their wild-type littermates/controls. The number of MOP-positive cells was significantly greater in all brain regions tested, with the highest level in the medial hypothalamic (MHT) region in PC2 null compared to their wild-type littermates/controls (Fig. 3). The staining was primarily over the cytoplasm. There were no detectable MOP- immunopositive cells in slices which were not exposed to the primary antibody (data not shown).

The role of PC2-derived peptides on the [3H]-naloxone specific binding sites

We also determined the level of [^{3}H]-naloxone specific binding in whole brain homogenate of mice lacking PC2 and their wild-type littermates/controls (Fig. 4). The result showed higher levels of specific [^{3}H]-naloxone binding sites in the brains of mice lacking the PC2 enzymes compared to their wild-type controls (t = 3.15, df = 4; p<0.05).

Discussion

The main finding of the present study is that PC2 null mice exhibited enhanced morphine-induced antinociception. Furthermore, these mice expressed blunted morphine tolerance and reduced signs of naloxone-precipitated withdrawal. Mice lacking PC2 had more MOP-immunopositive cells in crucial brain regions involved in drug reward/addiction compared to their wild-type controls. Together, the current results suggest that the absence of PC2 leads to MOP up-regulation and enhanced morphine-induced antinociception and reduced tolerance and dependence. Our results of decreased naloxone-induced withdrawal in PC2 mice are in agreement with Cpe fat/fat mice requiring higher doses of morphine to induce the same amount of withdrawal compared to wild-type controls (Decaillot et al., 2006). Our results in PC2 null mice are subject to the general caveat of null mice that changes may have occurred during development and do not necessarily reflect changes in the adult brain. Future experiments injecting PC2 siRNA or related approaches to reduce the expression of PC2 into different brain regions could address this research question.

We have previously shown that long-term morphine treatment increases the level of PC2 enzymes (Espinosa et al., 2008), raising the possibility that PC2-derived peptides may contribute to the action of morphine. PC2 null species have lowered levels of OFQ/N (Allen et al., 2001), CCK-8 (Vishnuvardhan et al., 2000) and FMRF-amide (Husson et al., 2006). These peptides are pronociceptive (Cesselin, 1995, Mogil et al., 1996a) and have been implicated in morphine tolerance (Wiertelak et al., 1992), and may explain the increase in morphine-induced antinociception and reduced antinociceptive tolerance in PC2 null mice reported in the present study. Interestingly, mice lacking PC2 exhibit enhanced opioid-mediated stress-induced antinociception (Croissandeau et al., 2006). A parsimonious explanation for the higher antinociceptive effect of morphine in PC2 null mice is the increased level of potent endogenous opioid β-endorphin$^{1–31}$ (Allen et al., 2001). Alternatively, the level of opioid receptor may be increased in these mice due to decreased levels of some of the opioid peptides, thereby leading to functional supersensitivity, as shown to previously (Yoburn et al., 1989, Lutfy and Yoburn, 1991). Consistent with this hypothesis, our immunohistochemical studies revealed that the level of MOP-immunopositive cells was up-regulated in the PAG, which is a key brain region mediating the antinociceptive effect of morphine (Noble and Cox, 1996) as well as other regions involved in processes related to drug addiction. We also observed increased levels of specific binding of [^{3}H]-naloxone in the brains of mice lacking PC2 compared to their wild-type controls, corroborating our immunostaining results and showing that the level of brain opioid receptors is increased in these mice.

Pan et al (Pan et al., 2006) used a quantitative peptidomics approach to characterize hypothalamic peptide levels in PC2 null mice, while Zhang et al. (Zhang et al., 2010) expanded this study to characterize peptide levels in several brain regions in these mice. Several peptides derived from prodynorphin, procholecystokinin, proneurotensin, proenkephaln, pro-OFQ/N, pro- neuropeptide Y and POMC were altered in different brain regions of PC2 null mice that could explain our findings of greater antinociception, decreased tolerance and decreased signs of naloxone-precipitated withdrawal in these mice. Interestingly, chronic morphine treatment has been shown to increase the level of pro-

nociceptive peptides (Yuan et al., 1999). Additionally, OFQ/N has been shown to be involved in tolerance induced by repeated microinjections of morphine into ventrolateral periaqueductal gray in rats (Ge et al., 2007). Likewise, endogenous enkephalins have also been implicated in analgesic tolerance to morphine using tail flick assay (Nitsche et al., 2002, Marquez et al., 2006). Thus, one can expect that the magnitude of tolerance to be reduced in mice lacking PC2 since the level of these peptides are reduced in the brain extracts of PC2 null mice (Allen et al., 2001). Interestingly, we also observed reduced signs of naloxone-precipitated withdrawal in PC2 null mice compared to their wild-type controls, showing that PC2-derived peptides may also play a functional role in the processes leading to morphine dependence. It is noteworthy that the level of PC2 enzyme is increased following repeated morphine treatment that leads to tolerance to and dependence on morphine (Espinosa et al., 2008). Thus, the increase in PC2 expression following repeated morphine treatment may be a mechanism through which morphine may induce tolerance and dependence. However, further studies are needed to test this possibility.

Conclusions

In summary, the current study reports that PC2 null mice had increased number of mu opioid receptors in different brain areas. These mice also exhibited enhanced morphine-induced antinociception but reduced tolerance to and dependence on morphine.

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Abbreviation

PC2	prohormone convertase 2
MOP	mu opioid receptor

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- Prohormone convertase 2 (PC2) processes prohormones to extended opioids.
- Chronic morphine treatment increases the expression of PC2.
- PC2 null mice had higher brain levels of mu opioid receptors.
- PC2 null mice exhibited enhanced morphine-induced antinociception.
- Morphine tolerance and naloxone-precipitated withdrawal were attenuated in these mice.

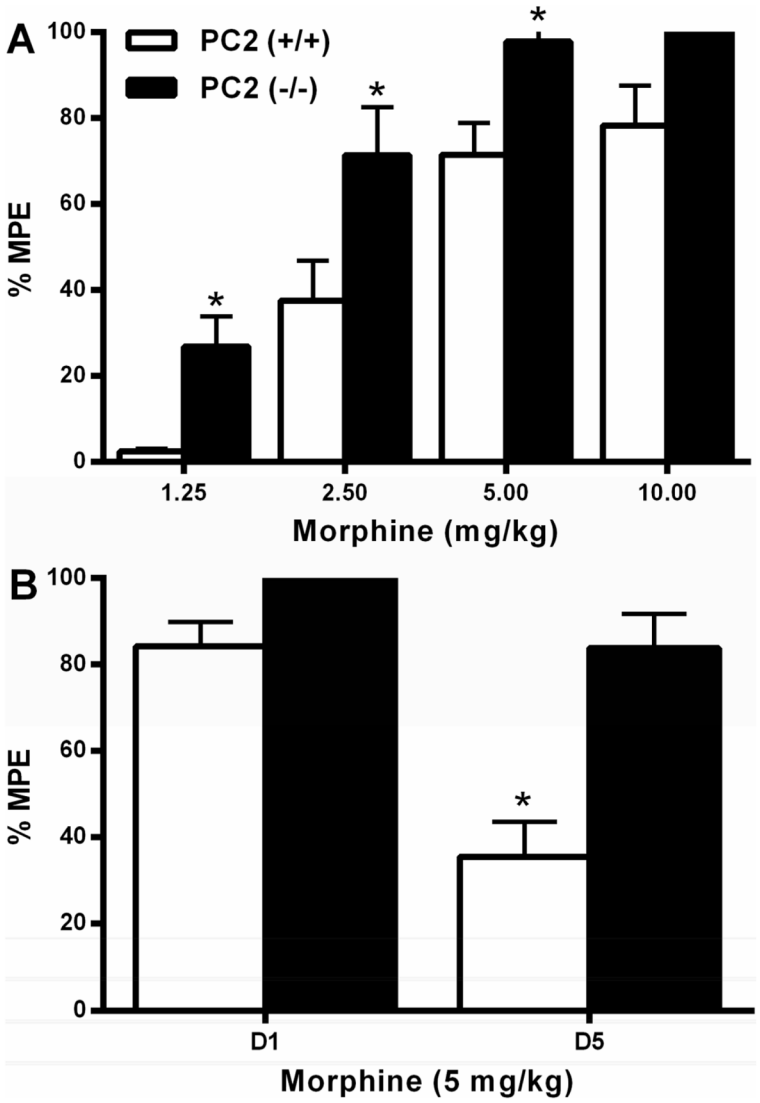

Fig. 1.

Morphine-induced antinociception (A) and tolerance (B) in PC2 null (−/−)] compared to their wild-type [PC2 (+/+)] littermates/controls (n = 6–20 mice per genotype for each dose and each time point). %MPE denotes percent maximum possible effect; *p<0.05 vs. PC2 (+/+) mice

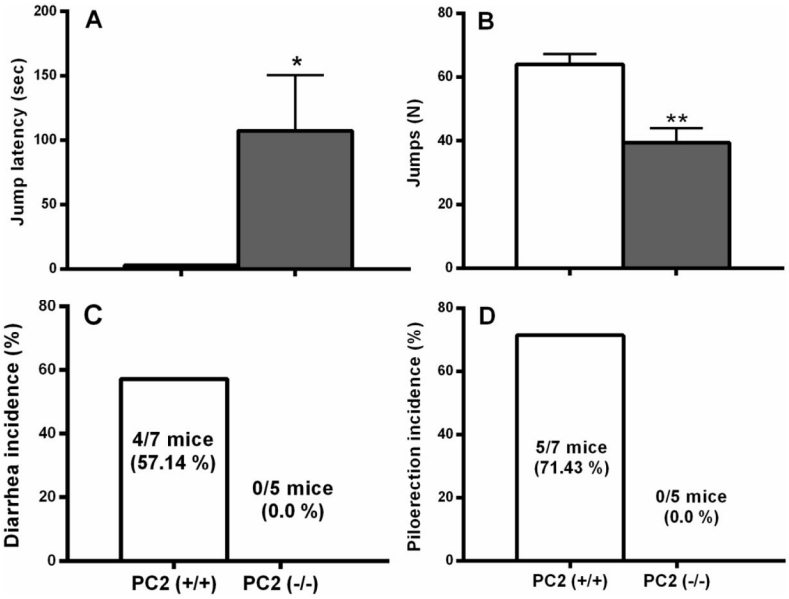

Fig. 2.

Signs of naloxone-precipitated withdrawal in PC2 null mice and their wild-type controls (n = 5–7 mice per genotype). Data represent mean ±S.E.M. of latency to jump (A), number of jumps, percent of animals with diarrhea (C) and percent of mice exhibiting piloerection (D) recorded for 30 min following a challenge dose of naloxone (10 mg/kg, s.c.). **p<0.01, significantly different from the control group

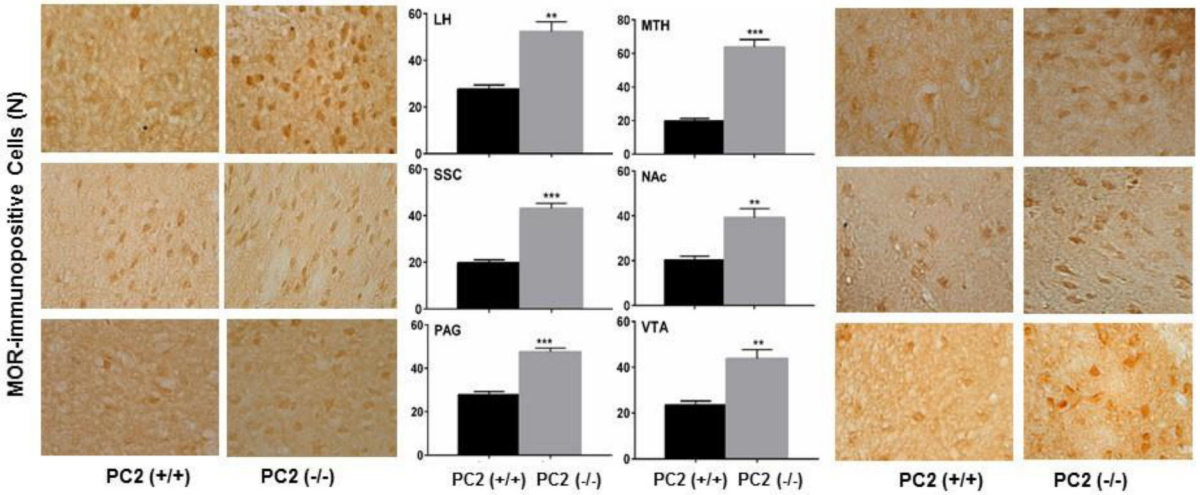

Fig. 3.

The number of MOP-immuno-positive cells in medial hypothalamus (MHT), nucleus accumbens (NAc, ventral tegmental area (VTA), lateral hypothalamus (LH), somatosensory cortex (SSC) and periaqueductal gray (PAG) of PC2 null mice and their wild-type controls (n = 4 mice per genotype). ***p<0.001, significantly different vs. all other measurements.

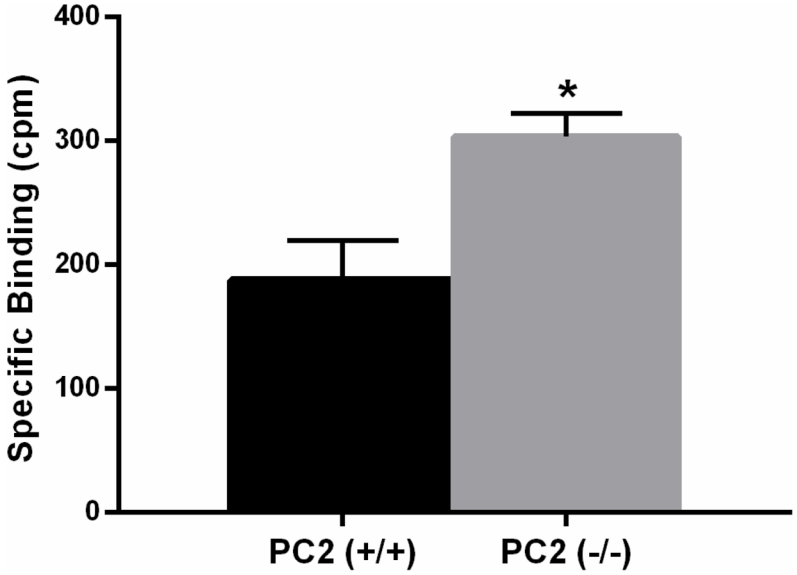

Fig. 4.

Specific binding of [^{3}H]-naloxone (cpm) in whole brain homogenates of mice lacking PC2 and their wild-type controls (n = 3 mice per genotype). *p<0.05, significantly different vs. the wild-type controls.