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The prolactin-releasing peptide antagonizes the opioid system through its receptor GPR10

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Prolactin-releasing peptide (PrRP) and its receptor G protein–coupled receptor 10 (GPR10) are expressed in brain areas involved in the processing of nociceptive signals. We investigated the role of this new neuropeptidergic system in GPR10-knockout mice. These mice had higher nociceptive thresholds and stronger stress-induced analgesia than wild-type mice, differences that were suppressed by naloxone treatment. In addition, potentiation of morphine-induced antinociception and reduction of morphine tolerance were observed in mutants. Intracerebroventricular administration of PrRP in wild-type mice promoted hyperalgesia and reversed morphine-induced antinociception. PrRP administration had no effect on GPR10-mutant mice, showing that its effects are mediated by GPR10. Anti-opioid effects of neuropeptide FF were found to require a functional PrRP-GPR10 system. Finally, GPR10 deficiency enhanced the acquisition of morphine-induced conditioned place preference and decreased the severity of naloxone-precipitated morphine withdrawal syndrome. Altogether, our data identify the PrRP-GPR10 system as a new and potent negative modulator of the opioid system.

Opiate drugs, the prototype of which is morphine, are used largely for the treatment of severe pain. However, the prolonged use of opiate drugs induces a behavioral adaptation that results in the development of tolerance and dependence[1]. Although these adaptive mechanisms have been known for decades, the underlying pathophysiological pathways have not been fully clarified. It has been proposed that the opioid receptor signaling pathway is adaptively regulated at the cellular level, although more recently a growing contribution of the plasticity of neuronal networks involving opioidergic neurons has been recognized. In support of this latter hypothesis, a number of neuropeptides, including cholecystokinin (CCK)[2], neuropeptide FF (NPFF)[3] and nociceptin (orphanin FQ)[4], have been proposed as modulators of the opioid system. These various peptidergic pathways are collectively designated as an anti-opioid system. However, the action of individual peptides on the opioid pathway and on the processing of nociceptive signals is complex and some aspects remain controversial, particularly as the behavioral consequences can vary depending on the injection site of these peptides and the precise experimental setup[5,6].

Among these peptides, NPFF, which belongs to the RF-amide peptide family, was shown (among other actions) to regulate blood pressure[7], prolactin release[8] and nociceptive signal processing[9]. Prolactin-releasing peptide (PrRP) has recently been identified as an additional member of the mammalian RF-amide peptide family, following its isolation as the natural agonist of the previously orphan GPR10 (ref. 10) (also known as hGR3 in human or UHR-1 in rat). Intracerebroventricular (i.c.v.) administration of PrRP affects feeding behavior[11], blood pressure[12] and neuroendocrine processes, such as corticotropin-releasing hormone (CRH)[13] and oxytocin[14] release.

In addition, recent observations suggest that PrRP and GPR10 are involved in the processing of nociceptive information *in vivo*. Indeed, PrRP and GPR10 expression is restricted to a limited number of structures in the rat central nervous system known to be involved in the processing of nociceptive signals[15,16]. These include the hypothalamus, amygdala and brainstem areas. In the same line, the coexpression of PrRP and proenkephalin has been demonstrated in amygdala neurons[17], and the activity of PrRP as a modulator of pain and allodynia has recently been described in rats[18]. In the present study, we generated *Gpr10*-deficient mice in order to determine the interaction of PrRP and its receptor GPR10 with the opioid system *in vivo*. Our observations identify the PrRP-GPR10 system as a new and potent negative modulator of the opioid system.

RESULTS

Knockout of GPR10 in mice

In our targeting vector, part of the *Gpr10* coding region was replaced by a tau-lacZ fusion gene (placed under control of the natural *Gpr10* promoter) and selection cassettes (**Fig. 1a–c**). After the generation of the knockout mice, *Gpr10* transcripts were amplified from brain by reverse transcription polymerase chain reaction (RT-PCR). As expected, no amplification occurred in knockout mice (**Fig. 1d**). *Gpr10*-knockout mice were fertile, transmitted the null allele with the expected mendelian frequency and did not show obvious morphological abnormalities.

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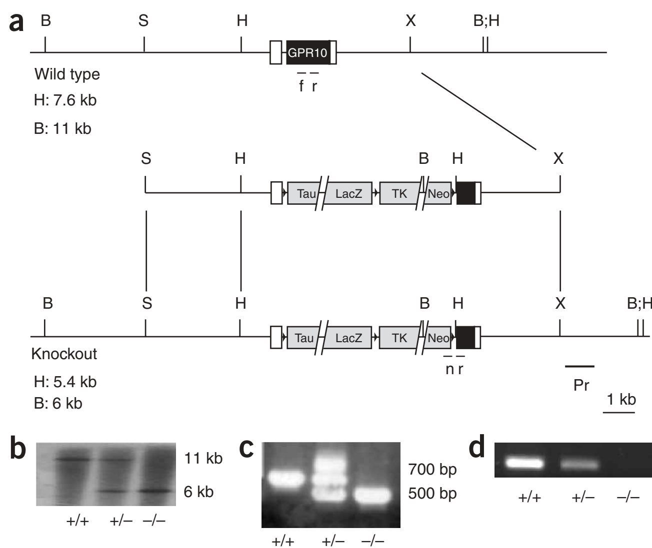

Figure 1 GPR10 gene targeting. (**a**) The structures of the wild-type *Gpr10* locus (wild type), the targeting vector and the locus resulting from homologous recombination (knockout) are shown. The location of the restriction sites—*Bam*HI (B), *Sac*I (S), *Xho*I (X) and *Hin*dIII (H)—of the probe used in Southern blotting experiments (Pr), and of the forward (f), reverse (r) and *Neo* (n) primers used for the PCR genotyping, are indicated. The size of the bands expected after *Hin*dIII digestion and hybridization with the probe are 7.6 kb and 5.4 kb for the wild-type and knockout alleles, respectively. After *Bam*HI digestion, the expected sizes are, respectively, 11.0 kb and 6.0 kb. (**b**) Southern blot illustrating the genotyping of wild-type (+/+), heterozygous (+/–) or knockout (–/–) mice, following digestion of genomic DNA by *Bam*HI. (**c**) Genotyping of mice by PCR using a three primers procedure. (**d**) Detection of GPR10 transcripts by RT-PCR in total RNA prepared from whole brain.

Hypothalamus pituitary adrenal axis

PrRP has been found to control the secretion of adrenocorticotropic hormone (ACTH), through corticotropin releasing hormone (CRH), and a number of stressful conditions have been reported to activate PrRP-containing neurons in rats, indicating a likely role in the control of the stress axis[13]. We therefore assessed the glucocorticoid levels in plasma, both under basal conditions and following a hypoglycemic stress, in wild-type and knockout mice. The basal corticosterone levels measured at 8 a.m. and at 4 p.m. were slightly lower in knockout mice, and the difference became highly significant following insulin-induced hypoglycemia (**Fig. 2a**). We measured CRH transcripts hypothalamus using quantitative RT-PCR. We did not observe significant differences in basal conditions, but when the mice were subjected to insulin-induced hypoglycemia, CRH transcripts were less abundant in knockout mice than in wild-type mice (**Fig. 2b**).

Behavioral characterization

In a first set of experiments, we explored anxiety-related behaviors and locomotor activity for both genotypes. The elevated plus maze and the light and dark box protocols were used to evaluate the anxiety-related responses. In the elevated plus maze test, the time spent (wild type: 35.2 ± 2.1 s; knockout: 38.5 ± 2.1 s, $n = 17$–20) and the number of entries (wild type: 17.8 ± 1.0; knockout: 14.7 ± 1.0; $n = 17$–20) in the open arms were not significantly different between genotypes ($P = 0.28$, $P = 0.051$, respectively). Similarly, in the light and dark box test, no difference was observed for the latency of the first entry (wild type: 16.9 ± 1.9 s; knockout: 14 ± 1.9 s, $n = 17$–18) or the total time spent in the lit compartment (wild type: 100.7 ± 5.2 s; knockout: 104.5 ± 3.9 s, $n = 17$–18). Motor coordination was not affected in knockout mice as evidenced by the rotarod test (wild type: 294 ± 22 s; knockout: 281 ± 23 s, $n = 15$). In actimetry boxes, the spontaneous locomotor activity (wild type: 457 ± 26 counts; knockout: 453 ± 32 counts, $n = 30$) and activity of mice administered morphine (10 mg kg^{-1}) were similar for both genotypes (wild type: 402 ± 22 counts; knockout: 396 ± 39 counts, $n = 10$) and were significantly greater than for mice administered saline (wild type: 221 ± 36 counts, $P < 0.001$; knockout: 229 ± 24 counts, $P < 0.0001$, $n = 10$).

In a second set of experiments, we measured the spontaneous thermal nociceptive threshold in wild-type and knockout mice using two tests reported to explore stimulus integration preferentially at spinal (tail-immersion) or supraspinal (hot-plate) levels. In the tail-immersion test, we observed similar nociceptive thresholds for both genotypes (**Fig. 3a**). In the hot-plate test (52 °C), we observed increased jump latency in knockout mice as compared to wild-type mice (**Fig. 3b**). Similar data were obtained when the hot-plate was set to 48 °C, 50 °C and 54 °C (data not shown), whereas no difference was seen at 56 °C. Corticosteroid supplementation did not reverse the modified nociceptive threshold of the knockout mice (**Supplementary Fig. 1** online). Administration of the opioid receptor antagonist naloxone (1 mg kg^{-1}, intraperitoneally (i.p.)) fully reversed the relative analgesia observed in knockout mice in the hot-plate test, but did not affect the nociceptive threshold of wild-type animals. Notably, naloxone administration also decreased the nociceptive threshold of knockout mice in the tail-immersion test below that of treated or untreated controls (**Fig. 3a**).

We then investigated the antinociceptive effects of morphine in the tail-immersion (52 °C) and hot-plate (56 °C) tests, conditions in which the basal nociceptive threshold is identical for both genotypes (**Fig. 3c,d**). As expected, morphine induced a dose-dependent antinociception in both genotypes, but this effect was significantly enhanced in knockout mice for the 10 mg kg^{-1} (i.p.) dose. To complement these observations, we also investigated mechanical and

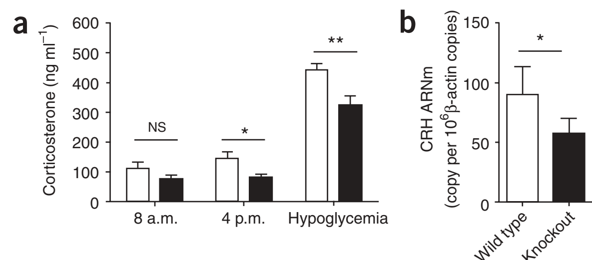

Figure 2 Activity of the hypothalamus-pituitary-adrenal (HPA) axis. (**a**) Corticosterone levels were measured in wild-type (open bars) and knockout (black bars) animals in resting conditions at 8 a.m. and 4 p.m., and during insulin-induced hypoglycemia. $n = 5$–13 depending on group. (**b**) Abundance of *CRH*-encoding transcripts, as determined by quantitative RT-PCR, in the hypothalamus of wild-type (open bar) and knockout (black bar) animals subjected to insulin-induced hypoglycemic stress. $n = 19$ or 20 animals depending on group. Values represent the mean ± s.e.m. $*P < 0.05$; $**P < 0.01$. Statistical significance was determined using the Student's *t*-test.

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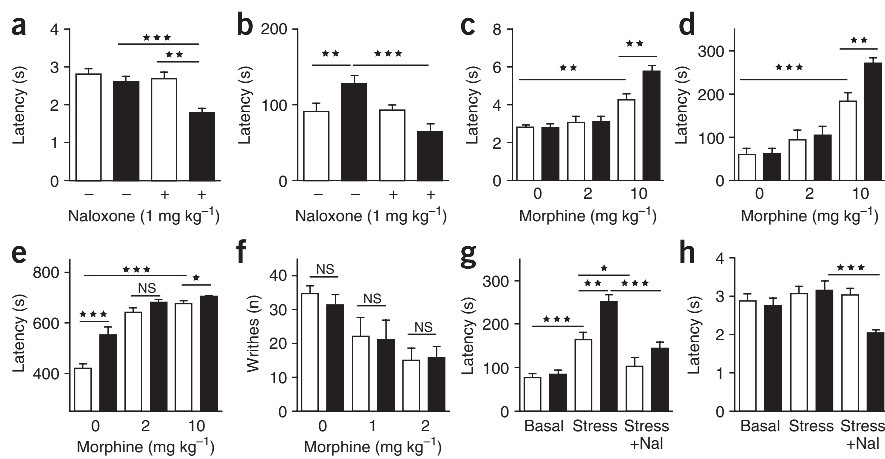

Figure 3 Nociceptive thresholds in GPR10-knockout mice. (**a–h**) The nociceptive thresholds were measured in the tail-immersion test (**a**,**c**,**g**), the hot-plate test (jumping response: **b**,**d**,**h**), the tail-pressure test (**e**) and the writhing test (**f**) on wild-type (open bars) and knockout (black bars) animals. (**a**,**b**) Basal nociceptive threshold and effect of naloxone treatment. (**c**,**d**) Antinociceptive response to morphine (2 mg kg^{-1} and 10 mg kg^{-1} i.p.).(**e**) Nociceptive threshold in the tail-pressure test and response to morphine analgesia (2 mg kg^{-1} and 10 mg kg^{-1} i.p.) (**f**) Number of writhes in the writhing test and response to morphine analgesia (1 mg kg^{-1} and 2 mg kg^{-1} i.p.). (**g**,**h**) Nociceptive threshold after a 5-min forced swim stress (water temperature: 32 ± 1 °C, 5 min) and effect of naloxone treatment. Values represent the mean ± s.e.m. $n = 7$–15 animals depending on group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (ANOVA followed by Dunnett *t*-test).

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visceral pain paradigms, using the tail-pressure and writhing tests, respectively (**Fig. 3e,f**). In the tail-pressure test, we observed a relative analgesia for knockout mice as compared to control animals in basal conditions (**Fig. 3e**). We observed the analgesic effect of morphine (2 mg kg^{-1} and 10 mg kg^{-1}) for both genotypes, although the relative increments in latency were difficult to assess as the values got close to the predefined cut-off of 800 s. In the writhing test, we obtained an analgesic effect of morphine, with no difference between the genotypes (**Fig. 3f**).

Environmental stress is known to promote potent inhibition of behavioral responses to nociceptive stimuli, and this stress-induced analgesia is mediated by the opioid system. As expected, a forced swim test[19] (5 min at 32 °C) induced a marked analgesia in the hot-plate test (56 °C), which was reversed by naloxone in both genotypes (**Fig. 3g**). In agreement with the response observed after morphine administration, the stress-induced analgesia was stronger in knockout mice. No analgesia was observed in the tail-immersion test, but the effect of naloxone on knockout mice was maintained (**Fig. 3h**).

Behavioral effects of centrally administered peptides

Together, the data reported in the previous sets of experiments suggest an interaction between the opioid system and *Gpr10*. To investigate this interaction further, we evaluated the consequences of centrally administered PrRP (5 nmol, i.c.v.) on the nociceptive threshold recorded in the tail-immersion test (50 °C, **Fig. 4a**). I.c.v. administration of PrRP promoted a hyperalgesia in wild-type mice and was able to reverse the analgesia induced by morphine. Administration of PrRP to knockout mice had no effects on nociceptive thresholds, showing that the effects of the peptide *in vivo* are exclusively mediated by the GPR10 receptor.

We also investigated the potential interaction of the PrRP-GPR10 system with previously characterized anti-opioid peptidergic systems, namely NPFF and nociceptin, using the tail-immersion test as readout (**Fig. 4b,c**). I.c.v. administration of nociceptin promoted hyperalgesia and reversed morphine-induced analgesia, without significant differences ($P = 0.29$, $P = 0.20$, respectively) between wild-type and knockout mice (**Fig. 4c**). In our conditions, NPFF did not modify the basal nociceptive threshold of wild-type mice, but antagonized the effects of morphine (**Fig. 4b**). Notably, in knockout animals, NPFF had an analgesic effect and was unable to reverse the effects of morphine. These observations suggest an interaction between the NPFF and PrRP systems, probably at the neuronal network level.

Opiate tolerance, dependence and rewarding properties

Repeated opiate administration leads to the development of tolerance of its analgesic effects. We evaluated morphine tolerance during a 5-d morphine exposure protocol in the tail-immersion test (52 °C, **Fig. 5a**). As expected, morphine (10 mg kg^{-1}, i.p.) induced a strong antinociception on day 1; from day 2 to day 5, the antinociceptive response to morphine decreased slowly, down to basal level in both genotypes. The kinetics of tolerance development were, however, delayed markedly in the knockout mice as compared to the wild-type mice.

Chronic morphine exposure also produces a strong physical dependence syndrome as assessed by the characteristic set of behavioral

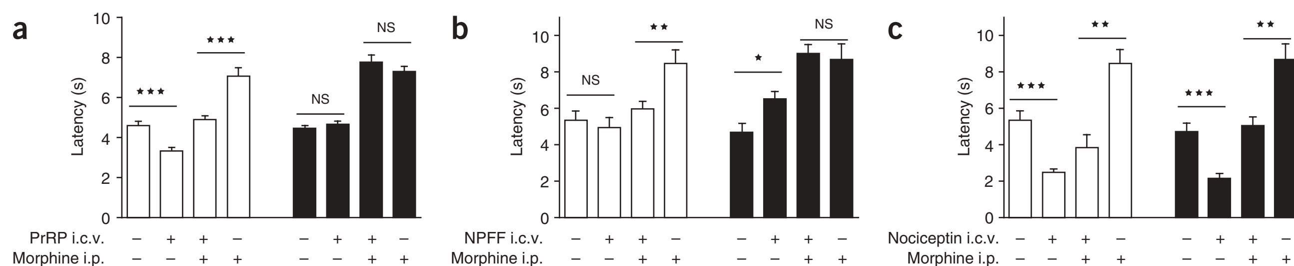

Figure 4 Anti-opiate properties of PrRP, NPFF and nociceptin. (**a–c**) Nociceptive thresholds were measured in the tail-immersion test. (**a**) PrRP (0 nmol or 5 nmol, i.c.v.) and morphine (0 mg kg^{-1} or 10 mg kg^{-1}, i.p.), (**b**) NPFF (0 nmol or 5 nmol, i.c.v.) and morphine (0 mg kg^{-1} or 10 mg kg^{-1}, i.p.) and (**c**) nociceptin (0 nmol or 25 nmol, i.c.v) and morphine (0 mg kg^{-1} or 10 mg kg^{-1}, i.p.) were coadministered to wild-type (open bars) and knockout (black bars) mice. Results are expressed as mean ± s.e.m. $n = 7$–14 animals depending on group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (ANOVA followed by Dunnett *t*-test).

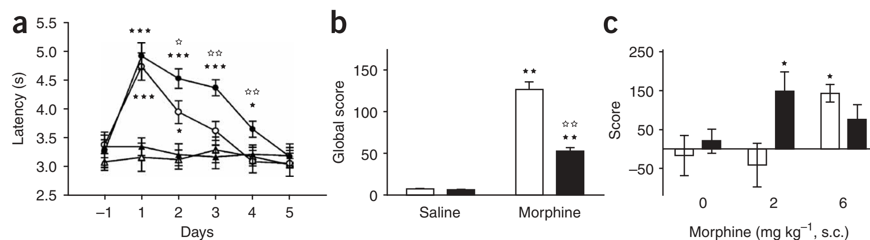

Figure 5 Effects of the PrRP-GPR10 system on morphine tolerance, dependence and rewarding. (**a**) Tolerance was evaluated in the tail-immersion test. Morphine (10 mg kg^{-1}, i.p, ●, ○) or vehicle (▲, △) was administered twice daily (7 a.m. and 7 p.m.) for 5 d to wild-type (○, △) or knockout (●, ▲) mice. The antinociceptive effect of morphine was evaluated every morning. (**b**) Dependence was evaluated in the withdrawal test. Wild-type (open bars) and knockout (black bars) mice were treated for 5 d with morphine (from 20 mg kg^{-1} to 100 mg kg^{-1}, i.p.) or with the saline vehicle as control, and withdrawal signs were evaluated after naloxone administration (1 mg kg^{-1}, s.c.). The global score was calculated for each mouse. (**c**) The morphine-induced rewarding response was evaluated in the place conditioning paradigm for wild-type (open bars) and knockout (black bars) mice. Morphine doses were 2 mg kg^{-1} or 6 mg kg^{-1}, s.c. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ when compared to the saline group of the same genotype. ☆$P < 0.05$; ☆☆$P < 0.01$ for the comparison between genotypes (ANOVA followed by Dunnett *t*-test). Results are expressed as mean ± s.e.m. $n = 8$–14 animals depending on group.

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responses to naloxone-evoked withdrawal. This includes a number of somatic signs such as jumps, wet-dog shakes, paw tremors, sniffing, ptosis, mastication, piloerection and body tremor, and vegetative signs such as diarrhea. We observed evidence of physical dependence for both genotypes during abstinence. However, the incidence of signs of withdrawal was significantly lower in knockout mice than in wild-type mice (**Supplementary Fig. 2** online). The global withdrawal score (**Fig. 5b**) illustrates the attenuation of the severity of naloxone-precipitated morphine withdrawal syndrome in knockout mice.

We investigated the rewarding properties of opiates in a conditioned place-preference paradigm. When morphine is administered to mice in this setting, the dose-response curve is reportedly bell shaped, with the maximal effect in mice at 6 mg kg^{-1} (s.c.) of morphine[20]. In our experiments (**Fig. 5c**), the maximal effect of morphine was indeed obtained for the 6 mg kg^{-1} dosage in wild-type animals. However, the knockout mice responded maximally to the lower dose of morphine (2 mg kg^{-1}, s.c.), whereas a higher dose did not induce any rewarding response. This observation suggests an increased sensitivity to the rewarding properties of morphine in the absence of GPR10.

Acute or chronic opiate administration affects gene expression in various brain areas. We measured the PrRP transcript levels using quantitative RT-PCR in hypothalamus and brainstem following morphine treatment. In the tolerance test, we observed that 3 h after the last morphine injection, the *PrRP* transcript levels were upregulated in the hypothalamus (**Supplementary Fig. 3** online).

Evaluation of the opioid and anti-opioid systems

GPR10 is expressed in enkephalin-containing neurons[21], suggesting that it may be involved in the control of proenkephalin expression. To assess whether the relative analgesia observed in knockout mice might be related to increased proenkephalin expression, we investigated the distribution of proenkephalin transcripts in the brain of both genotypes by *in situ* hybridization. We measured similar levels of proenkephalin transcripts in various brain areas of wild-type and knockout animals, including several pain-associated areas such as the parabrachial nucleus, the dorsal raphe nucleus, the bed nucleus of stria terminalis and the central amygdala (**Fig. 6a**). We observed a modest upregulation in basal ganglia (caudate-putamen and nucleus accumbens), in which GPR10 is poorly expressed, thus suggesting an indirect consequence of *Gpr10* inactivation. Using [^{3}H]DAMGO as radioligand, we also characterized the μ-binding sites in a saturation binding assay. Similar K_d (mean ± s.e.m.: 1.34 ± 0.25 nM versus 1.22 ± 0.36 nM) and B_{max} (155 ± 32 fmol mg^{-1} versus 163 ± 54 fmol mg^{-1} protein) values were found on whole brain membranes from wild-type and knockout mice, respectively. We also tested the δ-opioid receptor, using [^{3}H]naltrindole as tracer in a binding assay on whole brain membranes, but we found no difference between wild-type and knockout mice in K_D (0.39 ± 0.07 nM versus 0.44 ± 0.05 nM, respectively) or B_{max} (93 ± 9 fmol mg^{-1} versus 85 ± 12 fmol mg^{-1} of protein). The functional response of the μ and δ receptors was evaluated on whole brain membranes and on brain slices in [^{35}S]GTPγS binding assays. On whole brain membranes, the EC_{50} and E_{max} of the μ receptor (378 ± 15 nM versus 374 ± 13 nM, and 130 ± 8% versus 132 ± 12% relative to basal values, for wild-type and knockout mice, respectively) and the EC_{50} and E_{max} of the δ receptor (731 ± 45 nM versus 860 ± 43 nM; 121 ± 22% versus 128 ± 18% relative to basal values) were similar for both groups of mice. No differences in the binding distribution were detected on brain sections for the μ receptor (**Supplementary Table 1** online). We also evaluated pronociceptin and *NPFF* transcript levels by *in situ* hybridization, quantitative RT-PCR (**Fig. 6b,c**) or both. Both transcripts were found

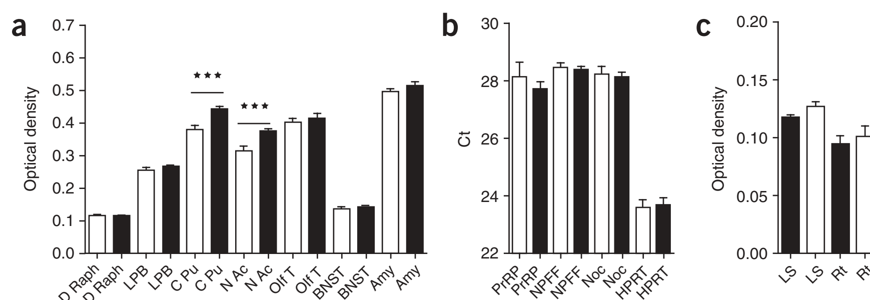

Figure 6 Modulation of the opioid and anti-opioid systems. (**a**) Proenkephalin transcript levels were evaluated in various brain areas of wild-type (open bars) and knockout (black bars) animals by *in situ* hybridization ($n = 5$). (**b**) PrRP, NPFF, nociceptin (Noc) and HPRT (as control) transcript levels were evaluated in the brainstem of wild-type (open bars) and knockout (black bars) mice by quantitative RT-PCR. "Ct" values represent the threshold cycle number at which fluorescence increases above a fixed threshold value. $n = 8$ wild-type and 14 knockout mice. (**c**) Nociceptin transcript levels were evaluated in two brain areas of wild-type (open bars) and knockout (black bars) mice by *in situ* hybridization ($n = 5$ mice). Results are expressed as mean ± s.e.m. $***P < 0.001$ (ANOVA followed by Dunnett *t*-test). D Raph, dorsal raphe; LPB, parabrachial nucleus; C Pu, caudate putamen; N Ac, nucleus accumbens; Olf T, olfactory tract; BNST, bed nucleus of stria terminalis; Amy, amygdala; LS, lateral septum; Rt, Reticular thalamus.

at similar levels in several brain areas of wild-type and knockout mice. From these experiments, therefore, it seems that no gross alteration of the opioid and anti-opioid systems in pain-associated areas occur as a compensatory mechanism following *Gpr10* knockout in mice.

DISCUSSION

Overall, our observations of knockout animals have confirmed that the PrRP-GPR10 system is involved in the control of neuroendocrine functions. Indeed, PrRP seems to regulate the level of CRH transcripts in hypothalamic structures, in agreement with the expression of the peptide precursor in catecholaminergic A1/A2 neurons. Together with the locus coeruleus, these neurons have been shown to control the release of CRH by the paraventricular nucleus (PVN) neurons[13,22], which are known to express *Gpr10*. These results are also in line with the previous observation that i.c.v. administration of PrRP enhances neuronal activity in the PVN[23], and increases ACTH and corticosterone levels in blood, through the release of CRH[13].

Moreover, we also demonstrated an important additional role of the PrRP-GPR10 system in the modulation of the various actions of opiates. Knockout mice showed a higher nociceptive threshold in some settings, increased analgesic and rewarding effects of morphine, increased stress-induced analgesia and reduced tolerance of morphine, as well as naloxone-precipitated withdrawal symptoms. Notably, naloxone reversed the analgesic effects of morphine, but also affected basal levels of the knockout animals in several tests, suggesting that *Gpr10* disruption generates or unmasks a basal opioid tone that is not detectable in wild-type animals. In line with these observations, we also observed that i.c.v. administration of PrRP results in hyperalgesia and reverses the antinociceptive effects of morphine in wild-type mice. The absence of effects of the peptide in knockout mice demonstrates that GPR10 is the sole target of PrRP *in vivo*. Altogether, these observations establish the involvement of GPR10 in the modulation of pain-signal processing.

The observed properties of the PrRP-GPR10 system are reminiscent of those of other opioid-modulating systems described previously. These other opioid-modulating systems (also referred to as anti-opioid systems) include the neuropeptides CCK, NPFF, nociceptin and dynorphin, and their respective receptors. Although the biological actions of these various peptides are not identical, they have all been reported to counteract some of the main behavioral effects of morphine in laboratory animals, including analgesia, tolerance, reward and dependence[6]. It is now well accepted that these neuropeptidergic systems have a major role in the adaptive mechanisms that characterize the chronic stimulation of the opiate system: namely, tolerance and dependence. Indeed, it is believed that these adaptive mechanisms involve not only regulations at the level of opioid receptors and their signaling cascades, but also long-term plasticity of neuronal networks[24]. As initially proposed[25], opioid receptor stimulation would result in the concomitant activation of anti-opiate neuronal networks, counteracting the various actions of opiate drugs. The delayed but long-lasting stimulation of these anti-opiate systems would, following chronic opioid stimulation, decrease the drug's effectiveness (tolerance) and contribute to adverse effects upon acute withdrawal (dependence).

Despite their overlapping actions, each opioid-modulating peptide has its own set of biological activities, as an obvious consequence of differences in receptor distributions and regulation of peptide release. In relation to the opiate system, NPFF and nociceptin show essentially the same panel of biological activities *in vivo*. Supraspinal administration of these peptides results in hyperalgesia, decrease of morphine- and stress-induced analgesia, reduction of rewarding properties (only demonstrated for nociceptin), enhancement of tolerance of morphine and precipitation of morphine withdrawal (although a matter of conflict for nociceptin)[5,6,26–28]. Spinal effects include analgesia and potentiation of morphine-induced analgesia[29]. Inactivation of the nociceptin receptor ORL1 leads to a markedly attenuated tolerance to the analgesic effects of morphine but without modification of the basal nociceptive threshold[30,31]. These actions are markedly similar to those we observed for PrRP. A previous report[18] described antinociceptive effects of PrRP following intrathecal administration. Despite the apparent contradiction with our findings, this observation also parallels the reported activities of NPFF and nociceptin. The activities of the two other anti-opioid peptides, dynorphin and CCK, are more dissimilar. Even though both peptides inhibit morphine-induced analgesia at the supraspinal level, they were reported to antagonize morphine action at the spinal level as well[32,33], in contrast to nociceptin, NPFF and PrRP. Other differences include the decrease in tolerance of morphine caused by dynorphin and the increase of rewarding effects of morphine caused by CCK, which contrasts with the action of the other anti-opioid peptides (reviewed in ref. 6). Also, knockout of the CCK_2 receptor gene led to spontaneous hyperalgesia and enhanced withdrawal signs[34], which might be explained by an increase in central endogenous opioid peptide secretion with a paradoxical coupling of the μ and δ receptors.

Given the similarity of the behavioral actions of PrRP, nociceptin and NPFF, we have investigated whether there might be interconnections between these systems. Inactivation of *Gpr10* did not result in significant modification of NPFF and nociceptin transcripts in the central nervous system. Nociceptin was also shown to retain its effects (hyperalgesia and reversion of morphine analgesia) in knockout animals, demonstrating that GPR10 is not required for the central effects of this peptide. In contrast, NPFF administration in knockout mice led to paradoxical analgesic effects and did not reverse morphine-induced analgesia (an activity seen in wild-type mice). These results indicate that some of the central actions of NPFF require a functional PrRP system and that inactivation of this system unmasks analgesic properties of NPFF that probably correspond to the spinal effect described in the literature[27]. It seems, therefore, that the PrRP-GPR10 system is located downstream of the NPFF-GPR10 system in a common central anti-opioid pathway.

The absence of a receptor during development might cause adaptive changes in the central nervous system. The agreement between the data obtained with the knockout mice and those resulting from PrRP administration suggest, however, that the observed phenotype is not the consequence of such long-term adaptive mechanisms. Our result do not show significant modification in the expression and coupling of the of μ- and δ-opioid receptors and in the abundance of NPFF and pronociceptin transcripts. Despite the modest upregulation of proenkephalin transcripts in basal ganglia, no major modifications were observed in brain areas involved in pain management. However, the upregulation in nucleus accumbens might contribute to the modifications of reward- and withdrawal-related behaviors observed in the knockout mice. As this area does not constitute a major site of expression of GPR10, it is likely that the proenkephalin overexpression is indirect and reflects a functional modification of the local neuronal networks.

Receptors involved in the action of opiate and anti-opiate peptides are essentially coupled to the same intracellular pathways. Indeed, opiate receptors, ORL1, $NPFF_2$ and GPR10 receptors are all coupled to the G_i class of G proteins. Although the actual downstream signaling events have not been formally demonstrated *in vivo* for each of these receptors, G_i proteins are known to stimulate K^+ channels, inhibit voltage-dependent Ca^{2+} channels and inhibit adenylyl cyclase, all of which result in an inhibition of signaling at the pre- or postsynaptic

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levels. The situation is somewhat more complex for CCK receptors, which have been shown to couple both to G_q and G_i family members. GPR10 has also been reported to couple to the G_q class of G proteins in some cell types[35]. The interference of anti-opioid systems with opiate peptide signaling therefore probably involves their action at different sites of the neuronal network, although interference between two receptors expressed in the same cell has been proposed as well[6].

Some of our observations might be explained by modulations of μ-agonist release or by a modified sensitivity of the network to similar levels of agonists. However, only this latter hypothesis accounts for all of our observations. Indeed, decreased tolerance for morphine is only compatible with a deficit of anti-opioid tone at a post-receptor level. Similarly, the morphine-induced analgesia experiments are not compatible with a presynaptic hypothesis. Indeed, a potentially elevated endogenous opioid tone is expected to be negligible with regard to the i.p. administration of 10 mg kg^{-1} of morphine. We believe therefore that the effects of PrRP are essentially mediated through counter-regulatory mechanisms acting post-synaptically in the opioid pathways. The loss of these counter-regulatory mechanisms in knockout mice would then reveal the weak opioid tone in basal situations and magnify the responses to endogenous or exogenous opioid agonists.

PrRP is highly expressed in A1 and A2 noradrenergic neurons of the caudal medulla, which are activated by pain signals and opioid withdrawal[36,37]. These noradrenergic neurons are well known to be anatomically connected with other brain areas involved in nociceptive and emotional pathways, which might explain the large array of effects observed. It has recently been shown that the lesion of the ascending A1 and A2 projections reduced the withdrawal syndrome, demonstrating the role of these projections in this process[38]. The precise *Gpr10* expression sites in which the observed anti-opioid effects of PrRP take place remain to be determined. However, potential sites include the amygdala, hypothalamus, brainstem and dorsal root ganglia, all regions of high *Gpr10* expression and key players in the processing of pain signals.

Altogether, the present study identifies the PrRP-GPR10 pathway as a new negative regulator of the opiate system and suggests that GPR10 might constitute a new pharmacological target for the clinical management of pain, opioid side-effects and addictive disorders.

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METHODS

Generation of GPR10-knockout mice. The targeting vector consisted in a 9-kb cassette containing a promoter-less *tau-lacZ* fusion gene, a *PGK-Neo* gene and a *HSV-TK* gene, flanked by 5 kb of 5′ and 3.5 kb of 3′ *Gpr10* gene fragments. The cassette replaced the first 194 codons of the *Gpr10* gene, encoding transmembrane segments 1–4. Homologous recombination was carried out in the R1 ES cell line, and the recombinant clone was aggregated with CD1 eight cell–stage embryos (**Supplementary Methods**). Heterozygous mutants were bred for seven generations on a CD1 background before generating the wild-type and knockout mice used in this study. Male mice (2–4 months of age) were used in all experiments.

RT-PCR analysis. Total RNA was purified from brain regions using the RNeasy Mini Kit (Qiagen). According to the specific experiment, the animals were killed 1 h after the injection of 1.5 IU kg^{-1} of human insulin (Novo Nordisc), 3–4 h after morphine or 1 h after naloxone injection. RNA samples were treated with RNase-free DNase I and reverse transcribed using the Superscript II kit (Invitrogen). The primers and methodological details are described in the **Supplementary Methods**.

Behavioral studies. The elevated plus maze test, rotarod test, light and dark box test, hot-plate test and tail-immersion test were performed as described[39,40]. For the tail-pressure test, increasing local pressure was applied until a withdrawal response was elicited (Basile analgesia meter, 1 mm tip diameter, 800 s cut-off, average of three responses, $n = 10$–12). For the writhing test, mice ($n = 6$–7) received an i.p. injection of 0.6% acetic acid, and the number of writhes was counted in 10-min periods starting 5 min and 15 min after the injection. For the forced swimming test, the hot-plate test was applied after a swimming session of 5 min ($n = 7$–11). For assessment of morphine tolerance, mice ($n = 10$–14) received 10 mg kg^{-1} of morphine i.p. twice daily. The withdrawal syndrome was promoted by two daily injections of morphine (20–100 mg kg^{-1} i.p. over 5 d) followed by a naloxone injection (1 mg kg^{-1}, s.c.), and the vegetative and somatic signs were scored over 30 min ($n = 8$–14). The rewarding effects of morphine were evaluated by using the conditioned place preference paradigm ($n = 8$–14)[39,40]. Additional details are available in the **Supplementary Methods**. PrRP (5 nmol), NPFF (5 nmol) or nociceptin (25 nmol) was administered i.c.v. into the left lateral ventricle in a volume of 4 μl using a modified Hamilton syringe.

***In situ* hybridization.** Coronal cryostat brain sections (15 μm) were hybridized with 3′-end-labeled [α^{35}S]dATP oligonucleotide probes for proenkephalin and pronociceptin as described in the **Supplementary Methods**. Quantification was performed for areas showing moderate to high expression levels. For each area, statistical analysis was performed for five mice of each genotype (3–9 slices per area and animal).

Radioligand binding assays and GTPγS binding assay. Saturation binding assays were performed on membrane preparations, using [^{3}H]DAMGO or [^{3}H]naltrindole as tracers and 10 μM naloxone for determination of nonspecific binding. Nonlinear regression analysis was performed by using the PRISM software (Biosoft) and a single-site model. Agonist-stimulated [^{35}S]GTPγS binding assays were performed on whole brain membrane preparations or on brain slices, using SNC80 or DAMGO as agonists. Details are provided in the **Supplementary Methods**.

Corticosterone assay. Blood samples were collected on ice within 2 min of the removal of mice from their cage. Corticosterone was assayed by using an RIA kit (Amersham Pharmacia). The stress procedures are described in the **Supplementary Methods**. For the corticosterone supplementation, corticosterone (12.5 μg ml^{-1}) was added to drinking water for 5 d. Blood samples were collected on day 3; the hot-plate test was performed on the afternoon of day 5.

Statistical analysis. The data were analyzed with the Instat software (GraphPad). For comparison with a theoretical value, one-sample *t*-test was used. For single comparisons, the two-tailed Student's *t*-test was used. For multiple comparisons, ANOVA was used, followed by two-tailed Dunnett *t*-test.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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