Neuropeptide B-deficient mice demonstrate hyperalgesia in response to inflammatory pain

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Neuropeptide B (NPB) and neuropeptide W (NPW) have been recently identified as ligands for the G protein-coupled receptor (GPR) 7 and GPR8. The precise *in vivo* role of this neuropeptide-receptor pathway has not been fully demonstrated. In this paper, we report that NPB-deficient mice manifest a mild adult-onset obesity, similar to that reported in GPR7-null mice. NPB-deficient mice also exhibit hyperalgesia in response to inflammatory pain. Hyperalgesia was not observed in response to chemical pain, thermal pain, or electrical stimulation. NPB-deficient mice demonstrated intact behavioral responses to pain, and learning from the negative reinforcement of electrical stimulation was unaltered. Baseline anxiety was also unchanged as measured in both the elevated plus maze and time spent immobile in a novel environment. These data support the idea that NPB is a factor in the modulation of responses to inflammatory pain and body weight homeostasis.

body weight | G protein-coupled receptor 7 | neuropeptide W | obesity

Neuropeptide B (NPB) and neuropeptide W (NPW) are members of a recently identified neuropeptide family that are ligands for G protein-coupled receptor (GPR) 7 and GPR8 (1–3). GPR7 recognizes both NPB and NPW with similar nanomolar affinities (with a slight preference for NPB), whereas GPR8 is moderately selective for NPW (1). Although both *NPB* and *NPW* have been found in mice, *GPR7* is the only receptor gene in this family to be positively demonstrated in rodents to date (4, 5). However, human chromosome 20 in the region encompassing *GPR8* has regions of strong homology with mouse chromosome 2, and further work is necessary before excluding the possibility that *GPR8* is expressed in the mouse (6). NPB is unique as a neuropeptide in that it is brominated at the N-terminal tryptophan residue (1). This feature was previously known to occur only in toxins from the *Conus* genus of predatory snails (7, 8).

The initial paper characterizing *GPR7* mRNA found, by Northern blot analysis, that humans had expression in the cerebellum, frontal cortex, pituitary, and hypothalamus (4). Later papers studying human expression patterns with RT-PCR found *GPR7* mRNA in the hippocampus, amygdala, pituitary, prostate, and trachea (3). *In situ* hybridization in rodents has revealed moderate to high levels of *GPR7* mRNA expression in taenia tecta, islands of Calleja, the olfactory tubercle, the primary olfactory cortex, suprachiasmatic nucleus, and in the parvocellular division of the hypothalamic paraventricular nucleus (5). Additional areas of expression have been observed in the supraoptic, dorsomedial, and ventromedial nuclei of the hypothalamus, as well as in the hippocampus (5). Particularly abundant expression was found in the medial amygdaloid nucleus (5). Some of the more extensive studies using RT-PCR on rat or mouse tissue have produced long lists of tissues that are positive for *GPR7* mRNA, including central nervous system and peripheral organs (2, 4). Unlike GPR7, GPR8 appears to be more limited in its pattern of expression in humans. *GPR8* mRNA is known to be expressed in the frontal cortex, parietal cortex hippocampus, caudate nucleus, thalamus, pituitary, adrenal gland, and lymph node (3, 4).

In situ hybridization has been used to examine detailed expression patterns of *NPB* mRNA in the mouse, and expression was found in the hippocampus (CA1, CA2, and CA3), lateral habenular nucleus, paraventricular hypothalamic nucleus, medial parvicellular part Edinger–Westphal (EW) nucleus, motor root of the trigeminal nerve, sensory root of the trigeminal nerve, lateral parabrachial nucleus internal part, mesencephalic trigeminal nucleus, subceruleus nucleus alpha part, locus ceruleus, noradrenergic cell group A5, and inferior olive subnucleus B (1). The strongest expression of *NPB* was observed in the EW nucleus (1). RT-PCR testing has detected various levels of *NPB* mRNA expression in rat and human tissues and is in agreement with the *in situ* hybridization data for the central nervous system, while adding several peripheral tissues (2, 3). By *in situ* hybridization, *NPW* mRNA has been detected in only a few confined areas: the periaqueductal gray matter, ventral tegmental area, EW nucleus, and dorsal raphe nucleus, especially the dorsal part of dorsal raphe nucleus (1).

Intracerebroventricular injection of NPB into rats enhanced locomotor activity, reduced responsiveness to pain in the formalin test, and induced a biphasic feeding effect at low doses (early, mild, orexigenic action followed by anorexia), whereas higher doses caused anorexia at all time points in rats (1). The administration of NPW by intracerebroventricular injection was reported to have both anorectic and orexigenic effects. The anorectic effect was observed after administration of NPW in both acute and long-term time points (9). The same study also demonstrated that administration of anti-NPW IgG stimulated cumulative food intake at both the 4-h and 12-h time points. However, two other groups have observed an orexigenic effect in rats with acute administration of NPW (10, 11). NPW has also been implicated in the indirect regulation of prolactin, corticosterone, and growth hormone (11). On the receptor side, GPR7-deficient mice have been generated and reported to have adult-onset obesity with associated metabolic defects (12).

Taken together, these studies imply that the neuropeptide–receptor pathway comprising NPB, NPW, and GPR7 (GPR8) may be involved in feeding, weight regulation, and pain response through either direct or indirect actions in the central nervous system. To discern the specific role that NPB plays separately from NPW in these diverse neurological and metabolic responses, we have generated NPB-deficient mice.

Methods

For a detailed description of the methods used in these studies please see our *Supporting Methods*, which is published as sup-

Abbreviations: NPB, neuropeptide B; NPW, neuropeptide W; GPR, G protein-coupled receptor; EW, Edinger–Westphal.

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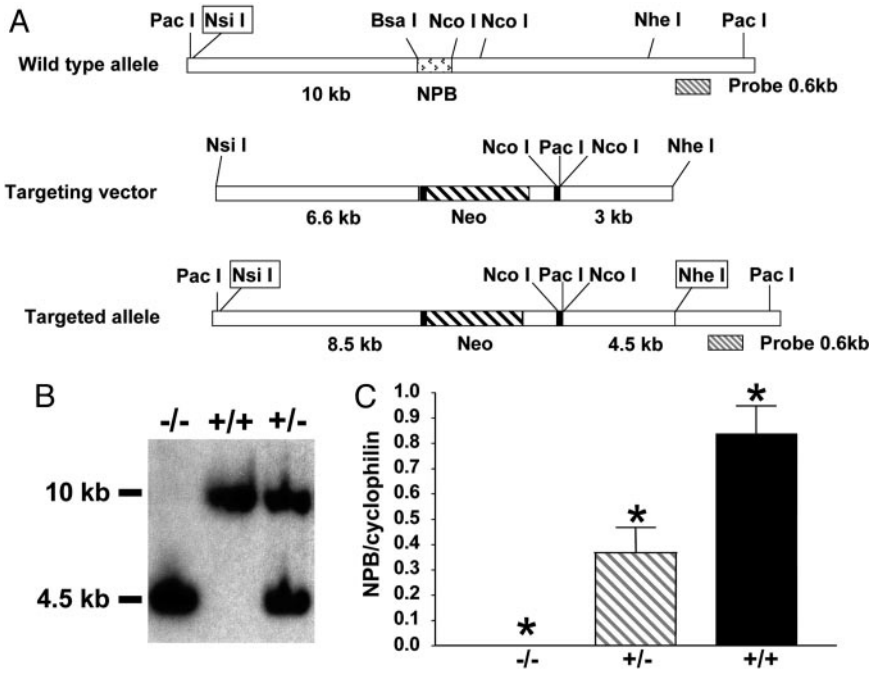

Fig. 1. Targeted disruption of the *NPB* gene. (*A*) Strategy for gene targeting. (*B*) Southern blot of tail DNA from the offspring of intercrossed heterozygotes. (*C*) Relative ratios of *NPB* to cyclophilin B mRNA in whole brain samples. The data are shown as the mean ± SEM. *, $P < 0.05$, Tukey post hoc comparisons.

porting information on the PNAS web site. The following is a brief description of the most relevant tests. The *NPB* gene was disrupted starting with a 15-kb genomic clone containing the entire coding region of the *NPB* gene that was isolated from a 129S6 (formerly 129/SvEv Tac) phage library, using a mouse cDNA hybridization probe (GenBank accession no. AI430753). The targeting vector had all of the coding region as well as the single intron deleted and replaced by a neomycin-resistance cassette (Fig. 1*A*). After electroporation into embryonic stem cells, microinjection, and breeding, the resultant mice produced F_2 offspring of all three possible genotypes on a mixed 129S6 × C57BL6/J background (Fig. 1*B*). The methods used for *in situ* hybridization have been described in ref. 13. The probe for *GPR7* was a generous gift from M. Ishii and J. Friedman (The Rockefeller University, New York) (12). Probes for *NPB* and *NPW* were as described in ref. 1. For the formalin test, male mice were injected s.c. on the dorsal surface of the left hind paw with 20 μl of a 5% formaldehyde solution (14). Observers who were blind to genotype scored mice for biting, licking, or scratching on the left hind leg and paw. A second group of mice, surgically implanted with electroencephalographic and electromyographic electrodes, were also tested in the formalin test ($-/-$ $n = 6$ and $+/+$ $n = 6$) and recorded as described in ref. 15. For the abdominal constriction tests, mice were injected i.p. with either 0.6% acetic acid or 120-mg/kg magnesium sulfate in a 10-ml/kg solution. The acetic acid group was scored for 30 min and the magnesium sulfate group for 5 min (for review and complete description see ref. 14). The observer was blinded to genotype and scored individual animals for the characteristic abdominal constrictions, or "writhes."

Statistical Analysis. Data were analyzed with JMP 5.01.2 software (SAS Institute, Cary, NC), by using the multivariate ANOVA, ANOVA, or least-squares method followed by Tukey post hoc analysis.

Results

Generation and Characterization of NPB-Deficient Mice. Homozygous *NPB* $-/-$ mice are viable, fertile, and display no overt abnormalities as young animals. Litters produced by homozygous crosses of *NPB* $-/-$ animals are not significantly different in number of pups born, pups surviving to weaning, or weaning weights (data not shown). Heterozygous male mice demonstrated an ≈2-fold decrease in *NPB* mRNA in whole brain by RT-PCR (ANOVA, $P < 0.0001$; Tukey post hoc, $P < 0.05$) (Fig. 1*C*). Homozygous NPB-deficient mice had no detectable *NPB* mRNA. *In situ* hybridization with the *NPB* probe confirmed the absence of *NPB* mRNA expression in the CA1, CA2, and EW nucleus, where robust expression is found in wild-type mice (Fig. 2 *A*, *B*, *E*, and *F*). Discrete expression patterns in the hippocampus were observed for each member of this neuropeptide receptor pathway, with *NPB* being expressed in the CA1 and CA2 regions, *NPW* in the CA3 region, and *GPR7* in the dentate gyrus. No changes were observed in the expression patterns of either *NPW* or *GPR7* mRNA in *NPB*-null mice (Fig. 2 *E–J*), indicating that loss of *NPB* exerts no influence in the expression of *NPW* or *GPR7* mRNA.

Although NPB has been reported to influence prolactin secretion (11), we saw none of the effects associated with elevated or decreased serum prolactin levels (16, 17). Urinalysis of mice on the 6% fat chow diet (see Table 1, which is published as supporting information on the PNAS web site) revealed that levels of creatinine, glucose, sodium, potassium, chloride, calcium, and osmolarity (data not shown) were not significantly different between genotypes.

Adult Onset of Mild Obesity in Male NPB-Deficient Mice. Because adult-onset obesity had been documented in GPR7-deficient males (12), our initial characterization of *NPB* $-/-$ mice sought to discern which ligand was involved in this phenotype. Although weight at weaning did not differ by sex or genotype, on low-fat chow (6% fat) male mice lacking one $(+/-)$ or both $(-/-)$ alleles encoding *NPB* became heavier (three-way multivariate ANOVA [time × sex × genotype], $P < 0.001$). *NPB* $-/-$ males were significantly heavier than $+/+$ male siblings by 30 weeks of age (Fig. 3*A*). Heterozygous males also became heavier at 36 weeks (Tukey post hoc, $P < 0.05$). Normal sexual dimorphism in weight gain was observed, but notably, female *NPB* $-/-$ mice

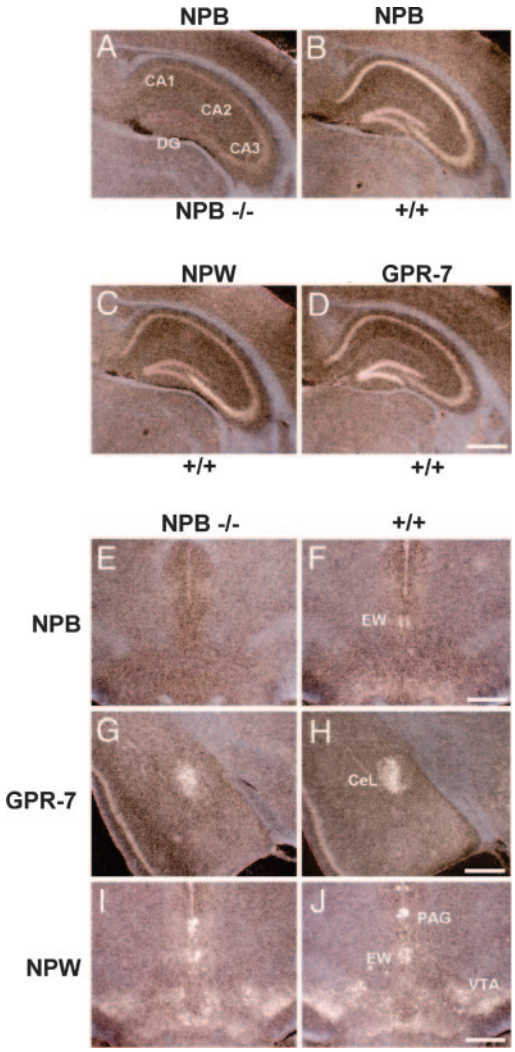

Fig. 2. Disruption of the *NPB* gene results in a loss of *NPB* mRNA expression as visualized by *in situ* hybridization with no observed changes in either *NPW* or *GPR7* mRNA expression patterns. Distribution of *NPB*, *NPW*, and *GPR7* mRNA expression in the adult mouse brain was revealed by *in situ* hybridization in both knockout (−/−) and wild-type (+/+) mice. (*A–D*) *NPB*, *NPW*, and *GPR7* mRNA were primarily found in one or two distinct areas of the hippocampus. The genotype of the mouse is displayed below each micrograph, and the probe is displayed above. (*E–J*) Direct comparison of *NPB* knockout (−/−) and wild-type (+/+) mice and demonstration of the preservation of *NPW* and *GPR7* expression patterns. DG, dental gyrus; CeL, central amygdaloid nucleus, lateral division; PAG, periaqueductal gray; EW, EW nucleus; VTA, ventral tegmental area. (Scale bars: 500 μm.)

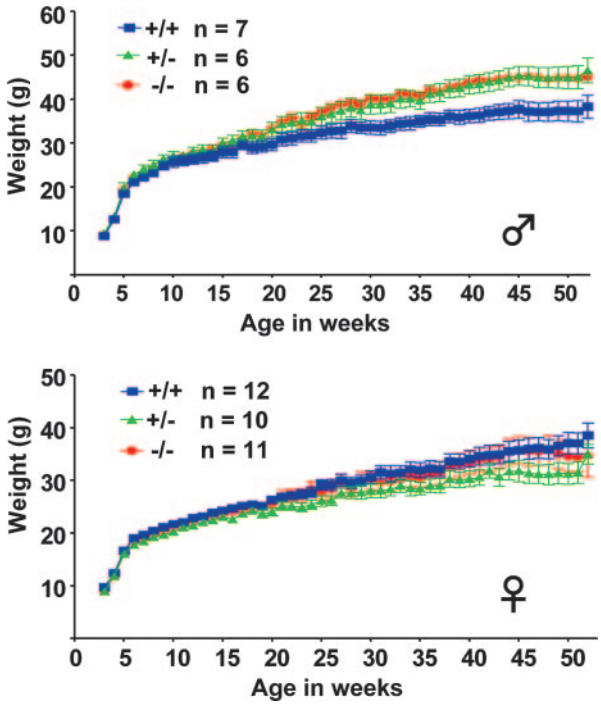

Fig. 3. Adult-onset obesity in *NPB* +/− and −/− male mice. F_2 mice were fed 6% fat chow from weaning through 1 year of age. Male −/− and +/− mice become significantly heavier by week 30 and 36, respectively. Each data point represents the mean ± SEM.

remained similar to female controls. The response to high-fat chow (60% fat) showed a trend toward male −/− mice becoming heavier than their siblings, but never achieved significance up to 53 weeks (Fig. 7, which is published as supporting information on the PNAS web site).

Hyperalgesia in NPB-Deficient Mice. The fact that the regions of the brain where *NPB*, *NPW*, and *GPR7* are expressed have been linked to pain processing pathways (1, 5, 18–20) led us to examine behavioral responses to pain in NPB-deficient mice. Hyperalgesic responses were observed during the formalin test in mice lacking NPB (Fig. 4). During the first 10 min (phase 1), NPB-deficient mice spent significantly more time biting, licking, or scratching at their left hind leg than +/+ littermates (one-way ANOVA by genotype, $P < 0.0006$; Tukey post hoc, $P < 0.05$). In the first 5 min, −/− mice accrued more time than both +/+ and ± mice (one-way ANOVA by genotype, $P < 0.0003$; Tukey post hoc, $P < 0.05$). From 11 to 60 min (phase 2), *NPB* −/− mice were not different from littermate controls. Significant differences were also found between the two parental strains, with 129S6 mice scoring significantly lower in both phases than C57BL6/J. Both parental strains (129S6 and C57BL6/J) as well as all three genotypes of littermates demonstrated the same degree of swelling in response to formalin (data not shown). No significant differences were found between the individually-housed and group-housed mice. No substantial differences between genotypes were found in comparisons of the electroencephalographic/electromyographic recordings for various stages of sleep during either the time period before or after the formalin test (data not shown). Because the animal's response to formalin test consists of several components and generally presents in two phases, we could not state with certainty which component of the formalin-induced pain caused the hyperalgesic response (for review and historical perspective on the formalin test see refs. 21 and 22).

To distinguish between two of the different types of pain, we used the acetic acid abdominal constriction test (a test for acute inflammatory pain) and the magnesium sulfate constriction test (an acute, noninflammatory, prostaglandin-independent nociception test) (14). In response to i.p. injection with acetic acid, an inflammatory agent, *NPB* −/− and +/− mice scored significantly more abdominal constrictions than +/+ controls [two-way ANOVA (genotype × sex); $P < 0.008$ for both; Tukey post hoc, $P < 0.05$] (Fig. 5*A*). Additionally, a significant difference in response was noted between the sexes, with males scoring higher than females (Tukey post hoc, $P < 0.05$). Homozygous mice of both sexes and +/− males scored significantly higher than +/+ animals of both sexes and +/− females (Tukey post hoc, $P < 0.05$). No significant difference was found between genotypes or sexes in the latency to initial response. In contrast,

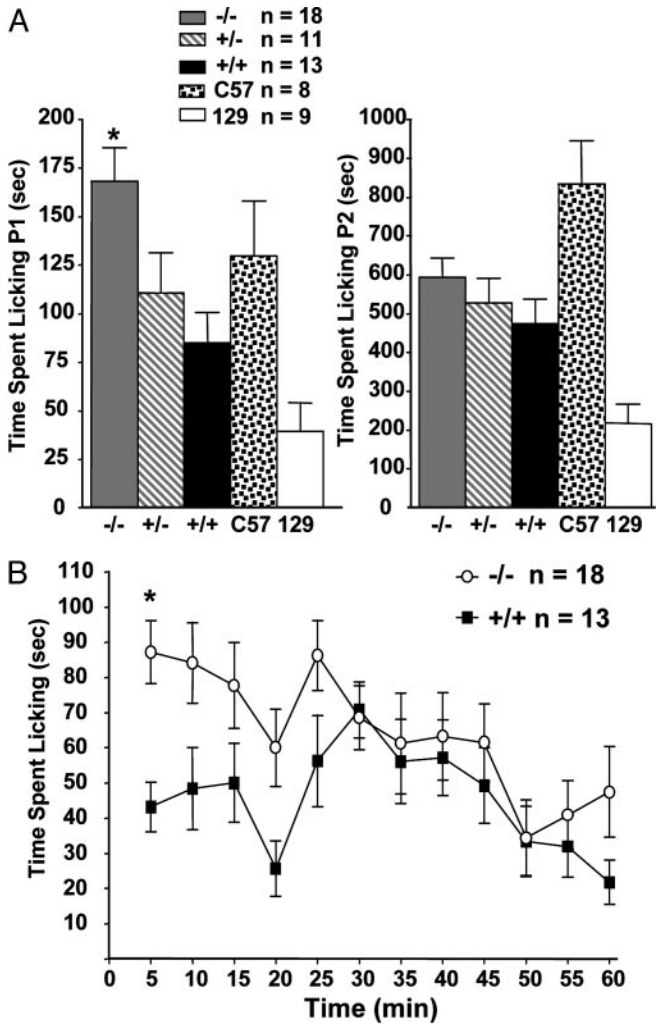

Fig. 4. NPB-deficient mice demonstrated hyperalgesia in response to the formalin test. Mice were injected with formalin on the dorsal surface of the left hind paw and scored for amount of time spent biting, licking, or scratching at the left hind leg. (*A*) Results for phase 1 (0–10 min, *Left*) and phase 2 (11–60 min, *Right*); different *y* axis for each time period. (*B*) Viewed over time, the differences between $-/-$ and $+/+$ siblings are even more apparent. Shown are 5-min cumulative scores. All data are shown as the mean ± SEM. *, $P < 0.05$, Tukey post hoc comparisons to $+/+$ siblings.

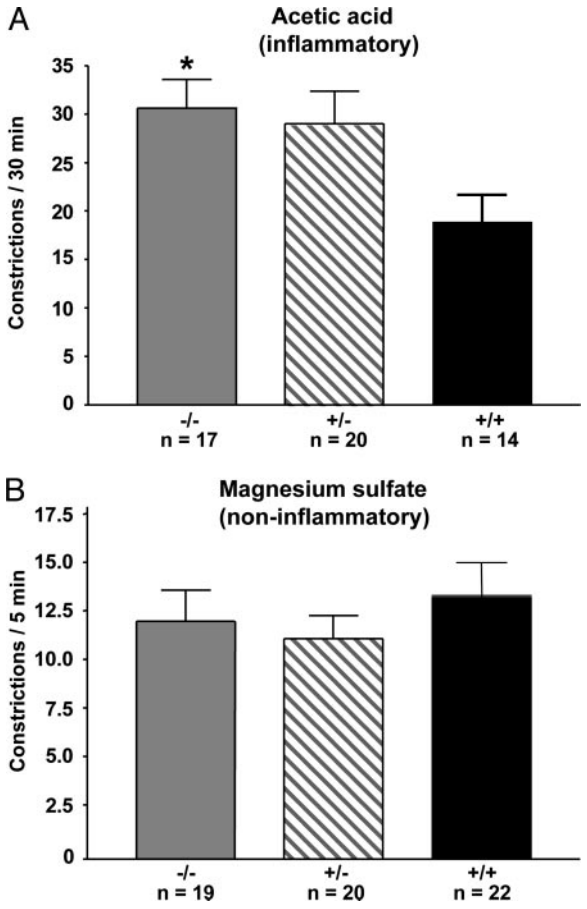

Fig. 5. An i.p. injection with acetic acid (an inflammatory agent), but not magnesium sulfate (a noninflammatory agent), causes hyperalgesia in NPB-deficient mice. Mice were injected with the designated agents and then scored for abdominal constrictions over the designated time period. All data are shown as the mean ± SEM. *, $P < 0.05$, Tukey post hoc comparisons to $+/+$ siblings.

injection i.p. with magnesium sulfate, a noninflammatory insult (23), resulted in responses that were not significantly different between *NPB* $-/-$ mice and their control groups (Fig. 5*B*). A small, but significant, difference was found between the sexes, with females scoring higher than males.

To further establish whether *NPB* $-/-$ mice had a more inclusive form of hyperalgesia, we tested for their response to thermal pain. Hot plate testing revealed that NPB deficiency had no effect on the responsiveness to acute thermal pain. *NPB* $-/-$ mice responded no more quickly than littermate controls (see Fig. 8, which is published as supporting information on the PNAS web site).

Assessments of Anxiety. Anxiety is considered a major component of pain responses and, thus, a more anxious mouse may be expected to respond differently to pain. To eliminate this anxiety as a contributing factor in the case of the NPB-deficient mouse, we used two different assessments of baseline anxiety: the cued and contextual fear test and the elevated plus maze. *NPB* $-/-$ mice were not distinguishable from their littermate controls in the cued and contextual fear assessment of baseline anxiety, responsiveness to electrical stimulation, and learning (as related to negative reinforcement by pain) (Fig. 6). As assessed by time spent immobile ("freezing"), $-/-$ mice were no more anxious in a novel environ-

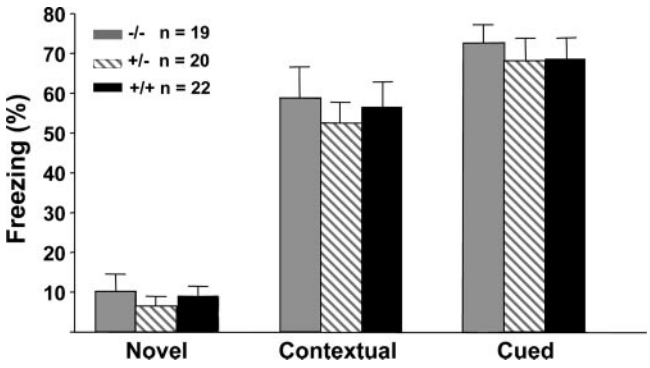

Fig. 6. Anxiety in response to a novel environment, the contextual environment, and the negatively reinforced cue is the same for *NPB* $-/-$ mice and their sibling controls. See *Supporting Methods* for a complete description. Shown is the percent of time spent immobile in one of the three situations. All data are shown as the mean ± SEM.

ment, in response to contextual cues, or in response to a learned cue. In all cases, they demonstrated appropriate responses and learning that was similar in pattern and amplitude to littermate controls. Additionally, *NPB* −/− mice were no more sensitive to electrical shock than were littermate controls (data not shown).

Using the elevated plus maze to further investigate the baseline level of anxiety revealed no significant changes in anxiety in *NPB* −/− mice when compared with littermate controls in this well established test (see Fig. 9, which is published as supporting information on the PNAS web site). *NPB* −/− mice explored the open arms and enclosed arms in the same proportions and for the same duration as their littermate controls.

Activity Levels and Circadian Rhythm. Despite reports of increased locomotor activity in response to intracerebroventricular injection of NPB, we found no significant differences in activity levels between *NPB* −/− mice and their littermate controls. In the open field arena, *NPB* −/− mice had normal activity levels on a par with their +/+ siblings. No significant differences were found in total horizontal distance traveled, time spent in motion, speed, or rearing (vertical movements) (data not shown).

Strong expression of *GPR7* mRNA in the suprachiasmatic nucleus, the central "clock" region of the brain, led us to assess the circadian behaviors in NPB deficient mice. However, *NPB* −/− mice displayed no deficits in maintenance of normal circadian rhythms in either the presence or the absence of external photo cues. Wheel running, duration of feeding, amount of food consumed (on a gram per gram of body weight), and drinking (in either amount or timing) were all indistinguishable from those of their +/+ littermates (Fig. 10, which is published as supporting information on the PNAS web site; also, data not shown).

Discussion

The present studies demonstrate that NPB plays important roles in both the maintenance of body weight homeostasis and the regulation of responses to inflammatory pain. We previously proposed, based on expression patterns and pharmacological responses, that this neuropeptide receptor pathway might be involved in the regulation of both feeding and nociception (1). We and other investigators have consistently found expression patterns for *NPB* and *GPR7* in several areas linked to either pain responses or body weight regulation (1, 5, 12). Long after its initial discovery (5), *GPR7* was isolated independently in a screen of mice with gold-thioglucose-induced lesions of the hypothalamus (12). These lesions resulted in hyperphagia and obesity that was partially replicated in GPR7-deficient mice (12). Despite this clear evidence of the involvement of GPR7 in body weight regulation, previous studies had not conclusively demonstrated whether this effect was mediated by NPB, NPW, or the two peptides in combination. Acute studies using NPW as the pharmacological agent had supported the idea that NPW was involved in feeding (9–11). In contrast, NPB had been shown in rats to have a biphasic feeding effect at low doses (early, mild, orexigenic action followed by anorexia), whereas higher doses caused anorexia at all time points in rats (1). It is evident that the pharmacological studies involving exogenous administration of NPB or NPW are unable to distinguish the separate roles of endogenous NPB and NPW, because both neuropeptides act through GPR7 with similar affinities in rodents.

Despite the biphasic nature of the pharmacological response to NPB, the significant weight gain on low-fat chow manifest by male *NPB* −/− mice (Fig. 3*A*) demonstrated that the loss of NPB resulted in dysregulation of body weight homeostasis. Indeed, as proven by the significant weight gains of male *NPB* +/− mice, *NPB* haploinsufficiency was sufficient to disrupt body weight homeostasis (Fig. 3*A*). Interestingly, however, NPW-deficient mice do not display any overt signs of dysregulation of body weight homeostasis (T. Motoike, personal communication), implicating the NPB/GPR7, but not the NPW/GPR7 axis in energy homeostasis. Because *NPB* −/− animals were not significantly different in either activity levels or food consumption from the littermate controls, we speculate that, like the GPR7-deficient mice, their metabolic rate is lower. We have examined the rates of O_2 consumption and CO_2 production of these animals at an early age when their weight was not different from that of control animals, but we did not find any substantial differences (data not shown). This issue will have to be revisited, in mice that are over 36 weeks of age, to definitively answer the question. The late onset of obesity, however, suggests that the decreased metabolic rate is slight and will be difficult to detect. Although the results of the GPR7 knockout support the idea that *NPB* −/− mice may have changes in their metabolic rate, other explanations for the obese phenotype of *NPB* −/− mice, including differences in nutrient absorption and decreased activity in the home cage in the absence of wheel running, cannot be ruled out at this time.

Several regions of the brain that have been linked to pain responses express *NPB*: the EW nucleus, the CA1 region of the hippocampus, as well as several brainstem nuclei that project to the amygdala such as the locus ceruleus, A5 noradrenergic cell group, nucleus parabrachialis, and nucleus of the solitary tract (1, 5). On the other hand, the amygdala, bed nucleus of stria terminalis, and hippocampus strongly express *GPR7* (1, 5). Because many neurons in the EW nucleus project to the spinal cord (especially to the superficial dorsal horn), this pathway may serve as a conduit for pain signaling (20, 24, 25). Additionally, CA1, the EW nucleus, and the amygdala have all been implicated in the modulation of pain responses (18–20). In addition to the expression patterns discussed earlier, two lines of evidence led us to investigate the effects of pain in NPB-deficient mice. First, our earlier pharmacological results showed an analgesic effect of intracerebroventricular NPB on rats during the formalin test (1). Second, a strong correlation between c-fos-positive cells in the spinal cord and in the CA1 region of the brain had been recently documented in rats during the formalin test (19). We tested our *NPB* −/− mice in paradigms designed to assess responses to inflammatory, chemical, and thermal pain. We found that *NPB* −/− mice demonstrated hyperalgesia in response to acute inflammatory pain, but not chemical or thermal pain. Only in the test of responsiveness to inflammatory pain (the acetic acid abdominal constriction test) did the *NPB* −/− mice manifest significant differences from sibling controls. In addition, the positive response of *NPB* −/− mice to the acute, noninflammatory nociception test (magnesium sulfate) was indistinguishable from that of their littermate controls. This directly comparable test has the same route of irritant administration, method of scoring, and behavioral responses. This test demonstrated that the ability of *NPB* −/− mice to respond to abdominal pain was unimpaired and that they did not have abnormal responses to all types of pain. The results of the inflammatory and noninflammatory abdominal constriction tests, taken together with the negative data from the thermal pain test and the shock threshold test, support the idea that NPB deficiency results in hyperalgesia only in response to a specific paradigm of insult, including acute inflammatory pain. Interestingly, NPW-deficient mice do not demonstrate hyperalgesia (T. Motoike, personal communication), suggesting that the NPB/GPR7, but not the NPW/GPR7, pathway serves as a negative regulatory mechanism for pain processing. It is important to note that many antiinflammatory drugs inhibit pain responses primarily in the second phase of the formalin test, but that this finding does not rule out an inflammatory component to the first phase (1–10 min). The continuum of the inflammatory response begins within seconds to minutes of the initial injury and should be thought of as one of the body's most rapid protective responses (26).

Because anxiety is thought to be a major component of the pain response, we used two different tests that provide insight into the anxiety levels of mice: cued and contextual fear and the elevated

plus maze. *NPB* −/− mice were neither more nor less anxious than their littermate controls. In both paradigms, the *NPB* −/− animals responded in appropriate ways; they learned from pain, became more anxious in response to pain, and avoided "danger" to the same degree as their littermate controls. The results of the cued and contextual fear and elevated plus maze testing help to eliminate anxiety as a source of difference in pain responsiveness between *NPB* −/− animals and their littermate controls.

NPB expression has been reported in a number of peripheral tissues, including the kidney, stomach, intestinal tract, and uterus, but we did not observe any overt abnormalities in these tissues (2, 3, 5). It is possible that only under certain stimuli or pathological conditions a lack of NPB would result in a detectable phenotype. In summary, the several components of the NPB/NPW/GPR7 (GPR8) peptide-receptor pathway might serve as a target for pharmacological modulation of body weight homeostasis and inflammatory pain responses.

Note Added in Proof. These results are supported by the recent work of Yamamoto *et al.* (27) on the "Anti-hyperalgesic effects of intrathecally administered neuropeptide W-23, and neuropeptide B, in tests of inflammatory pain in rats."

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