Critical Role of Neuropeptides B/W Receptor 1 Signaling in Social Behavior and Fear Memory

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Abstract

Neuropeptide B/W receptor 1 (NPBWR1) is a G-protein coupled receptor, which was initially reported as an orphan receptor, and whose ligands were identified by this and other groups in 2002 and 2003. To examine the physiological roles of NPBWR1, we examined phenotype of Npbwr1−/− mice. When presented with an intruder mouse, Npbwr1−/− mice showed impulsive contact with the strange mice, produced more intense approaches toward them, and had longer contact and chasing time along with greater and sustained elevation of heart rate and blood pressure compared to wild type mice. Npbwr1−/− mice also showed increased autonomic and neuroendocrine responses to physical stress, suggesting that impairment of NPBWR1 leads to stress vulnerability. We also observed that these mice show abnormality in the contextual fear conditioning test. These data suggest that NPBWR1 plays a critical role in limbic system function and stress responses. Histological and electrophysiological studies showed that NPBWR1 acts as an inhibitory regulator on a subpopulation of GABAergic neurons in the lateral division of the CeA and terminates stress responses. These findings suggest important roles of NPBWR1 in regulating amygdala function during physical and social stress.

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Introduction

NPB and NPW were recently identified as endogenous ligands for two closely related G-protein coupled receptors, GPR7 (NPBWR1) and GPR8 (NPBWR2) [1,2,3]. The NPBWR1 gene is highly conserved between the humans and rodents, while NPBWR2 is not found in rodent genomes [4,5]. Npbwr1 mRNA is localized in discrete brain regions in rodents, including the hypothalamus (dorsomedial hypothalamus and suprachiasmatic nucleus), hippocampus, ventral terminal area (VTA) and extended amygdala (CeA and bed nucleus of the stria terminalis; BST) [3,6]. The particularly strong expression of Npbwr1 in the CeA, together with the robust projection of NPW-containing axons to the CeA [7], suggests that this receptor might be an important modulator of the output signal from the amygdala. NPBWR1 is also abundantly expressed in other limbic regions, including the hippocampus, suggesting its roles in emotion and memory [3,5].

In this study, we investigated potential physiological roles of NPBWR1 by studying mice with a battery of behavioral tests [8] (Table 1). While Npbwr1−/− mice showed normal results in many of these tests, the screening pointed to obvious abnormality of social interaction and contextual fear in these mice. Histological and electrophysiological studies revealed that NPBWR1 was expressed in GABAergic neurons in the CeA, and acted as a neuroinhibitory regulator of these neurons. These findings suggest that NPBWR1 is an important modulator of amygdala function, and that NPBWR1 may be implicated in responses to stressful social and environmental stimuli.

These observations suggest that the NPB/W system plays important roles in regulating emotion and fear memory.

Results

Abnormalities in Social Behaviour in Npbwr1−/− Mice

In the resident-intruder test, male Npbwr1−/− mice showed significantly shorter latency to initial physical contact with the intruder and a significantly longer time in contact with the intruder compared with wild type male mice (C57BL/6j) (Fig. 1A,B). The resident-intruder test also revealed that...
Altered Stress Responses of Npbwr1+/− Mice

The behavioral and autonomic abnormality of Npbwr1+/− mice in threatening circumstances induced by social interaction suggests that NPBWR1 plays an important role in regulation of behavioral arousal and autonomic output induced by social emotional stress in mice. To examine the roles of NPBWR1 in evoking stress responses to physical environmental challenges, we further examined the autonomic and neuroendocrine responses of Npbwr1+/− mice to physical stresses. We found that stress-induced hyperthermia, which is often used to examine stress responses in mice [10,11], was significantly higher in Npbwr1+/− mice than in wild type mice (Fig. 2A). We also found that basal corticosterone-releasing hormone (CRH) mRNA level in the hypothalamus was higher in Npbwr1+/− mice than in controls (Fig. 2B). Furthermore, although the basal serum corticosterone level in Npbwr1+/− mice was comparable to that in control mice, possibly due to tight feedback regulation of this hormone in the basal state, it increased to a higher level after application of restraint stress for 10 min as compared with that in wild type controls (Fig. 2B). These observations further support an inhibitory role of NPBWR1 in stress-induced neuroendocrine and autonomic responses.

The increased responses to various stresses in Npbwr1+/− mice suggest the possibility that these mice show high anxiety. However, in the open-field test, Npbwr1+/− mice exhibited no abnormality in the percentage of time spent in the center of the arena (thigmotaxis), and showed no significant difference in the percentage of time spent in the open arms in the elevated-plus maze test (Fig. 2C), suggesting that the basal level of anxiety was unaltered in Npbwr1+/− mice. However, Npbwr1+/− mice showed a significantly shorter latency to first entry into the dark chamber in the light-dark exploration test (Fig. 2C). As Npbwr1+/− mice had normal thigmotaxis and a normal response in the elevated plus maze, this response to light-dark exploration might reflect increased impulsivity of Npbwr1+/− mice to a novel physical environment rather than heightened anxiety. This is consistent with the aforementioned results of the resident-intruder test, which may be interpreted as increased impulsivity to a social challenge.

Abnormality of Contextual Fear in Npbwr1+/− Mice

The amygdala and hippocampus have long been thought to play an important role in establishment of emotional memory. We tested whether Npbwr1 plays a role in this process using classical cued and contextual fear conditioning paradigms. Mice were placed in a conditioning chamber for 2 min before being given an auditory-cued conditioned stimulus (CS), a tone, which lasted for 30 sec. The last 2 sec of the CS was paired with a mildly aversive shock unconditioned stimulus (US). For contextual fear testing, mice were tested in the absence of both CS and US in the same experimental context at 24 hr after training [12]. Although wild

Table 1. Summary of behavioral phenotypes of NPBWR1 knockout mice.

| Behavioral test                  | Parameter     | Results                                                                 |
|----------------------------------|---------------|------------------------------------------------------------------------|
| Open field test                  | Anxiety       | Normal time spent in center of arena                                   |
| Elevated plus maze test          | Anxiety       | Normal time spent and number of entries in open arms                   |
| Light-dark exploration test      | Anxiety       | Decrease in escape latency and time spent in light box                 |
| Porsolt forced swim test         | Depression, learning helplessness | Normal time spent swimming                                              |
| Prepulse inhibition test         | Sensory motor reactivity | Normal percentage of prepulse inhibition                              |
| Marble burying behavior test     | Compulsive behavior | Normal number of marbles buried                                       |
| Cued and contextual fear conditioning test | Fear and memory | Decrease in time of freezing behavior during contextual testing while normal during auditory-cued testing |
| Morris water maze test           | Spatial memory | Normal escape latency                                                  |
| Resident-intruder test           | Social interaction | Abnormal social interaction                                            |
| Stress-induced hyperthermia      | Stress response | Exaggerated hyperthermia                                              |
| Daily locomotor activity         | Circadian rhythm | Normal in both light/dark cycle and constant dark condition. Normal entrainment by food or light |
| Sleep-wake behavior (EEG/EMG)    | Sleep/wake cycle | Normal in each episode duration, times spent in each state in hourly sleep/wake analysis |
Figure 1. Increased impulsiveness and contact time with associated increased autonomic responses in Npbwr1−/− mice during resident-intruder test. (A) Male naive 8-week-old mice were housed individually for 4 weeks before the procedure. The behavior of mice was recorded with a CCD video camera. A randomly chosen male intruder (C57BL/6J) was used only once in each session. The intruder was introduced into the resident cage, and behavior was recorded for 10 min. A variety of social behaviors were scored including the latency to the first aggressive contact (left panel) and time spent in aggressive contact (sniffing, rattling, chasing, mounting, wrestling and fighting) (right panel). Npbwr1−/− mice showed a shorter latency time to contact with the intruder (F1,12 = 5.304, p = 0.040), and longer physical contact with the intruder compared with wild type mice (F1,12 = 6.068, p = 0.030). Data are presented as mean ± SEM (WT n = 6, KO n = 8). Also see movies S1 and S2, which show typical examples of behavior observed during this test. (B) Video tracking system shows traces of intruder (white) and resident (green) during 10 min session of resident-intruder test, showing that Npbwr1−/− mice exhibited more sustained and persistent contact and chasing behavior. Note that the trace of Npbwr1−/− is very similar to that of the intruder, reflecting the persistent chasing. (C) Locomotor and cardiovascular responses during resident-intruder test in radiotelemetry-implanted freely moving mice. Activity (upper panels), heart rate (HR; middle panels) and mean arterial pressure (MAP; lower panels) of resident mice (Npbwr1−/− or wild type littermates) during the time course of the resident-intruder test are shown. Intruders (male C57BL/6J mice) were put in the cages at 0 min. Horizontal solid bar indicates the presence of an intruder. Baseline values were defined as the average values of parameters obtained during 10 min immediately prior to the resident-intruder test. Data are presented as mean ± SEM (wild type; n = 4, Npbwr1−/−; n = 5) (*p<0.05, **p<0.01, compared to wild-type). (D) Real time PCR analysis showed that Neuropeptide B (NPB) and Neuropeptide W (NPW) mRNAs in whole brain were upregulated after the resident-intruder test for 60 min. Each level of expression was normalized by the level of Gapdh mRNA (wild type; n = 45, Npbwr1−/−; n = 5).

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Discussion

Abnormality in Behavioral and Neuroendocrine Responses of Npbwr1−/− Mice

Our present study showed that Npbwr1−/− mice have abnormal behavioral and neuroendocrine responses to social and physical stresses (Figs. 1, 2). The abnormal behavior toward the intruder possibly reflects increased impulsivity to potential danger and/or inability to appropriately recognize unknown conspecifics as a threat. We hypothesized that this abnormal behavior is due to abnormal neurotransmission in the amygdala, firstly because NPBWR1 is abundantly expressed in the CeAl, the only region in which the lesioned animals showed less tension-related behavior (Fig. 4C, D). These observations demonstrate that NPB/W acts on projection neurons in the CeAl. We also observed some cells with shorter axons that ended within the CeAl. Because these studies were done using slice preparations, we cannot conclude that these axons ended within the CeAl, but it is plausible that some NPBWR1-positive neurons could be GABAergic interneurons in the CeAl.
NPBWR1 and Amygdala Function

$NPbwr1^{-/-}$ mice have a lack of personal space regulation, which may be similar to that seen in a human with bilateral amygdala lesions, who showed a lack of discomfort at close distances to others [17]. Our observations suggest that $NPbwr1^{-/-}$ mice have an abnormality in evoking normal caution and/or fear when confronted with strange mice.

Our findings in $NPbwr1^{-/-}$ mice of exaggerated neuroendocrine responses to various physical stresses are consistent with a neuroinhibitory effect on Npbwr1 on amygdala function, which leads to strong and sustained activation of the sympathetic division of the autonomic outflow in response to environmental stimuli.

Abnormality of Contextual Fear in $NPbwr1^{-/-}$ Mice

Abnormality of the behavior evoked by the resident-intruder paradigm and the results of contextual fear conditioning suggest that Npbwr1 might play a role in evoking proper behavior to
Figure 3. *Npbwr1"/"* mice showed abnormality in contextual fear conditioning. (A) Fear conditioning was performed to examine the ability of *Npbwr1"/"* mice to learn and remember an auditory cue or context that predicted electric shock. Bars show the mean percentage of time spent freezing (defensive tonic immobility) during 30 s observation. For contextual fear test, mice were tested in the absence of cues in the same context at 24 hr after training. For cued test, mice were tested in new cages and the auditory cue applied. Freezing behavior of mice was counted before (pre-CS) and during application of the cue. There was a significant difference in duration of freezing behavior between *Npbwr1"/"* mice and wild type mice during the contextual fear task (wild type; n = 13, *Npbwr1"/"*; n = 18, F1,29 = 114.15, p < 0.001), while no significant difference in freezing behavior was observed during auditory-cued testing under the altered context. (B) Alternative protocols for fear and safety conditioning [13]. Upper panel,
schematic representation of training and testing protocols. Mice were put in the conditioning chamber for 2 min before the first stimulus. Conditioning sessions consisted of 5 CS (20 s) (interval, mean 130 s, range, 100–140 s). In safety conditioning sessions, the US was explicitly unpaired and occurred during the inter-CS interval (five US per session, separated by 20–80 s from each CS). Training sessions were conducted for three days (one session per day). In fear conditioning, the US was applied for the last two sec of the CS, which was applied at the same protocol as the safety conditioning. In the test sessions, CS was delivered at the same protocol as conditioning, and no US was delivered. Freezing times in the 20 s periods before and during CS application were scored as context and cued conditioning, respectively. Lower panels, Times spent freezing during 20 s CS and 20 s prior to CS in safety conditioning (left panel) and fear conditioning (right panel) are shown. In safety conditioning, wild type mice displayed freezing to the context, which invariably accrued with US exposure. The freezing was reduced by the arrival of the CS. However, Npbwr1−/− mice did not show fear responses to the context, and exhibited freezing to the CS. In fear conditioning, we obtained virtually the same results as those with the classical protocol (A).

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relatively complex polymodal environmental cues, such as contextual information for danger and social interaction. On the other hand, Npbwr1−/− mice can respond to simple sensory cues as shown by cued fear conditioning.

Another very interesting pair of results is the impairment of contextual fear conditioning and “inversion” of safety conditioning. In safety conditioning, mice received unpaired presentations of a tone or light CS and a shock US. Because the shock never occurred during the CS, wild type mice learned to treat the CS as a safety signal, so that fear-related behaviors, such as freezing, were inhibited during presentation of the CS. Npbwr1−/− mice showed “inversion” of this learning in that the safety-trained CS elicits fear rather than a reduction in fear (Fig. 3). Npbwr1−/− mice apparently undergo trace conditioning rather than safety conditioning in this procedure, in that they associate the CS and US across a long “trace” interval, while the control mice treat CS and US as unpaired. Npbwr1−/− mice seem to be more “stimulus bound”, meaning that they preferentially attend to discrete stimuli, to the exclusion of more complex, conjunctive stimuli such as contexts. This hypothesis would also explain the deficit in contextual fear conditioning, and should be confirmed in future studies.

The abnormality in contextual fear conditioning tests suggests that Npbrw1 might be involved in hippocampal function, since both the amygdala and hippocampus are necessary for establishing contextual fear memory, although we did not find any abnormality in the Morris water maze test, a hippocampus-dependent memory task (Fig. S3). We also did not find any difference in long-term potentiation in CA3 pyramidal neurons (N.F., unpublished results). Together with the strong expression of Npbrw1 in the CeAl, and the fact that NPW-i fibers were exclusively observed in the CeA in mice [7], this supports the notion that the abnormality of contextual fear memory in Npbwr1−/− mice is likely to stem from abnormal neurotransmission in the amygdala.

The LA/BLA regions of the amygdala are believed to be a principal storage site for emotional memory (US-CS association), while the CeA is implicated in output regulation. Activity of CeAl neurons could affect the level of inhibitory control in the CeAl-CeAm circuit, thereby controlling CeAm output. Since Npbrw1 is expressed in the CeA, but not LA/BLA, Npbrw1 is not likely to be involved in the storing of emotional memory. Npbrw1 might instead play an important role in evoking and controlling proper behavioral and neuroendocrine responses. Npbrw1−/− mice consistently showed normal freezing behavior in auditory-cued testing (Fig. 3A).

However, with the recent evidence suggesting that CeA also participates in the acquisition or expression of fear memory [14,18], Npbrw1 may play a role in this process. Coordinated control of CeA neurons by Npbrw1 might contribute to both memory storage and proper expression of behavioral and neuroendocrine responses according to complex environmental conditions. Recent studies have shown that the CeA neurons receive input from various regions, including the sensory thalamus, BLA and insular cortex. Therefore, this region appears to be important for coordinating and processing various sensory and internal information in establishing fear memory.

However, the involvement of the NPBWR1 in hippocampal function should not be disregarded. Indeed, the deficit in contextual fear conditioning with normal cued fear conditioning is consistent with hippocampal damage, and the neuroendocrine phenotype could be related to either hypothalamic or hippocampal abnormalities both of which express Npbrw1 mRNA [5]. A further study would be needed to confirm the involvement of the amygdala in these abnormalities using spatially-restricted deletion of NPBWR1, knockdown or a rescue experiment using Npbrw1−/− mice or electrophysiological experiments using the hippocampus of mutant mice.

The results in the social interaction test are surprising as Npbrw1−/− mice showed an increased neuroendocrine response. In addition, the increased corticosterone response to stress suggests that these mice would be anxious, which was not obvious in the open field or elevated plus maze test, but was evident in the light-dark test. Factorial analysis of behavior in anxiety-related experiments in animals has shown that different tests reflect different underlying factors [19]. Therefore, the fact that Npbrw1−/− mice showed such a specific phenotype in the light-dark exploration test and not in others might simply reflect the fact that these tests measure different dimensions of anxiety-related behaviors, such as impulsivity. Abnormality of stress-induced autonomic changes may also have contributed to the finding in the resident-intruder test, because feedback of autonomic responses through the vagal nerve may contribute to overall behavioral responses in animals [20].

Peptidergic neuromodulation is a relatively slow and sustained process as compared to the glutamatergic and GABAergic systems. We speculate that the NPB/W system might play a role in regulating amygdala function over a relatively longer time scale. Both Npb and Npw mRNAs were increased under stressful conditions induced by the resident-intruder paradigm, suggesting that this system might work as a feedback regulator of the amygdala by inhibiting projection neurons in the CeA (Fig. 1D). In addition, some of the GABAergic interneurons within the CeA also expressed Npbrw1 (Fig. 4C, D). This suggests an intriguing possibility that NPB/W regulates amygdala networks by inhibiting some specific outputs while disinhibiting others, thereby helping to select proper behavioral and neuroendocrine responses [14] (Fig. 5). This model may explain why Npbrw1−/− mice showed decreased fear-related behavioral responses to complex contexts such as social interaction and contextual fear, but showed increased sympathetic responses to various stresses. Russel and Mehrabian classified emotions into three dimensions of factors; valence (pleasure-displeasure), arousal (autonomic response), and dominance [21]. The NPB/W system might regulate emotions in the arousal and dominance scales according to the animal’s environment which contains a relatively complex context.
Figure 4. Function of NPBWR1 in regulation of CeA neurons. (A) Left panel, Dual-label In situ hybridization histochemistry showed co-localization of Npbwr1 mRNA (blue) with Gad67-expressing neurons (red) in the CeAl of mice. Scale bar equals 250 μm. Middle panel, higher power view of yellow rectangle region in the left panel. Right panel, high power view of yellow rectangle region in the middle panel. Opt, optic tract. (B) Left panels, typical examples of whole cell patch-clamp recording from GAD67-expressing neurons in Gad67-gfp brain sections, showing that bath-application of NPB (upper panel, 500 nM) or NPW (lower panel, 500 nM) potently inhibited neuronal activity. Right panel, numbers of GFP-positive neurons activated or inhibited by NPB/W application. We did not observe any effects in neurons of Npbwr1−/− mice. (C) A typical example of morphology of NPB/W-inhibited GABAergic neurons as revealed by neurobiotin injection after patch-clamp recordings. This cell resides in the medial region of the CeAl and sends long projections to outside of the amygdala. (D) Schematic drawings of axonal projections of NPW and/or NPB-inhibited neurons in the CeAl. Left panel shows three neurons depicted in different colors that send axons within the CeAl. Right panel shows four neurons that send axons outside of the CeA.

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In human and primate genomes, there is a closely related receptor, NPBWR2, which also receives NPB and NPW as its ligands. This gene duplication during primate evolution might result in adaptation to more complex social contexts in humans and primates compared to those in rodents. Analyzing NPBWR2 function in primate social behavior is also obviously necessary to fully understand this neuropeptide system. Our ongoing study also suggests that NPW, one of the ligands for NPBWR1, plays an essential role in modulating amygdala function under stress in mice (T.M. et al., unpublished data).

In summary, we carried out behavioral characterization of Npbwr1-deficient mice. These mice showed an intriguing pattern of behavioral abnormalities, including impaired contextual fear conditioning, impaired safety conditioning, and increased social...
interaction in the resident-intruder paradigm. We also observed electrophysiological changes in GABAergic neurons of the CeA, although we cannot exclude the possibility that the phenotype may be attributable to developmental loss of the gene. However, to determine the neural mechanisms and the possible developmental and/or extra-amygdalar origins of the phenotype, further investigation using spatially-restricted knockout mice and/or genetic rescue of the phenotype of these mice by expressing NPBWR1 in a region-specific manner will be clearly required in the near future. It is also clear that our findings could provide novel therapeutic targets for disorders induced by social stress, which are among the most prevalent mental health problems in the world today.

Materials and Methods

Animals

All experimental procedures involving animals were approved by the Animal Experiment and Use Committee of University or Kanazawa University (AP-101567), and were in accordance with NIH guidelines. Npbwr1+/− mice [8], in which the NPBWR1-coding region in exon 1 is disrupted by inserting a tau-LacZ cassette, used in the experiments were obtained from the mating of heterozygous Npbwr1+/− mice, which were backcrossed to wild type C57BL/6j mice for more than 10 generations. Their littersmates with Npbwr1+/− genotype were used as wild-type control. Gad67-gff.ANeo; Npbwr1+/− with C57BL/6j background were used for electrophysiological and histological studies. Mice were maintained under a strict 12 hour light:dark cycle in a temperature and humidity controlled room and fed ad libitum.

Behavioral Experiments

All behavioral experiments (Table 1) were performed during the light phase (13:00–17:00) using 8- to 14-week-old male mice. We used wild type littersmates as control mice. The experimenters were blind to the genotypes until all data had been gathered and analyzed. Behavioral experiments in this study were basically performed according to protocols previously described [23]. The behavior of mice was recorded with a charge coupled device (CCD) video camera.

Resident-Intruder Test

Male naive mice were housed individually for 4 weeks before the procedure. Isolation started at 8 weeks old. A randomly chosen intruder used only once in each session was introduced in the resident cage, and time spent in aggressive behaviors including chasing, rattling, wrestling, biting and aggressive grooming were recorded for 10 min.

Measurement of Blood Pressure and Heart Rate in Fully Behaving Mice

Radio telemetry implants (PA-C10; Data Sciences International; St. Paul, MN, U.S.A.) were used to monitor locomotor activity, heart rate (HR) and mean arterial pressure (MAP) in freely moving animals. The PA-C10 catheter was implanted in the left carotid artery and the transmitter body was placed in a subcutaneous pocket. All implanted mice were kept isolated, and tests were performed 7 days post-surgery to allow the mice to fully recover and their HR and MAP to return to pre-surgical levels. Before starting the test, HR, MAP and activity were recorded for 1 hour (baseline). Wild type C57BL/6j mice were used as intruders. A randomly chosen intruder was introduced in the resident cage, and HR, MAP and activity of the resident were recorded for 60 min.

Open-Field Test

We used an open field apparatus consisting of a circular (75 cm diameter, 43 cm height) gray Plexiglas. The arena was set up under a CCD camera, and data were collected using a video tracking system, Compact VAS ver 3.0x (Muromachi Kikai, Tokyo, Japan). The floor was divided into 25 quadrants on the computer. A single mouse was placed in the center of the open field arena and its behavior was recorded for a 5-min test session. Times spent in the central quadrants and in behaviors such as rising, rearing, grooming, and voiding were evaluated as indexes of anxiety in mice. Data are presented as mean ± SEM (n = 23 and 17 for wild type and Npbwr1−/−, respectively).

Elevated Plus-Maze Test

We used an elevated plus-maze, constructed of Plexiglas and raised 40 cm above the floor, consisting of two opposite enclosed arms with 14 cm high opaque walls and two opposite open arms of the same size (30 cm × 3 cm). Data were collected using a video tracking system compact VAS ver 3.0x. A single session lasted for 5 min. To begin a trial, the test animal was placed on the central platform facing an open arm. Anxiety levels of mice were evaluated by the percentage of entries into the open and closed arms, time spent in the open arms and distance traveled. Data are presented as mean ± SEM (n = 20 and 24 for wild type and Npbwr1−/−, respectively).

Light-Dark Exploration Test

The light-dark exploration test measures the tendency of mice to explore a novel environment versus the aversive properties of a brightly lit open field. The light/dark exploration test was performed using a cage (45 × 27 × 26 cm) equally divided into two (dark and light) compartments by a black partition containing a small opening. The total number of transitions, time spent in the light side, and latency until mice escaped to the dark side were recorded for 10 min after a single mouse was placed in the light compartment. Data are presented as mean ± SEM (n = 20 and 15 for wild type and Npbwr1−/−, respectively).

Cued and Contextual Fear Conditioning

Experiments were performed essentially as previously described [12]. On the training day, the mouse was placed in the conditioning chamber for 2 min before giving the conditioned stimuli (CS), a tone, which lasted 30 sec at 2900 Hz, 70 dB. The training was performed three times. The last 2 sec of the CS was paired with the unconditioned stimuli (US), a mild foot shock of 0.6 mA. After an additional 30 sec in the chamber, the mouse was returned to its home cage. Mice were tested 24 hr after training. Context testing was conducted in the same chamber, and freezing behavior was scored during a 30-sec testing session. Cued testing was conducted by placing the mouse in a novel environment (altered context) with the same 30 sec tone that was presented during the training day. Data are presented as mean ± SEM.

In Situ Hybridization

Preparation of coronal brain sections and single in situ hybridization were performed according to procedures previously described [24]. For double in situ hybridization, each combination of two antisense riboprobes labeled with either fluorescein-UTP (Gadl) or digoxigenin-UTP (for Npbwr1) was hybridized to sections simultaneously. Following the chromogen reaction of the first color (blue) obtained with anti-digoxigenin-alkaline phosphatase (AP) Fab fragments, 5-bromo-4-chloro-3-indolyl phosphate (Roche) and nitroblue tetrazolium (Roche), sections were rinsed three times.
with TBS, treated twice with 0.1 M glycine pH 2.2 and 0.1% Tween 20 for 5 min, washed, and then incubated with anti-fluorescein-alkaline phosphatase (AP) Fab fragments. For the chromogenic reaction of the second color (orange), 5-bromo-4-chloro-3-indolyl phosphate (Roche) and 2-[4-iodophenyl]-3-[4-nitrophenoxy]-5- phenyl-tetrazolium chloride (Roche) were used. Antisense riboprobes were synthesized from plasmids containing the coding regions of mouse Npbwr1 (Transmembrane domain 1–5, nucleotides, 323–764) and mouse Gad1 (NM_000877, nucleotides 281–821) cDNAs.

Electrophysiology

Gad67-gfp(Neo) mice were used for whole cell intracellular recordings. Mice were anesthetized with intraperitoneal administration of Forane (Abbott, Osaka, Japan). The mice were decapitated under deep anesthesia. The brain was isolated in ice-cold cutting solution consisting of (mM): 280 sucrose, 2 KCl, 10 HEPES, 0.5 CaCl2, 10 MgCl2, 10 glucose, pH 7.4, bubbled with 100% O2. Brains were cut coronally into 300-μm slices with a vibratome (VTA-1000S, Leica, Germany). Slices containing the CeAl were transferred for 1 hr to an incubation chamber at room temperature filled with physiological solution containing (mM): 140 NaCl, 2 KCl, 1 NaHCO3, 1 MgCl2, 10 HEPES, 10 glucose, pH 7.4 with NaOH. The slices were transferred to a recording chamber (RC-27L, Warner Instrument Corp., CT, USA) at room temperature on a fluorescence microscope stage (BX51WI, Olympus, Tokyo, Japan). Neurons that showed EGFP fluorescence in the CeAl region were used for patch-clamp recordings. The fluorescence microscope was equipped with an infrared camera (C-3077 78, Hamamatsu Photonics, Hamamatsu, Japan) for infrared differential interference contrast (IR-DIC) imaging and a CCD camera (JK-TU53H, Olympus) for fluorescent imaging. Each image was displayed separately on a monitor (Gawin, EIZO, Tokyo, Japan). Recordings were carried out with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) using a borosilicate pipette (GC150-10, Harvard Apparatus, Holliston, MA) prepared using a micropipette puller (P-97, Sutter Instruments, Pangbourne, UK) and filled with intracellular solution (4–10 MΩ consisting of (mM): 125 K-glucosate, 5 KCl, 1 MgCl2, 10 HEPES, 1.1 EGTA-Na2, 5 MgATP, 0.5 Na2GTP, 0.1% neurobiotin, pH 7.3 with KOH). Osmolarity of the solution was checked with a vapor pressure osmometer (model 5520, Wescor, Logan, UT). The osmolarities of the internal and external solutions were 280–290 and 320–330 mOsm/l, respectively. The liquid junction potential of the patch pipette and perfused extracellular solution was estimated to be ~16.2 mV and was applied to the data. The recording pipette was under positive pressure while it was advanced toward individual cells in the slice. Tight seals of 0.5–1.0 GΩ were made by applying negative pressure and ZAP procedure. The membrane patch was then ruptured by suction. The series resistance during recording was 10–25 MΩ and was compensated. The reference electrode was an Ag-AgCl pellet immersed in bath solution. During recordings, cells were superfused with extracellular solution at a rate of 1.0–2.0 ml/ min using a peristaltic pump (Miniplus3, Gilson, Paris, France) at room temperature.

Statistical Analysis

Data were expressed as mean±SEM. One-way analysis of variance (ANOVA) followed by Bonferroni method as a post-hoc test or student’s t-test using Origin 6.1 software was used for statistical comparison among the various treatment groups. Differences were considered significant at p<0.05.

Supporting Information

Figure S1 Supplemental data for resident intruder test. (A) Increased impulsiveness and contact time in Npbwr1−/− mice during resident-intruder test when intruders were wild type or Npbwr1+/- mice. Male naive 8-week-old wild type mice were housed individually for 4 weeks before the procedure. The behavior of mice was recorded with a CCD video camera. A randomly chosen male intruder Npbwr1−/− or wild type (WT) mouse (C57BL/6J) was used only once in each session. The intruder was introduced into the resident cage, and behavior was recorded for 10 min. A variety of social behaviors were scored, including the latency to the first aggressive contact (left panel) and time spent in aggressive contact (sniffing, rattling, chasing, mounting, wrestling and fighting) (right panel). Npbwr1−/− intruder mice showed a shorter latency time to contact with the resident compared with wildtype (wild type; n = 6, Npbwr1−/−; n = 7, F1,11 = 5.162, p = 0.044) and longer contact time (wild type; n = 6, Npbwr1−/−; n = 7, F1,11 = 4.643, p = 0.050). Data are presented as mean ± SEM. (B) Locomotor activity of Npbwr1−/− (n = 5), Npbwr1+/- (n = 5), and WT (Npbwr1+/+) mice (n = 5) monitored by radiotratometry system during resident-intruder test. Horizontal solid bar indicates the presence of an intruder. Baseline values were defined as the average of parameters obtained during 10 min immediately prior to resident-intruder test. Data are presented as mean ± SEM. * indicates p<0.05. (TIFF)

Figure S2 CeA and BST is major effector site for neuropeptide W. (A) In situ hybridization histochemistry combined with GFP-immunostaining showing that Npbwr1 mRNA is colocalized with GFP in the bed nucleus of the stria terminalis (BST) of Gad67-gfp(Neo) mice. Brown staining shows GFP immunoreactivity. Blue staining demonstrates NPW-ir fibers in the CeAl in both wild type and Npbwr1−/− mice. Upper panels show sections from wild type mice and lower panels show sections from Npbwr1−/− mice. Rectangles in the left panels are shown as high power views in the right panels. Similar staining was also observed in the BST. (TIFF)

Figure S3 Npbwr1−/− mice show normal spatial memory as measured by Morris water maze test. Npbwr1−/− mice did not show a significant difference compared with wild type mice, even in the retention phase, transfer phase, and probe trial [WT n = 15, KO n = 13]. Data are presented as mean ± SEM. The apparatus consisted of a circular pool (40 cm high×120 cm diameter) filled with water maintained at 25°C and made opaque by addition of nontoxic white paint. Visual cues were placed around the pool. The escape platform, made of Plexiglas, was positioned such that its top surface was 1 cm below the surface of the water. Data were collected using a video tracking system Compact VAS vers 3.0x (Muromachi Kikai, Tokyo, Japan). The experiment was conducted in four phases. The first phase consisted of 2 days with the platform visible. This tested the ability of the animal to successfully conduct the task, particularly its visual ability to see the room cues and its motor ability to swim in the pool. For each trial during the training phase, the platform was hidden in the same quadrant and the test mouse was placed in the pool from different quadrants, and its time to reach the platform was...
recorded. At the end of training, a probe trial was performed where each mouse was tested to see if it could identify its spatial location. For the probe trial, the hidden platform was removed from the pool, and then the mouse was placed in the pool as before. The time each mouse spent in the quadrant that formerly contained the platform for 60 sec was recorded. Five days after the training phase, a retention phase was conducted to test for long-term memory. Finally, after the retention phase, the hidden platform was placed in the opposite quadrant and each mouse was retrained to the new platform location (transfer phase). This trial tested reversal learning in the mice. For each phase, four trials per day were given to each mouse. Each trial lasted a maximum of 90 sec (except the probe trial) with a 15 min interval between trials.

(TIF)

Movie S1 Typical behavior of wild type C57Bl/6J mouse during resident-intruder test. The wild type resident mouse maintained an appropriate personal space, exercising caution towards the intruder. (MPG)

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Movie S2 Typical behavior of Npbwr1−/− mouse during resident-intruder test. The resident Npbwr1−/− mouse showed insistent chasing of the intruder. They abandoned their normal caution and tendency to withdraw when confronted with a strange mouse. Instead, they impulsively approached the intruder and showed a greater frequency and duration of contact. (MPG)

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Author Contributions
Conceived and designed the experiments: TS. Performed the experiments: RKN NF JH TA MH NI TM MM TK. Analyzed the data: TM MY MY TS. Contributed reagents/materials/analysis tools: YY. Wrote the paper: TS.

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