初めて報告していた．2012年度の本研究では，一般住民のなかでも40歳以上を対象として被養育体験としての主観的な両親の養育スタイルを調べると，父親において，最も悪いとされる冷淡と過干渉の養育スタイルで慢性疼痛の有症リスクが約2倍になるという結果が得られ，具体的なリスクが算出できた．

養育については，「高い過干渉」は，依存的なパーソナリティ，対人過敏性，強迫性との関連が報告されている．そのため，能動的問題解決や対人関係・時間マネージメントに問題を抱えやすく，疼痛を悪化させない作業負荷・ペースの調整や健康管理が適切に行えないことで疼痛を慢性化させる可能性がある．

「低いケア」と「高い過干渉」は，自己評価の低さや成人後の抑うつ症状との関連が報告されており，養育と慢性疼痛の関連の一部は抑うつ症状を介している可能性がある．

父親の「低いケア・高い過干渉」のなかに，身体的な被虐待経験を有するものが認められるという報告が複数あり，父親が「冷淡と過干渉」の群には潜在的な被虐待経験者が他の群より多く含まれている可能性がある．

今回，父親の養育スタイルが慢性疼痛の有症率に与える影響と同様の傾向を有するものの，2011年度の研究結果と異なり母親の養育スタイルで有意な差が得られなかった．その原因として，久山町一般住民の母親の養育スタイルが比較的望ましい群が多かったことが考えられる．久山町一般住民と心療内科を受診した患者群では，母親において，心療内科を受診した患者群の被養育体験で望ましい養育スタイルが有意に少ないという結果が得られている．そのため，母親の養育スタイルが悪いサンプルを比較的多く含む住民集団を対象にすると，母親の養育スタイルも有意に影響するという結果が得られる可能性もあり，母親の養育スタイルの重要性は無視されるべきではないと考えられる．

最後に，日本国民全体の慢性疼痛の有症リスクという観点で今回の結果を概観すると，養育スタイルと慢性疼痛の有症率に有意な結果が得られたということは，将来の日本の慢性疼痛有症人口を減少させるための予防策につながる情報として重要であると考えられる．つまり，現代日本の重大なテーマである少子化社会において，養育は過干渉に偏りやすく，将来をゆだねる子の進路に関しては両親の希望が優先で本人の気持ちを聞かないという「冷淡と過干渉」の養育スタイルに陥りやすい状況にある．また，本研究の対象集団が養育を受けていた42年以上前の日本よりも，本研究で有意に慢性疼痛の有症率を上げていた“ケアなし／過干渉あり”の養育スタイルは現代ではより広く一般化していると考えられる．
さらに，大学病院の心療内科を受診するまでになる慢性疼痛の重症感は冷淡と過干渉の養育スタイルと関連しているという昨年の研究結果を合わせると，今後慢性疼痛の難治化が懸念される．その対策としては，養育と慢性疼痛との関連に視点をあてた患者対策とともに，予防策として，「幼少期に受けた冷淡と過干渉の親の養育スタイルが成人後の慢性疼痛の有症率をあげる」という結果を，養育スタイルを視野に入れて現在こどもを養育中である市民へ情報提供していくことが必要であると思われる．現時点でのエビデンスをもとに，今後は情動に影響をあたえる養育がどのような心理学的および生理学的メカニズムで慢性疼痛有症リスクをあげるかについてのさらなる実証的研究が必要である．

E. 結論

一般人口において，幼少期の両親の低いケアと高い過干渉といった養育態度が成人後の慢性疼痛発症に影響していた．特に，父親の養育スタイルも影響することが示唆された．

G．研究発表

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H. 知的財産権の出願・登録状況

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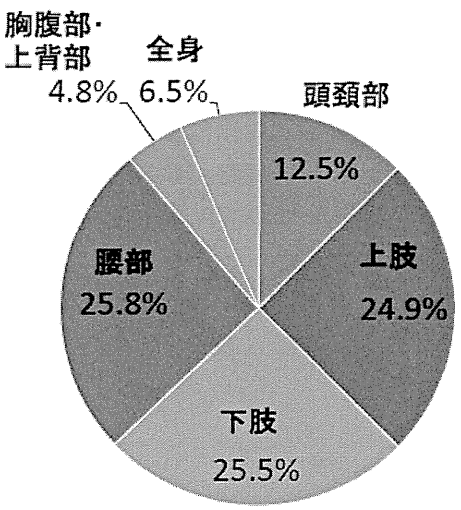
図１　　慢性疼痛群における主要な疼痛部位と疼痛の強さ



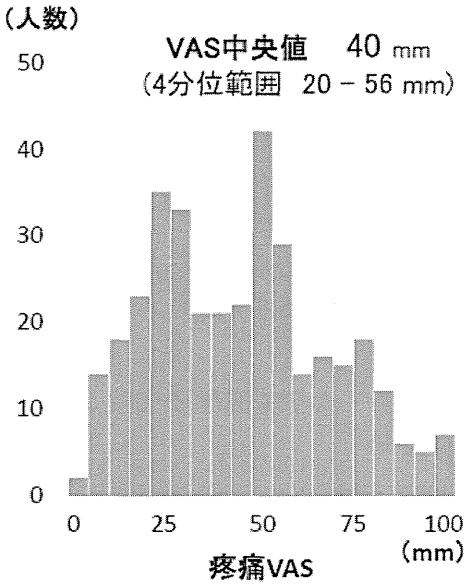


表１　　慢性疼痛有無別に見た背景因子と **PBI** スコア

	慢性疼痛なし（*n* = 407）		慢性疼痛あり（*n* = 353）	
年齢	59.0	±10.9（歳）	59.7	±12.1（歳）
性別（女性）	60.2	%	64.9	%
婚姻（ﾊﾟｰﾄﾅｰ有）	80.1	%	81.9	%
教育（≦中学）	11.5	%	15.9	%
経済（苦）	**17.0**	**%**	**24.9***	**%**
＜PBIスコア＞				
父 ケア (0–36)	**29.0**	**(22–34)**	**27.0 ** **	**(21–33)**
父 過干渉 (0–39)	**7.0**	**(3–12)**	**9.0 *** **	**(5–14)**
母 ケア (0–36)	31.0	(26–35)	31.0	(24–35)
母 過干渉 (0–39)	**7.0**	**(3–12)**	**9.0 ***	**(4–14)**

値は平均値±SD / 中央値(4分位範囲) / %　　* $p < 0.05$　** $p < 0.01$　*** $p < 0.001$

図２　ＰＢＩスコア 3 分位 別にみた慢性疼痛の頻度

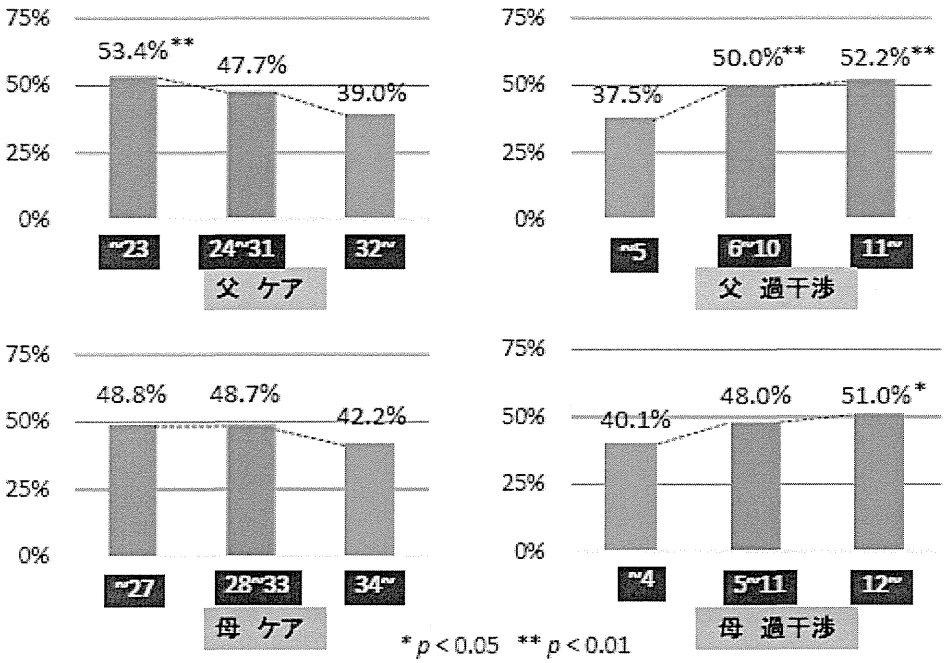


図３　ＰＢＩスコア 3 分位別にみた慢性疼痛のオッズ比

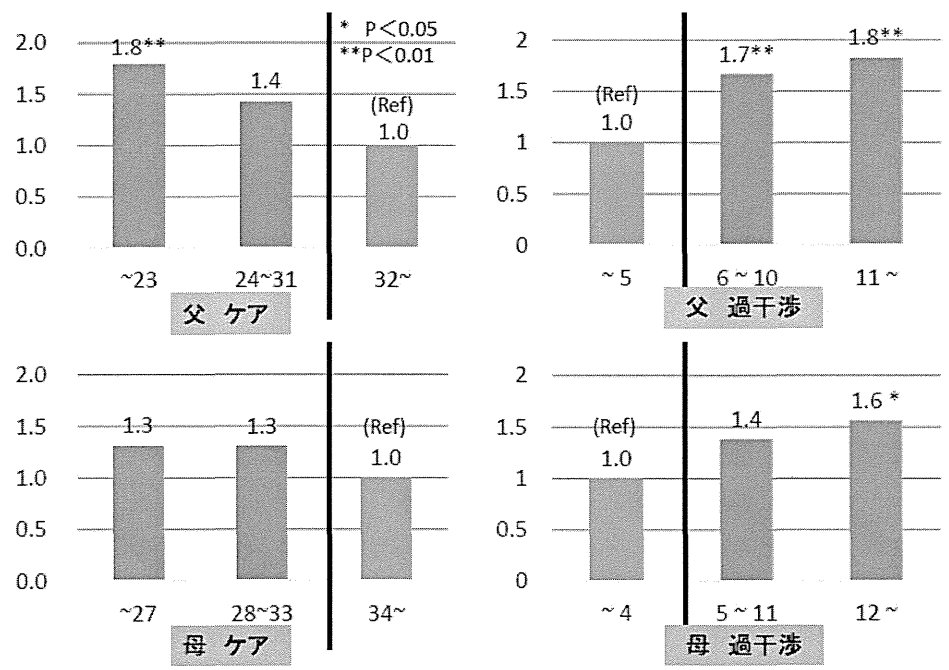


図４　ＰＢＩによる養育態度の４分類

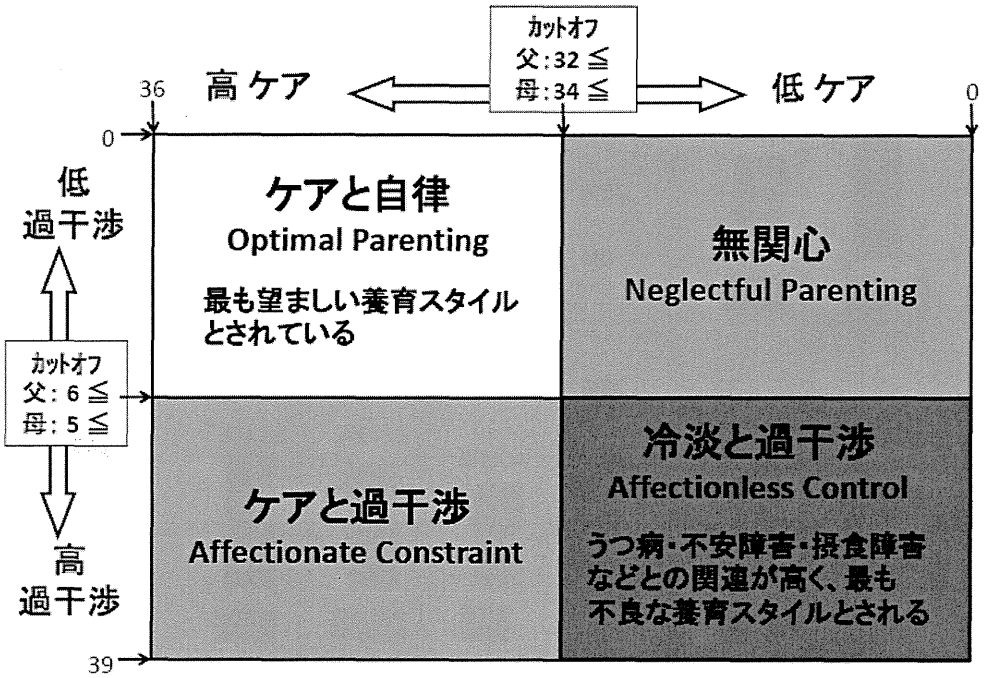


図５　養育態度の４分類別にみた慢性疼痛の頻度

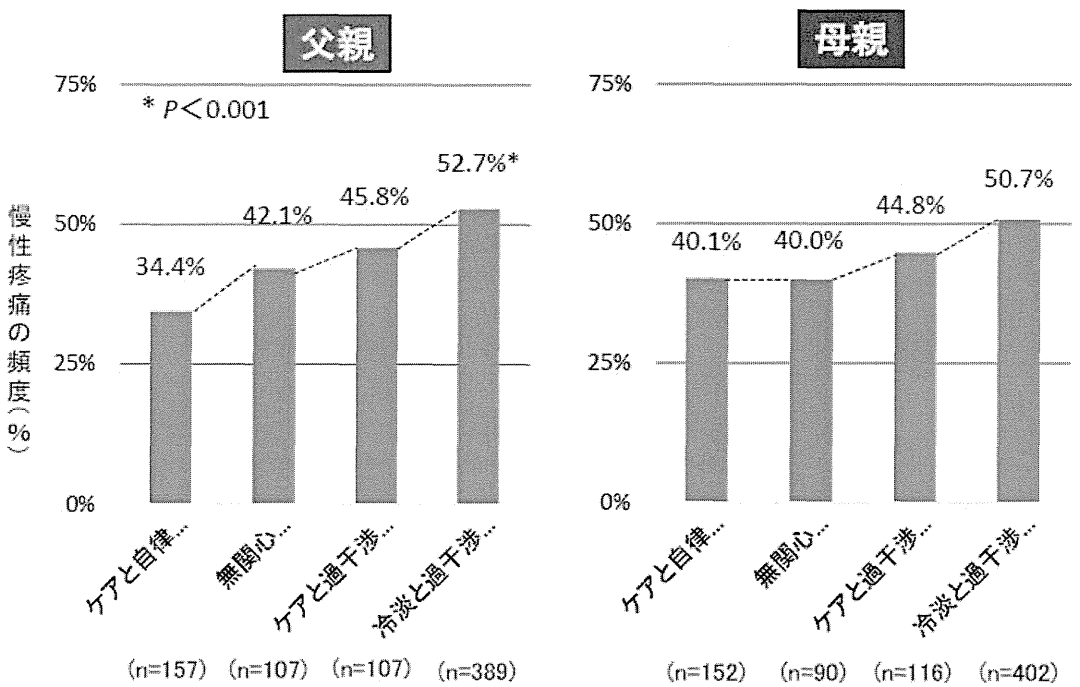
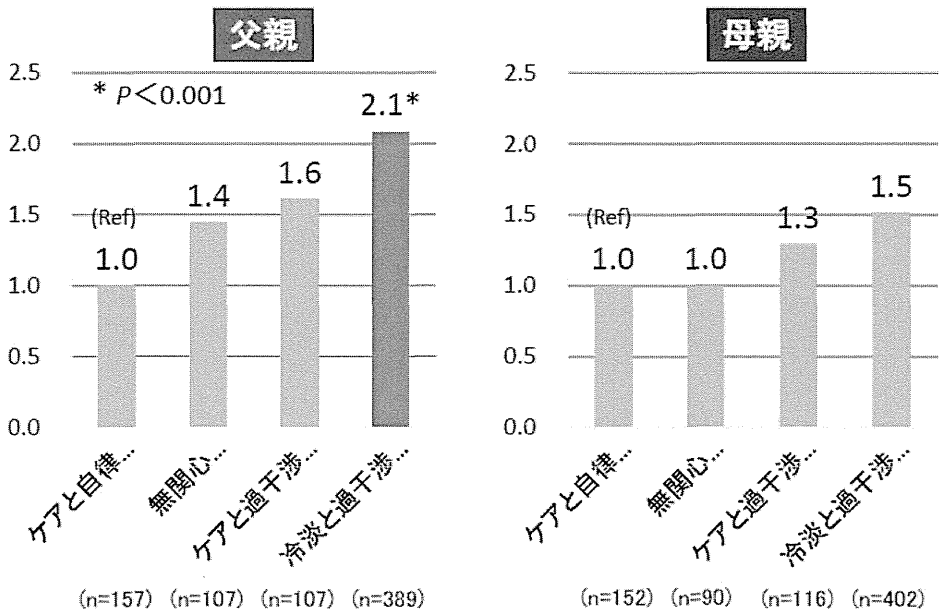


図６　養育態度の４分類別にみた慢性疼痛のオッズ比
（年齢・性別・婚姻・教育年数・経済状況で調整）



ＩＩＩ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Naka T, Ide S, Nakako T, Hirata M, Majima Y, Deyama S, Takeda H, Yoshioka M, Minami M	Activation of β-adrenoceptors in the bed nucleus of the stria terminalis induces food intake reduction and anxiety-like behaviors.	Neuropharmacology	67	326-330	2013
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YM, Miyamoto T, Koyama T, Shirato H					
Inoue T, Tanaka T, Nakagawa S, Nakato Y, Kameyama R, Boku S, Toda H, Kurita T, Koyama T	Utility and limitations of PHQ-9 in a clinic specializing in psychiatric care.	BMC Psychiatry	12(1)	73	2012

ＩＶ．研究成果の刊行物・別刷

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Activation of β-adrenoceptors in the bed nucleus of the stria terminalis induces food intake reduction and anxiety-like behaviors

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Noradrenaline

ABSTRACT

We previously demonstrated the critical role of noradrenergic transmission within the ventral part of the bed nucleus of the stria terminalis (vBNST) in pain-induced aversion. We showed that activation of β-adrenoceptors in this brain region by intra-vBNST injection of isoproterenol, a β-adrenoceptor agonist, produced aversive responses. In the present study, we examined the effects of a β-adrenoceptor agonist injected into the vBNST on food intake and anxiety-like behaviors in male Sprague-Dawley rats. Bilateral intra-vBNST injection of isoproterenol (3 and 10 nmol/side) caused a dose-dependent decrease in food intake; this suppressive effect was reversed by co-administration of timolol, a β-adrenoceptor antagonist. Dose-dependent (10 and 30 nmol/side) induction of anxiety-like behaviors by isoproterenol was observed in the elevated plus maze (EPM) test, which was also reversed by co-administration of timolol. Off-site control injections of isoproterenol into the lateral ventricle did not show any significant effect in the food consumption and EPM tests. These results suggest that the vBNST is one of the neuroanatomical substrates which may be involved in the close relationship between negative affective states and reduction of food intake, and that noradrenergic transmission within this brain region plays a critical role in inducing anxiety-like behaviors and reduced food intake.

1. Introduction

A close relationship between anorexia and anxiety has been suggested. Pollice et al. (1997) showed a significant relationship between anorexia and anxiety disorders. Godart et al. (2000) reported that 83% of patients with anorexia nervosa had at least one lifetime diagnosis of an anxiety disorder. These findings suggest that common neuroanatomical substrates and neurotransmission mechanisms may mediate reduction of food intake and anxiety. The bed nucleus of the stria terminalis (BNST) is a limbic structure involved in stress responses and the regulation of negative affective states, such as anxiety and fear (Walker et al., 2003). The BNST has also been implicated in the regulation of food intake (Ciccocioppo et al., 2003). Thus, this brain region may be involved in the close relationship between negative affective states and reduction of food intake.

The BNST, especially its ventral part (vBNST), is densely innervated by noradrenergic fibers arising mainly from the nuclei of the solitary tract (including the A2 cell group) and caudal ventrolateral medulla (including the A1 cell group) (Woulfe et al., 1990; Forray et al., 2000). Noradrenergic transmission within the vBNST is important for mediating negative affective states. Specifically, suppression of noradrenergic neurotransmission in the vBNST attenuates fox odor-induced freezing behaviors (Fendt et al., 2005) and morphine withdrawal-induced conditioned place aversion (CPA) (Delfs et al., 2000) in rats. We previously demonstrated the crucial role of enhanced noradrenergic transmission via β-adrenoceptors within the vBNST in pain-induced CPA in rats (Deyama et al., 2008, 2009). We showed that activation of β-adrenoceptors in this brain region by intra-vBNST injection of isoproterenol, a β-adrenoceptor agonist, produced aversive responses. In the present study, we examined the effects of β-adrenoceptor activation within the vBNST on food intake and anxiety-like behaviors in rats.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (190–300 g; Japan SLC, Hamamatsu, Japan) were used. Three to four rats were housed per cage. After implantation of guide

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cannulae, the rats were individually housed in cages. All rats were housed under standard conditions (temperature 23 ± 1 °C, 12 h light/dark cycle, water available *ad libitum*). Food was also available *ad libitum*, except during the food-deprivation period. Naïve animals were used for each experiment. All experiments were approved by the institutional animal care and use committee of Hokkaido University.

2.2. Drugs

Isoproterenol (β-adrenoceptor agonist) and timolol (β-adrenoceptor antagonist) were purchased from Sigma (St Louis, MO). These drugs were dissolved in phosphate-buffered saline.

2.3. Microinjection

Under sodium pentobarbital anesthesia (50 mg/kg, i.p.), each rat was bilaterally implanted with 25-gauge stainless steel guide cannulae 1.5 mm above the vBNST (−0.3 mm rostral, 1.6 mm lateral, 6.0 mm ventral to the bregma) or lateral ventricle (−0.8 mm rostral, 1.4 mm lateral, 2.7 mm ventral to the bregma) according to the brain atlas (Paxinos and Watson, 1998). The rats were allowed to recover for at least 5 days and were handled for 1–2 min each day for habituation. Microinjection was performed using 33-gauge stainless steel injection cannulae inserted bilaterally into the guide cannulae. The injection cannulae protruded 1.5 mm from the tip of the guide cannulae to reach the vBNST or lateral ventricle. The injection cannulae were attached to a microinjection pump (CMA, Stockholm, Sweden) via PE 8 tubing. Drugs or vehicle were bilaterally administered into the vBNST in a volume of 0.5 μl/side at a rate of 0.5 μl/min, and the injection cannulae were left in place for 1 min after the injection to prevent backflow.

2.4. Food consumption test

Food intake was measured for 24 h, and then the animals were deprived of food for 20 h. Five min after the injection of isoproterenol (3 or 10 nmol/side), isoproterenol (10 nmol/side) plus timolol (10 nmol/side), or vehicle into the bilateral vBNST or lateral ventricle, food was given to the food-deprived rats, and food intake during 30 min was measured. The data are expressed as the food intake adjusted by the body weight of each rat (g/kg).

2.5. Elevated plus maze test

The apparatus used for the elevated plus maze (EPM) test was a cross-shaped maze composed of two open arms (10 × 50 cm) and two closed arms (10 × 50 cm with walls 45 cm in height) connected by a central platform (10 × 10 cm). The apparatus was elevated 50 cm from the floor, and illuminated by dim light (~20 lux at the central platform). Five minutes after microinjection, each rat was placed on the central platform facing an open arm. Then the animals were allowed to freely explore the apparatus for 10 min. The movement of each animal was recorded by video camera and analyzed using the software package LimeLight2 (Actimetrics, Wilmette, IL) to determine the time spent in open arms and the number of entries to closed arms.

2.6. Rotarod test

The effect of intra-vBNST isoproterenol injection on coordinated motor function was assessed using an accelerating rotarod apparatus (Med-Associates, St. Albans, VT). The rotarod accelerated constantly from 4 to 40 rpm over 5 min. Each rat was trained once a day for 2 days on the apparatus before the test day. On the test day, isoproterenol (30 nmol/side) or vehicle was injected into the bilateral vBNST. Five minutes after microinjection, each rat was placed on the rod and time spent on the rod was measured.

2.7. Histology

After behavioral experiments, histological analyses were performed to examine the placements of injection cannulae. Briefly, rats were decapitated and the brain was rapidly removed and frozen in powdered dry ice. Coronal sections (50 μm) were prepared on a cryostat, and stained with thionin. These sections were examined by light microscopy (40×). Data from rats with correct placements of the bilateral injection cannulae (Fig. 1) were used for statistical analyses.

2.8. Statistical analyses

The data are expressed as means ± SEM. Food intake and the time spent in the open arms and the number of closed arm entries in the EPM test were analyzed using one- or two-way analysis of variance (ANOVA) followed by the Newman–Keuls *post hoc* test, or using Student's *t*-test. Time spent on the rod in the rotarod test was analyzed using Student's *t*-test. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Effect of intra-vBNST injection of isoproterenol on food intake

Before food deprivation, no significant differences were observed in food intake for 24 h among the three groups (3, 10 nmol/side isoproterenol and vehicle; 95.1 ± 3.5, 97.0 ± 2.9 and 95.5 ± 3.1 g/kg/24 h, respectively). After food deprivation for 20 h, isoproterenol (3 or 10 nmol/side) or vehicle was injected into the bilateral vBNST, and then food intake was measured for 30 min. As shown in Fig. 2A, intra-vBNST injection of isoproterenol reduced food intake in a dose-dependent manner. One-way ANOVA showed a significant difference among groups ($F_{2, 16} = 12.86$, $P < 0.001$). *Post hoc* comparisons by Newman–Keuls test indicated that isoproterenol at a dose of 10 nmol/side significantly decreased food intake (7.61 ± 0.67 g/kg/30 min, $P < 0.001$) compared to the vehicle-injected group (16.4 ± 1.6 g/kg/30 min).

Off-site control experiments were carried out by injecting isoproterenol into the lateral ventricle at a dose of 10 nmol/side. Before food deprivation, no significant differences were observed in food intake for 24 h between the two groups (10 nmol/side isoproterenol and vehicle; 100.8 ± 2.8 and 101.0 ± 1.3 g/kg/24 h, respectively). After food deprivation for 20 h, isoproterenol (10 nmol/side) or vehicle was injected intracerebroventricularly, and then food intake was measured for 30 min. As shown in Fig. 2B, there was no significant difference in food intake between the isoproterenol- and vehicle-injected groups (16.0 ± 3.0 and 14.4 ± 1.0 g/kg/30 min, respectively; $P > 0.05$, Student's *t*-test).

3.2. Effect of co-administration of timolol on the reduction of food intake by isoproterenol

Next, we examined the effect of co-administration of timolol, a β-adrenoceptor antagonist, on the reduction of food intake induced by intra-vBNST injection of isoproterenol (Fig. 3). Two-way ANOVA indicated a significant main effect of isoproterenol ($F_{1, 26} = 13.1$, $P < 0.01$) and a significant interaction between the effects of isoproterenol and timolol ($F_{1, 26} = 5.05$; $P < 0.05$). Reduction of food intake induced by isoproterenol (7.31 ± 1.16 g/kg/30 min) was significantly reversed by co-administration of timolol (14.4 ± 1.1 g/kg/30 min, $P < 0.01$, Newman–Keuls *post hoc* test). No significant effect was observed in the rats injected with timolol alone (16.3 ± 1.6 g/kg/30 min), compared to the vehicle-injected rats (15.5 ± 1.5 g/kg/30 min).

3.3. Effect of intra-vBNST injection of isoproterenol on anxiety-like behaviors in the EPM test

To investigate β-adrenergic neurotransmission within the vBNST related to anxiety-like behaviors, we examined the effect of intra-vBNST isoproterenol injection in an EPM test. Intra-vBNST isoproterenol injection reduced the time spent in open arms in a dose-dependent manner (Fig. 4A). One-way ANOVA indicated a significant difference among groups ($F_{2, 32} = 4.90$, $P < 0.05$), and *post hoc* comparisons by Newman–Keuls test showed that isoproterenol at a dose of 30 nmol/side significantly decreased the time spent in open arms (50.0 ± 13.0 s, $P < 0.05$) compared to the vehicle-injected group (135.4 ± 21.4 s). Intra-vBNST injection of isoproterenol did not affect the number of closed arm entries in the EPM test (Fig. 4B; $F_{2, 32} = 1.06$, $P > 0.05$, one-way ANOVA), suggesting no effect of intra-vBNST isoproterenol injection on locomotor activity.

Off-site control injections of isoproterenol (30 nmol/side) into the lateral ventricle did not show any significant effect in the EPM test (Fig. 4C, D). Specifically, the time spent in open arms of the

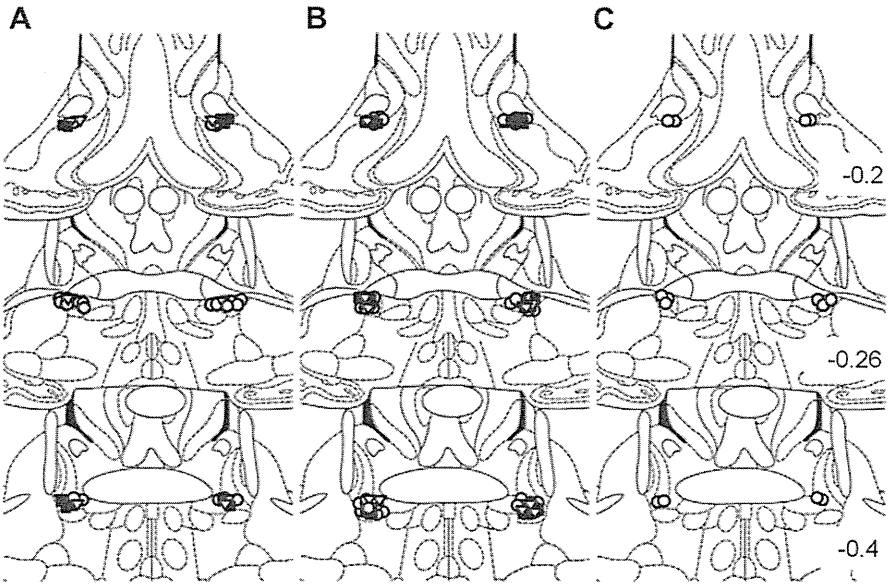

Fig. 1. Illustrations demonstrating the placement of the tips of microinjection cannulae in the food consumption test (A), EPM test (B), and rotarod test (C). (A) 10 nmol isoproterenol: open circles; 10 nmol timolol: open triangles; 10 nmol isoproterenol plus 10 nmol timolol: filled squares. (B) 30 nmol isoproterenol: open circles; 30 nmol timolol: open triangles; 30 nmol isoproterenol plus 30 nmol timolol: filled squares. (C) 30 nmol isoproterenol: open circles. The illustrations of coronal sections were taken from the atlas of Paxinos and Watson (1998); 0.2, −0.26, and −0.4 indicate distances (mm) from bregma.

isoproterenol-injected rats (70.9 ± 15.7 s) was not significantly ($P > 0.05$ ($P = 0.19$, $t = 1.38$), Student's t-test) different from that of the vehicle-injected group (109.0 ± 23.4 s). The number of closed arm entries was not different between the isoproterenol- and vehicle-injected rats ($P > 0.05$, Student's t-test).

3.4. Effect of co-administration of timolol on the anxiogenic effect of isoproterenol

The effect of co-administration of timolol on isoproterenol-induced anxiety-like behaviors in the EPM test was examined (Fig. 5). Two-way ANOVA indicated a significant main effect of isoproterenol ($F_{1, 48} = 18.1$, $P < 0.001$) and a significant interaction between the effects of isoproterenol and timolol ($F_{1, 48} = 6.65$; $P < 0.05$) on the time spent in open arms (Fig. 5A), but not on the number of closed arm entries (main effect of isoproterenol; $F_{1, 48} = 3.75$; $P > 0.05$) (Fig. 5B). The isoproterenol-induced reduction of the time spent in open arms (54.3 ± 11.4 s) was significantly reversed by the co-administration of timolol (127.0 ± 13.4 s, $P < 0.05$, Newman–Keuls *post hoc* test). No significant effect was observed in the rats injected with timolol alone (163.7 ± 34.6 s), compared to the vehicle-injected rats (203.9 ± 26.4 s).

3.5. Effect of intra-vBNST injection of isoproterenol on coordinated motor function in the rotarod test

The effect of intra-vBNST isoproterenol injection on motor function was determined using a rotarod test (Fig. 6). Rats that were bilaterally injected with isoproterenol (30 nmol/side) showed no

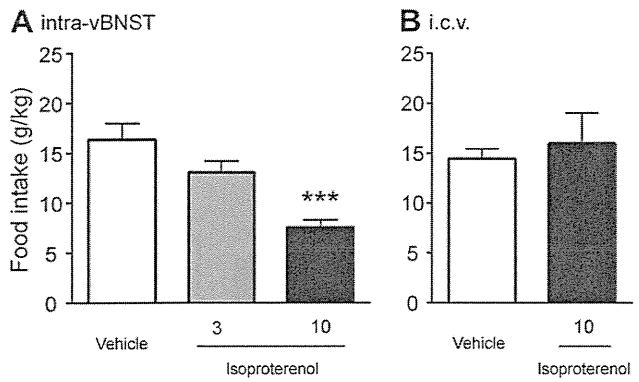

Fig. 2. The effect of intra-vBNST injection of isoproterenol on food intake in food-deprived rats. (A) The columns show food intake for 30 min after the injection of vehicle ($n = 5$) or isoproterenol (3 nmol, $n = 8$; 10 nmol, $n = 6$) into the vBNST. (B) The columns show food intake for 30 min after the injection of vehicle ($n = 6$) or isoproterenol (10 nmol, $n = 5$) into the lateral ventricle. Data are expressed as means ± SEM. ***$P < 0.001$ compared to the vehicle-injected rats (Newman–Keuls *post hoc* test).

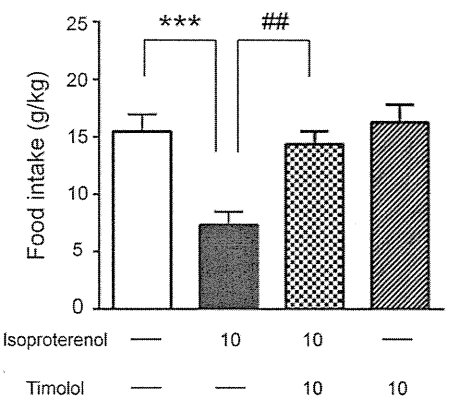

Fig. 3. The effect of co-administration of timolol on the reduction of food intake by isoproterenol. The columns show food intake for 30 min after the injection of vehicle ($n = 9$), 10 nmol/side isoproterenol ($n = 7$), 10 nmol/side isoproterenol plus 10 nmol/side timolol ($n = 7$), or 10 nmol/side timolol ($n = 7$). Data are expressed as means ± SEM. ***$P < 0.001$ compared to the vehicle-injected rats; ##$P < 0.01$ compared to the isoproterenol-injected rats (Newman–Keuls *post hoc* test).

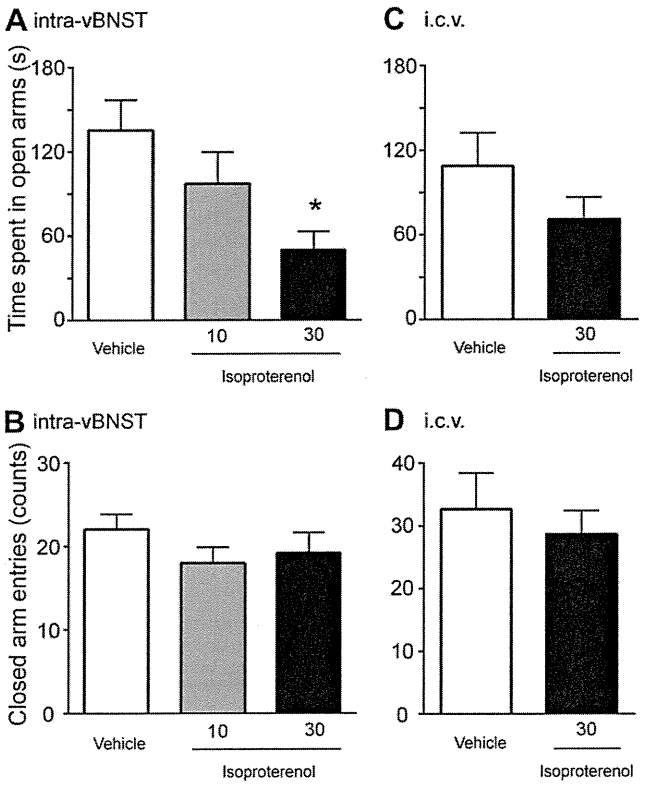

Fig. 4. The effect of intra-vBNST injection of isoproterenol on anxiety-like behaviors in the EPM test. (A, B) The columns show the time spent in open arms (A) and the number of closed arm entries (B) in rats injected with vehicle ($n = 15$) or isoproterenol (10 nmol, $n = 9$; 30 nmol, $n = 11$) into the vBNST. (C, D) The columns show the time spent in open arms (C) and the number of closed arm entries (D) in rats intracerebroventricularly injected with vehicle ($n = 9$) or isoproterenol (30 nmol, $n = 10$). Data are expressed as means ± SEM. $^{*}P < 0.05$ compared to the vehicle-injected rats (Newman–Keuls *post hoc* test).

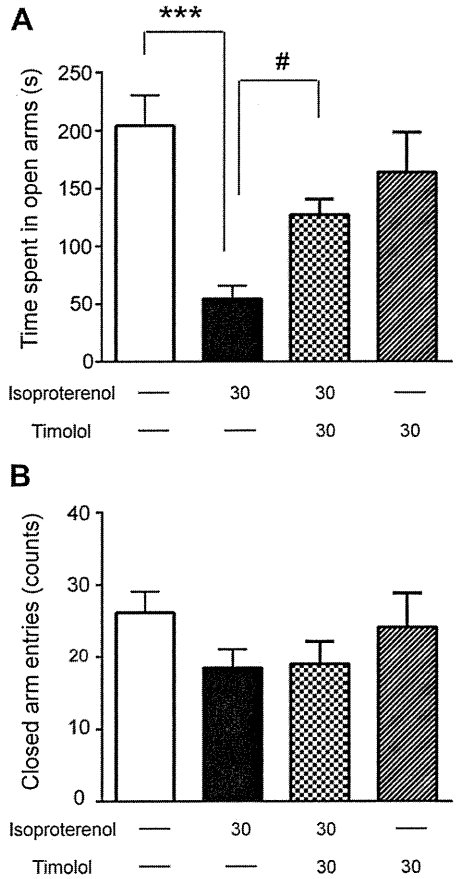

Fig. 5. The effect of co-administration of timolol on the anxiogenic effect of isoproterenol. The columns show the time spent in open arms (A) and the number of closed arm entries (B) in rats injected with vehicle ($n = 14$), 30 nmol/side isoproterenol ($n = 13$), 30 nmol/side isoproterenol plus 30 nmol/side timolol ($n = 15$), or 30 nmol/side timolol ($n = 10$). Data are expressed as means ± SEM. $^{***}P < 0.001$ compared to the vehicle-injected rats; $^{\#}P < 0.05$ compared to the isoproterenol-injected rats (Newman–Keuls post hoc test).

significant difference in the time spent on the rod compared to the vehicle-injected animals ($P > 0.05$, Student's *t*-test).

4. Discussion

Using food consumption and EPM tests, the present study demonstrates that intra-vBNST injection of isoproterenol, a β-adrenoceptor agonist, induced reduction of food intake and anxiety-like behaviors. Although behavioral tests can be affected by alterations in locomotor activity and motor function, the data of closed arm entries in the EPM test and the time spent on the rod in the rotarod test revealed that locomotor activity and motor function were not affected by intra-vBNST isoproterenol. Off-site control injections of isoproterenol into the lateral ventricle did not show any significant effect in the food consumption and EPM tests, suggesting that the vBNST is the likely site of action of isoproterenol although the contribution of bordering regions, including the dorsal BNST cannot be ruled out.

Important roles of intra-BNST noradrenergic transmission in negative emotional states have been reported. Fendt et al. (2005) reported that exposure to trimethylthiazoline, a component of fox odor, increased noradrenaline release in the BNST, and that intra-BNST administration of clonidine, an α_2-adrenoceptor agonist, suppressed both noradrenaline release and trimethylthiazoline-induced freezing behaviors. Delfs et al. (2000) reported that microinjection of β-antagonists or an α_2-agonist into the BNST markedly attenuated opiate withdrawal-induced CPA in rats. We previously demonstrated that intraplantar formalin- and intraperitoneal acetic acid-induced noxious stimuli increased noradrenaline release in the vBNST, and that intra-vBNST administration of timolol, a β-adrenoceptor antagonist, suppressed pain-induced CPA (Deyama et al., 2008, 2009). In addition, we recently reported that intra-vBNST injection of clonidine suppressed both pain-induced

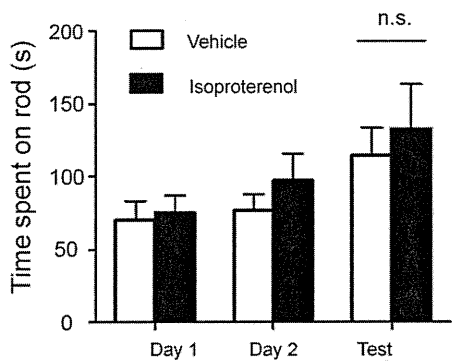

Fig. 6. The effect of intra-vBNST injection of isoproterenol on coordinated motor function in the rotarod test. The columns show the time spent on the rod in the rats injected with vehicle ($n = 9$) or 30 nmol/side isoproterenol ($n = 7$) on the test day. Data are expressed as means ± SEM.

noradrenaline release and CPA (Deyama et al., 2011). We also demonstrated that intra-vBNST injection of isoproterenol induced aversive responses in the CPA test (Deyama et al., 2008). These findings strongly suggest a pivotal role of intra-vBNST noradrenergic transmission via β-adrenoceptors in negative affective states. Our current study extends those findings by showing that intra-vBNST injection of isoproterenol induced anxiety-like behaviors in an EPM test.

We previously demonstrated that intra-vBNST injection of ICI118551, a β_2-adrenoceptor antagonist, significantly suppressed pain-induced aversive behaviors in a CPA test. However, betaxolol, a β_1-adrenoceptor antagonist, attenuated the aversive behaviors, but it was only a partial effect even at a high dose. Recently, Hott et al. (2012) reported that freezing behaviors in the conditioned contextual fear paradigm was suppressed by CGP20712, a β_1-adrenoceptor antagonist, but not by ICI118551. These findings suggest the differential contributions of β_1- and β_2-adrenoceptors to distinct emotional states. Further studies are needed to clarify the contributions of β_1- and β_2-adrenoceptors to reduced food intake and anxiety-like behaviors induced by intra-vBNST injection of isoproterenol.

Food intake is often suppressed by various kinds of stresses. However, we are only beginning to understand the mechanisms underlying the negative regulation of food intake by stresses. Reportedly, corticotropin-releasing factor (CRF), one of the key stress-response molecules, is involved in restraint stress- or emotional stress-induced inhibition of food intake (Sekino et al., 2004). Ciccocioppo et al. (2001) demonstrated that reduction of food intake by restraint stress or intracerebroventricular injection of CRF was attenuated by nociceptin. They also showed that reduction of food intake by CRF was reversed by intra-BNST injection of nociceptin, suggesting that the BNST is a neuroanatomical substrate for the suppressive effect of CRF on food intake and for its reversal by nociceptin (Ciccocioppo et al., 2003). In the present study, we found that intra-vBNST injection of a β-agonist, isoproterenol, suppressed food intake. This suggests that intra-BNST noradrenaline, another key molecule for stress responses in addition to CRF, plays an important role in the regulation of food intake.

The current study demonstrates that activation of β-adrenoceptors within the vBNST induces reduction of food intake and anxiety-like behaviors. In addition to the suppressive effect on food intake (Ciccocioppo et al., 2001, 2003), CRF induces anxiety-like behaviors in the EPM test when injected into the BNST (Sahuque et al., 2006). These findings suggest that the BNST is one of the neuroanatomical substrates which may be involved in the close relationship between negative affective states and reduction of food intake, and that two major stress-related molecules, noradrenaline and CRF, within this brain region play a critical role in inducing anxiety-like behaviors and reduced food intake.

Acknowledgments

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Behavioral/Cognitive

Opposing Roles of Corticotropin-Releasing Factor and Neuropeptide Y within the Dorsolateral Bed Nucleus of the Stria Terminalis in the Negative Affective Component of Pain in Rats

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Pain is a complex experience composed of sensory and affective components. Although the neural systems of the sensory component of pain have been studied extensively, those of its affective component remain to be determined. In the present study, we examined the effects of corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) injected into the dorsolateral bed nucleus of the stria terminalis (dlBNST) on pain-induced aversion and nociceptive behaviors in rats to examine the roles of these peptides in affective and sensory components of pain, respectively. *In vivo* microdialysis showed that formalin-evoked pain enhanced the release of CRF in this brain region. Using a conditioned place aversion (CPA) test, we found that intra-dlBNST injection of a CRF_1 or CRF_2 receptor antagonist suppressed pain-induced aversion. Intra-dlBNST CRF injection induced CPA even in the absence of pain stimulation. On the other hand, intra-dlBNST NPY injection suppressed pain-induced aversion. Coadministration of NPY inhibited CRF-induced CPA. This inhibitory effect of NPY was blocked by coadministration of a Y_1 or Y_5 receptor antagonist. Furthermore, whole-cell patch-clamp electrophysiology in dlBNST slices revealed that CRF increased neuronal excitability specifically in type II dlBNST neurons, whereas NPY decreased it in these neurons. Excitatory effects of CRF on type II dlBNST neurons were suppressed by NPY. These results have uncovered some of the neuronal mechanisms underlying the affective component of pain by showing opposing roles of intra-dlBNST CRF and NPY in pain-induced aversion and opposing actions of these peptides on neuronal excitability converging on the same target, type II neurons, within the dlBNST.

Introduction

Pain is a complex experience consisting of sensory and affective components. Although the neural systems of the sensory component of pain have been studied extensively, those of the negative affective component are only beginning to be understood. Recently, some behavioral studies using a conditioned place paradigm have revealed neural mechanisms underlying the negative affective component of pain. Johansen et al. (2001) reported the crucial role of the anterior cingulate cortex in conditioned place aversion (CPA) induced by the intraplantar injection of formalin. We reported that the central amygdaloid nucleus and basolateral amygdaloid nucleus (BLA) were differently involved in intraplantar formalin-induced and intraperitoneal acetic acid-induced CPA (Tanimoto et al., 2003). In addition to these brain areas, we found that the excitotoxic lesions of the bed nucleus of the stria terminalis (BNST) reduced pain-induced aversion without reducing nociceptive behaviors (Deyama et al., 2007). Moreover, we recently demonstrated that noradrenergic neurotransmission within the ventral part of BNST mediated the negative affective component of pain (Deyama et al., 2008, 2009, 2011).

The dorsolateral part of the BNST (dlBNST) is densely innervated with corticotropin-releasing factor (CRF)-containing fibers (Sakanaka et al., 1986; Morin et al., 1999) and expresses CRF receptors (Van Pett et al., 2000). Intra-BNST CRF has been implicated in negative affective states, such as anxiety, fear, and aversion. Intra-BNST infusion of CRF has been shown to elicit anxiety-associated behaviors in the elevated plus maze test (Sahuque et al., 2006) and to enhance startle responses (Lee and Davis, 1997). Furthermore, it has been reported that intra-BNST administration of CRF produced CPA (Sahuque et al., 2006). However, the role of CRF-mediated neurotransmission within the dlBNST in the negative affective component of pain remains unclear.

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Neuropeptide Y (NPY)-containing fibers are also observed in the dlBNST (Walter et al., 1991), and its receptors, Y_1, Y_2, Y_4, and Y_5 subtypes, are expressed in this brain region (Parker and Herzog, 1999). Although a large body of literature exists on the anxiolytic and anti-aversive effects of NPY (Heilig, 1995; Kask et al., 1997; Nakajima et al., 1998; Gutman et al., 2008), the role of this peptide in pain-induced aversion remains to be determined.

In this study, we examined the effects of intra-dlBNST injection of CRF and NPY on pain-induced aversion and nociceptive behaviors to examine the roles of these peptides in affective and sensory components of pain, respectively, and revealed their opposing roles in the negative affective component of pain. Furthermore, whole-cell patch-clamp electrophysiology in dlBNST slices showed opposing actions of these peptides on neuronal excitability specifically in type II dlBNST neurons.

Materials and Methods

Animals. Male Sprague Dawley rats (Japan SLC) (180–280 g or 20 to 50 d old) were used for the behavioral or electrophysiological experiments, respectively. The rats were maintained at a constant ambient temperature (22 ± 1°C) under a 12 h light/dark cycle with food and water available *ad libitum*. All experiments were performed with the approval of the Institutional Animal Care and Use Committee at Hokkaido University.

Drugs. NBI27914 (a selective CRF_1 receptor antagonist), antisauvagine-30 (AS-30; a selective CRF_2 receptor antagonist), and L-152,804 (a selective NPY Y_5 receptor antagonist) were purchased from Tocris Bioscience. CRF was purchased from Peptide Institute or Bachem AG. NPY and BIBP3226 (a selective NPY Y_1 antagonist) were from Abgent and Bachem AG, respectively. Tetrodotoxin (TTX) was from Wako Pure Chemical Industries and SR95531, ZD7288, and kynurenic acid were from Sigma.

For the behavioral experiments, AS-30 was dissolved in PBS, pH 7.4, containing 0.1% bovine serum albumin (BSA; Sigma). NBI27914 was dissolved in dimethylsulfoxide (DMSO), then diluted with PBS containing 0.1% BSA; the solution contained DMSO at a final concentration of 8.4%. The final concentration of these antagonists was 0.3 nmol/0.5 μl or 1.0 nmol/0.5 μl. NPY was dissolved in PBS containing 0.1% BSA at a concentration of 0.1 nmol/0.5 μl or 0.3 nmol/0.5 μl. CRF was dissolved in saline containing 0.1% BSA and 0.03% acetic acid, then mixed with PBS containing 0.1% BSA at a ratio of 2:1. The final concentration of CRF was 0.1 nmol/0.6 μl or 0.3 nmol/0.6 μl. When the effects of NPY in the presence or absence of NPY antagonists on CRF-induced CPA were examined, CRF solution was mixed with these drugs dissolved in PBS containing 0.1% BSA at a ratio of 2:1. L-152,804 was dissolved in DMSO, then diluted with PBS containing 0.1% BSA; the solution contained DMSO at a final concentration of 16.7%.

For the electrophysiological experiments, the stock solutions for CRF and NPY were prepared at concentrations of 1 mM in H_2O containing 0.1% BSA. The stock solutions for NBI27914 and AS-30 were prepared at a concentration of 300 μM in DMSO and in H_2O containing 0.1% BSA, respectively. The stock solutions for BIBP3226 and L-152,804 were prepared at a concentration of 1 mM in H_2O and in DMSO, respectively. The stock solution for ZD7288 was prepared at a concentration of 10 mM in H_2O. These stock solutions were stored at −30°C until use. Before bath application, these stock solutions were diluted to final concentrations with normal Ringer's solution containing 0.01% BSA. We confirmed that bath application of DMSO alone at the final concentration (0.1%) did not affect either membrane potential or input resistance (data not shown).

In vivo *microdialysis. In vivo* microdialysis was performed using a peptide microdialysis system (AtmosLM; Eicom). Under sodium pentobarbital anesthesia (50 mg/kg, i.p.), each rat was implanted unilaterally with a microdialysis guide cannula (outer diameter, o.d.; 0.72 mm, PEG-6; Eicom) 1.0 mm above the dlBNST (−0.3 mm rostral, 1.7 mm lateral, 5.5 mm ventral to bregma) (Paxinos and Watson, 1998). After surgery, rats were housed individually in cages. One day after the implantation of the guide cannula, microdialysis experiments were performed in unanesthetized and freely moving rats. Microdialysis probes (dialysis membrane: 1000 kDa molecular weight cutoff polyethylene membrane, length 1.0 mm; o.d., 0.44 mm; PEP-6–01; Eicom) were inserted through the guide cannula and continuously perfused with Ringer's solution (Na^+ 147 mM, K^+ 4 mM, Ca^{2+} 2.3 mM, and Cl^- 155.6 mM) containing 0.15% BSA at a flow rate of 10 μl /min. The rats were then placed in a Plexiglas chamber (30 × 30 × 35 cm: width × length × height) for the 2 h preconditioning period. After the preconditioning period, the flow rate was changed to 1 μl/min. After an additional stabilization period of 1 h, 11 15 min dialysates were collected in polypropylene tubes at 4°C. The first three samples were taken as baseline samples. Immediately after collection of the last baseline sample, each rat was administered an intraplantar injection (right hindpaw) of 100 μl of 2% formalin. Dialysate samples were stored at −20°C. CRF concentrations in the samples were measured using a competitive enzyme immunoassay kit (Phoenix Pharmaceuticals).

Surgery and microinjection. Under sodium pentobarbital anesthesia (50 mg/kg, i.p.), rats were implanted bilaterally with 25 gauge stainless steel guide cannulae (o.d., 0.5 mm; inner diameter, i.d., 0.22 mm) 1.5 mm above the dlBNST (−0.3 mm rostral, 1.7 mm lateral, 5.0 mm ventral to bregma) or lateral ventricle (−0.3 mm rostral, 1.4 mm lateral, 2.7 mm ventral to bregma) (Paxinos and Watson, 1998). After surgery, rats were housed individually in plastic cages with woodchip bedding, allowed to recover for at least 5 d, and handled for 1–2 min each day for 3 consecutive days before behavioral experiments. For microinjection, 33 gauge stainless steel injection cannulae (o.d., 0.2 mm; i.d., 0.08 mm) were inserted bilaterally into the guide cannulae. The injection cannulae protruded 1.5 mm from the tip of the guide cannulae to reach the dlBNST or lateral ventricle. Injection cannulae were attached to a microinjection pump (CMA) via PE 8 polyethylene tubing. Drug or vehicle was administrated bilaterally in a volume of 0.5–0.6 μl/side at a rate of 0.5 μl/min, and the injection cannulae were left in place for an additional 1 min after microinjection to prevent backflow.

Conditioned CPA. CPA tests were conducted as described previously (Deyama et al., 2007, 2008, 2009). A shuttle box composed of two equal-sized compartments (30 × 30 × 30 cm) with distinct visual and tactile cues (one compartment was black with a smooth floor, and the other was white with a textured floor) under dim illumination (25 ± 5 lux at the center of the box) was used for a 4 consecutive day experimental procedure. On day 1 (habituation session) and day 2 (preconditioning session), rats explored the two compartments *ad libitum* for 900 s; the time spent in each compartment during the exploring period was measured automatically (KN-80; Natsume Seisakusho). Rats that spent >80% (>720 s) of the total time (900 s) in one side on day 2 or showed a difference of >200 s in the time spent in one side between days 1 and 2 were eliminated from subsequent procedures. Additionally, after behavioral tests, histological analyses were performed, and data from rats with misplacement of both or either of the bilateral microinjection cannulae were eliminated from statistical analyses. Both before and after such eliminations, no significant difference ($p > 0.05$ ($n = 353$) and $p > 0.05$ ($n = 197$), respectively) was observed between the time spent in the black (442.6 ± 6.3 s and 445.8 ± 7.1 s, respectively) and white (457.4 ± 6.3 s and 454.2 ± 7.1 s, respectively) compartments, indicating the absence of any significant bias in compartment preference before conditioning.

Formalin-induced CPA was used to evaluate the affective component of pain (Johansen et al., 2001; Tanimoto et al., 2003; Johansen and Fields, 2004; Deyama et al., 2008, 2011). In this CPA test, we used a bias-like protocol (Tzschentke, 1998). Specifically, we designated the compartment in which each rat spent more time (>450 s) on day 2 (preconditioning session) as each animal's pain-paired compartment. This type of protocol was successfully used to examine the CPA induced by opioid withdrawal (Kosten, 1994; Rafieian-Kopaei et al., 1995; Nakagawa et al., 2005) and by visceral and somatic pain (Tanimoto et al., 2003; Deyama et al., 2007, 2008, 2009). On day 3 (conditioning session), place conditioning was performed as follows. In the vehicle control session (conducted between 08:00 and 12:00), each rat was given an intraplantar injection of saline (100 μl) into the left hindpaw and then immediately confined in the nonpain-paired compartment for 1 h. After at least 4 h, in the pain-conditioning session (conducted between 14:00 and 18:00), each rat was injected with NBI27914 (0.3 nmol/side or 1 nmol/side), AS-30 (0.3 nmol/side or 1 nmol/side), NPY (0.1 nmol/side or 0.3 nmol/side), or

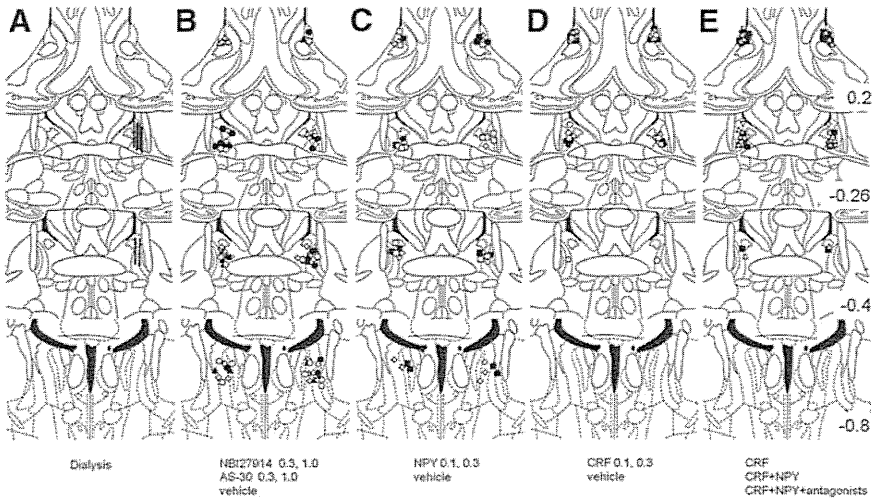

Figure 1. The placements of the tips of the microdialysis probes (***A***) and microinjection cannulae (***B–E***). ***B***, NBI27914 1.0 nmol, closed circles; 0.3 nmol, open circles; AS-30 1.0 nmol, closed triangles; 0.3 nmol, open triangles; vehicle, open rhomboids. ***C***, NPY 0.3 nmol, closed squares; 0.1 nmol, open squares; vehicle, open rhomboids. ***D***, CRF 0.3 nmol, closed circles; 0.1 nmol, open circles; vehicle, open rhomboids. ***E***, CRF, closed circles; CRF+NPY, closed squares; CRF+NPY+BIBP3226, open reversed triangles; CRF+NPY+ L-152,804, open triangles. The illustrations of coronal sections were taken from the atlas of Paxinos and Watson (1998); 0.2, −0.26, −0.4, and −0.8 indicate distances (mm) from bregma.

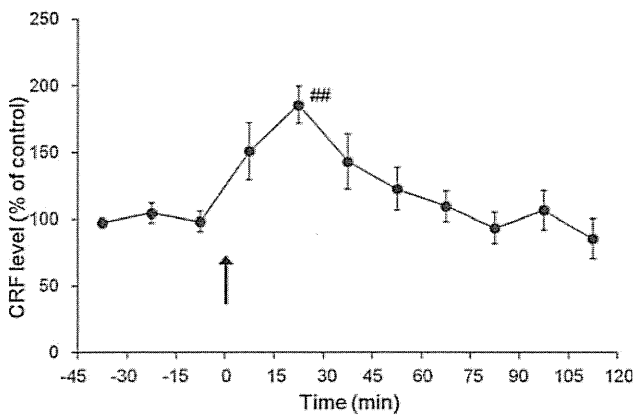

Figure 2. Effects of intraplantar injection of formalin ($n = 7$) on extracellular CRF levels in the dlBNST were examined. The arrow indicates the time point of intraplantar injection. Data are expressed as means ± SEM the percentage baseline control value, calculated as an average of three consecutive dialysates before intraplantar injection. $^{\#\#}p < 0.01$ compared with the value just before intraplantar injection (Newman–Keuls *post hoc* test).

vehicle into the bilateral dlBNST or lateral ventricle. At 10 min after the intra-dlBNST injection, the rats were given an intraplantar injection of 2% formalin (100 μl) into the right hindpaw and then confined in the pain-paired compartment for 1 h. On day 4 (test session), each rat was allowed to explore the two compartments *ad libitum*, and the time spent in each compartment during the exploring period (900 s) was recorded automatically. CPA scores were calculated by subtracting the time spent in the pain-paired compartment during the test session from the time spent in this compartment during the preconditioning session.

In the experiments investigating intra-dlBNST CRF-induced aversion (a 6 consecutive day experimental protocol), we designated the compartment in which the rat spent more time (>450 s) on day 2 (preconditioning session) as the drug-paired compartment for each animal. On days 3–5, place conditioning was performed over 3 consecutive days as follows. The rats were divided into two groups (Groups 1 and 2). In the morning session (conducted between 08:00 and 12:00), rats in Group 1 were given an intra-dlBNST injection of drugs or vehicle and were immediately confined in the drug-paired compartment for 30 min. On the other hand, rats in Group 2 were confined in the nondrug-paired compartment for 30 min without being given an intra-dlBNST injection. In the afternoon session (conducted between 13:00 and 18:00), rats in Group 1 were confined in the nondrug-paired compartment for 30 min without being given an intra-dlBNST injection, and rats in Group 2 were given an intra-dlBNST injection of drugs or vehicle and were immediately confined in the drug-paired compartment for 30 min. On day 6, in the test session, each rat was allowed to explore the two compartments freely, and the time spent in each compartment during the exploring period (900 s) was recorded. CPA scores were calculated as in the CPA test for pain-induced aversion.

Formalin test. Formalin-induced nociceptive behaviors were examined to evaluate the sensory component of pain (Johansen et al., 2001; Tanimoto et al., 2003; Johansen and Fields, 2004; Deyama et al., 2008, 2011). Each rat was placed in a Plexiglas cylinder (30 cm diameter; 30 cm height) for 30 min to acclimatize it to the experimental environment. Drugs or vehicle were injected bilaterally into the dlBNST of each rat, and the animals were returned to the cylinder. At 10 min after the intra-dlBNST injection, the rats were given an intraplantar injection of 2% formalin (100 μl) into the right hindpaw and immediately returned to the cylinder. The amount of time the rat spent lifting, licking, shaking, or biting the injected paw was measured for each 5 min period over 60 min. Measurement of nociceptive behaviors was done live by an observer blind to the treatment conditions. Nociception was quantified using a rating scale method by assigning weights to the following categories of nociceptive behaviors: category 0 = weight is evenly distributed among all paws; category 1 = injected paw is lifted; and category 2 = injected paw is licked, shaken, or bitten. The nociceptive score was calculated for each 5 min (300 s) period using the following formula: nociceptive score = [{time (s) spent with lifting the injected paw} × 1 + {time (s) spent with licking, shaking, or biting the injected paw} × 2]/300 (s).

Histology. After the *in vivo* microdialysis experiments and behavioral tests, histological analyses were performed. Rats were decapitated, and brains were removed rapidly and frozen in powdered dry ice. Coronal sections (50 μm) including the BNST were prepared with a cryostat, thaw-mounted onto slides, stained with thionine, and examined under a microscope (×40). Data from the rats with extensive tissue damage, misplacement of the microdialysis probes, or misplacement of both or either of the bilateral injection cannulae were excluded from statistical analyses.

Slice preparation for electrophysiology. Rats were decapitated under isoflurane anesthesia, and the brains were quickly removed and submerged in ice-cold artificial CSF containing the following (in mM): 130 NaCl, 3.5 KCl, 1.1 KH_2PO_4, 1.0 $CaCl_2$, 6.0 $MgCl_2$, 30 $NaHCO_3$, 10 glucose, and 2 kynurenic acid, saturated with 95% O_2/5% CO_2. Coronal slices (250 μm thick) containing the BNST region were prepared using a microslicer (VT1200S; Leica). Slices were incubated in a chamber containing normal Ringer's solution containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 26 $NaHCO_3$, and 25 glucose, saturated with 95% O_2/5% CO_2 at 33–35°C for 0.5 h, and then placed at room temperature for >0.5 h before recording.

Recording procedures for electrophysiology. Slices were placed in a recording chamber on an upright microscope (BX-51WI; Olympus) and continuously superfused with normal Ringer's solution (34°C) saturated with 95% O_2/5% CO_2 at a flow rate of 1–1.5 ml/min. Pipettes were pulled from thin-walled borosilicate glass capillaries with a micropipette puller (Model P-1000; Sutter Instrument). Tip resistance was 5–8 MΩ when pipettes were filled with an internal solution containing the following (in mM): 150 KOH, 2 $MgCl_2$, 10 KCl, 0.2 EGTA, 2 Na_2-ATP, 0.3 Na_2-GTP, 10 HEPES, and 0.1 spermine. The pH was adjusted to 7.3–7.4 with gluconic