Fig. 1. NPCs derived from E14 telencephalon express $\alpha_{1A}$-adrenoceptor ($\alpha_{1A}$), $\alpha_{1B}$-adrenoceptor ($\alpha_{1B}$) and $\alpha_{1D}$-adrenoceptor ($\alpha_{1D}$). Total RNA isolated from cultured NPCs and E14 mouse telencephalon (TEL) was reverse-transcribed, and the resultant cDNA was used in RT-PCR analysis. (A) As indicated on the left, the PCR products corresponding to the $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$ adrenoceptor genes and *GAPDH* were separated on a 3% agarose gel. The size of PCR products are indicated on the right. (B) Gene expression levels of the $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$ adrenoceptor were analyzed by quantitative RT-PCR, and the expression levels relative to *hprt* are presented. The results are representative of three separate experiments that yielded similar results.

telencephalon and E14 telencephalon-derived cultured NPCs (Fig. 1A). Semi-quantitative analysis of expression levels of the $\alpha_1$-adrenoceptor genes (normalized to the internal control, *hprt*) showed that the isolated NPCs highly expressed $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$ adrenoceptor genes as compared with the E14 telencephalon (Fig. 1B). Among the $\alpha_1$-adrenoceptor genes, the $\alpha_{1A}$ and $\alpha_{1B}$ genes were highly expressed in the cultured NPCs, whereas a low level of expression was detected for the $\alpha_{1D}$ gene in both the E14 telencephalon and cultured NPCs (Fig. 1B).

### 3.2. Effect of the $\alpha_1$-adrenoceptor agonist phenylephrine on NPC differentiation

E14 telencephalon-derived NPCs have potencies to differentiate into multiple neural cell types, including neurons, astrocytes and oligodendrocytes, in the absence of bFGF (Fig. 2). The effect of phenylephrine, an $\alpha_1$-adrenoceptor-selective agonist, on NPC differentiation was examined using the neural cell differentiation marker tuj1 for neuronal cells, glial fibrillary acidic protein for astroglial cells and galactocerebroside for oligodendrocytes. At 72 h after bFGF deprivation, 41.8±1.0% of the NPCs had differentiated into tuj1-positive (tuj1^{+}) cells, 40.6±6.0% of the NPCs had differentiated into glial fibrillary acidic protein-positive (GFAP^{+}) cells and 9.4±1.1% were galactocerebroside-positive (GC[+]) (Fig. 2A and B). Phenylephrine treatment did not significantly change the percentages of neuronal and glial cells (tuj1[+], 38.1±3.2%; GFAP[+], 28.7±3.1%; and GC[+], 6.2±1.0%) in cultures lacking bFGF (Fig. 2A and B). These results indicate

that phenylephrine did not affect NPC differentiation induced by bFGF deprivation. We also examined the effect of phenylephrine on neural differentiation of NPCs in cultures containing bFGF and again found no effect on neuronal or glial differentiation (data not shown).

### 3.3. $\alpha_1$-adrenoceptor agonists protect NPCs from cell death

It has been reported that activation of $\alpha_1$-adrenoceptors stimulates DNA synthesis of embryonic NPCs in mixed culture conditions (Pabbathi et al., 1997). Primary NPC cultures prepared from the telencephalon contain a considerable number of neuronal cells (>10%) that also express $\alpha_1$-adrenoceptors (Papay et al., 2006). To determine whether the previously reported activation of DNA synthesis was indicative of NPC proliferation or proliferation by secondary effect via other cell types contaminating the culture, we re-seeded cultured NPCs from a primary culture to prepare highly purified secondary
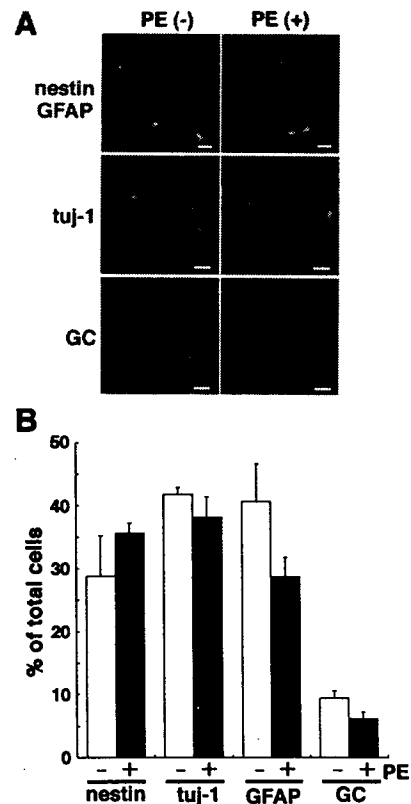


Fig. 2. Effect of the $\alpha_1$-adrenoceptor agonist phenylephrine on NPC differentiation. (A) Immunofluorescence staining was carried out after 72 h on NPC cultures with or without phenylephrine. Fluorescence microscopic images of cells labeled with anti-nestin (green), anti-GFAP (red), anti-tuj1 (green) and anti-galactocerebroside (green) are shown; nuclei are stained with Hoechst (blue). Scale bar=20 µm. Similar results were obtained in two independent experiments. (B) Secondary cultured NPCs from the E14 mouse telencephalon were maintained *in vitro* for 72 h without or with 10 µM phenylephrine (PE). After 72 h, cells were fixed and immunostained for tuj1, glial fibrillary acidic protein (GFAP), nestin and galactocerebroside (GC). The number of nestin[+], tuj1[+], GFAP[+] and GC^{+} cells were counted, and the percentages are presented. Nestin was used as a marker for undifferentiated NPCs. No significant differences were observed.
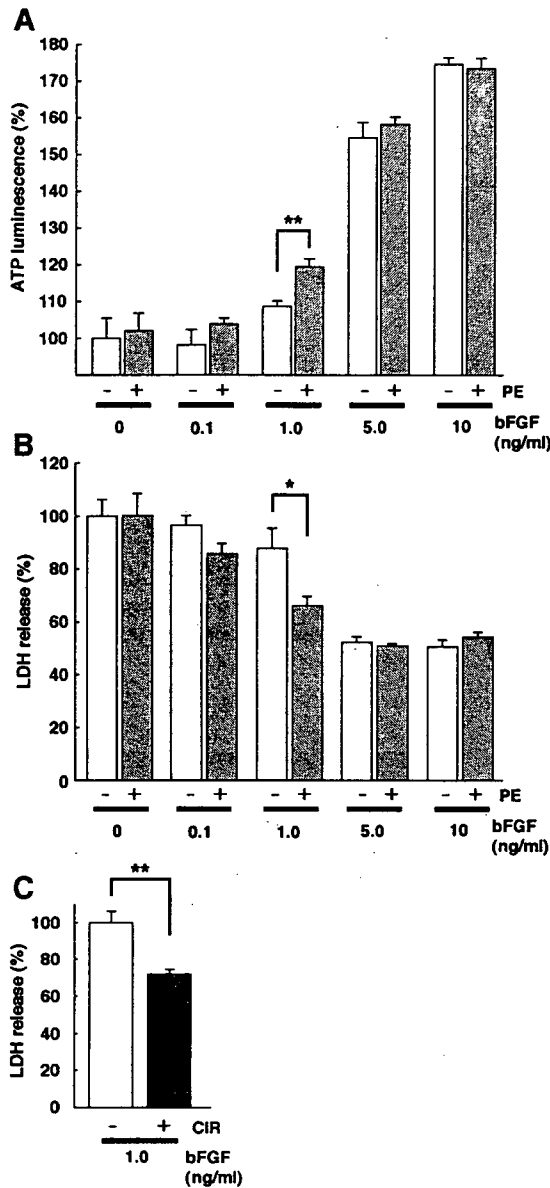
only at a moderate dose (1.0 ng/ml) of bFGF ($P<0.01$; Fig. 3A) and had no significant effect at high doses (5–10 ng/ml), a low dose (0.1 ng/ml) of bFGF, or no bFGF. To determine whether the effect of phenylephrine on NPCs at 1 ng/ml bFGF was due to promotion of cell growth or cell survival, we examine the effect of phenylephrine on cell death using the LDH release assay (Decker and Lohmann-Matthes, 1988), which measures destruction of the plasma membrane. Phenylephrine also significantly decreased LDH release at 1 ng/ml bFGF ($P<0.05$; Fig. 3B), indicating that the difference between ATP luminescence (cell numbers) of phenylephrine-treated and



Fig. 3. Effects of α₁-adrenoceptor agonists on the number of cells and cell death of NPCs at various doses of bFGF. Secondary cultured NPCs from the E14 mouse telencephalon were treated without or with 10 μM phenylephrine at different concentrations of bFGF for 24 h. (A) The number of NPCs was examined with an ATP luminescence assay. (B) The viability of NPCs was examined with the LDH release assay. (C) Secondary cultured NPCs from the E14 mouse telencephalon were treated without or with 10 μM cirazoline in 1 ng/ml bFGF for 24 h. Cell survival was assessed by the ATP luminescence assay. Bars represent mean±S.E.M. ($n=4$). Significant differences are indicated by single or double asterisks (*$P<0.05$, **$P<0.01$, Student's $t$-test).

NPC cultures that contained over 99±0.4% nestin[+] undifferentiated NPCs and no more than 0.5% tuj1^{+} neuronal cells. Using the secondary NPC culture, we examined whether phenylephrine increased the number of NPCs at various doses of bFGF (0~10 ng/ml) using an intracellular ATP luminescence assay (Crouch et al., 1993; Petty et al., 1995). We found that phenylephrine significantly increased the number of NPCs
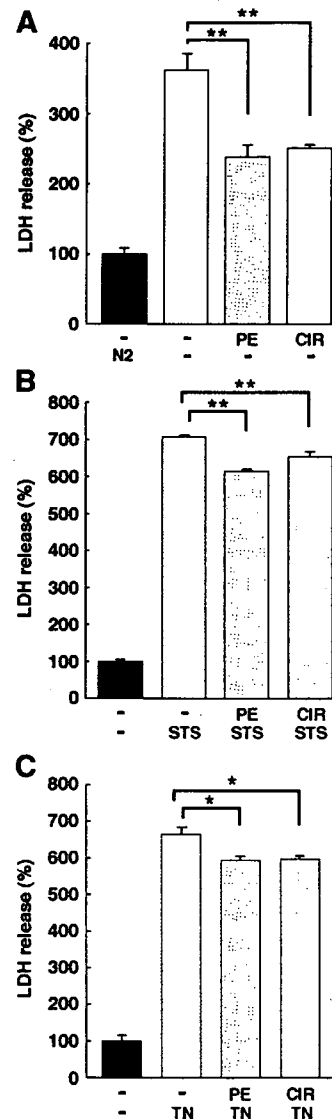
Fig. 4. Phenylephrine and cirazoline promote survival of NPCs under various stress conditions. Secondary cultured NPCs from the E14 mouse telencephalon were incubated in medium lacking N2 (A), 50 nM staurosporine (STS) (B) or 30 ng/ml tunicamycin (TN) (C) in the presence or absence of 10 μM phenylephrine (PE) or 10 μM cirazoline (CIR) for 24 h. Quantification of cell death was performed with the LDH release assay. Bars represent mean±S.E.M. ($n=3~5$). Significant differences are indicated by single or double asterisks (*$P<0.05$, **$P<0.01$, Dunnett's test).
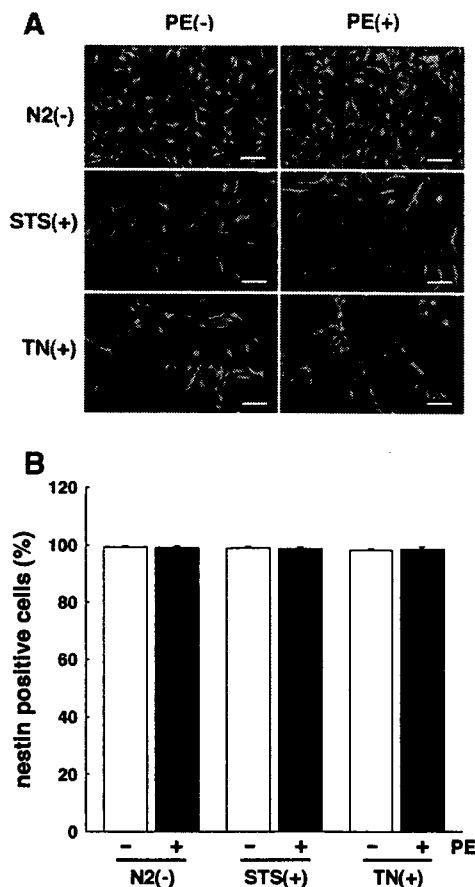
**A**



**B**



Fig. 5. Effect of phenylephrine on the proportion of nestin-positive cells under various stress conditions. Secondary cultured NPCs were exposed for 24 h to stress conditions without or with 10 µM phenylephrine: N2 deprivation medium, or exposure to 50 nM staurosporine (STS) or 30 ng/ml tunicamycin (TN). After 24 h, the NPCs were fixed and stained with anti-nestin and Hoechst. (A) Fluorescence microscopic images of cells labeled with anti-nestin (green) and Hoechst (blue). Scale bar=50 µm. (B) The percentages of nestin-positive cells in the cultures were quantified. No significant differences were seen among the groups.

untreated NPCs correlated with the difference in the amount of cell death. These results also indicated that phenylephrine protected NPCs from death during bFGF deprivation-induced stress. Moreover, we confirmed that $\alpha_1$-adrenoceptor agonists specifically protected NPCs from death using another $\alpha_1$-adrenoceptor agonist, cirazoline. Cirazoline treatment of NPCs under the same culture conditions resulted in a significant decrease in LDH release ($P<0.01$; Fig. 3C), indicating that $\alpha_1$-adrenoceptor agonists promote survival of NPCs cultured in 1 ng/ml bFGF. A [$^3$H]thymidine incorporation assay showed that phenylephrine and cirazoline did not induce DNA synthesis of NPCs (data not shown).

### 3.4. $\alpha_1$-adrenoceptor agonists prevent NPC death upon exposure to various stresses

To determine the extent to which $\alpha_1$-adrenoceptor agonists could prevent NPC death, we employed other stress conditions:

N2 deprivation, or exposure to 50 nM staurosporine or 30 ng/ml tunicamycin. The results of the LDH release assay for these stress conditions showed that N2 deprivation, staurosporine treatment, and tunicamycin treatment induced LDH release from NPCs ($361\pm24\%$, $706\pm5\%$ and $664\pm21\%$, respectively, relative to the controls; Fig. 4). However, application of the agonists to the NPC cultures under these stress conditions significantly reduced LDH release (N2 deprivation+phenylephrine, $238\pm18\%$ $P<0.01$; N2
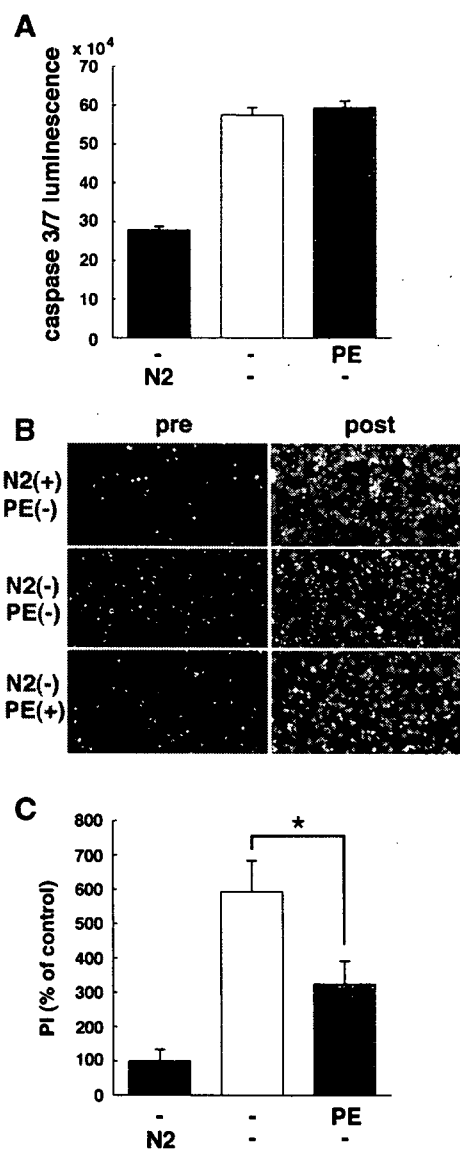
**A**



**B**



**C**



Fig. 6. Phenylephrine does not alter cellular caspase activity but changes propidium iodide penetration into NPCs. Secondary cultured NPCs were incubated without or with 10 µM phenylephrine (PE) under N2 deprivation conditions for 24 h. (A) Caspase-3 and caspase-7 activities in cell lysates were measured by luminometry. (B) Fluorescence microscopy images of dead cells stained with propidium iodide are presented (left). For positive controls, cells were fixed and stained with PI after permeabilization with 0.02% Triton X-100 (right). Scale bar=100 µm. (C) The intensity of propidium iodide staining was measured by fluorometry. Bars represent mean±S.E.M. ($n$=4). Significant differences are indicated by an asterisk (*$P<0.05$, Dunnett's test).

deprivation+cirazoline, 251±5% $P<0.01$; staurosporine+phen-
ylephrine, 614±9% $P<0.01$; staurosporine+cirazoline, 654±
16% $P<0.01$; and tunicamycin+phenylephrine, 594±13%
$P<0.05$; tunicamycin+cirazoline, 597±10% $P<0.05$) (Fig. 4).
Under the three stress conditions, the differences in the efficacies
of phenylephrine and cirazoline on LDH release did not differ
greatly. However, both agonists were most effective against N2
deprivation stress, where phenylephrine and cirazoline reduced
LDH release 34% and 31%, respectively, as compared with the
unstressed control (Fig. 4A). These data suggest that $\alpha_1$-
adrenoceptor agonists protected NPC death under several
different stress conditions but exhibited different efficacies
depending on the particular stress.

### 3.5. $\alpha_1$-adrenoceptor agonists do not modulate the proportion of nestin-positive NPCs under stress conditions

Phenylephrine did not affect differentiation of NPCs in cultures
with or without bFGF (Fig. 1), and the high proportion (over 98%)
of nestin-positive cells remained for ~24 h even in 1 ng/ml bFGF
(data not shown). The ability of the agonists to prevent cell death in
1 ng/ml bFGF was thus a direct effect on the nestin-positive NPCs
and not an artifact due to contaminating cells. We also examined
the effects of stress induced by N2 deprivation, 50 nM
staurosporine or 30 ng/ml tunicamycin on NPC differentiation to
exclude the possibility that the increased cell numbers observed
following $\alpha_1$-adrenoceptor agonist exposure, as measured by ATP
production, were derived from the differentiated cells induced by
the stresses. The proportion of nestin-positive NPCs was not
changed by N2 deprivation, staurosporine-or tunicamycin-induced
stress, and treatment of NPCs with phenylephrine or cirazoline
under these stress conditions did not decrease the proportions of
nestin-positive cells (Fig. 5).

### 3.6. Phenylephrine inhibits caspase-3/7-independent cell death

Of the three stress conditions tested, $\alpha_1$-adrenoceptor agonists
most effectively suppressed stress caused by N2 deprivation, as
measured by LDH release (Figs. 3 and 4). Whereas cell death
induced by bFGF deprivation, staurosporine treatment or
tunicamycin treatment probably was caused by activation of
various death signaling pathways, the stress caused by N2
deprivation mainly induced caspase-3/7-dependent cell death in
NPC cultures (Fig. 6). To ascertain whether $\alpha_1$-adrenoceptor
agonists protect against caspase-3/7-dependent cell death, we
examined the effects of $\alpha_1$-adrenoceptor agonists on cellular
caspase-3/7 activities. Stress caused by N2 deprivation induced
elevated cellular caspase-3 and caspase-7 activities as compared
with non-stress conditions (>2 fold; Fig. 6A). However, the
elevated activities of caspases were not changed by treatment of
NPCs with phenylephrine (Fig. 6A). Despite a lack of modulation
of the cellular caspase activity, phenylephrine suppressed
propidium iodide penetration into NPC cytoplasm (45.4%;
$P<0.05$, as compared with untreated cells deprived of N2)
(Fig. 6B and C). These data indicate that $\alpha_1$-adrenoceptor agonists
selectively protect against caspase-3/7-independent death of NPCs
exposed to stress.

## 4. Discussion

In this study, we prepared highly purified embryonic NPCs
(>99% nestin$^+$ cells) from the E14 mouse cortex and found that
the cortical embryonic NPCs highly express $\alpha_{1A}$- and $\alpha_{1B}$-
adrenoceptor genes but express the $\alpha_{1D}$-adrenoceptor gene at low
levels. Our pharmacological experiments also revealed that $\alpha_1$-
adrenoceptor agonists are protective against NPC death induced by
various stresses without any modification of the cell differentiation
state of the NPCs. Moreover we demonstrated that $\alpha_1$-adreno-
ceptor agonists reduced NPC death caused by the N2 deprivation
stress without modulation of intracellular caspase-3/7 activities.

The $\alpha_1$-adrenoceptor is expressed in the ventricular zone and
subventricular zone of the embryonic rat forebrain (Pabbathi et al.,
1997). The ventricular zone of the embryonic cerebral cortex
contains both undifferentiated NPCs and differentiated nascent
neuronal cells. Our quantitative RT-PCR analysis showed that
purified cortical NPCs express $\alpha_{1A}$- and $\alpha_{1B}$-adrenoceptor genes at
high levels compared with the E14 embryonic telencephalon,
suggesting that the undifferentiated NPCs highly expressed the
$\alpha_{1A}$- and $\alpha_{1B}$-adrenoceptor genes. We could not confirm the
expression of $\alpha_1$-adrenoceptor proteins in NPCs because of low
specificity of commercially available antibodies against $\alpha_1$-
adrenoceptor in immunocytochemical experiments with NPCs
(data not shown). However, we demonstrated that $\alpha_1$-adrenoceptor
agonists have protective effects against cell death in NPCs,
indicating the presence of the $\alpha_1$-adrenoceptors in NPCs.

The chemical structure of phenylephrine ($(R)$-3-[1-hydroxy-
2-(methylamino) ethyl] phenol) differs from that of cirazoline
(2-[(2-cyclopropylphenoxy) methyl]-4, 5-dihydro-1H-imidaz-
ole). However, both $\alpha_1$-adrenoceptor agonists had the same
effect on NPC death induced by a moderate concentration (1 ng/
ml) of bFGF, nutritional deprivation (no N2 supplementation),
staurosporine treatment or endoplasmic reticulum stress (tuni-
camycin treatment), indicating that the effects were specifically
mediated by $\alpha_1$-adrenoceptors. It is well known that cell death
can be induced via multiple apoptosis signaling pathways that
are specifically activated by different stresses. Although the $\alpha_1$-
adrenoceptor agonists were able to protect NPCs from death
induced by the stresses we tested, the molecular mechanism that
underlies this broad protection is unknown. However, treatment
of NPCs with $\alpha_1$-adrenoceptor agonists failed to decrease the
activities of caspase-3 and caspase-7, which are activated in the
apoptosis pathway. Instead, $\alpha_1$-adrenoceptor agonists reduced
propidium iodide incorporation induced by stress caused by N2
deprivation. These data suggest that $\alpha_1$-adrenoceptor agonists
protect against necrotic NPC death but not apoptotic NPC death.

Phenylephrine or cirazoline protects against cell death
induced by bFGF deprivation as well as by STS and nutritional
deprivation, or treatment with staurosporine or tunicamycin.
Niidome et al. (Niidome et al., 2006) showed that NPC death
resulting from growth factor deprivation is caused by both
caspase-dependent and -independent pathways in concert with
oxidative stress, suggesting that $\alpha_1$-adrenoreceptor agonists may
also protect against NPC death induced by oxidative stresses. On
the other hand, application of phenylephrine is effective only
against cell death induced by 1.0 ng/ml bFGF. Complete

deprivation of growth factors activates multiple cell death pathways, including apoptosis, necrosis and the oxidative cell death pathway, in cultured NPCs (Niidome et al., 2006). Therefore, the application of phenylephrine is unlikely to be effective against NPC death induced by the overlapping activation of multiple death pathways after complete loss of growth factor support.

In a recent study, Hiramoto et al. (2006) reported that the stimulation of $\alpha_1$-adrenoreceptors by phenylephrine or by L-epinephrine induces the proliferation of NPCs derived from cultured neurospheres. Our data indicate that $\alpha_1$-adrenoreceptor agonists do not induce proliferation of purified NPCs. It is known that the neurospheres have the 3D organization in which nestin-positive (progenitor) cells surround a large core of differentiated GFAP-positive (glial) and $\beta$-tubulin III-positive (neuronal) cells (Campos, 2004). As such, the NPC cultures prepared from neurospheres could also contain glial and neuronal cells. Thus, the difference between these data may have resulted from differences in the NPC preparation methods and from the purity of the nestin-positive NPCs.

Transplantation of neural stem and progenitor cells into patients with intractable neurological diseases is considered an effective strategy for neural regeneration therapy reviewed in Lindvall et al., (2004). Studies on cell transplantation/implantation for CNS disorders have indicated that neural stem cells and progenitor cells have the ability to replace lost neurons and to repair the damaged nervous system (Chu et al., 2004). However, a large proportion of grafted cells is lost due to early necrotic death. Thus, the low rate of graft survival reduces the effectiveness of such therapies (Emgard et al., 2003). Here we demonstrated that $\alpha_1$-adrenoceptor agonists protect against NPC death (probably necrotic death) induced by various stresses. We also demonstrated that $\alpha_1$-adrenoceptor agonists with this protective effect did not modify the cell differentiation state of the NPCs. Thus, $\alpha_1$-adrenoceptor agonists may be useful for the preparation and maintenance of neural stem and progenitor cells for transplantation therapy, as they are likely to increase cell viability without induction of unexpected cell differentiation. Moreover, phenylephrine is a popular and safe drug that is used as a non-prescription decongestant (Chua and Benrimoj, 1988) Therefore, phenylephrine may be easily applied as an additional reagent in the cultivation medium of neural stem and progenitor cells for transplantation therapy.

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# Neurotensin type 2 receptor is involved in fear memory in mice

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## Abstract

Neurotensin receptor subtype 2 (Ntsr2) is a levocabastine-sensitive neurotensin receptor expressed diffusely throughout the mouse brain. Previously, we found that Ntsr2-deficient mice have an abnormality in the processing of thermal nociception. In this study, to examine the involvement of Ntsr2 in mouse behavior, we performed a fear-conditioning test in Ntsr2-deficient mice. In the contextual fear-conditioning test, the freezing response was significantly reduced in Ntsr2-deficient mice compared with that of wild-type mice. This reduction was observed from 1 h to 3 weeks after conditioning, and neither shock sensitivity nor locomotor activity was altered in Ntsr2-deficient mice. In addition, we found that Ntsr2 mRNA was predominantly expressed in cultured astrocytes and weakly expressed in cultured neurons derived from mouse brain. The combination of in situ hybridization and immunohistochemistry showed that Ntsr2 mRNA was dominantly expressed in glial fibrillary acidic protein positive cells in many brain regions including the hypothalamus, while Ntsr2 gene was co-expressed with neuron-specific microtubule associated protein-2 in limited numbers of cells. These results suggest that Ntsr2 in astrocytes and neurons may have unique function like a modulation of fear memory in the mouse brain.

Keywords: astrocytes, fear memory, neuron, neurotensin, neurotensin receptor subtype 2.

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Neurotensin (NT) is a bioactive tridecapeptide first isolated from the bovine hypothalamus, and is widely distributed in the central nervous system and peripheral tissues (Carraway and Leeman 1973). There are three subtypes of neurotensin receptors (NTRs): neurotensin receptor subtype 1 (Ntsr1) (Tanaka et al. 1990), neurotensin receptor subtype 2 (Ntsr2) (Mazella et al. 1996), and neurotensin receptor subtype 3 (Ntsr3) (Mazella et al. 1998). Ntsr1 and Ntsr2 are high- and low-affinity receptors, respectively; both are G-protein coupled receptors (GPCRs) with seven-transmembrane domains. Two forms of Ntsr2, a full-length form and a truncated form, have been isolated from rodent brain, and it is thought that these forms are generated by alternative splicing (Botto et al. 1997). In cultured cells isolated from rat brain, expression of the full-length form is observed in astrocytes, whereas mRNA for the truncated form is expressed in cultured neurons (Nouel et al. 1997, 1999). Ntsr3 is a NT-binding protein having a single transmembrane

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structure, but the mechanism of signal transduction through this receptor is not clear (Mazella *et al.* 1998).

Neurotensin is associated with a wide spectrum of biological activities including analgesia, hypothermia, hypotension, stimulation of anterior pituitary hormone release, and cell proliferation (Maeno *et al.* 2004). In addition, NT may be involved in psychiatric disorders and drug abuse (Holsboer 2003). For example, the level of NT in the cerebrospinal fluid is lower in some patients with schizophrenia than in controls. Recently, to better understand the function of the NT/NTR system *in vivo*, Ntsr1- and Ntsr2-deficient mice have been established and characterized (Pettibone *et al.* 2002; Remaury *et al.* 2002; Leonetti *et al.* 2004; Maeno *et al.* 2004). Ntsr1-deficient mice show a small increase in body weight and a higher food intake compared with wild-type control mice (Remaury *et al.* 2002). By contrast, Ntsr2-deficient mice have an abnormality in the processing of thermal nociception (Maeno *et al.* 2004). Centrally administered NT stimulates the hypothalamic–pituitary–adrenal axis in rodents, suggesting that NT can modulate the stress response or other aspects of emotional status (Rowe *et al.* 1995). Recently, we reported an anti-stress effect of the Ntsr2 agonist β-lactotensin (β-LT), using restrained mice in a hole-board test (Yamauchi *et al.* 2006). Mice subjected to acute restraint stress exhibited a decreased number of head-dips and increased head-dip latency compared with non-stressed controls in the hole-board test. However, the prior administration of β-LT improved the behaviors caused by stress. Furthermore by the administration of β-LT, the duration of freezing responses by cued fear conditioning was significantly reduced. These findings indicate that Ntsr2 may have an important role in modulation of the emotional behavior.

*In situ* hybridization (ISH) and immunohistochemistry (IHC) have been performed; however, the distribution of Ntsr2, particularly at the cellular level, remains unclear. For example, rat Ntsr2 mRNA was expressed in glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes both *in vivo* and *in vitro* (Walker *et al.* 1998; Nouel *et al.* 1999). However, using an N-terminal-specific anti-Ntsr2 antibody, no Ntsr2 immunostaining was observed in astrocytes in adult rat brain (Sarret *et al.* 2003).

In this study, to elucidate the effect of the NT/Ntsr2 system in fear memory, we performed a fear-conditioning test in Ntsr2-deficient mice. Furthermore, we identified the property of cells expressing Ntsr2 mRNA by quantitative RT-PCR and a combination of ISH and IHC.

## Materials and methods

### Animals

Male Ntsr2-deficient mice and wild-type littermates (3–7 months of age) were used for behavioral studies. Male Ntsr2-deficient mice and wild-type littermates were produced in our laboratory by mating Ntsr2 heterozygous males with Ntsr2 heterozygous females or intercrossing with the same genotype (Ntsr2-deficient male mice × Ntsr2-deficient female mice, and wild-type male mice × wild-type female mice, respectively). All mutant mice used in this study were backcrossed to the C57BL/6J mice at least eight times.

Mice were kept in a temperature- and humidity-controlled room with a 12-h light–dark cycle (lights on at 08:00 hours). Food and water were available *ad libitum*. All behavioral experiments were performed between 11:00 and 16:00 hours during the light cycle. All animal experiments were performed in strict accordance with the guidelines of the National Institutes of Neuroscience, National Center of Neurology and Psychiatry (Japan) and were approved by the Animal Investigation Committee of the Institute.

### Fear-conditioning test

Fear-conditioning tests were performed using a computerized fear-conditioning system (O'Hara & Co. Ltd, Tokyo, Japan) as previously described (Yamauchi *et al.* 2006). On the conditioning day, each mouse was placed in the conditioning chamber for 2 min before the onset of a tone (70 dBs, 10 kHz) that lasted for 30 s. A foot shock (0.5 mA) was delivered during the last 2 s of the tone. Mice received the foot shock twice, with a 1 min interval in-between. For contextual fear-conditioning test, freezing behavior was examined for 3 min in the same chamber without the tone or foot shock. Whereas in a novel environment, after the habituation period, the same tone (70 dBs, 10 kHz) used during conditioning was given for cued fear-conditioning (CS) test. Freezing score (%) is presented as the ratio of time during the experiment. For long-term experiment, contextual tests were examined using the same mice continuously at 24 h, and 8, 22, and 43 days, and CS tests continuously at 48 h, and 9, 23, and 44 days after conditioning. Consequently, each mouse was investigated for both contextual and CS test at 0, 1, 3, and 6 weeks after conditioning. For short-term experiment, contextual fear-conditioning test was performed using another group of mice at 1 and 5 h after conditioning.

### Spontaneous activity test

Spontaneous activity test was performed using an activity monitoring system (Neuroscience Co., Osaka, Japan) as previously described (Yamada *et al.* 2000). Each mouse was placed in the test chamber and locomotor activity, vertical movements (rearing and leaning on the wall), and stereotypic movements (repeated back and forward movements) were measured for 30 min.

### Shock sensitivity test

The shock sensitivity test was performed as previously described (Yamada *et al.* 2003). Individual animals were placed on a floor consisting of parallel stainless steel rods (5 mm diameter with gaps of 10 mm) and covered with a 1 L glass beaker. Six series of six shocks (1 s duration, 20, 40, 60, 80, 100, and 130 μA) were delivered at a 15-s interval through the grid floor. Series of shock were administered in ascending and then descending order of magnitude. The least amount of electricity that causes an animal's hind foot left the floor, shock threshold, was defined as the lowest shock sensitivity (μA). For each mouse, a mean shock threshold value was calculated as the average of the six thresholds recorded in the series.

## Glial cell cultures

Astrocyte cultures were prepared from post-natal 1-day-old C57BL/6J mice. The cortex and hippocampus were dissected and mechanically dissociated by trituration. Cell suspensions were plated on 10 cm plates at an approximate density of $5 \times 10^6$ cells/plate. Cells were incubated in Hepes-buffered Dulbecco's Modified Eagle's Medium/10% fetal bovine serum for 2 weeks, and half of the medium was replaced twice a week.

Oligodendrocyte cultures (CG4 cells) were kindly provided by Drs K. Ikenaka and A. Espinosa de los Monteros. CG4 cells were incubated in glial-defined medium as described (Yonemasu et al. 1998).

## Neuron cultures

Neuron cultures were prepared from embryonic day 17 (E17) C57BL/6J mice as previously described (Okabe et al. 1998). The cell suspension (about $10^7$ cells) was plated on 10 cm-diameter poly-L-lysine-coated dish. Cells were incubated in minimal essential medium containing 2% B-27 supplement and 5% fetal bovine serum for 2 weeks.

## SYBR Green-based real-time quantitative RT-PCR

Total RNAs of astrocytes and neurons were treated with RNase-free DNase and reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA, USA). Quantification of Ntsr2 mRNA by real-time PCR quantification system (ABI PRISM 7900HT; Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master mix (Applied Biosystems) was performed according to the supplier's protocol. For standardization and quantification, hypoxanthine phosphoribosyltransferase was amplified. The following primer pairs were used: 5'-CCGATGATGGATGGACTGATG-3' (forward) and 5'-AAGGAGGAAGACACGGCATTG-3' (reverse) for amplification of Ntsr2; and 5'-GTAATGATCAGTCAACGGGGGAC-3' (forward) and 5'-CCAGCAAGCTTGCAACCTTAACCA-3' (reverse) for amplification of hypoxanthine phosphoribosyltransferase.

## In situ hybridization with $^{35}$S-labeled probe

In situ hybridization using a $^{35}$S-labeled probe was performed as described (Maeno et al. 2004).

## Double staining of Ntsr2 mRNA and either MAP2 or GFAP protein

A combination of ISH for Ntsr2 and IHC was performed using a Ventana Discovery Automated ISH System and a RiboMap Kit (Ventana HX system, Ventana, Tucson, AZ, USA) following the manufacturer's instructions. Paraffin sections fixed with formalin were prepared from mouse brain according to a standard protocol. Paraffin sections were automatically deparaffinized, fixed, and treated with protease.

Digoxigenin (DIG)-labeled anti-sense and sense RNA probes were prepared by in vitro transcription of a fragment of mouse Ntsr2 cDNA (nucleotides 1239–1483 of GeneBank accession number NM_008747) in pBluescript II SK (−) using T3 and T7 RNA polymerase, respectively. Quality and concentration of DIG-labeled probes were confirmed using a dot-blot assay.

Sections were hybridized with DIG-labeled anti-sense or sense probes at 69°C for 3 h, at a concentration of 25 ng/slide. After hybridization, sections were incubated with a horseradish peroxy-dase-conjugated anti-DIG antibody (anti-DIG/horseradish peroxydase, 1 : 500, Dako, Glostrup, Denmark). Signals were then enhanced using a tyramide signal amplification (TSA) system using AmpMap with TSA (Ventana). After incubation with alkaline phosphatase, signals were detected in the presence of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate by a Blue Map Kit (Ventana).

Brain sections labeled for ISH with Ntsr2 anti-sense or sense probes were immunostained. Hybridized sections were treated with citric acid and blocking solution. Sections were then incubated with a mouse monoclonal antibody to GFAP (anti-GFAP, 1 : 400, Chemicon, Temecula, CA, USA) or microtubule associated protein-2 (anti-MAP2, 1 : 25, Chemicon) for 60 min at 37°C. Secondary antibody to mouse IgG, at a dilution of 1 : 500, was applied to the sections, and signals were enhanced using the TSA system. Finally, peroxidase activity was detected using 3,3'-diaminobenzidine.

## Statistical analysis

The values are presented as the means ± SEM. Statistical analyses were performed using an unpaired Student's $t$-test or ANOVA (followed by Bonferroni's multiple comparison test). $p$-values $< 0.05$ were considered statistically significant in all statistical tests.

## Results

### Contextual and cued fear conditioning

We performed fear-conditioning tests with Ntsr2-deficient and wild-type mice to determine the involvement of Ntsr2 in fear memory. During conditioning, the freezing behavior of Ntsr2-deficient mice was not different from that of wild-type control mice (wild type: $10.7 \pm 3.0\%$, $n = 9$; deficient: $5.0 \pm 2.5\%$, $n = 9$; $p > 0.05$).

In the contextual fear-conditioning test, at 24 h after conditioning, the ratio of freezing (%) was significantly decreased in Ntsr2-deficient mice compared with that in wild-type mice (wild type: $49.8 \pm 5.9\%$, $n = 9$; deficient: $27.7 \pm 4.1\%$, $n = 9$; $p < 0.05$, Fig. 1a). This difference in freezing behavior was still evident at 1, 3, and 6 weeks (all $p < 0.01$) after conditioning. In wild-type mice, freezing responses at 1, 3, and 6 weeks after conditioning were reduced compare to that at 24 h after conditioning but not significant (all $p > 0.05$). In contrast, in Ntsr2-deficient mice, freezing responses at 1, 3, and 6 weeks after conditioning were reduced significantly compare with that at 24 h after conditioning (all $p < 0.05$).

In the CS test, at 48 h after conditioning, increased freezing response was observed at the onset of the tone (CS) as compared with the freezing prior to the tone (pre-CS) in both wild-type (pre-CS: $6.2 \pm 3.5\%$, CS: $22.6 \pm 6.2\%$, $n = 8$; $p < 0.05$) and Ntsr2-deficient (pre-CS: $1.9 \pm 1.0\%$, CS: $32.3 \pm 4.3\%$, $n = 9$; $p < 0.001$) mice. The ratio of freezing in Ntsr2-deficient mice was not different from that
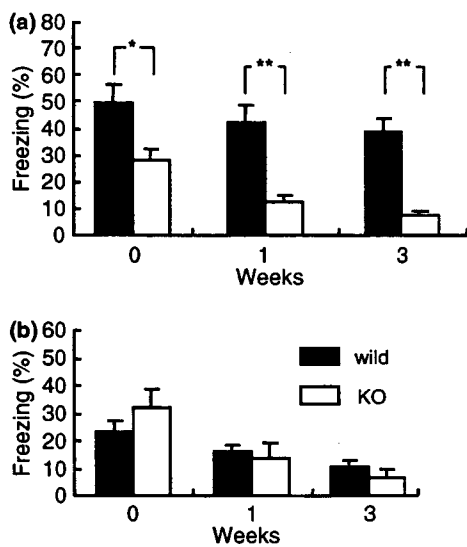
**Fig. 1** Contextual (a) and cued (b) fear-conditioning tests in neurotensin receptor subtype 2 (Ntsr2)-deficient (open bar, KO) and wild-type (filled bar) mice. The rate of freezing (%) was calculated to indicate fear memory. The values shown are means ± SEM ($n = 9$). $*p < 0.05$ and$**p < 0.01$ versus control are considered as significant using the unpaired Student's $t$-test.

of wild-type mice at 0, 1, 3, and 6 weeks after conditioning (all $p > 0.05$, Fig. 1b).

To investigate the freezing response at an early stage, contextual fear-conditioning tests were performed at 1 and 5 h after conditioning. The freezing response in the contextual test at 1 h after conditioning was significantly reduced in Ntsr2-deficient mice compared with that of wild-type mice (wild type: $46.5 \pm 2.9\%$, $n = 9$; deficient: $31.9 \pm 5.4\%$, $n = 9$; $p < 0.05$, Fig. 2). At 5 h after conditioning, the freezing response of Ntsr2-deficient mice was slightly reduced, but not significantly, compared with that of wild-type mice (wild type: $28.5 \pm 7.3\%$, $n = 9$; deficient:
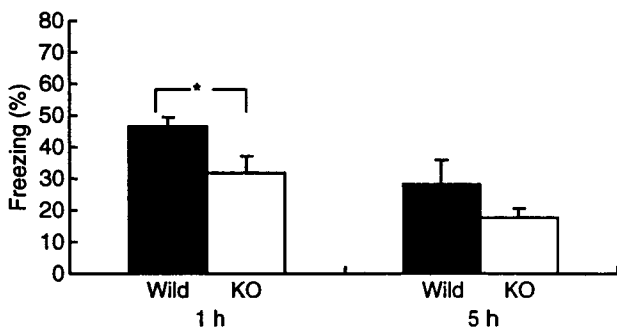


**Fig. 2** Contextual fear-conditioning test performed at 1 and 5 h immediately after conditioning, in neurotensin receptor subtype 2 (Ntsr2)-deficient (open bar, KO) and wild-type (filled bar) mice. The rate of freezing (%) was calculated to indicate fear memory. The values shown are means ± SEM ($n = 10$). $*p < 0.05$ versus control are considered as significant using the unpaired Student's $t$-test.
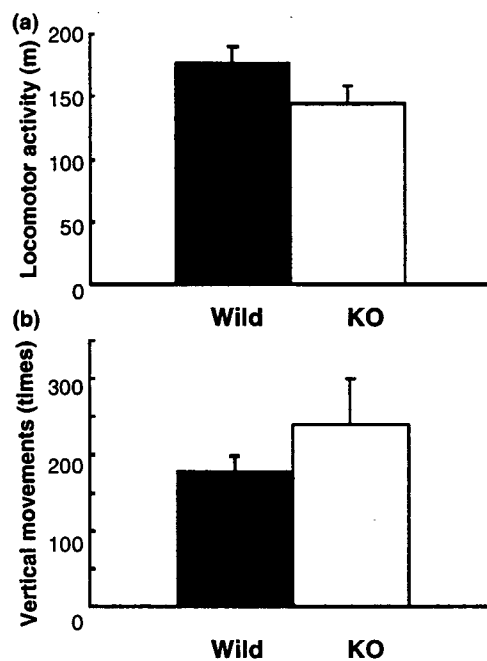


**Fig. 3** Total locomotor activity (a) and vertical movements (b) in neurotensin receptor subtype 2 (Ntsr2)-deficient (open bar, KO) and wild-type (filled bar) mice. The values shown are means ± SEM ($n = 8$).

$18.0 \pm 2.5\%$, $n = 9$; Fig. 2). These results suggest that Ntsr2 is involved in contextual fear memory.

### Spontaneous activity

The decreased freezing responses of Ntsr2-deficient mice in the contextual fear-conditioning test may be caused by the decrease in spontaneous activities. With the spontaneous activity test, there were no differences between wild-type and Ntsr2-deficient mice in locomotor activity (wild type: $17759.4 \pm 1278.1$ cm, $n = 8$; deficient: $14469.2 \pm 1390.7$ cm, $n = 8$, $p > 0.05$, Fig. 3a) and vertical movements (wild type: $180.3 \pm 17.8$, $n = 8$; deficient: $240.1 \pm 59.9$, $n = 8$, $p > 0.05$, Fig. 3b). Stereotypic movements in Ntsr2-deficient mice were similar to that in wild-type mice (wild type: $6717.4 \pm 271.5$, $n = 8$; deficient: $7012 \pm 334.9$, $n = 8$, $p > 0.05$). These results suggest that spontaneous activity was not altered in Ntsr2-deficient mice.

### Shock sensitivity

Recently, we reported that Ntsr2-deficient mice showed altered thermal nociception in the hot plate test (Maeno *et al.* 2004). To confirm if these mice percept the electrical shock, we measured electrical-shock sensitivity in Ntsr2-deficient and wild-type mice. There was no difference in shock sensitivity between Ntsr2-deficient and wild-type mice (wild type: $52.0 \pm 3.9$ µA, $n = 8$; deficient: $45.1 \pm 3.7$ µA, $n = 9$; $p > 0.05$, Fig. 4). These results suggest that deficiency of Ntsr2 is not responsible for electrical shock sensitivity.
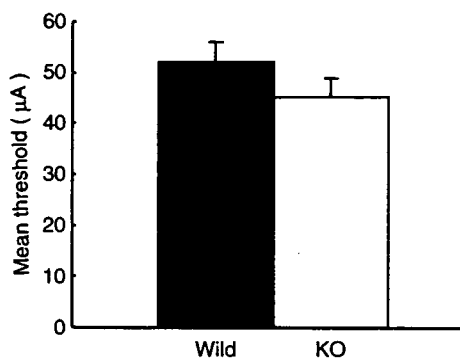
**Fig. 4** Shock sensitivity in neurotensin receptor subtype 2 (Ntsr2)-deficient (open bar, KO) and wild-type (filled bar) mice. The values shown are mean thresholds ($\mu$A) ± SEM ($n$ = 9).
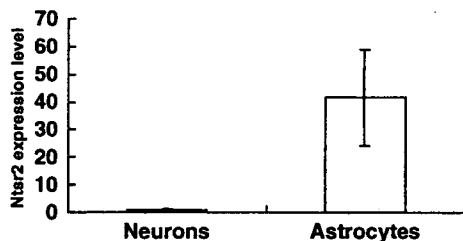


**Fig. 5** Neurotensin receptor subtype 2 (Ntsr2) mRNA expression levels in neurons (filled bar) and astrocytes (open bar) in C57BL/6J mice brain. The values are the amount of expression levels of quantitative RT-PCR products ± SEM ($n$ = 4).

### Ntsr2 gene expression in primary cultured cells

The distribution of Ntsr2 mRNA has been observed in the mouse brain (Sarret *et al.* 1998; Walker *et al.* 1998). However, the cells that express Ntsr2 mRNA have not been clearly characterized. To examine the subpopulation of cells expressing Ntsr2 in the mouse brain, we performed real-time quantitative RT-PCR on total RNA extracted from primary-cultured astrocytes, neurons and oligodendrocytes.

Neurotensin receptor subtype 2 mRNA was predominantly expressed in cultured astrocytes (Fig. 5), but only weakly expressed in cultured neurons. The amount of Ntsr2 mRNA expression in astrocytes was about 40 times that in neurons. Furthermore, Ntsr2 mRNA was not expressed in rat oligo-dendrocytes (CG4 OL) (data not shown). These results suggest that Ntsr2 mRNA is mainly expressed in astrocytes *in vitro*.

### Ntsr2 gene expression in the mouse brain

To confirm the distribution of Ntsr2 mRNA in the mouse brain, ISH was performed with $^{35}$S-labeled cRNA probe. As previously reported, moderate hybridization signals for Ntsr2 anti-sense probe were observed diffusely throughout the brain, including the cortex, thalamus, hypothalamus, and hippocampus. Although hybridization signals were observed diffusely throughout the brain, intense signals were observed
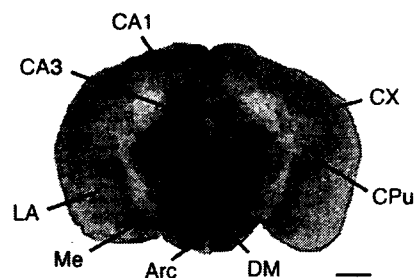


**Fig. 6** Neurotensin receptor subtype 2 (Ntsr2) gene expression in the mouse brain. An autoradiogram of a representative coronal section hybridized with a $^{35}$S-labeled anti-sense cRNA probe for Ntsr2 is shown. Arc, arcuate nucleus; CA1, field CA1 of Ammon's horn; CA3, field CA3 of Ammon's horn; CPu, caudate–putamen; CX, cortex; DM, dorsomedial hypothalamic nucleus; LA, lateral amygdaloid nucleus; Me, medial amygdaloid nucleus. Scale bar indicates 1 mm.

in the hypothalamic region including pre-optic nuclei and hypothalamic nuclei, pyramidal cell layer of Ammon's horn, and globus pallidus (Fig. 6). By contrast, very weak signals were observed in the internal capsule and fimbria hippo-campus. No hybridization signal above background was observed with the Ntsr2 sense probe.

### Characterization of cells expressing Ntsr2 mRNA in the mouse brain

To characterize the cells expressing Ntsr2 mRNA, sections were hybridized with DIG-labeled anti-sense or sense Ntsr2 cRNA probes. The pattern of hybridization with a DIG-labeled probe was consistent with that of signals obtained by using a $^{35}$S-labeled probe. Hybridized sections were then immunostained with cell type-specific antibodies, including the neuronal nuclei-specific anti-MAP2 antibody and the astrocyte-specific anti-GFAP antibody (Fig. 7). In the hypo-thalamus and globus pallidus, many Ntsr2 mRNA positive cells were stained with anti-GFAP antibody (Fig. 7f). In the Ammon's horn, Ntsr2 mRNA was expressed in GFAP-immunopositive cells in the oriens layer and radiatum layer, whereas cells expressing Ntsr2 mRNA were stained with anti-MAP2 antibody in the pyramidal cell layer (Fig. 7a–e). These results suggest that, *in vivo*, Ntsr2 mRNA is dominantly expressed in astrocytes, whereas gene expression of Ntsr2 was not as prominent in neurons.

### Discussion

Neurotensin has various biological activities, including modulation of feeding and learning behaviors and anti-nociception. Among its various functions, Ntsr1 mediates learning and memory. For example, rats treated with the Ntsr1-specific antagonist, SR 48692, make more working memory errors in spatial learning (Tirado-Santiago *et al.* 2006). In mice, SR 48692 significantly modifies the risk assessment aspect of defensive behavior (Griebel *et al.*
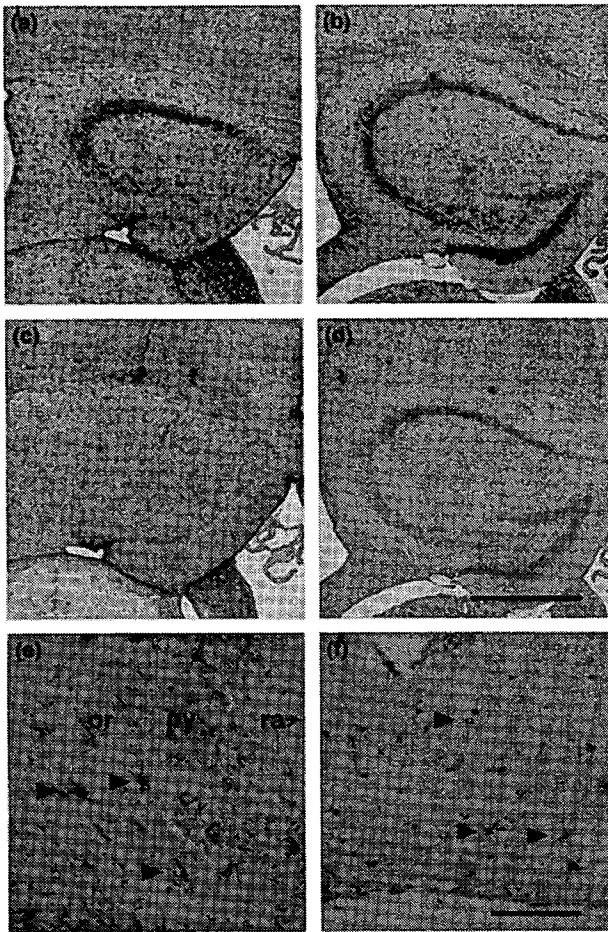
**Fig. 7** A combination of *in situ* hybridization (ISH) for neurotensin receptor subtype 2 (Ntsr2) and IHC for glial fibrillary acidic protein (GFAP) or microtubule associated protein-2 (MAP2) in the hippocampus (a–e) and hypothalamus (f). Expression of Ntsr2 mRNA was demonstrated by ISH using digoxigenin (DIG)-labeled anti-sense (a, b, e, and f) or sense (c, and d) probes. In addition to ISH, sections were stained with anti-GFAP antibody (a, c, e, and f), or anti-MAP2 antibody (b, and d). Arrowheads show Ntsr2 gene expression in GFAP-positive cells. or, oriens layer of Ammon's horn; py, pyramidal cell layer of Ammon's horn; ra, radiatum layer of Ammon's horn. Scale bars indicate 500 μm (d) and 100 μm (f).

2001). In contrast to Ntsr1, the biological effects of Ntsr2 on learning and memory have not been reported, although the spinal nociceptive effects of Ntsr2 have been studied well (Maeno *et al.* 2004). In this study, we showed that the freezing response of Ntsr2-deficient mice during contextual fear-conditioning test was decreased compared with wild-type mice. Recently, we reported that the administration of Ntsr2 agonist, β-LT, promotes the extinction of cued fear memory, but not affects the contextual fear conditioning (Yamauchi *et al.* 2006). β-LT has affinity to Ntsr2, but it also binds to Ntsr1 as well. Indeed we reported that ileum-contracting activity of β-LT is mediated by Ntsr1 (Yamauchi

*et al.* 2003). In addition, the effects of β-LT on fear-conditioning test were not blocked by Ntsr2 antagonist levocavastine in our previous study. These findings suggest that the effects of β-LT on the fear memory might not be mediated by Ntsr2 only.

Fear memory, examined by the fear-conditioning test, is comprised of three different stages of acquisition, consolidation, and extinction (Sotres-Bayon *et al.* 2006). During the acquisition stage, fear memory for the context and shock is established. During conditioning, there was no difference in freezing behavior between Ntsr2-deficient and wild-type mice, suggesting that acquisition of fear memory is not altered in Ntsr2-deficient mice. In the consolidation stage that comes after the acquisition stage, the fear memory is consolidated when the mouse is returned to its homecage (Abel and Lattal 2001). In the present study, a significant decrease in freezing response was observed in Ntsr2-deficient mice compared with that of wild-type mice at the first exposure to the context (24 or 1 h). These results indicate that Ntsr2 may be involved in the consolidation processes of fear memory. After consolidation, fear memory becomes labile when the animal is exposed again to the conditioning context (retrieval), where memories for the context and shock are associated. During retrieval, the fear memory becomes either reconsolidated or extinct. In general, a short-term exposure to the context leads to reconsolidation, whereas a longer exposure leads to extinction by producing new memory of context without shock (Abel and Lattal 2001; Suzuki *et al.* 2004; Zushida *et al.* 2007). In wild-type mice, freezing ratios to context decreased gradually with time but not significant. On the other hand, in Ntsr2-deficient mice, freezing responses were significantly reduced after second exposure to the context at 1, 3, and 6 weeks after conditioning compared with the first exposure to the contextual. This suggests that Ntsr2 may modulate the formation of extinction learning.

Dopamine (DA) is one of the most important neurotransmitters in the amygdala that plays important roles involved in the mechanisms of fear and anxiety. By activating DA $D_1$ receptor subtype, a potentiation of conditioned fear responses is introduced. An inhibitory action on conditioned fear may be mediated via the $D_2$ receptor (Pezze and Feldon 2004). Associations between NT and DA have been well investigated (Binder *et al.* 2001; St-Gelais *et al.* 2006). In many brain regions, NT increases the firing rate of DAergic neurons and has a depolarizing effect. These responses induced by NT are mediated at least in part by Ntsr1 (St-Gelais *et al.* 2006). Although at present the neurotransmitter systems that interact with Ntsr2 have not been well investigated, Ntsr2 may modulate fear memory via $D_1$ and/or $D_2$ receptors.

We showed that Ntsr2 gene expression was more predominant in primary astrocyte cultures compared with that in primary neuron cultures. To confirm the purity of

astrocytes in the primary astrocyte cultures, immunocyto-chemistry was performed (data not shown). In the primary astrocyte culture, more than 95% of cultured cells were stained with the anti-GFAP antibody and few cells were stained with the anti-MAP2 antibody or antibodies for other glial cells such as microglia or oligodendrocytes (data not shown). Nouel et al. (1999) reported that, of the two forms of Ntsr2, the full-length form is expressed in astrocytes in the rat brain. In fact, we obtained similar results in cultures from mouse brain (data not shown). In addition to in vivo studies, we presented the finding that Ntsr2 mRNA is diffusely expressed throughout the mouse brain, which is in good agreement with earlier studies (Sarret et al. 1998; Walker et al. 1998). By a combination of ISH and IHC, we showed that Ntsr2 mRNA is expressed dominantly in astrocytes in the regions where we observed. By contrast, in the pyramidal cell layer of Ammon's horn, Ntsr2 mRNA was co-localized with MAP2 immunostaining. This finding indicates that in the layer where neurons packed densely like pyramidal cell layer, Ntsr2 gene is apparently expressed higher level in MAP2 positive cells. Sarret et al. (2003) reported that Ntsr2-like immunoreactivity is broadly distributed throughout the rat brain, using an N-terminal-specific anti-Ntsr2 antibody. However, no Ntsr2 signal was observed in astrocytes of rat brain, as confirmed by double immunostaining for the astrocyte marker, calcium-binding protein S100β. These disparities may be caused by a difference in the sensitivity of IHC and ISH techniques, and/or a difference that may exist between rat and mouse.

Astrocytes are traditionally thought to provide physical and structural support for neurons. Recently, many investigators have reported that astrocytes play an important role in the central nervous system (Miller 2005). For example, mice devoid of S100β showed enhanced long-term potentiation in the hippocampal CA1 region, suggesting that S100β secreted from astrocytes may modulate a signal cascade involved in neuronal synaptic plasticity (Nishiyama et al. 2002). On the other hand, the importance of astrocytic receptors, especially GPCRs, has not been well investigated. Ntsr2 is a member of a GPCR superfamily that is one of the largest classes of receptors in mammalian genomes. GPCRs mediate diverse physiological functions and are the target of more than 50% of all clinical drugs (Lundstrom 2006). Our findings demonstrate the possibility that GPCRs expressed in astrocytes may play an important role in the modulation of higher brain functions such as emotion and fear.

Regarding the involvement of astrocytes in fear memory, there is a report that suggests the involvement of astrocytes in fear-memory formation. Mei et al. (2005) reported gene expression profiles in hippocampus of mouse brain after fear conditioning. After fear conditioning, gene expression of phosphoprotein enriched in astrocytes 15 which blocks extracellular signal-regulated kinase-dependent transcription

and proliferation was shown to be decreased. Besides, gene expression of glutamine synthetase which is expressed in astrocytes specifically was increased. These observations suggest that astrocytes may be involved in fear memory formation.

Antonelli et al. (2002, 2004) reported that NT enhances glutamate release and modifies the function of glutamate receptors. In addition, astrocytes can release glutamate in a calcium-dependent manner to activate $N$-methyl-D-aspartate and α-amino-3-hydoxy-5-methyl-4-isoxazole propionate receptors expressed in adjacent neurons, and modulate synaptic transmission (Parpura and Haydon 2000). These observations raise the possibility of physiological interaction between glutamate and the NT/Ntsr2 system.

Furthermore, in addition to the alterations seen in Ntsr2-deficient mice, Ntsr1-deficient mice show alterations of long-term potentiation in the amygdala which plays a critical role in emotional learning and social cognition (T. Amano et al. unpublished data). This finding suggests that Ntsr1-deficient mice may have emotional disorders, such as those involving fear memory. Our observation is the first finding that the NT/NTR system may have an important role in modulating emotional behavior including fear memory.

## Acknowledgements

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