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**Regulation of the neurotensin NT₁ receptor in the developing rat brain
following chronic treatment with the antagonist SR 48692**

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Running title: Regulation of NT₁ receptors in the developing rat brain

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ABSTRACT

The aim of the present study was to investigate the role of neurotensin in the regulation of NT₁ receptors during postnatal development in the rat brain. Characterization of the ontogeny of neurotensin concentration and [¹²⁵I]neurotensin binding to NT₁ receptors in the brain at different embryonic and postnatal stages showed that neurotensin was highly expressed at birth, reaching peak levels at postnatal day 5 (P5), and decreasing thereafter. The transient rise in neurotensin levels preceded the maximal expression of NT₁ receptors, observed at P10, suggesting that neurotensin may influence the developmental profile of NT₁ receptors. Using primary cultures of cerebral cortex neurons from fetal rats, we showed that exposure to the neurotensin agonist JMV 449 (1 nM) decreased (−43%) the amount of NT₁ receptor mRNA measured by reverse transcription-PCR, an effect that was abolished by the non-peptide NT₁ receptor antagonist SR 48692 (1 μM). However, daily injection of SR 48692 to rat pups from birth for 5, 9 or 15 days, did not modify [¹²⁵I]neurotensin binding in brain membrane homogenates. Moreover, postnatal blockade of neurotensin transmission did not alter the density and distribution of NT₁ receptors assessed by quantitative autoradiography nor NT₁ receptor mRNA expression measured by *in situ* hybridization in the cerebral cortex, caudate-putamen and midbrain. These results suggest that although NT₁ receptor expression can be regulated *in vitro* by the agonist at an early developmental stage, neurotensin is not a major factor in the establishment of the ontogenetic pattern of these receptors in the rat brain.

KEY WORDS: postnatal development; JMV 449; *in situ* hybridization; RT-PCR

INTRODUCTION

Neurotensin, a 13 amino-acid neuropeptide, is heterogeneously distributed in the mammalian brain (Alexander *et al.*, 1989). In the central nervous system (CNS), neurotensin acts as a neurotransmitter and neuromodulator of several neuronal systems; in particular, neurotensin has been shown to play an important role in the modulation of dopamine transmission (Lambert *et al.*, 1995). Neurotensin is also involved in nociception, thermoregulation and modulation of neuroendocrine systems (for reviews, see Nemeroff and Kitabgi, 1992; Rostène and Alexander, 1997).

Two types of neurotensin receptors, NT₁ and NT₂, have been cloned and shown to belong to the family of G protein-coupled receptors (Tanaka *et al.*, 1990; Vita *et al.*, 1993; Chalon *et al.*, 1996; Mazella *et al.*, 1996). These two receptors are distinguished by their affinity for neurotensin and their capacity to bind levocabastine, a histamine H₁ receptor antagonist (Schotte *et al.*, 1986). The levocabastine-insensitive, high-affinity neurotensin receptor (NT₁) mediates most of the physiological functions ascribed to neurotensin. In contrast, only limited data are available concerning the functional role of the levocabastine-sensitive, lower-affinity neurotensin

receptor (NT₂). Using an anti-sense strategy in mice, the NT₂ receptor was implicated in the analgesic effects induced by neurotensin (Dubuc *et al.*, 1999). A third neurotensin receptor (NT₃) was recently cloned from human brain (Mazella *et al.*, 1998); it does not belong to the superfamily of G protein-coupled receptors, corresponds to the previously cloned gp95/sortilin and could be involved in intracellular trafficking.

Previous studies on the ontogeny of NT₁ receptors in the rat brain using autoradiography (Palacios *et al.*, 1988), binding experiments in brain homogenates (Schotte and Laduron, 1987) and *in situ* hybridization (Sato *et al.*, 1992; Lépée-Lorgeoux *et al.*, 1999), reported marked regional differences in their developmental profile. Thus, the cerebral cortex exhibits a high and transient expression of NT₁ receptors during the early postnatal period, which declines to reach adult levels by the third postnatal week, whereas other brain areas, including the midbrain, show a gradual increase in NT₁ receptor expression from late gestation to the second week of life. Interestingly, neurotensin is also highly expressed in the brain at birth, followed by a dramatic decrease to adult levels during the second and third weeks of postnatal life (Hara *et al.*, 1982; Sato *et al.*, 1991; Bennett *et al.*, 1998).

The role of endogenous ligands in establishing the developmental pattern of expression of their corresponding receptors has been suggested for several neurotransmitter systems, including neuropeptides (Kudlacz *et al.*, 1991; Hill *et al.*, 1994; Sircar *et al.*, 1996; Liu *et al.*, 1998). However, the possible involvement of neurotensin in the regulation of NT₁ receptors during ontogeny has not been studied. In a recent series of experiments, we used the non-peptide antagonist of NT₁ receptors SR 48692 to investigate the role of endogenous neurotensin in the regulation of NT₁ receptors in the adult rat brain. We showed that chronic administration of SR 48692 induces an up-regulation of NT₁ receptor binding sites and mRNA expression, suggesting a tonic inhibitory control of neurotensin on NT₁ receptors in the mature brain (Azzi *et al.*, 1994, 1996; Najimi *et al.*, 1998).

The aim of the present study was to investigate the role of neurotensin in the regulation of NT₁ receptors during postnatal development in the rat brain. We first examined concomitantly the ontogeny of neurotensin content and NT₁ receptor expression, since previous studies examining separately either parameter rendered difficult their comparison at a given time point. Second, we used primary cultures of embryonic neurons from rat cerebral cortex, the brain region with the highest expression of NT₁ receptors during early development, to investigate whether these receptors could be regulated by the agonist at an early developmental stage. The amount of NT₁ receptor mRNA transcripts in the cultures was measured by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Finally, we studied the effects of *in vivo* blockade of neurotensinergic transmission during postnatal development in rat pups injected with the NT₁ receptor antagonist SR 48692. [¹²⁵I]neurotensin binding to NT₁ receptors was examined using brain homogenates and autoradiography, and NT₁ receptor mRNA expression was assessed by *in situ* hybridization histochemistry.

MATERIALS AND METHODS

Animals and treatments

Wistar rats bred in our laboratory were maintained on a 12-hour dark/light cycle and were given food and water *ad libitum*. To study the ontogeny of neurotensin and NT₁ receptor expression in the brain, rats were analyzed at different prenatal and postnatal ages (E15, E20, P0, P5, P10, P15, P21 and P30). The day of the vaginal plug was counted as E1 and the day of birth as P0. All experiments were performed in accordance with the European Communities Council Directive for the care and use of laboratory animals.

In order to examine the role of endogenous neurotensin in the developmental regulation of NT₁ receptors, rat pups received a daily subcutaneous (s.c.) injection of the non-peptide NT₁ receptor antagonist, SR 48692 (Gully *et al.*, 1993; Sanofi Recherche, Toulouse, France), for different periods of time: P1-P5, P1-P9 or P1-P15 (n = six per group). SR 48692, administered at the dose of 1 mg/kg, was solubilized in Tween 20 and dissolved in sterile 0.9% NaCl solution. This dose of SR 48692 was chosen because it was previously shown to induce an up-regulation of NT₁ receptors in the adult rat (Azzi *et al.*, 1996; Najimi *et al.*, 1998). Control pups were injected with vehicle (Tween 20 1% in NaCl 0.9%). In a separate group of animals, prenatal treatment with SR 48692 was started at E15; pregnant dams received a daily injection of the antagonist until the day of birth, after which pups were injected with SR 48692 from P1 to P9. In all experiments, rats were decapitated 24 h after the last injection.

Preparation of fetal brain cortical cultures and treatment with neurotensin agonist and/or antagonist

Cultures were prepared from the cerebral cortex of Wistar rat fetuses at E17 following the procedure previously described for cultures of hypothalamic neurons (Scarcériaux *et al.*, 1996). The cerebral cortex was excised under sterile conditions and microscopic control, and cells were grown in serum-free medium consisting of a mixture of Dulbecco modified Eagle's medium and Ham-F12 (1vol/1vol, Gibco), 15 mM HEPES, NaHCO₃ as indicated by the manufacturer, 0.5 U/ml penicillin, 20 µg/ml streptomycin, 5 µg/ml insulin, 100 µg/ml human transferrin, 2.10⁻⁸ M progesterone, 10⁻¹² M 17 β-estradiol, 1 µg/ml arachidonic acid, 0.5 µg/ml docosahexaenoic acid, 3.10⁻⁸ M selenium, 10⁻⁴ M putrescine and 0.5 mM glutamine. Cells were plated at a density of 400,000 cells per cm² in six-well plastic culture plates (Costar) previously coated with poly-D-lysine. Cultures were maintained in a moist atmosphere (95% air, 5% CO₂) at 37°C and medium was renewed after 5 days. Cytosine arabinoside (1 µM) was added from the first change onward to prevent further glial proliferation. After 5 days of culture, neurons were incubated in the presence of the neurotensin agonist JMV 449 (Doulut *et al.*, 1992; Neosystem), 1 nM; the NT₁ receptor antagonist SR 48692, 1 µM; or both drugs. SR 48692 was dissolved in dimethyl sulfoxide (DMSO); incubation of cortical neurons in the presence of DMSO (final concentration 0.1%) showed that the solvent alone had no effect on NT₁ receptor mRNA expression. After 48 h, the culture medium was removed and the cells were rinsed with ice-cold phosphate-buffered saline. Total RNA

was extracted by the acid guanidium thiocyanate-phenol chloroform method (Chomczynski and Sacchi, 1987), followed by ethanol precipitation.

Quantitative RT-PCR of NT₁ receptor mRNA

Internal standard preparation and quantitative RT-PCR were carried out as described (Souazé *et al.*, 1997). Briefly, a plasmid pNTR18 was constructed by inserting the entire coding sequence of rat NT₁ receptor cDNA (nt: -7 to 1301) into the *SmaI/BamHI* sites of p T7/T3 α 18. A 34-nucleotide deletion was created by removing the *NcoI-NheI* fragment. A polyA₍₄₅₎ oligonucleotide was then inserted at the *Sall/BamHI* sites. For RT-PCR quantitation, mixtures of 100 ng of total RNA and a known number of internal control (cRNA) molecules were reverse transcribed in 30 μ l of reaction buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 10 mM dithiothreitol, 1 mM of each dNTP, 1 pmol/ μ l of specific primer, 1 unit/ μ l RNasin (Promega) and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies). The PCR amplification was performed in an automatic thermal cycler (DNA thermal cycler 480, Perkin Elmer Cetus). The amplification profile included an initial denaturation at 95°C for 7 min, followed by 26 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 1 min 30 s. The amplification was completed with an additional extension step at 72°C for 10 min. The different PCR products were separated on 5% polyacrylamide gel. Guided by ethidium bromide staining, the bands were excised and the radioactivity counted in a β scintillation counter (Beckman). Radioactivity (counts/min) was plotted against the amount of template (cRNA or target molecule). Results were expressed as molecules of NT₁ receptor mRNA per μ g of total RNA.

Radioimmunoassay of neurotensin

Brains were homogenized and sonicated in ice-cold 0.1 N HCl. Homogenates were boiled for 10 min and centrifuged at 10,000 *g* for 15 min at 4°C. Supernatants were separated and frozen at -20°C until use. The neurotensin radioimmunoassay was performed as described previously (Scarcériaux *et al.*, 1995), with a specific antibody directed against neurotensin diluted at 1:15,000 and [¹²⁵I]neurotensin as the tracer.

Brain membrane preparation and binding assays

Whole brains (minus cerebellum) were homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.4, centrifuged at 60,000 *g* for 35 min at 4°C, resuspended in the same buffer and recentrifuged. The pellets were then resuspended in 50 mM Tris-HCl buffer containing 0.1% bovine serum albumin, 0.5 mM 1-10 orthophenantroline, 1 mM EDTA and 4 mg/ml bacitracin, in a final concentration of 10-15 mg protein/ml. Membrane preparations were aliquoted, frozen in liquid nitrogen and stored at -80°C. Binding experiments were performed as described previously (Azzi *et al.*, 1994). Brain membrane homogenates, 0.1 mg protein/ml, were incubated at room temperature in a final volume of 300 μ l in 50 mM Tris-HCl buffer containing 0.2% bovine serum albumin, 5 mM MgCl₂, 0.5 mM 1-10 orthophenantroline, and 0.1 nM [¹²⁵I]Tyr₃-neurotensin. All experiments were

conducted in the presence of 1 μ M levocabastine (Janssen, Beerse, Belgium), which selectively inhibits neurotensin binding to NT₂ receptors (Schotte *et al.*, 1986). Non-specific binding was determined in the presence of 1 μ M unlabeled neurotensin (Neosystem Laboratories, Strasbourg, France). After incubation for 30 min, bound and free ligands were separated by centrifugation at 12,000 g for 4 min at 4°C. The bound radioactivity in the pellet was determined with a γ counter. Competition studies were conducted with a single concentration of [¹²⁵I]neurotensin (0.1 nM) and increasing concentrations of neurotensin or SR 48692. Data from competition studies were analyzed with a non-linear regression program (Munson and Rodbard, 1980). *K_i* values were calculated according to the Cheng and Prusoff (1973) equation.

Autoradiography of [¹²⁵I]neurotensin binding sites

Slide-mounted coronal brain sections (20 μ m) were preincubated at 4°C in 50 mM Tris-HCl buffer for 45 min to remove the endogenous ligand and then incubated at 4°C for 60 min in the same buffer used in the binding assays, containing 0.1 nM [¹²⁵I]neurotensin and 1 μ M levocabastine (Betancur *et al.*, 1998). Additional sections were incubated with 1 μ M unlabeled neurotensin, to determine non-specific binding. After incubation, the sections were washed four times for 2 min each in 40 mM Tris-HCl buffer, pH 7.4, at 4°C, dipped in distilled water and dried. Autoradiograms were generated by apposition of labeled sections to Hyperfilm β max (Amersham), for two or five days (6- and 16-day-old rats, respectively).

In situ hybridization histochemistry

In situ hybridization histochemistry of NT₁ receptor mRNA was performed as described (Lépée-Lorgeoux *et al.*, 1999), using four oligodeoxynucleotides complementary to nucleotides 128-169, 810-843, 1152-1184, 1375-1411 of the rat NT₁ receptor cDNA (Tanaka *et al.*, 1990). The oligonucleotides were 3' end-labeled with α -[³⁵S]deoxyadenosine-5'-triphosphate (specific activity: >1000 Ci/mmol, Amersham), using terminal transferase (Boehringer Mannheim). The mixture containing the labeled probes (0.25 pmol/ml of each oligonucleotide; 10⁷ cpm/ml) was diluted in hybridization solution (50% vol/vol formamide, 4 x SSC, 1% sarcosyl, 0.1 M potassium phosphate, pH 7.4, 250 μ g/ml yeast tRNA, 250 μ g/ml herring sperm DNA, 10% dextran sulfate, 1 x Denhardt's solution, 50 μ g/ml polyA, 10 mM dithiothreitol), applied onto each slide and overlaid with a coverslip. Hybridization was allowed to proceed for 18 h at 42°C in humidified chambers. Film autoradiograms were obtained by apposition of radiolabeled sections to Hyperfilm β max for 20 or 30 days (6- and 16-day-old rats, respectively).

Quantitative analysis of autoradiograms

Quantitative optical density measurements of film autoradiograms were carried out using a computer-based image analysis system (HISTO-RAG, Biocom, Les Ulis, France). Optical densities of non-specific signal for *in situ* hybridization were determined in the corpus callosum, a brain area which does not contain NT₁ receptor mRNA, or in sections incubated with unlabeled neurotensin for [¹²⁵I]neurotensin autoradiography, and subtracted from all values to obtain

specific values. The analysis was performed bilaterally on six brain sections per animal at each level.

RESULTS

Ontogeny of neurotensin and NT₁ receptors in the rat brain

Neurotensin concentration and [¹²⁵I]neurotensin binding to NT₁ receptors were measured in brain homogenates at different embryonic and postnatal stages (Figure 1). Neurotensin was first detected at moderate levels at E20. At birth, neurotensin levels were already elevated and further increased to reach peak levels at P5. The concentration of the peptide decreased thereafter, and adult levels were observed at P21. [¹²⁵I]neurotensin binding to NT₁ receptors was detected at very low levels at E20, followed by a very high and transient expression during the first postnatal week. NT₁ receptor expression peaked at P10 and decreased subsequently, reaching adult levels around P21.

In addition, we studied the binding properties of NT₁ receptors in the neonate (five-day-old) rat brain. Experiments were performed in the presence of levocabastine to block the binding of neurotensin to NT₂ receptors. Analysis of competition experiments revealed a dissociation constant (K_d) of 0.2 ± 0.05 nM and a maximal bound capacity (B_{max}) of 120 ± 20 fmol/mg of protein (mean \pm S.E.M. of three independent experiments). The inhibition constant (K_i) of SR 48692 to inhibit neurotensin binding to NT₁ receptors was 2.6 ± 0.52 nM. These K_d and K_i values are equivalent to the binding constants observed previously in the adult rat brain (Gully *et al.*, 1993), whereas the number of receptors in the newborn brain is five-times higher than in the adult (Azzi *et al.*, 1996).

Effect of the neurotensin agonist JMV 449 on NT₁ receptor mRNA expression in fetal brain cortical cultures

Using quantitative RT-PCR, we examined the *in vitro* effect of a stable neurotensin agonist, JMV 449, on the amount of NT₁ receptor mRNA transcripts in fetal brain cortical cultures. Exposure of cortical neurons to JMV 449 (1 nM) for 48 h resulted in a 42% decrease in NT₁ receptor mRNA, when compared to control cultures (Figure 2). The effect of JMV 449 was abolished by coadministration of the NT₁ receptor antagonist SR 48692 (1 μ M). No effect was observed after treatment with SR 48692 alone.

Effect of chronic treatment with the antagonist SR 48692 on the ontogeny of NT₁ receptor expression

The contribution of endogenous neurotensin to the ontogeny of NT₁ receptors was studied in rat pups exposed to the NT₁ receptor antagonist SR 48692 during the third week of pregnancy or during early postnatal life. Daily SR 48692 treatment during pregnancy had no significant effect on litter size, mean body weight, and brain weight of the offspring (data not shown). Furthermore,

there was no difference in the body and brain weight of pups treated postnatally with SR 48692 or vehicle.

Administration of the antagonist (1 mg/kg s.c.) was started at P1 and continued until P5, P9 or P15, corresponding respectively to the period of increase, the peak or the whole period of high transient expression of NT₁ receptors in the brain. No significant difference was found in [¹²⁵I]neurotensin binding in brain membrane homogenates between SR 48692- and vehicle-treated rats, whatever the treatment length (Table 1). Treatment with a higher dose of SR 48692, 10 mg/kg, from P1 to P5, also failed to modify neurotensin binding (not shown). In addition, exposure to SR 48692 during the prenatal period, by administration of the antagonist to pregnant dams from gestational day 15 (1 mg/kg i.p.), followed by injection of SR 48692 to pups from P1 to P9 (1 mg/kg s.c.), was also without effect on the level of [¹²⁵I]neurotensin binding to NT₁ receptors (Table 1).

Similarly, postnatal treatment with SR 48692 (1 mg/kg s.c.) for five days (not shown) or 15 days (Figure 3C) did not modify the density of [¹²⁵I]neurotensin binding sites measured by quantitative autoradiography in different brain regions, including the cingulate and parietal cortices, caudate-putamen, substantia nigra compact part, and ventral tegmental area. Furthermore, administration of the NT₁ receptor antagonist for five days (not shown) or 15 days (Figure 3F) did not modify the expression pattern or the density of NT₁ receptor mRNA in any of the brain regions studied.

DISCUSSION

In the present study, we investigated the role of neurotensin in the regulation of NT₁ receptors in the developing rat brain. We showed that exposure to the neurotensin agonist JMV 449 induced a down-regulation of NT₁ receptor mRNA in primary cultures of fetal rat cerebral cortex neurons, indicating that NT₁ receptor gene expression can be regulated by the agonist during the developmental period. However, chronic blockade of endogenous neurotensin by administration of SR 48692 to rat pups did not modify the postnatal expression of NT₁ receptor mRNA or binding sites in the brain, suggesting that neurotensin is not essential for the establishment of the ontogenetic pattern of these receptors.

The concomitant study of the ontogeny of brain neurotensin concentration and [¹²⁵I]neurotensin binding in the rat showed that a marked and transient rise in the levels of neurotensin (at P5) preceded the increase in the expression of NT₁ receptors (at P10). The presence of high levels of neurotensin during the perinatal period is consistent with previous studies showing that neurotensin appears early in development and reaches maximal levels within the first postnatal week, decreasing thereafter (Hara *et al.*, 1982; Bisette *et al.*, 1984; Kiyama *et al.*, 1992; Sato *et al.*, 1991; Bennett *et al.*, 1998). The peak in [¹²⁵I]neurotensin receptor binding observed in brain homogenates during the second postnatal week corresponds mainly to the increased expression of NT₁ receptors in the cerebral cortex, the brain region with the highest

expression of these receptors during the perinatal period (Palacios *et al.*, 1988; Sato *et al.*, 1992; Lépée-Logeoux *et al.*, 1999). Although only few neurotensin-containing neurons and fibers are present in the cerebral cortex, it has been suggested that neurotensin synthesized in other brain areas and/or present in the cerebrospinal fluid could exert a paracrine action on cortical receptors (Palacios *et al.*, 1988; Sato *et al.*, 1992).

The ability of agonists to regulate the expression of their own receptors *in vitro* has been described for numerous receptor systems. Previous studies on the regulation of the NT₁ receptor in cell cultures showed that exposure to neurotensin results in receptor internalization, receptor down-regulation and regulation at the second messenger level (reviewed in Hermans and Maloteaux, 1998). In order to investigate whether cortical NT₁ receptors could be regulated by the agonist at an early developmental stage, we studied the effect of a peptidase-resistant neurotensin agonist, JMV 449, on NT₁ receptor mRNA expression in primary cultures of fetal rat cerebral cortex neurons. Exposure of cortical neurons to JMV 449 for 48 h induced a significant decrease in NT₁ receptor mRNA, and this effect was abolished by the antagonist SR 48692. These results are in agreement with our previous studies on hypothalamic neuronal cultures, demonstrating that release of endogenous neurotensin induced by dexamethasone and forskolin down-regulates NT₁ receptor mRNA (Scarcériaux *et al.*, 1996). These data suggest that NT₁ receptors in the developing neocortex and hypothalamus can be regulated by the agonist, despite inherent differences in the maturational profile of NT₁ receptors in these two brain regions (Palacios *et al.*, 1988; Sato *et al.*, 1992). In addition, these findings illustrate the ability of SR 48692 to suppress agonist-induced down-regulation of NT₁ receptors.

Despite the demonstration of a potential modulation of NT₁ receptors by the agonist early in development, blockade of neurotensin transmission during the perinatal period with SR 48692 did not affect the ontogenetic pattern of [¹²⁵I]neurotensin binding sites in brain homogenates. Likewise, the density and distribution of NT₁ receptor binding sites and mRNA expression was similar in SR 48692- and vehicle-treated pups. These results are in contrast with those obtained previously in the adult rat, showing that chronic administration of SR 48692, at the same dose used in the present study, results in up-regulation of NT₁ receptor gene expression in numerous brain regions, including the anterior cingulate and perirhinal cortices, substantia nigra and ventral tegmental area (Azzi *et al.*, 1994, 1996). Thus, whereas endogenous neurotensin appears to exert a tonic inhibitory control on NT₁ receptors in the mature CNS, the results of the present study indicate that it does not play a major role in establishing the developmental profile of NT₁ receptors during the early postnatal period.

The lack of effect of SR 48692 is not due to undeveloped intracellular pathways of NT₁ receptors transiently expressed in the brain of fetal and infant rats, since NT₁ receptor gene expression in primary cell cultures prepared from E17 cerebral cortex was down-regulated after agonist-induced receptor activation. In addition, the differential effect of SR 48692 on the regulation of NT₁ receptors in the immature and adult rat brain can not be ascribed to different binding properties of these receptors, because NT₁ receptors in five-day-old rat brain have the

same affinity for SR 48692 and neurotensin as NT₁ receptors present in the adult (Gully *et al.*, 1993; Azzi *et al.*, 1996). Hence, these findings suggest that NT₁ receptors expressed during the perinatal period represent functional receptors having the same ligand affinity as NT₁ receptors in the adult and may thus be involved in neuronal signaling.

In the neocortex, NT₁ receptors are abundantly expressed in the molecular layer, the cortical plate and the developing cortical layer VI during late gestation and the first postnatal week, followed by a dramatic decrease to the low levels seen in the adult at the end of the third postnatal week (Palacios *et al.*, 1988). The transient expression of NT₁ receptors in the cerebral cortex could be related to the reorganization of cortical neurons during postnatal development, including neuronal death, which is a prominent feature of the normal development of the neocortex (Ferrer *et al.*, 1992). Moreover, the formation of the pattern of cortical connectivity, the regression of particular synapses and the stabilization of others, can act upon the level of gene expression in cortical cells. Therefore, the decline in the amount of cortical NT₁ receptors during the maturation of the cerebral cortex could be explained by the programmed cell death of neurons bearing NT₁ receptors, or the influence of cortical inputs on the expression of NT₁ receptors. This in turn may explain the absence of effect of perinatal SR 48692 treatment on the developmental regulation of NT₁ receptors in the cortex.

Interestingly, the time frame of increased expression of neurotensin and NT₁ receptors in the cortex coincides with a critical period of cortical development, involving laminar organization, determination of neuronal migration pattern and synapse formation (O'Leary and Koester, 1993). It is possible therefore that neurotensin and NT₁ receptor-mediated signaling play a role in the synaptic plasticity associated with the development of the cerebral cortex. In support of this idea, studies in non-neuronal cells have shown that neurotensin exerts mitogenic and trophic effects in normal and cancer cells in the intestine, liver, pancreas, lung and prostate (Evers *et al.*, 1992; Hasegawa *et al.*, 1994; Sehgal *et al.*, 1994). The proliferative effect of neurotensin on intestinal cells is mediated by NT₁ receptors (Ehlers *et al.* 1998). In addition, neurotensin has been shown to stimulate proliferation of astrocytic cell lines (Camby *et al.*, 1996). It is noteworthy that the regionally specific and transient appearance of receptors during brain development has been reported for other neurotransmitters and peptides, including cholecystokinin, substance P, and vasopressin (Parnavelas and Cavanagh, 1988; Pélaprat *et al.*, 1988; Tribollet *et al.*, 1991; Zhang and Harlan, 1994; Taoka *et al.*, 1996), thus supporting the role of neurotransmitters and peptides as growth regulatory signals during maturation of the CNS (Lauder, 1993).

Other brain areas, including the substantia nigra and ventral tegmental area, show a developmental pattern of NT₁ receptors radically different from the one observed in the neocortex, characterized by a gradual increase during the postnatal period (Palacios *et al.*, 1988; Sato *et al.*, 1992; Lépée-Lorgeoux *et al.*, 1999). These regions are also characterized by the presence of neurotensin terminals at birth and in the adult (Kiyama *et al.*, 1992), therefore suggesting the possible regulation of NT₁ receptors in the developing brain following the

administration of SR 48692, as shown in the adult (Azzi *et al.*, 1994, 1996). However, as mentioned above, chronic blockade of NT₁ receptors during fetal and postnatal development did not affect the expression of receptors in the midbrain, suggesting that during this period, peptide release and connection formation are probably not sufficient for the regulation of NT₁ receptors by the endogenous ligand.

Early exposure of developing postsynaptic receptors to the appropriate agonist has been suggested to be important for establishing the ontogenetic pattern of several receptor systems. For instance, postnatal treatment with a vasoactive intestinal polypeptide (VIP) antagonist increases expression of VIP receptors in the rat brain (Hill *et al.*, 1994). Furthermore, prenatal or postnatal exposure to GABA(A) or NMDA receptor antagonist selectively decrease the expression of certain receptor subunits in the developing brain (Sircar *et al.*, 1996; Liu *et al.*, 1998). However, lack of agonist involvement in receptor ontogeny has also been described for some other members of the family of G protein-coupled receptors. Thus, prenatal administration of the β -adrenergic antagonist propranolol does not modify the expression of its receptors in the newborn rat brain (Kudlacz *et al.*, 1991; Speiser *et al.*, 1991). Moreover, using the knockout approach, it was recently shown that deletion of the oxytocin gene does not alter the expression of oxytocin receptors in the CNS (Nishimori *et al.*, 1996). Similarly, mice unable to synthesize dopamine after targeted inactivation of the tyrosine hydroxylase gene in dopaminergic neurons show normal development of midbrain dopaminergic neurons and the pattern and intensity of D₁ and D₂ receptor mRNA expression in the striatum are unaltered (Zhou and Palmiter, 1995). Our results indicate that NT₁ receptors represent another instance of receptors for which the agonist, while being involved in receptor regulation in the adult rat brain, does not influence the pattern of expression during ontogeny.

In conclusion, these findings indicate that NT₁ receptor regulation in the developing nervous system differs from that seen in adulthood and that endogenous neurotensin is not required for the normal expression of NT₁ receptors during neonatal development. The transiently high expression of neurotensin and NT₁ receptors during the early postnatal period overlaps with a critical period of development of the brain, suggesting a physiological role for neurotensin and NT₁ receptors in the development of the CNS.

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Table 1. Effect of chronic treatment with SR 48692 during perinatal development on NT₁ receptor binding sites

Treatment length	Bound [¹²⁵ I]neurotensin (fmol/mg protein)	
	Vehicle	SR 48692
E15-P9	163.5 ± 7.1	161.1 ± 4.6
P1-P5	85.9 ± 5.2	96.4 ± 16.4
P1-P9	181.8 ± 13.1	190.4 ± 6.6
P1-P15	67.2 ± 3.6	68.4 ± 5.5

Rat pups were injected daily with the neurotensin NT₁ receptor antagonist SR 48692 (1 mg/kg s.c.) or vehicle for different lengths of time starting at postnatal day 1 (P1). In another group, exposure to SR 48692 was started during the prenatal period, by daily administration of the antagonist to pregnant females from gestational day 15 (E15). Binding of [¹²⁵I]neurotensin to NT₁ receptors was determined in brain membrane homogenates in the presence of levocabastine, to inhibit binding to NT₂ receptors. Values represent mean ± S.E.M. (n = six rats per group).

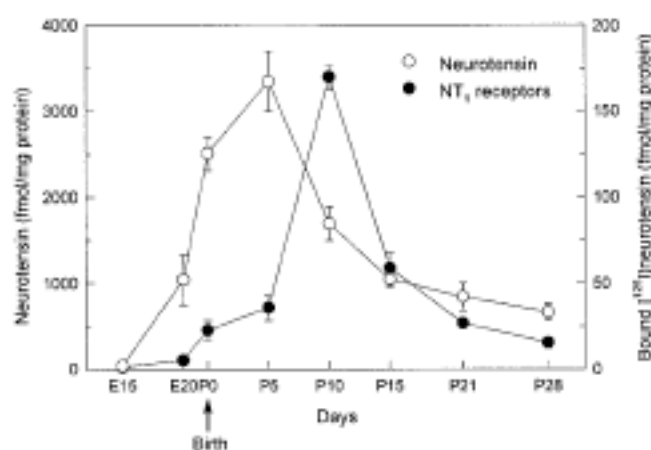


Figure 1. Ontogeny of neurotensin and NT₁ receptors in the rat brain. Left axis represents neurotensin levels, measured by radioimmunoassay, in whole brain homogenates. Right axis shows the developmental pattern of NT₁ receptor binding sites, measured by [¹²⁵I]neurotensin binding in brain membrane homogenates. Each point represents mean ± S.E.M. of four rats at different developmental stages: gestational day 15 (E15), E20, postnatal day 0 (P0, at birth), P5, P10, P15, P21, and P28.

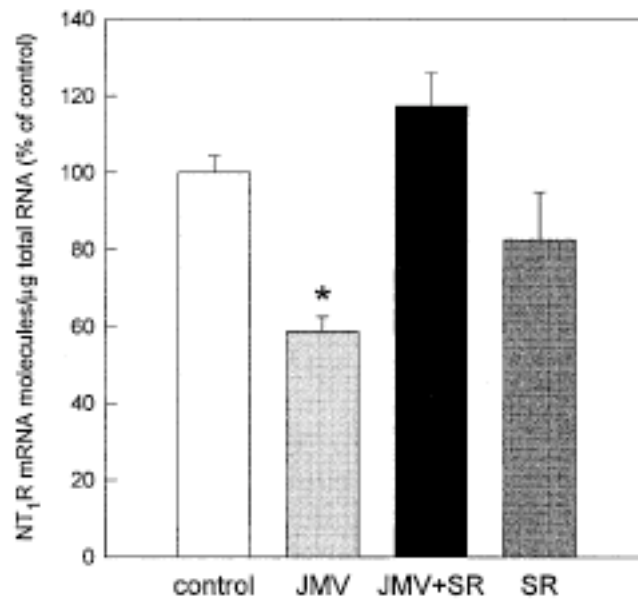


Figure 2. Regulation of NT₁ receptor mRNA in brain cortical cultures after exposure to the neurotensin agonist JMV 449, and the NT₁ receptor antagonist SR 48692. Cortical neurons were treated for 48 h with JMV 449 1 nM, SR 48692 1 µM, or both. NT₁ receptor mRNA was quantified with RT-PCR. Values represent mean ± S.E.M. of four cultures. * $p < 0.05$ vs. control (Student's *t*-test).

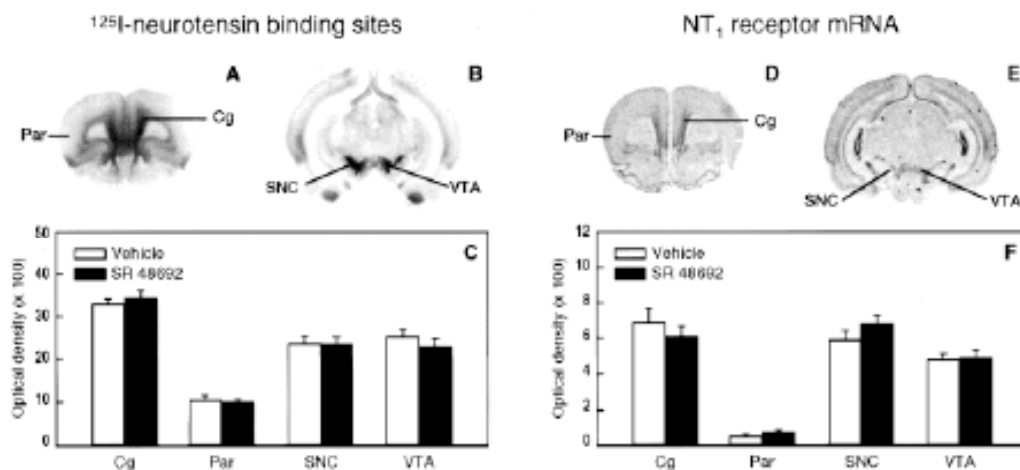


Figure 3. Effect of chronic administration of the antagonist SR 48692 during postnatal development on NT₁ receptor binding sites and mRNA expression in the rat brain. Pups received a daily injection of SR 48692 (1 mg/kg s.c.) or vehicle from P1 to P15 and were killed 24 h after the last injection. Autoradiograms illustrate the brain areas where the optical density was quantified. *A,B,C*: [¹²⁵I]neurotensin binding to NT₁ receptors was assessed by quantitative autoradiography. *D,E,F*: NT₁ receptor mRNA expression was measured using *in situ* hybridization. Bars represent mean ± S.E.M. of six rats. Cg, cingulate cortex; Par, parietal cortex; SNC, substantia nigra, compact part; VTA, ventral tegmental area.