Specific involvement of neurotensin type 1 receptor in the neurotensin-mediated in vivo dopamine efflux using knock-out mice

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Abstract
Neurotensin is a tridecapeptide neurotransmitter known to be involved in psychiatric disorders, various physiological processes and several different neurobiological mechanisms, including modulation of accumbal dopamine release. Two neurotensin extracellular binding sites, namely NT1- and NT2-receptor (NT1R and NT2R), have been cloned from the rat brain. These receptors are distinguishable by their different in vitro pharmacological properties but the available pharmacological tools have weak in vivo potency and specificity. The use of genetically engineered knock-out mice has provided a powerful alternative to the classical pharmacological approach to investigate their respective roles. In this study, using in vivo differential pulse amperometry, we show that, in wild-type mice, neurotensin application into the ventral tegmental area dose-dependently evokes dopamine efflux in the nucleus accumbens. This neurotensin-mediated efflux is dramatically decreased in mice lacking NT1R while it is unaffected in NT2R-deleted mice. This finding indicates that a large part of the dopamine efflux evoked by neurotensin in the nucleus accumbens of wild-type mice is mediated via NT1R present in the ventral tegmental area.

Keywords: dopamine, in vivo electrochemistry, knock-out mice, neurotensin, nucleus accumbens, ventral tegmental area.

J. Neurochem. (2004) 89, 1–6.

The abnormalities in dopaminergic transmission observed in the pathophysiology of psychiatric disorders may be intrinsic to dopamine (DA) neurones and/or secondary to alterations in other afferent systems that intimately interact with the DA systems. In this respect, there is a large body of anatomical, physiological and behavioural evidence that neurotensin (NT) interacts with DA systems and particularly with the mesolimbic system (Kalivas 1993; Binder et al. 2001). In the ventral tegmental area (VTA), NT has been localized within neuronal perikarya, some of them colocalizing DA (Hökfelt et al. 1984). Moreover, the VTA cells are surrounded by a dense plexus of NT fibres arising mainly from the pre-optic region and from the rostral parts of the lateral hypothalamus (Zahm et al. 2001). Among the different physiological effects triggered by NT in the mesolimbic system, it has been shown that NT applied in the rat VTA dose-dependently increases DA neurone firing activity and consequently enhances the DA efflux in the caudal part of the nucleus accumbens (NAcc) (Sotty et al. 1998).

Despite the identification of three NT-receptor subtypes, the subtype involved in this mesolimbic effect of the peptide has not yet been identified. Three NT binding sites, named NT1-, NT2- and NT3-receptor (NT1R, NT2R and NT3R), have been cloned (Tanaka et al. 1990; Chalon et al. 1996; Mazella et al. 1998). The involvement of the NT3 site is unlikely because of its intracellular localization (Mazella et al. 1998). NT1R and NT2R are extracellular binding sites...
and distinct by their pharmacological properties (Tanaka et al. 1990; Chalon et al. 1996). They are both widely expressed throughout the brain but transcripts of both NT-receptor subtypes are abundantly coexpressed only in the ventral mesencephalic region, which includes the VTA (Boudin et al. 1996; Walker et al. 1998). While NT2R is found in all cell types, including neurones, glia and ependymal cells (Walker et al. 1998), interestingly NT1R is preferentially associated with neurones (Boudin et al. 1996). However, a recent study revealed that NT2R was exclusively associated with neurones (Sarret et al. 2003).

Pharmacological studies failed to reveal the role of the NT-receptor subtypes on mesolimbic DA activity. Neurotensin itself exhibits unusual pharmacodynamic features at the NT1R and NT2R. It shows agonistic activities at NT1R (Vincent 1995), characterized as a high affinity site for NT insensitive to levocabastine (a histamine H1 receptor antagonist) (Tanaka et al. 1990; Vita et al. 1993), whereas, in some in vitro conditions, NT can behave as a neutral antagonist at the NT2 site (Botto et al. 1998; Vita et al. 1998; Yamada et al. 1998a; Richard et al. 2001), characterized as a low affinity site for NT (Chalon et al. 1996). The two chemically related compounds, SR 48692 and SR 142948 A, described as potent, selective and competitive non-peptide ligands of the NT-receptor subtypes (Gully et al. 1993, 1997) are not specific for one or the other extracellular NT-receptor subtype. Moreover, the existence of a new extracellular NT binding site, not yet cloned but often suggested by the use of the two non-peptide ligands (Gully et al. 1997; Trudeau 2000; Leonetti et al. 2002), should be taken into account in the NT-mediated in vivo DA efflux.

The present study was designed to characterize NT-receptor subtypes involved in the control of DA efflux in the mesolimbic system. For this purpose, we used mice knock-out for NT1R (NT1R-KO), mice knock-out for NT2R (NT2R-KO) and corresponding wild-type mice (WT1 and WT2, respectively). For the first time, the DA efflux evoked in the NAcc by NT application into the VTA and measured by differential pulse amperometry combined with carbon fibre electrodes (CFE) was compared between all types of mice.

Materials and methods

Animals

Experiments were performed in accordance with French (act no. 87-848; Ministère de l’Agriculture et de la Forêt) and European Economic Community (act no. 86-6091) guidelines for the care and use of laboratory animals. Mice lacking NT1R subtype were generated as previously described (Remaury et al. 2002). Mice lacking NT2R subtype were generated as below. The A129/Ola mouse genomic lambda 2001 library was screened with an NT2R cDNA probe to isolate cloned DNA for targeting vector construction. A ~16-kb XhoI fragment was subsequently recovered in a pbblueScrip plasmid containing the entire gene and, from this, sequences were defined for the construction of a replacement type targeting vector designed to create a null allele of the NT2R gene. The vector contained approximately 8 kb of cloned genomic DNA sequence to provide 5’ and 3’ homology arms of equal length with a region between a Smal site in exon 1 and a HinfIII site in intron 2 removed and substituted with the reporter/selection cassette TAG3/IRES/lacZ/SV40pA/MC1neo/PA. An MC1-th dimer cassette was appended to the end of the 5’ homology arm at a Sall site for negative selection. E14TG2a embryonic stem cells were electroporated with NotI linearized targeting vector and selected in G418 and ganciclovir. Targeted embryonic stem (ES) cells were injected into C57BL/6 blastocysts by a standard procedure and the resulting male chimeras subsequently test-crossed with C57BL/6 females. Germ-line transmission of the targeted allele using chimeras derived from two independent ES cell clones was confirmed in agouti coat coloured test-cross offspring by Southern blot analysis of DNA obtained from tail biopsy. Male and female offspring heterozygous for the null allele were intercrossed and WT (+/–), heterozygous (+/–) and homozygous (–/–) pups born at a normal Mendelian ratio. The NT2R-deficient strain was maintained by back-crossing heterozygous males with C57BL/6 females at each generation.

Drugs

Urethane and pargyline (Sigma, St Quentin Fallavier, France) were dissolved in saline solution (0.9% NaCl). NMDA (Sigma) was dissolved in water and 10⁻² M aliquots were stored at –20°C. Neurotensin (Sigma) was dissolved in phosphate-buffered saline [containing (g/l): NaCl, 8; KCl, 0.2; Na₂HPO₄, 2H₂O, 1.44; KH₂PO₄, 0.2; pH 7.4] containing CaCl₂.₂H₂O (132 mg/L) and 10⁻³ M aliquots were stored at –20°C. Final solutions were freshly prepared in phosphate-buffered saline containing CaCl₂.₂H₂O.

Surgery and electrochemical techniques

Male homozygote NT1R-KO, NT2R-KO and their corresponding WT mice, weighing 27–31 g, were anaesthetized with urethane (1.8 g/kg, i.p.) and treated with pargyline (75 mg/kg, i.p.). They were mounted in a stereotaxic frame according to the atlas of Franklin and Paxinos (1997): 1.8 mm anterior to bregma, 0.8 mmlateral to the midline and 3.9 mm below the cortical surface. Their active part was the surface of one pyrolytic carbon fibre electrode (CFE) was compared between all types of mice.
rats (Gonon 1988; Suau-Chagny et al. 1992; Sotty et al. 2000; Leonetti et al. 2002) and mice (Benoit-Marand et al. 2000). The final potential was adjusted to +80 mV (vs. Ag/AgCl) because DA oxidizes at this potential on the surface of these treated CFE (Suau-Chagny et al. 1992). In the absence of stimulation conditions, the measured oxidation current corresponds to the basal level of DA. The chemical stimulation of DA neurones, by NMDA or NT ejection into the VTA, induces an immediate and transient increase in the measured current followed by a rapid recovery. This increase reflects the oxidation of DA efflux evoked by the stimulation.

**Pneumatic ejection**

NMDA and NT were ejected into the VTA by means of a double-barrelled pipette (Leonetti et al. 2002) which consisted of two glass tubings (internal diameter 0.32 mm and calibrated at 15 mm/μL; Assistent® ref. 555/5; Hoecht, Sondheim-Rhoen, Germany) glued and pulled together and broken back to an external diameter of 100 μm (50 μm for each tip). Just before use, one barrel of the pipette, used as internal standard, was filled by applying negative pressure with NMDA (10^{-4} M) and the other was filled with NT at different concentrations. The double-barrelled pipettes were implanted just above the VTA at the following coordinates adjusted from the atlas of Franklin and Paxinos (1997): 3.3 mm posterior to bregma, 0.4 mm lateral to the midline. NMDA and NT were successively and alternately ejected every 15 min at the same VTA depth. The same ejection procedure was performed after moving the double pipette 150 μm down in the VTA from 4.4 to 5.75 mm below the cortical surface. Substances were ejected by applying air pressure. The ejected volume and the speed (65 nL/20 s) of ejection were determined by the movement of the meniscus of the solution in the pipette.

**Histological controls**

To visualize the track of the recording CFE in the NAcc and of the ejection pipette in the VTA, electrolytic lesions were performed, at the end of each experiment and before the removal of the brain, by applying a continuous potential of 5 V for 5 s through the CFE. For this, the recording CFE was kept in the NAcc while the ejection pipette was carefully removed from the CFE and replaced by a new CFE. Coronal sections (25 μm) throughout the NAcc and the VTA were cut on a cryostat, mounted on slides and stained with cresyl violet. The recording sites were checked according to the atlas of Franklin and Paxinos (1997).

**Data analysis**

To allow comparisons between the efficacy of NT in evoking DA release in genotype groups we used NMDA, a robust and reproducible excitatory agent of the DA mesolimbic system (Suau-Chagny et al. 1992; Rodriguez et al. 2000), as a calibrator. For each animal, the DA efflux evoked by NT ejection, at each depth explored at the level of the VTA, was expressed as a percentage of the maximal effect evoked by NMDA in the same experiment. To minimize the variability in the stereotaxic approaches of the VTA, the ejection depth corresponding to the maximal effect evoked by NT was considered as depth zero. The other depths explored were expressed relative to this point. As a control of DA release between genotype groups, the internal standard (NMDA) effect was expressed as a percentage of its maximal effect and the corresponding VTA depth was referenced as depth zero. All results are expressed as mean ± SEM. A Student’s t-test was used to assess the significance of the differences between each gene-deleted group and its corresponding WT group in each experiment.

**Results**

**Stereotaxic placements of electrochemical electrodes and ejection pipettes**

The placements of the electrochemical recording electrodes and ejection pipettes used in the present study are shown in Fig. 1. The active part of the CFE was seen to be confined within the core part of the NAcc in the range of 1.34–1.78 mm anterior to the bregma for all genotype groups (Fig. 1a). The tip of the ejection pipette was confined in the VTA, i.e. the parabrachial and paranigral nuclei, in the range of 3.08–3.40 mm posterior to the bregma for all genotype groups (Fig. 1b). The stereotaxic coordinates that we needed to use (see Materials and methods) differed from the theoretical coordinates given by Franklin and Paxinos (1997) particularly concerning the rostro-caudal position of the NAcc (Fig. 1a) and the depth position of the VTA (Fig. 1b). This may be related to the mouse strains used in the present study.

**Effects of NMDA and neurotensin ejection**

The pneumatic ejection of NMDA (10^{-4} M, 65 nL) or NT (10^{-8}–10^{-6} M, 65 nL) into the mouse VTA induced an immediate and transient increase in the extracellular DA level recorded within the NAcc by differential pulse amperometry (Fig. 2). To allow comparisons between genotype groups, each NT-evoked response was expressed as a percentage of the maximal response evoked by NMDA. These local ejections yielded parallel DA changes according to the penetration course of the ejection pipette throughout the VTA (Fig. 3). Vehicle alone did not trigger any significant effect (data not shown).

At any ejection depth explored, the amplitudes of the effects evoked by NT never differed between WT2 and NT2-KO mice (NT 10^{-7} M, p > 0.05; Fig. 3a).

In WT1 mice, NT (10^{-8}–10^{-6} M) ejected into the VTA evoked a concentration-dependent increase of DA release recorded into the NAcc. In NT1R-KO mice, the effects evoked by NT, in the same concentration range, were largely decreased compared with the corresponding effect in WT1 mice but a remaining effect was still observed (Fig. 4). For example, in WT1 mice the effects evoked by NT (10^{-7} M), ejected from three different depths into the VTA, corresponded, respectively, to 46.5 ± 7.7, 78.6 ± 9.4 and 59.7 ± 9.0% of the maximal effect evoked by NMDA, whereas in NT1R-KO mice they corresponded, respectively, to 10.6 ± 4.3, 21.7 ± 5.2 and 10.9 ± 6.4% (p < 0.01, Fig. 3b).
Fig. 2 Typical recordings of the effects of a pneumatic ejection of NMDA (10^{-4} M, 65 nL, black arrow) and neurotensin (NT) (10^{-7} M, 65 nL, white arrow) into the ventral tegmental area of mice knock-out for NT1-receptor (NT1R-KO), wild-type (WT)1, mice knock-out for NT2-receptor (NT2R-KO) and WT2 mice on the dopamine (DA) efflux in the nucleus accumbens. Neurotensin and NMDA ejections were performed successively 15 min apart. DA efflux was measured as the oxidative current recorded by differential pulse amperometry (1 measurement/s) combined with carbon fibre electrode.

Fig. 1 Representative coronal sections of the mouse brain showing the placement (a) of the carbon fibre electrode in the nucleus accumbens (NAcc) (n = 16) and (b) of the ejection pipettes in the ventral tegmental area (VTA) (n = 16) corresponding to each experiment performed. The positions of electrolytic lesions observed on coronal brain slices were schematically drawn on coronal sections from the atlas of Franklin and Paxinos (1997). Symbols correspond to the placements in mice knock-out for neurotensin (NT)1-receptor (●), wild-type (WT)1 (○), mice knock-out for NT2-receptor (●) and WT2 (○). aca, anterior commissure; core, core part of the NAcc; shell, shell part of the NAcc.

Fig. 3 Amplitude of the dopamine (DA) efflux evoked by NMDA and neurotensin (NT) ejections into the ventral tegmental area (VTA) of (a) mice knock-out for NT2-receptor (NT2R-KO) and wild-type (WT)2 mice and (b) mice knock-out for NT1-receptor (NT1R-KO) and WT1 mice, according to the three relative localizations of the ejection pipette throughout the VTA. In each mouse NMDA (10^{-4} M, 65 nL) and NT (10^{-7} M, 65 nL) were successively and alternately ejected (every 15 min) at various depths below the cortical surface at the level of the VTA from 4.4 to 5.75 mm (150-μm steps). The successive evoked effects on DA efflux were monitored in nucleus accumbens (NAcc) with differential pulse amperometry and expressed as a percentage of the maximal response evoked by NMDA (100%). The depth of the ejection pipette into the VTA, allowing NMDA and NT to evoke respectively a maximal effect into the NAcc, was quoted as depth zero. The other depths explored are expressed relative to this point (–, above; +, below). Results are expressed as mean ± SEM of the effects observed from four distinct mice in each group. **p < 0.01 compared with the WT1-NT group (Student’s t-test).
**Discussion**

In the present study, the application of NT into the VTA was shown, for the first time in the mouse, to induce an immediate and transient increase in the DA efflux recorded within the core part of the NAcc. The relative involvement of each NT-receptor subtype in mediating this effect was demonstrated by using mice deleted for NT1R or NT2R.

Differential pulse amperometry combined with electrochemically treated CFE is a technique suitable for monitoring extracellular neurochemical changes when they are rapid and transient as evoked by chemical or electrical stimulations. This approach has been extensively used to monitor DA efflux on a 1-s time scale. The characterization of the electrochemical signal in rats (Suaud-Chagny et al. 1992; Sotty et al. 1998; Leonetti et al. 2002) as well as in mice (Benoit-Marand et al. 2000, 2001; Dumartin et al. 2000) strongly suggests that, in the present study, the chemically evoked changes in oxidation current recorded in the mouse NAcc correspond to changes in DA efflux.

The excitatory effect of NT on the mouse DA mesolimbic system reported in this study is in agreement with (i) the increase in the DA firing activity and in the DA efflux evoked in the rat NAcc by NT administration into the VTA (Sotty et al. 1998) and (ii) the localization of NT-receptors on the surface of the DA perikarya and dendrite throughout the VTA (Boudin et al. 1996; Walker et al. 1998; Rodriguez et al. 2000; Sarret et al. 2003).

In order to standardize the NT-evoked responses between mice, NMDA, a robust and reproducible excitatory agent of the DA mesolimbic system (Suaud-Chagny et al. 1992; Rodriguez et al. 2000), was used as a calibrator. Variations in the amplitude of the responses evoked by NT and NMDA according to the penetration course of the ejection pipette were correlated (Fig. 3). This experimental paradigm allowed us to demonstrate that the excitatory effect of NT was largely reduced in NT1R-KO mice compared with WT1 at any VTA site requested and at any NT concentration tested. This result indicates that the NT-evoked DA efflux in the core part of the NAcc of WT mice is mediated via NT1R present in the VTA. Considering a recent result revealing that, in the VTA, a few neurones bore NT2R (Sarret et al. 2003), the slight remaining effect observed in NT1R-KO mice may be due to their participation in the evoked-DA efflux. In this hypothesis, the persistence of the NT excitatory effect in NT2R-KO versus WT2 experiments could be due to an NT1R up-regulation thus masking any small NT2R contribution. However, we cannot exclude the implication of an unknown NT-receptor, an adaptive mechanism appearing in NT1R-KO mice or a non-specific effect.

This study proves, for the first time, that the in vivo regulation of DA mesencephalic activity by NT is mainly mediated via NT1R. Our data extend the major central role demonstrated for NT1R in mediating thermal regulation, food intake, NT-induced hypolocomotion and rotarod performance effects (Pettibone et al. 2002; Remaury et al. 2002) revealed by NT1R knock-out mice. The role which we demonstrated for NT1R could not be revealed by the use of the non-peptide ligands available, possibly due to their pharmacodynamic and pharmacokinetic features (Leonetti et al. 2002). In this context, mutant mice for NT1R or NT2R have provided a powerful alternative to the classical pharmacological approaches. These preparations may be of interest in characterizing the NT-receptor subtypes involved in the numerous interactions between NT and DA systems and more generally in precisely defining the role of NT in various physiological processes and physiopathologies.

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