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NEUROTENSIN REGULATES INTRACELLULAR CALCIUM IN VENTRAL TEGMENTAL AREA ASTROCYTES: EVIDENCE FOR THE INVOLVEMENT OF MULTIPLE RECEPTORS

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Abstract—Recent evidence suggests that some types of neurotensin receptors may be expressed by astrocytes. In order to explore the function of neurotensin receptors in astrocytes, the effect of a neurotensin receptor agonist, neurotensin(8–13), on intracellular Ca^{2+} dynamics in mixed neuronal/glial cultures prepared from rat ventral tegmental area was examined. It was found that neurotensin(8–13) induces a long-lasting rise in intracellular Ca^{2+} concentration in a subset of glial fibrilary acidic protein-positive glial cells. This response displays extensive desensitization and appears to implicate both intracellular and extracellular Ca^{2+} sources. In the absence of extracellular Ca^{2+}, neurotensin(8–13) evokes only a short-lasting rise in intracellular Ca^{2+}. The neurotensin-evoked intracellular Ca^{2+} accumulation is blocked by the phospholipase C inhibitor U73122 and by thapsigargin, suggesting that it is initiated by release of Ca^{2+} from an inositol triphosphate-dependent store. The Ca^{2+}-mobilizing action of neurotensin(8–13) in astrocytes is dependent on at least two receptors, because the response is blocked in part only by SR48692, a type 1 neurotensin receptor antagonist, and is blocked completely by SR142948A, a novel neurotensin receptor antagonist. The finding that the type 2 neurotensin receptor agonist levocabastine fails to mimic or alter the effects of neurotensin(8–13) on intracellular Ca^{2+} makes it unlikely that the type 2 neurotensin receptor is involved.

In summary, these results show that functional neurotensin receptors are present in cultured ventral tegmental area astrocytes and that their activation induces a highly desensitizing rise in intracellular Ca^{2+}. The pharmacological profile of this response suggests that a type 1 neurotensin receptor is involved but that another, possibly novel, non-type 2 neurotensin receptor is also implicated. If present *in vivo*, such signalling could be involved in some of the physiological actions of neurotensin. © 2000 IBRO. Published by Elsevier Science Ltd.

Key words: neurotensin, astrocytes, calcium, ventral tegmental area, desensitization, phospholipase C.

Neurotensin (NT) is a 13-amino acid peptide that, although widely expressed in the CNS, is found to be closely associated with the major dopaminergic nuclei.[13,41,55,60] In the ventral tegmental area (VTA), NT is found in both dopaminergic neurons and in nerve terminals.[2,32,36,56,70,71] A clear physiological role for this peptide in the CNS has not been established. However, direct application of NT in the brain, either i.c.v. or *in situ* by local application, produces a wide range of effects.[5,10,11,21,33,39,48,50,54,58,65–67,69] The effects of NT are thought to be mediated by two or more NT receptors.[73] A first NT receptor was cloned in 1990 and found to be G-protein linked and coupled to phosphoinositide hydrolysis, inositol triphosphate (IP_3) production and intracellular Ca^{2+} release.[15,30,72] This receptor, termed NT1, is widely expressed in the CNS, with high concentrations in the VTA and substantia nigra.[7,8,49] This receptor can be activated by either NT itself or by neuromedin N, a peptide originating from the same precursor giving rise to NT.[19] Two non-peptide antagonists that effectively block NT1 have been identified, namely SR48692 ($K_d \sim 3$–10 nM)[25,42] and more recently SR142948A ($K_d \sim 1$ nM).[26,47] While SR48692 is able to block some of the physiological effects of NT, such as the increase in potassium-evoked dopamine release in slices,[27] the turning behaviour induced by intra-VTA application of NT[68] or the hypolocomotion induced by i.c.v. injections of NT,[20] other effects of NT are insensitive to this antagonist. For example, the enhancement of dopamine release induced by intra-VTA application of NT[9] and analgesia and hypothermia induced by centrally administered NT are insensitive to SR48692.[26] The newer antagonist, SR142948A, has the same effects as SR48692 but, in addition, can reduce both NT-induced hypothermia and analgesia.[26] It can bind to both NT1 and to NT2, the second cloned NT receptor, with low nanomolar affinity.[4,16,44] In comparison, SR48692 also binds to NT2, but with much lower affinity (80–300 nM, depending on the preparation).[16,26,44] In addition to SR142948A, the binding of NT to NT2 can be very effectively displaced by levocabastine (K_d of 2–10 nM) in transfected cell lines.[44] Although initially considered a possible antagonist, it has been reported that levocabastine appears to act as an agonist on NT2 receptors expressed in heterologous expression systems.[44]

Evidence from a number of systems points toward the existence of additional NT receptors. For example, the enhancement of dopamine release in the nucleus accumbens induced by intra-VTA NT is resistant to both SR48692 and SR142948A, and the antagonism of NT-induced hypothermia by SR142948A is only partial.[26] These and other results suggest that other subtypes of NT receptors may exist. Recently, a third class of NT binding proteins has been identified.[45] This protein, named NT3, displays very little affinity for SR48692 and levocabastine, and does not have the structure of either G-protein-coupled or ionotropic plasma

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Abbreviations: EGTA, ethyleneglycolbis(amino-ethyl ether)tetra-acetate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; IP_3, inositol triphosphate; NT, neurotensin; NT(8–13), 8–13 fragment of neurotensin; NT1, type 1 neurotensin receptor; NT2, type 2 neurotensin receptor; NT3, type 3 neurotensin receptor; PLC, phospholipase C; VTA, ventral tegmental area.

membrane receptors.[45] It has been shown to be usually localized to intracellular compartments, although indirect evidence suggests that it may be recruited to the plasma membrane following stimulation of cells with NT.[14] The coupling of this receptor to second messenger systems has not been determined.

The physiological effects of NT in the CNS are thought to be mostly mediated by NT receptors located on neurons, as suggested by early binding studies.[46,56] A higher level of resolution was afforded by studies using cultured cells and electron microscopy. Using explant cultures of rat VTA, it was found that, besides neurons, astrocytes also displayed specific NT binding sites as well as NT-evoked hyperpolarizing responses.[34] These were blocked by SR48692, suggesting that astrocytes can express functional NT1 receptors. Two studies using rat cortical astrocytes in culture also showed NT binding sites, but these were exclusively levocabastine sensitive, suggesting the expression of NT2 receptors.[51,52] Finally, a recent study has taken advantage of the availability of anti-NT1 antibodies to examine the ultrastructural localization of NT1 receptors in the VTA.[8] This study confirmed that most receptors were indeed localized on dopaminergic neurons, but a subset appeared to be localized on glial membranes. The function and functional coupling of these glial receptors is unknown. However, if, as in transfected cell lines, astrocytic NT receptors couple to phospholipase C (PLC) activation and IP_3 production, it is to be expected that receptor activation will lead to mobilization of intracellular Ca^{2+}. This increase in Ca^{2+} could regulate a number of physiological processes in astrocytes and perhaps trigger glial–neuronal signalling.[1] Although it has been reported previously that NT can cause a rise in intracellular Ca^{2+} in endothelial cells,[64] in amacrine cells[6] and in cultured cortical cells,[62] a detailed characterization of the mechanism of these responses and a pharmacological dissection of the receptors involved has not been reported previously for any primary cultured cells. Here, the role of NT receptors on VTA astrocytes is explored. It was found that, indeed, NT causes a rise in intracellular Ca^{2+} in these cells. Surprisingly, the present results suggest that, besides NT1, another non-NT2 receptor is involved.

EXPERIMENTAL PROCEDURES

Cell culture

Primary cultures of rat VTA were prepared from neonatal animals (postnatal days 1–3). Two small blocks of tissue containing the left and right portions of the VTA were dissected out using a custom tissue micropunch from a coronal slice with a thickness of approximately 1.5 mm and localized rostrocaudally at the level of the midbrain flexure. The tissue was incubated in papaïn for 30 min and dissociated according to a protocol modified from Cardozo.[12] Modifications to the protocol included the use of collagen/poly-L-lysine-coated coverslips instead of poly-L-lysine alone, and the use of 5% fetal calf serum and Mito + serum additive (VWR Canlab, Montréal, Québec, Canada) instead of 10% serum. Dissociated cells were plated on 15-mm-diameter round coverslips (Ted Pella, Reading, PA, U.S.A.). The culture medium was replaced in part with fresh medium twice a week. For most experiments, cultures were used between 12 and 19 days after plating. In a small subset of experiments, VTA neurons and astrocytes were cultured on small drops of poly-D-lysine sprayed onto agarose-coated glass coverslips.[3] This permitted the establishment of small groups of isolated cells, some of which contained only astrocytes. All experiments conformed to the Canadian Council on Animal Care guidelines and were approved by the Université de Montréal's animal ethics committee. All efforts were made to minimize the number of animals used and their suffering.

Fluorescence imaging and immunolabelling

For experiments using dynamic fluorescence imaging of intracellular Ca^{2+} levels, cells were loaded at room temperature with either Fura-2/AM or Calcium Green-1/AM and imaged in a custom grease-free chamber set-up on a Nikon Eclipse inverted microscope. Calcium concentrations were calculated using an *in situ* calibration protocol and Grynkiewicz *et al.*'s equation.[24] Most experiments were performed in normal extracellular saline consisting of (in mM): NaCl 140, $MgCl_2$ 2, $CaCl_2$ 2, KCl 5, HEPES 10, sucrose 6, glucose 10 (pH 7.35). Nominally Ca^{2+}-free saline contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 4, EGTA 0.1, HEPES 10, sucrose 4, glucose 10 (pH 7.35). Fluorescence images were captured using Axon Imaging Workbench software (Axon Instruments, Foster City, CA, U.S.A.), a Gen-III + intensified CCD camera (Stanford Photonics, Salt Lake City, UT, U.S.A.) and a DX-1000 fast filter changer (Stanford Photonics). In these experiments, it was easy to distinguish between astrocytes and neurons based on morphological criteria alone. In most experiments, high-potassium saline was perfused at the end of the experimental protocol in order to depolarize cells and elicit large Ca^{2+} influx in neurons through voltage-gated Ca^{2+} channels. Under these experimental conditions, astrocytes were unresponsive to such a stimulation (results not shown). In Fura-2 imaging experiments, astrocytes were considered to have responded to NT agonists if they displayed a rise in intracellular Ca^{2+} of at least 25 nM for at least 30 s. Immunolocalization of tyrosine hydroxylase was used to identify dopamine neurons (monoclonal mouse antibody). Glial fibrillary acidic protein was localized using a monoclonal antibody and permitted identification of astrocytes. Fluorescent secondary antibodies were coupled to Alexa-488 or Alexa-546. Cells were fixed with 4% formaldehyde and permeabilized with Triton X-100 to facilitate the penetration of antibodies.

Materials

Most chemicals and primary antibodies were obtained from Sigma Chemicals (St Louis, MO, U.S.A.). Fura-2/AM, Calcium Green-1/AM and secondary antibodies were obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). The NT receptor antagonists SR48692 and SR142948A were obtained from Sanofi Recherche (France). Levocabastine was obtained from the Janssen Research Foundation (Belgium).

RESULTS

*Calcium-mobilizing action of neurotensin(*8–13*) on ventral tegmental area astrocytes*

Experiments were performed on primary cultures of rat VTA. These preparations contained both neurons and astrocytes. Dopaminergic neurons comprised approximately 50% of the neuronal population, other neurons being mostly GABAergic. A glial cell monolayer covered most of the coverslip surface. These cells were exclusively astrocytes as suggested by their immunoreactivity to glial fibrillary acidic protein (not shown).

Intracellular Ca^{2+} concentration in astrocytes was monitored by fluorescence imaging using Fura-2. Brief applications of the 8–13 fragment of neurotensin [NT(8–13)] induced a rise in intracellular Ca^{2+} in a proportion of astrocytes (Fig. 1A, B). This response was robustly elicited with concentrations of NT(8–13) between 10 and 1000 nM, while 1 nM occasionally induced detectable responses (Table 1). On average, 39 ± 3% of astrocytes in the field of view responded to 100 nM NT(8–13) ($n = 12$ coverslips; Table 1). Although the magnitude of the rise in Ca^{2+} was variable, it was often quite substantial and, on average, concentrations of intracellular Ca^{2+} reached maximal levels of 553 ± 54 nM above baseline [$n = 50$ cells; 100 nM NT(8–13)]. Astrocytes could be similarly activated by the parent peptide [NT(1–13)] or by neuromedin N (both tested at 100 nM; $n = 3$ and 6 coverslips, respectively; not shown). The development of these astrocyte responses to NT(8–13) was determined by comparing the ability of 100 nM NT(8–13) to elicit a rise in intracellular Ca^{2+} after different periods

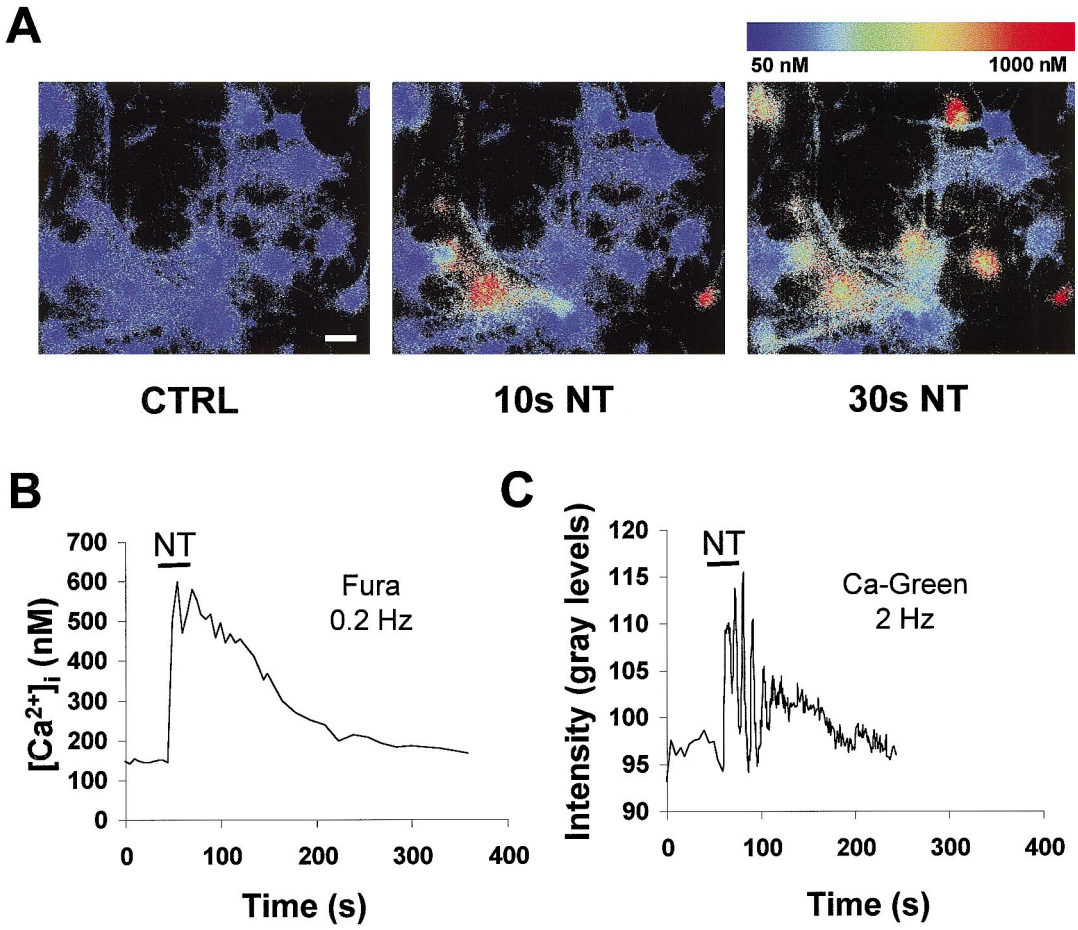

Fig. 1. Calcium-mobilizing action of NT(8–13) in astrocytes. (A) Intracellular Ca^{2+} was imaged using Fura-2/AM. The false-coloured image sequence illustrates that NT(8–13) usually caused a rise in intracellular Ca^{2+} in a subset of astrocytes. The effect was not synchronous in all cells and often gave the appearance of a propagating Ca^{2+} wave. (B) Time-course of the rise in intracellular Ca^{2+} in an astrocyte during a 30-s exposure to 100 nM NT(8–13). Image ratios were acquired every 5 s. (C) Time-course of the rise in intracellular Ca^{2+} in an astrocyte loaded with Calcium Green-1/AM and exposed to 100 nM NT. Images were acquired at a rate of 0.2 Hz during the first 60 s, and then at 2 Hz during and after application of NT(8–13). Note the expression of intracellular Ca^{2+} oscillations within the imaged astrocyte. Scale bar = 15 μm.

Table 1. Dose–response relationship of the mobilization of intracellular calcium by neurotensin(8–13) in ventral tegmental area astrocytes

	Concentration			
	1 nM	10 nM	100 nM	1 μM
Responsive astrocytes (%)	7 ± 4.5 (3)	41 ± 9.7 (3)	39 ± 4.3 (12)	40 ± 5.9 (11)

The different concentrations of NT(8–13) were tested in separate experiments. Data are expressed as mean ± S.E.M. The number of coverslips tested is given in parentheses.

of time in culture. It was found that astrocytes were completely unresponsive after three to four days in culture. Although a small percentage were responsive after seven to eight days, maximal effects were obtained after two weeks in culture (Table 2).

In most experiments using Fura-2, ratio image pairs were acquired every 5 s. Using this frequency of acquisition, it was possible in many astrocytes to detect that NT-evoked Ca^{2+} rises displayed biphasic kinetics. This was usually manifested as an initial rapid phase followed by a slow return to baseline (Fig. 1B). Using faster acquisition rates with a single-wavelength Ca^{2+} indicator (Calcium Green-1; two images per second), it was possible to observe multiphasic kinetics in most cells (seven of nine astrocytes which displayed a rise in Ca^{2+}; $n = 3$ coverslips). Multiple closely occurring peaks could be detected, suggesting that intracellular Ca^{2+} oscillations were induced (Fig. 1C).

To determine whether the Ca^{2+} mobilization caused by NT(8–13) in astrocytes was dependent on the expression of NT receptors directly on astrocytes or rather depended on NT-evoked activation of neurons which were co-cultured with astrocytes, an additional series of experiments was performed on small groups of VTA astrocytes cultured in isolation on microdots of substrate. For these experiments, only groups of astrocytes free of all neurons were chosen. Compatible with the idea of the localization of NT receptors on astrocytes, it was found that 28 ± 10% of such neuron-free astrocytes demonstrated a rise

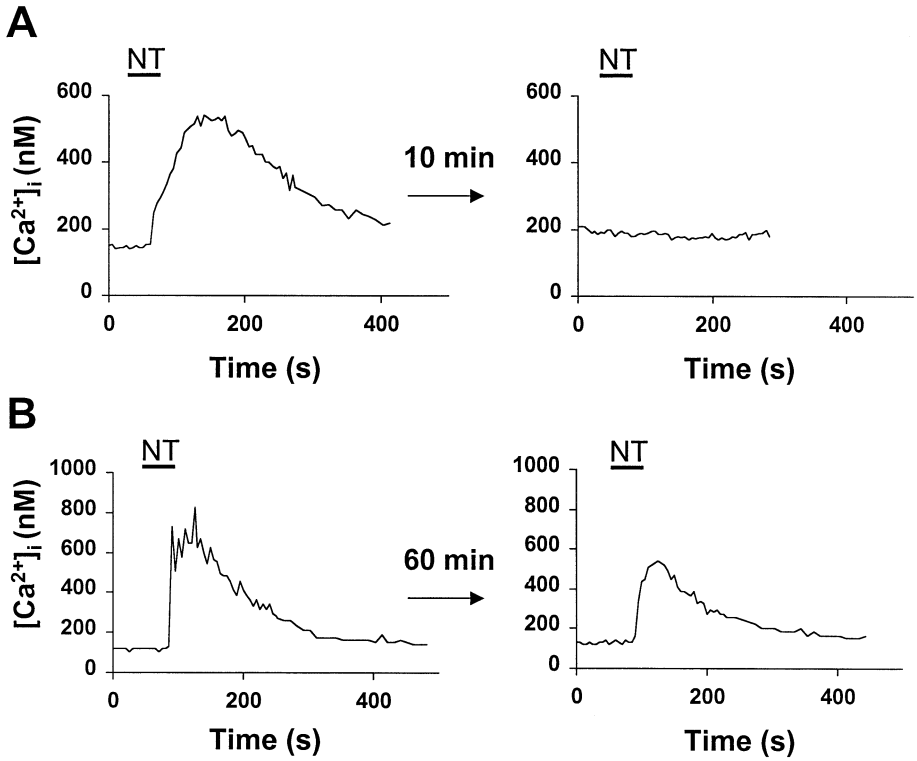

Fig. 2. Pronounced desensitization of NT-evoked calcium responses in astrocytes. (A) Time-course of the rise in intracellular Ca^{2+} in an astrocyte exposed to NT(8–13) (100 nM) twice with an interval of 10 min. The second application caused no response. (B) Two applications of NT(8–13) (100 nM) separated by a 60-min interval permitted significant recovery of the desensitized response.

Table 2. Development of neurotensin(8–13)-evoked mobilization of intracellular calcium in ventral tegmental area astrocytes

	Days in culture			
	3–4	7–8	14–15	30
Responsive astrocytes (%)	0 (2)	10.5 ± 6.1 (4)	39 ± 4.3 (12)	25 ± 3.6 (2)

The effectiveness of 100 nM NT(8–13) to mobilize intracellular Ca^{2+} was tested after a variable number of days in culture. The data are expressed as mean ± S.E.M. The number of coverslips tested is given in parentheses.

in Ca^{2+} caused by 100 nM NT(8–13) ($n = 8$ coverslips). This proportion was not significantly different from that in standard cultures containing both neurons and astrocytes (*t*-test, $P > 0.05$).

Extensive desensitization of neurotensin-evoked Ca^{2+} *mobilization*

It has been suggested that NT receptors undergo internalization and prolonged sequestration following their activation.[29] NT1 receptors expressed in a heterologous expression system also show evidence of desensitization.[28] If an NT1 receptor is involved in the Ca^{2+} mobilization induced by NT(8–13) in astrocytes, this response should also show some evidence of desensitization. Astrocytes were exposed to two sequential applications of NT(8–13) (100 nM) with a variable interval. Using an interval of 10 min, extensive desensitization was evident. In many cells, no response was detected on the second application of NT(8–13) (Fig. 2A). On average, the second Ca^{2+} response was 18 ± 6.8% of the first ($n = 21$ cells; Table 3). Similar results were found with an interval of 20 min (17.8 ± 7.4%, $n = 9$; Table 3). This desensitization was long lasting, because a washout time of 30–60 min was required to detect significant recovery of the NT-evoked rise in Ca^{2+}. The second response was then 53.2 ± 16.2% (30 min; $n = 11$) or 56.5 ± 5.5% (60 min) of the first ($n = 28$; Table 3). These desensitization characteristics were compared to those of ATP receptors, which are also known to mobilize intracellular Ca^{2+} in astrocytes.[61] Two applications of ATP (5 μM) were performed with a 30-min interval. Very little long-term desensitization was evident in these experiments. On average, the second rise in intracellular Ca^{2+} was 90 ± 7% of the first ($n = 19$ cells; Table 3).

Involvement of extracellular and intracellular calcium

In order to determine the mechanism by which NT(8–13) induces a rise in Ca^{2+} in astrocytes, it was first tested whether the responses could be obtained in the absence of extracellular Ca^{2+}. Cells were washed with nominally Ca^{2+}-free saline for 1 min and then exposed to 100 nM NT(8–13) for 30 s, also in Ca^{2+}-free saline. Although the proportion of astrocytes that

Table 3. Long-term desensitization of neurotensin(8–13)-evoked mobilization of intracellular calcium in ventral tegmental area astrocytes

	Application interval (min)				
	10	20	30	60	30 (ATP)
Rise in intracellular calcium (% control)	18 ± 6.8 (21)	17.8 ± 7.4 (9)	53.2 ± 16.2 (11)	56.5 ± 5.5 (28)	90 ± 7 (19)

NT(8–13) was applied twice with a variable interval. Calcium mobilization evoked by ATP was tested for comparison. The data are expressed as mean ± S.E.M. The number of cells tested is given in parentheses.

Table 4. Mechanism of neurotensin(8–13)-evoked mobilization of intracellular calcium in ventral tegmental area astrocytes

	Condition					
	Control	Ca^{2+}-free	Gd^{3+}	Thapsigargin	U73122	U73123
Responsive astrocytes (%)	39 ± 4.3 (12)	29.3 ± 8.4 (4)	29.8 ± 7.2 (5)	0 (4)	0 (5)	38.8 ± 17.1 (4)

The effect of different conditions or antagonists was evaluated in separate experiments. The data are expressed as mean ± S.E.M. The number of coverslips tested is given in parentheses.

responded to NT(8–13) under these conditions was not significantly different than in normal saline (29 ± 8%, $n = 6$ coverslips; *t*-test, $P > 0.05$; Table 4), the kinetics of the response in Ca^{2+}-free saline were markedly different. Instead of a long-lasting, usually biphasic response, the rise in Ca^{2+} was always monophasic and short lasting (Fig. 3A).

As an alternative approach to test whether influx of external Ca^{2+} was necessary, NT(8–13) was applied in the presence of 50 μM gadolinium, an agent known to block a variety of Ca^{2+} and cationic channels.[40] The proportion of astrocytes that responded to NT(8–13) under these conditions was again not significantly different than in normal saline (30 ± 7%, $n = 5$ coverslips; *t*-test, $P > 0.05$; Table 4). As for cells responding in Ca^{2+}-free saline, the response to NT(8–13) in the presence of gadolinium was short lasting and always monophasic (Fig. 3B). Together, these two sets of results suggest that extracellular Ca^{2+} is necessary to sustain a component of the NT-evoked intracellular Ca^{2+} response and that release of Ca^{2+} from intracellular stores is probably responsible for the initial phase of the response.

To confirm the involvement of intracellular Ca^{2+} stores, these stores were perturbed by using thapsigargin, a blocker of the Ca^{2+}–ATPase found on the endoplasmic reticulum. Cells were incubated for 60 min in 1 μM thapsigargin and then challenged with 100 nM NT(8–13). Under such conditions, NT(8–13) failed to induce any detectable rise in intracellular Ca^{2+} in astrocytes (none of 51 astrocytes in five coverslips; Table 4). This suggests that intact intracellular Ca^{2+} stores are necessary to initiate the NT-induced rise in intracellular Ca^{2+} and that the influx of extracellular Ca^{2+} does not occur independently of the mobilization of intracellular stores.

If intracellular Ca^{2+} release is necessary, this is likely to result from an activation of IP_3 receptors located on intracellular Ca^{2+} stores. If this is the case, then it should be possible to block NT's action by blocking the production of IP_3. This was attempted by blocking the activity of PLC using U73122, a specific blocker of this enzyme.[38] Cells were exposed to U73122 (5 μM) for 10 min and then challenged with NT(8–13) (100 nM). Much like after thapsigargin, astrocytes treated with U73122 were unresponsive to NT(8–13) (none of 60 astrocytes in five coverslips; Table 4). The inactive analogue U73123 (5 μM) was without effect and did not prevent astrocytes from showing an NT-evoked Ca^{2+}-mobilizing response (eight of 20 astrocytes were responsive, $n = 4$ coverslips; Table 4). This proportion was not different from control cells (*t*-test, $P > 0.05$).

Agonist and antagonist pharmacology

In order to characterize the type of receptor involved in the Ca^{2+}-mobilizing action of NT(8–13), the ability of NT receptor antagonists or agonists to interfere with this response was investigated. Because it was reported that rat cortical astrocytes in primary culture expressed mainly levocabastine-sensitive NT receptors,[51] this ligand of NT2 receptors was tested first. Cells were exposed to 1 or 10 μM levocabastine for 2 min prior to the addition of 100 nM NT(8–13). By itself, levocabastine failed to cause any rise in intracellular Ca^{2+} in astrocytes (Fig. 4A). It also failed to prevent the effect of NT(8–13) on intracellular Ca^{2+} in astrocytes (Fig. 4A). The percentage of astrocytes showing an NT-evoked rise in Ca^{2+} was 33 ± 11% in the presence of 1 μM levocabastine ($n = 22$ cells in four coverslips) and 46 ± 5% in the presence of 10 μM levocabastine ($n = 13$ cells in three coverslips). The average maximal rise in Ca^{2+} was 429 ± 63 nM in the presence of 1 μM levocabastine and 456 ± 85 nM in the presence of 10 μM, not significantly different from control coverslips (*t*-tests, $P > 0.05$). These results suggest that NT2 receptors are not involved in the Ca^{2+}-mobilizing action of NT on astrocytes.

The involvement of NT1 receptors was investigated by using SR48692 and SR142948A. In the presence of a saturating concentration of SR48692 (1 μM), cells still responded to 100 nM NT(8–13), but usually with a considerable delay and with much prolonged kinetics (Fig. 4B). On average, the responses in the presence of SR48692 reached a peak 395 ± 58 s after the introduction of NT(8–13) ($n = 15$ cells in three coverslips). In comparison, responses under control conditions reached a peak after 42 ± 4 s ($n = 32$ cells in seven coverslips). The average maximal rise in Ca^{2+} was

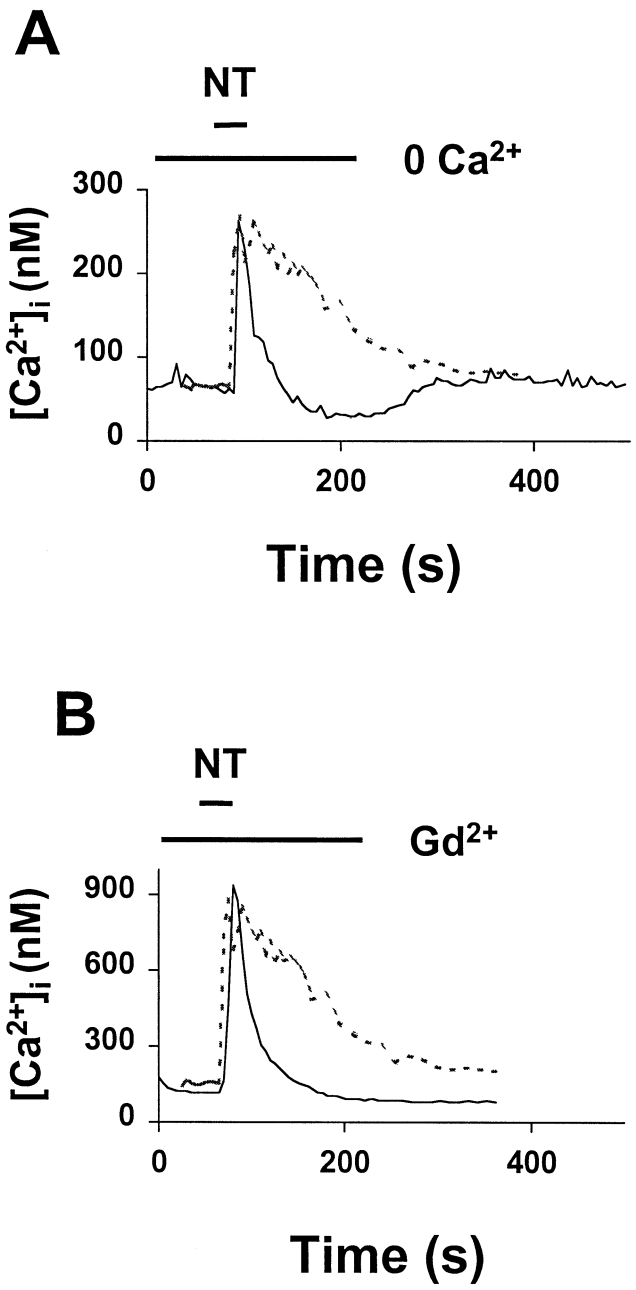

Fig. 3. Involvement of extra- and intracellular calcium in the action of NT(8–13) on astrocytes. (A) Time-course of the rise in intracellular Ca^{2+} in single astrocytes caused by NT(8–13) (100 nM) in the presence of nominally Ca^{2+}-free saline. Note that the response was monophasic and of much shorter duration than in Ca^{2+}-containing medium. The scaled response of an astrocyte in normal saline (from Fig. 1B) is shown for comparison (dotted line). (B) Time-course of the rise in intracellular Ca^{2+} in an astrocyte caused by NT(8–13) in the presence of 50 μM gadolinium. Note that, as in Ca^{2+}-free saline, the response was monophasic and again of much shorter duration than in Ca^{2+}-containing medium. The scaled response of an astrocyte in normal saline (from Fig. 1B) is shown for comparison (dotted line).

491 ± 52 nM. Because the concentration of SR48692 used (1 μM) should have blocked all available NT1 receptors ($K_d \sim 3–10$ nM), one explanation for the partial block is that, at 100 nM, NT(8–13) activates more than one receptor subtype, the second being insensitive or less sensitive to SR48692. If the second receptor is of lower affinity for NT, one prediction is that the block of NT(8–13)-evoked rise in Ca^{2+} by SR48692 should be more extensive using lower concentrations of NT(8–13), which predominantly activate the higher affinity NT1 receptor. It was first tested whether 1 μM SR48692 produced a more extensive block of the NT-evoked rise in Ca^{2+} when 10 nM NT(8–13) was used instead of 100 nM. Indeed, this was the case as, under such conditions, 1 μM SR48692 completely blocked the rise in Ca^{2+} in all cells (none of 61 astrocytes in five coverslips were responsive; Fig. 4B). To find whether the putative second receptor is insensitive or simply less sensitive to SR48692, it was then tested whether the effect of 100 nM NT(8–13) could be blocked by an elevated concentration of SR48692 (10 μM). It was found that, at this very high concentration, only two of 43 astrocytes (4.6%; $n = 4$ coverslips) still demonstrated a rise in Ca^{2+}. The size of the response was also very small (average of 60 nM increase for the two cells). Taken together, these results suggest that NT1 receptors are involved in mediating only part of the response induced by NT(8–13), and that a second receptor with a lower affinity for NT(8–13) and for SR48692 is involved.

SR142948A, the newer NT receptor antagonist, was also tested. It was found that, at a concentration of 100 nM, SR142948A completely blocked the Ca^{2+}-mobilizing effect of 100 nM NT(8–13) (Fig. 4C). In the presence of SR142948A, no responsive astrocytes could be detected (none of 52 astrocytes). These results suggest that NT(8–13) acts to increase intracellular Ca^{2+} by acting through at least two different NT receptors.

Because it has been suggested that, *in vivo*, the NT analogue [D-Tyr^{11}]NT may activate a different subset of NT receptors than NT(8–13),[69] it was tested whether this modified peptide agonist could also trigger a rise in Ca^{2+} in VTA astrocytes. At concentrations between 10 nM and 1 μM, no detectable effect on intracellular Ca^{2+} was found ($n = 4$ coverslips; results not shown).

DISCUSSION

Identity of neurotensin receptors on astrocytes

Although previous reports have already suggested that astrocytes can express NT receptors,[34,51,52] the identity of the receptor(s) and the functional consequences of the activation of such receptors were unclear. The present work clarifies these issues by providing a detailed characterization of NT-evoked Ca^{2+} signalling in cultured VTA astrocytes. In line with the report of Hösli *et al.*,[34] the present results are compatible with the idea that cultured VTA astrocytes express NT1 receptors. In addition, it is shown here that these are functionally coupled to PLC hydrolysis and the release of Ca^{2+} from intracellular stores (Figs 1 and 3). The presence of NT1-like receptors was revealed in the present work by the ability of SR48692 to block part of the NT-evoked rise in intracellular Ca^{2+} in astrocytes (Fig. 4). At a concentration of SR48692 high enough to saturate all NT1 receptors (1 μM for an antagonist with a $K_d \sim 3–10$ nM),[25] only a partial block of the response evoked by 100 nM NT(8–13) was obtained (Fig. 4B). It was intriguing to find that the same concentration of antagonist was able to completely block the response to 10 nM NT(8–13) (Fig. 4B) and that 10 μM SR48692 produced an almost complete block of the response evoked by 100 nM NT(8–13). This suggests that a second NT receptor with a lower affinity for NT(8–13) and for SR48692 was

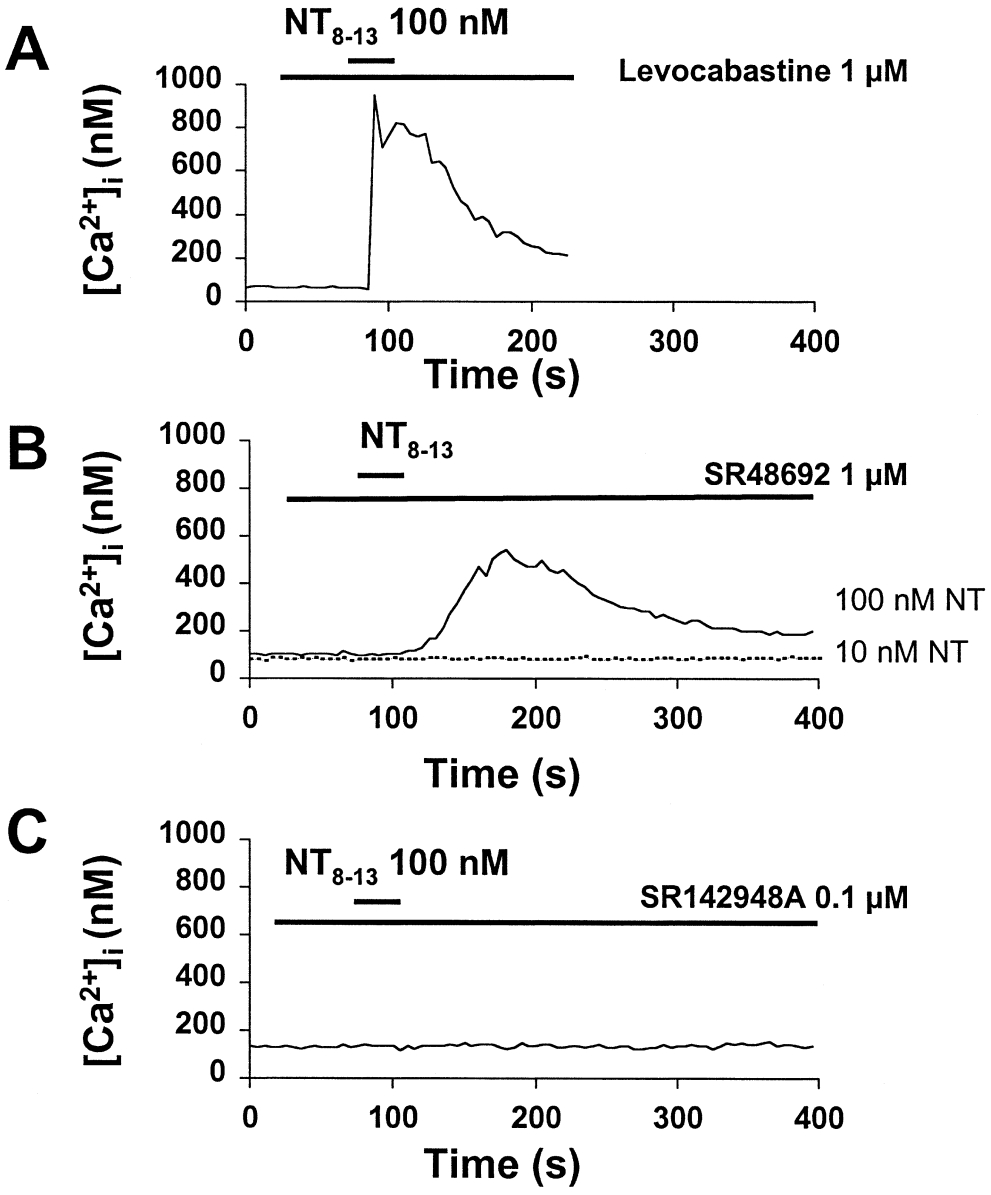

Fig. 4. Antagonist pharmacology suggests the existence of more than one receptor. (A) Time-course of the rise in intracellular Ca^{2+} in an astrocyte in response to NT(8–13) (100 nM). The presence of 1 μM levocabastine, an NT2 receptor ligand, did not prevent the action of NT. (B) Time-course of the rise in intracellular Ca^{2+} in an astrocyte in response to NT(8–13) (100 nM). A response was still detectable in the presence of 1 μM SR48692, an NT1 receptor antagonist. Note that the response was delayed and of longer duration then in control cells. However, the antagonist was capable of completely blocking the action of 10 nM NT(8–13) in another astrocyte (dotted trace). (C) The broader spectrum antagonist SR142948A (100 nM) was effective at completely blocking the Ca^{2+}-mobilizing action of NT(8–13) (100 nM).

also involved in mediating the NT-evoked rise in Ca^{2+}. Considering that SR48692 displays affinity, albeit low, for NT2, this second receptor could be NT2. However, this is not likely because the NT2 ligand levocabastine was not an effective agonist and neither could it reduce the NT-evoked rise in Ca^{2+} (Fig. 4A), even at concentrations as high as 10 μM. To better characterize the pharmacological profile of the NT receptors involved, additional experiments were performed with SR142948A, a new antagonist which blocks more NT-evoked physiological effects[26] and has an affinity for NT1 receptors only slightly higher than SR48692 (~1 nM vs ~3–10 nM).[4] It was found that, contrarily to SR48692, SR142948A completely blocked the rise in Ca^{2+} evoked by 100 nM NT(8–13) (Fig. 4C). This result was obtained at a concentration of 100 nM, which is 10 times less that the concentration of SR48692 that was used initially. These data are compatible with the idea that only part of the rise in Ca^{2+} evoked by NT(8–13) was mediated by NT1 and that an additional non-NT2 NT receptor type or subtype must be involved. Alternatively, in the context of recent work which has suggested that many G-protein-coupled receptors can exist as homo- or heterodimers,[74] and considering that heterodimers could display pharmacological properties different from those of the individual receptors, it remains possible that the responses evoked by NT(8–13) in astrocytes display a novel pharmacological profile because of the existence of NT1/NT2 heterodimers. No data are currently available to address this possibility.

Although the possibility that NT3 was involved in the NT-evoked rise in intracellular Ca^{2+} cannot be rejected, this is unlikely for three reasons. First, NT3 is not a G-protein-coupled

receptor and it is unclear how it could cause PLC activation and a mobilization of intracellular Ca^{2+} stores.[45] Second, this receptor does not appear to be usually localized to the plasma membrane.[45] Finally, although it has been suggested that this receptor could be recruited to the plasma membrane after stimulation with NT, this process takes a few minutes to occur.[14] Receptors recruited in this way would be unlikely to be involved in the relatively rapid Ca^{2+} mobilization caused by NT(8–13) under the experimental conditions used here (Fig. 1). In addition, it would seem that receptors putatively recruited through such a mechanism should become available in the few minutes following a first NT(8–13) challenge. This is unlikely to have happened under the conditions used here because NT usually failed to cause any detectable Ca^{2+} mobilization 10 min after a first application (Fig. 2). In conclusion, although it has not been directly tested whether VTA astrocytes express NT3 receptors, these are unlikely to be involved in the mobilization of intracellular Ca^{2+} caused by NT(8–13).

Intracellular mechanisms

Although more than one NT receptor is likely to be involved in the NT-evoked rise in intracellular Ca^{2+} observed in the present experiments, both of these receptors are likely to be coupled to PLC and appear to cause a rise in intracellular Ca^{2+} through the same mechanism. This conclusion can be derived from the observation that emptying intracellular Ca^{2+} stores with thapsigargin completely blocked the response to NT(8–13) (Table 4). Also, blocking PLC with U73122 abolished the response (Table 4). The source of the Ca^{2+} is, however, unlikely to be completely from intracellular stores, because it was found that, in the absence of extracellular Ca^{2+} (Fig. 3A) or when Ca^{2+} and cationic channels were blocked with the broad spectrum antagonist gadolinium (Fig. 3B), the NT-evoked rise in Ca^{2+} was still detectable but much shortened in duration. This suggests that, although mobilization of Ca^{2+} from intracellular stores is necessary to initiate the Ca^{2+} signal, a secondary influx of Ca^{2+} from the extracellular medium occurs. In many cells, a non-monophasic rise in Ca^{2+} was actually induced (Fig. 1B, C), with part of the secondary phase likely to represent influx from the extracellular medium. As reported previously, oscillatory intracellular Ca^{2+} responses stimulated with agonists that activate PLC and IP_3 production are likely to be maintained by cycles of intracellular Ca^{2+} release followed by Ca^{2+} influx that contributes to replenishing the intracellular stores.[23,63] The Ca^{2+} influx could occur through membrane channels activated by intracellular Ca^{2+} store depletion.[35,53] These are known to be blocked by trivalent cations like gadolinium.[59] However, the current results do not allow the exact mechanism of Ca^{2+} entry to be pinpointed.

Developmental profile

The observation that the proportion of astrocytes that demonstrated a detectable NT-evoked rise in intracellular Ca^{2+} increased with time in culture (Table 2) is similar to the reported developmental profile of NT binding sites in cultured neurons.[18] Although it is unclear whether the absence of response at three to four days in culture indeed reflects an absence of NT receptors on astrocytes at this time, it was otherwise observed that a proportion of three- to four-day-old VTA astrocytes in culture are able to mobilize intracellular Ca^{2+} in response to ATP (results not shown). This is compatible with the idea that the developmental profile of NT-evoked Ca^{2+} mobilization in astrocytes reported here probably reflects the developmental profile of NT receptor expression in these cells.

Desensitization

Many receptors of the seven-transmembrane segment family undergo desensitization and/or internalization following stimulation by agonists.[22] NT receptors are no exception. Both in cell lines expressing NT1 heterologously and *in vivo*, NT-evoked responses show evidence of some form of desensitization.[29] In the current report, the observation of extensive and long-lasting desensitization of NT-evoked rises in Ca^{2+} in astrocytes (Table 3) is fully in line with previous data. The observation that the Ca^{2+} mobilization evoked by activating ATP receptors on astrocytes shows very little desensitization using a 30-min interval suggests that the long-term decrease in NT(8–13)-evoked Ca^{2+} mobilization observed here did not result from deterioration of the cells during the washout period. Rather, these results are compatible with a long-term removal of functional NT receptors from the cell surface, presumably by receptor sequestration. Considering these findings as well as previous data, it is puzzling that a number of NT-evoked physiological effects show evidence of little desensitization. Effects which show little if any desensitization include the enhancement in the firing rate of VTA dopamine neurons,[47,54] the inward current evoked by NT,[37] the cationic single-channel openings induced by NT[17] and the enhancement of dopamine release in the nucleus accumbens induced by application of NT in the VTA.[68] Because such non-desensitizing responses are not predicted from the properties of NT1 and of the putative additional receptor present on astrocytes, it remains possible that yet other NT receptors exist in neurons. NT-evoked astrocyte-mediated physiological responses could conceivably be differentiated from more direct neuronal responses by their desensitization characteristics.

CONCLUSIONS

This work provides clear evidence for the existence of functional NT receptors in astrocytes. These receptors cause a mobilization of intracellular Ca^{2+} and display pharmacological properties that are difficult to explain with the existence of a single type of NT receptor in astrocytes. Because the present work was performed in cultured cells, it remains unclear whether the reported observations have direct relevance for an understanding of the actions of NT in the intact brain. The first question is whether NT receptors are expressed in astrocytes *in vivo* as they clearly are *in vitro*. Recent electron microscopy work looking at NT1 immunolabelling in VTA slices from rat brain has revealed that, although the larger proportion of labelling is associated with neuronal membranes, a significant fraction of the labelling is found along glial membranes.[8] This observation is compatible with the idea that glial cells such as astrocytes may indeed express NT receptors *in vivo*. As suggested previously, it may also explain in part the observation that, following destruction of the cell bodies of dopamine neurons in the substantia nigra, residual NT labelling can still be detected in this structure.[31,43] Because the present work suggests that astrocytes may express an additional receptor subtype which would be different from the three cloned NT receptors, it is also possible

that this putative receptor will be found to be more abundantly expressed in glial cells than in neurons. The second question is whether the activation of glial cells by NT could be implicated in some of the actions of NT *in vivo*. Although this is certainly a difficult question to answer, much recent work has suggested that, both in culture and in more intact brain tissue, astrocytes and other glial cells play active roles in regulating synaptic transmission.[1,57] It is thus conceivable that NT-evoked Ca^{2+} signalling in astrocytes could be implicated in a number of the physiological actions of NT.

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