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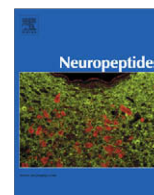

Brain derived neurotrophic factor and neurotrophin-4 employ different intracellular pathways to modulate norepinephrine uptake and release in rat hypothalamus

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ABSTRACT

Classical actions of the neurotrophin family are related to cellular survival and differentiation. Moreover, acute effects of neurotrophins have been reported. Although neurotrophins effects on synaptic transmission at central nervous system level have been largely studied, acute effects of neurotrophins on hypothalamic noradrenergic transmission are still poorly understood. Thus, we have studied the effects of the neurotrophin family members nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) on norepinephrine (NE) neuronal uptake and its evoked release, as well as the receptor and the intracellular pathways involved in these processes in rat hypothalamus.

Present results indicate that BDNF increased NE uptake and decreased its evoked release through a mechanism that involve Trk B receptor and phospholipase C. Moreover, NT-4, also through the Trk B receptor, decreased NE uptake and its evoked release by activating phosphatidylinositol 3-OH-kinase. These effects were observed in whole hypothalamus as well as in the anterior hypothalamic zone. On the other hand, NGF did not modify noradrenergic transmission.

In conclusion, we showed for the first time that BDNF and NT-4 activate two different intracellular signalling pathways through a Trk B receptor dependent mechanism. Furthermore, present findings support the hypothesis that BDNF and NT-4 acutely applied, could be considered as modulators of noradrenergic transmission and thus may regulate hypothalamic physiological as well as pathophysiological responses.

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1. Introduction

Noradrenergic neurotransmission is a complex process which includes norepinephrine (NE) biosynthesis, storage, release, interaction with its specific postsynaptic and presynaptic receptors, uptake and catabolism (Eisenhofer, 2001; Burgoyne and Morgan, 2003). Furthermore, NE uptake and release are the main processes that acutely regulate NE availability at the synaptic cleft and thus can modulate postsynaptic NE effects (Barclay et al., 2005; Bönisch and Brüss, 2006; Kubista and Boehm, 2006; Mandela and Ordway, 2006).

We have previously reported that different vasoactive peptides such as angiotensin II, angiotensin 1–7 and natriuretic peptides (Vatta et al., 1992, 1996; Gironacci et al., 2000; Rodríguez Fermepin et al., 2000) acutely applied regulate NE neuronal uptake and its release in the hypothalamus. Moreover, these peptides can be considered as neuromodulators of noradrenergic transmission. On the other hand, besides their acute effects, this group of vasoactive peptides is strongly related to cardiac hypertrophy, where they exert long term effects on cellular growth and proliferation (Barry et al., 2008; Katovich et al., 2008; Kumar et al., 2008). These antecedents allow us to hypothesise that other peptides which regulate cellular growth and proliferation, like neurotrophins (Lu et al., 2005), could also exert acute actions on noradrenergic transmission.

The neurotrophin family, which belongs to the neurotrophic factor superfamily, includes nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-4 (NT-4) and neurotrophin-3. The members of this family exert their actions by binding to two types of cellular membrane receptors named tropomyosin-related kinase receptor family (Trk) and p75 neurotrophic receptor ($p75^{NTR}$) (Chao et al., 2006). Likewise, three members of Trk receptors have been described (Trk A, Trk B and Trk C), showing all of them different affinities for each neurotrophin. For instance Trk A presents higher affinity to NGF, Trk B to BDNF and NT-4, and Trk C to NT-3. On the other hand, every neurotrophin binds to $p75^{NTR}$ with similar affinity (Hempstead, 2002; Reichardt, 2006).

The interaction between neurotrophins and Trk receptors leads to the activation of different intracellular signalling pathways (Huang and Reichardt, 2003). The specificity of downstream Trk

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receptor-mediated signalling depends on the expression and membrane trafficking of intermediates that finally regulate the activity of phospholipase C (PLC), phosphatidylinositol-3-OH-kinase (PI3K), mitogen-activated protein kinases (MAPKs) and Rho family GTPases (Reichardt, 2006).

The classical actions of neurotrophins are related to survival, growth, differentiation and proliferation of neuronal and glial cells (Lu et al., 2005). Moreover, after the early reports of Poo's group (Lohof et al., 1993; Stoop and Poo, 1995, 1996) many articles have emerged describing acute effects of neurotrophins on membrane excitability, activity-dependent synaptic plasticity and synaptic transmission (for review see Schuman (1999); Poo (2001); Lu (2004); Blum and Konnerth (2005)). Most of these studies were carried out in neuromuscular synapses, hippocampal neurons and visual cortex. Although neurotrophins expression was demonstrated in the hypothalamus (Nishio et al., 1994; Kawamoto et al., 1996; Yan et al., 1997), there are scarce reports describing acute effects of neurotrophins at this level (Tapia-Arancibia et al., 2004; Blum and Konnerth, 2005).

The hypothalamus is a brain area which contains integrative neural centres and receives abundant sympathetic input innervation (Kumar et al., 2007). Moreover, neuropeptides modulate the synaptic transmission in the hypothalamus and exert a critical role in the regulation of diverse output pathways closely related to the control of several physiological functions such as blood pressure, water and salt balance, thirst, body temperature and hormone secretion, as well as the modulation of pathophysiological responses to stress.

Thus, considering that neurotrophins family members could acutely modulate a sympathetic network involved in the control of physiological processes regulated by the hypothalamus, the aim of the present study was to analyze BDNF, NT-4 and NGF effects on NE neuronal uptake and release and to elucidate the intracellular signalling pathways involved in the modulation of noradrenergic neurotransmission.

2. Experimental procedures

Sprague–Dawley young adult male rats (2–3 month old) from the Department of Pathophysiology, Faculty of Pharmacy and Biochemistry, Buenos Aires, Argentina were used for the experiments. Female rats were discarded in order to avoid sexual cycle influence on NE metabolism (Renner et al., 1984). The animals were housed in steel cages, maintained in a controlled room at 22–24 °C with 12 h light/dark cycles (light from 7:00 am to 7:00 pm) and free access to tap water and food. Experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.1. Norepinephrine neuronal uptake

In order to determine the effects of BDNF, NT-4 and NGF on NE uptake in the hypothalamus, the experimental protocols were carried out according to techniques previously developed in our laboratory (Rodríguez Fermepin et al., 2000). Briefly, the animals were killed by decapitation between 10:00 and 11:30 am to avoid circadian changes. Each hypothalamus was quickly removed, dissected according to Palkovits and Brownstein (1988), cooled, minced and then transferred into a glass tube with a mesh of nylon fitted at the bottom to allow free interchange with the medium.

Several evidences indicated that anterior hypothalamic zone (AHZ) exerts opposite physiological effects than those of posterior hypothalamic zone (PHZ). In this way, the AHZ exerts a sympathoinhibitory regulation on central cardiovascular activity, while the PHZ induces sympathoexcitatory effects (Oparil et al., 1995). Then, in other set of experiments, hypothalami were divided in AHZ which included the paraventricular, periventricular and anterior hypothalamic nuclei, a segment of the ventromedial nucleus, the preoptic area and a portion of the median eminence, and in PHZ, which included the arcuate, supramammillary, mammillary and posterior hypothalamic nuclei, the dorsomedial and ventromedial nuclei, a segment of the lateral hypothalamic nucleus and the median eminence (Paxinos and Watson, 1986).

The tissues were pre-incubated at 37 °C for 30 min in a modified Krebs solution (MKS, pH 7.4) containing (mM) NaCl 118, KCl 4.7, $MgCl_2$ 1.2, NaH_2PO_4 1.0, $CaCl_2$ 2.5, EDTA-Na 0.004, dextrose 11.1, $NaHCO_3$ 25.0 and ascorbic acid 0.11, continuously bubbled with 95% O_2/5% CO_2. All reagents used were of analytical grade and obtained from standard sources.

To avoid ^{3}H-NE degradation and ^{3}H-NE non neuronal uptake, 100 μM of pargyline and 100 μM hydrocortisone (Sigma Chemical Co., St. Louis, USA) were respectively added in the incubation medium.

In order to determine which neurotrophin concentration was able to modified NE neuronal uptake, the tissues were incubated for 5 min in MKS containing 125 pM L-7-^{3}H-NE (New England Nuclear, Boston, MA, USA) in absence (control group) or presence (experimental groups) of increasing concentrations of BDNF, NT-4 or NGF (0.5; 5; 50 and 100 ng/ml) (Alomone Labs, Jerusalem, Israel). This concentration-range was based on the literature according to effective neurotrophin concentrations which show stimulating or inhibiting effects in the central nervous system, in *in vitro* experiments (Helke and Verdier-Pinard, 2000; Li and Keifer, 2008; Vaz et al., 2008).

Afterwards, to stop NE uptake and to avoid the possibility that remainder amounts of ^{3}H-NE could be uptaken by norepinephrine transporter (NET), the tissues were washed immediately four times for 15 min at 4 °C in cold MKS containing 15 μM desipramine (Sigma Chemical Co., St. Louis, MO, USA) to inhibit NET activity, and 4–6 μM nonradioactive NE (generously supplied by Dr. Lancelotti, Richet Laboratory, Buenos Aires, Argentina). After the washing period, tissues were homogenized and two aliquots were kept, one of them to determine tritium activity by scintillation counting method (Wallac 1214 RackBeta), and the other to measure protein content by Lowry's technique (Lowry et al., 1951). Results were expressed as dpm/μg prot ± SEM.

Once the concentration able to modify NE neuronal uptake for each neurotrophin was determined from the concentration–response curve, the receptor and intracellular pathways involved in these effects were studied. A similar protocol to that previously mentioned was used, except for it included an additional 15 min-inhibition period between the pre-incubation and incubation periods. The inhibitors and the concentrations used in these experiments were chosen according to previous reports of the literature (Li and Keifer, 2008; Busche et al., 2001; Secondo et al., 2003). Thus, to block Trk receptors and to inhibit PLC and PI3K activity, 200 nM K252a, 10 μM U 73122 and 25 μM LY 294002 (Alomone Labs, Jerusalem, Israel) dissolved in DMSO were respectively added to the incubation medium in the course of all inhibition and incubation periods. DMSO vehicle did not affect NE uptake or release.

2.2. Evoked norepinephrine neuronal release

Evoked ^{3} H-NE release was assessed according to slight modifications of the technique described by Vatta (Vatta et al., 1996). Briefly, minced rat hypothalami were pre-incubated at 37 °C for 30 min in MKS in the presence of 100 μM pargyline (to inhibit monoamine oxidase activity) and 100 μM hydrocortisone (to inhibit non neuronal NE uptake), and then NE stores were labelled with 125 pM L-7-^{3}H-NE for 30 min. After 6 consecutive 15-min washing periods with MKS, minced tissues were incubated for 5 min in MKS

containing 15 μM desipramine (to avoid neuronal NE re-uptake). This period corresponded to spontaneous release (S). Subsequent a 5 min period was carried out to evoke NE release (E) by incubating hypothalami in 25 mM KCl Krebs solution in absence (control) or presence of 100 ng/ml BDNF, NT-4 and NGF (experimental groups). The results of evoked NE release were expressed as the ratio between the radioactivity present in the incubation medium in the E and S periods (E/S) ± SEM.

In order to establish the receptor as well as the intracellular signalling pathway involved in neurotrophin effects on evoked NE release, tissues were incubated in absence (control) or in presence of the respective Trk, PLC and PI3K inhibitors 200 nM K252a, 10 μM U 73122 and 25 μM LY 294002 (experimental groups). The inhibitors were added to the incubation medium for 15 min between S and E periods and were also present during the E period.

2.3. Statistical analysis

Results were expressed as the mean ± SEM of 6–10 individual experiments. Two-tailed Student's *t*-test was used to compare different groups, and one way ANOVA followed by Bonferroni post test was used to compare multiple groups. *F* and *P* values corresponding to ANOVA test were calculated and they are expressed in the legend of the figures. *P* values of 0.05 or less were considered statistically significant.

3. Results

3.1. BDNF and NT-4 but not NGF modified NE neuronal uptake

Fig. 1 shows the concentration-response curves to different neurotrophins on NE uptake in rat hypothalamus. It can be observed that 100 ng/ml of BDNF and NT-4 was the concentration able to modify NE uptake ($P < 0.05$). While BDNF increased neuronal NE uptake, NT-4 produced its decrease (Fig. 1a and b). On the other hand, NGF did not modify NE uptake at any tested concentration (Fig. 1c).

When neurotrophins effects were determined in AHZ and PHZ, the effects of BDNF and NT-4 were restricted to the AHZ ($P < 0.05$). That is, BDNF increased and NT-4 decreased NE uptake in the AHZ but not in the PHZ. Meanwhile, NGF did not modify NE uptake either in AHZ or in PHZ (Fig. 2a–c).

3.2. BDNF and NT-4 modified NE neuronal uptake through the Trk receptor but employing different intracellular signalling pathways

In order to determine whether Trk receptors are involved in BDNF and NT-4 effects on NE uptake in the AHZ, 200 nM K252a was added to the incubation medium. This blocker did not modify NE uptake *per se*. However, the incubation of K252a together with BDNF or NT-4, prevented the enhancing effect of BDNF ($P < 0.05$) and the decreasing action of NT-4 ($P < 0.05$) on NE uptake (Fig. 3a and b, respectively).

To determine whether PLC and PI3K participate as intracellular signalling pathways activated by Trk receptors, BDNF and NT-4 effects on NE uptake were tested in presence of the specific inhibitors of PLC and PI3K (U 73122 and LY 294002, respectively). In Fig. 4a, it can be appreciated that 10 μM U 73122 did not alter NE uptake by itself, but its incubation jointly with BDNF reversed the increasing effect developed by this neurotrophin, reaching similar levels to those of the control group ($P < 0.01$ vs BDNF). On the other hand, 25 μM LY 294002 neither modified NE uptake nor altered BDNF effect on NE uptake.

Fig. 4b shows the results obtained by incubating 100 ng/ml NT-4 plus U 73122 or LY 294002. In this case, U 73122 did not modify the decrease in NE uptake elicited by NT-4, but in contrast to BDNF, LY 294002 reversed NT-4 effect ($P < 0.05$).

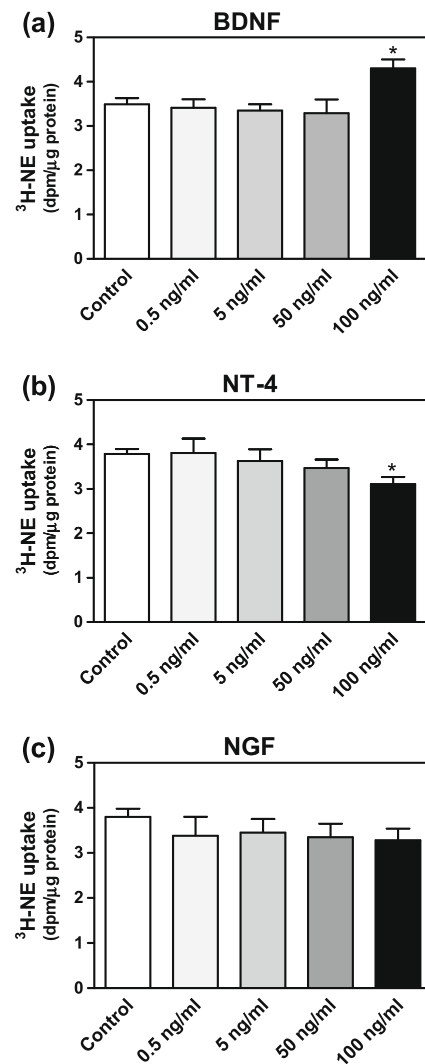

Fig. 1. Concentration–response curves (0.5–100 ng/ml) of the neurotrophins (a) BDNF, (b) NT-4 and (c) NGF, on neuronal ^{3}H-Norepinephrine (^{3}H-NE) uptake in whole minced hypothalamus. Results are expressed as mean ± SEM of 8–10 individual experiments. $^*P$ values $\leqslant 0.05$ vs control group.

3.3. BDNF and NT-4, but not NGF, modified evoked NE neuronal release

Fig. 5a and b shows that 100 ng/ml BDNF and NT-4 decreased evoked NE release ($P < 0.05$) in whole hypothalamus as well as in AHZ. On the other hand, NGF did not modify evoked NE release in any region studied (Fig. 5c). In PHZ, any neurotrophin studied was able to modify evoked NE release (data not shown).

3.4. BDNF and NT-4 decreased evoked NE neuronal release through the Trk receptor but signalling through different intracellular pathways

As shown in Fig. 6a and b, K252a did not exert *per se* any effect on evoked NE release. In addition, the fact that K252a blocked BDNF ($P < 0.05$) and NT-4 ($P < 0.01$) induced decrease of NE release in AHZ suggests that these neurotrophins exert their effects by interacting with Trk B receptor.

The effect of BDNF on evoked NE release was abolished by the presence of PLC inhibitor U 73122 ($P < 0.05$), suggesting that BDNF signals through PLC activation. In addition, the PI3K inhibitor LY

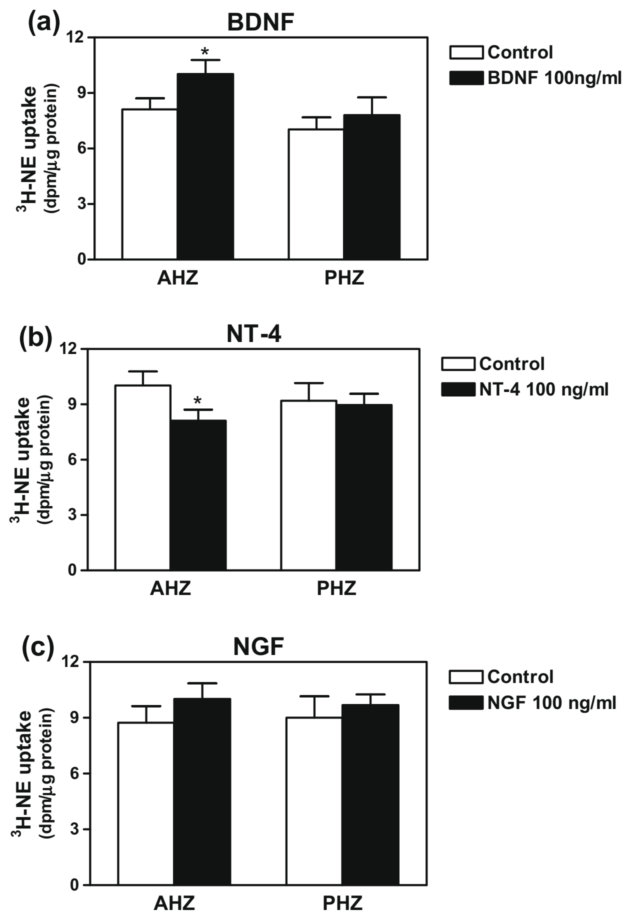

Fig. 2. Effects of 100 ng/ml of the neurotrophins (a) BDNF, (b) NT-4 and (c) NGF, on neuronal ^{3}H-Norepinephrine (^{3}H-NE) uptake in anterior and posterior hypothalamic zone (AHZ and PHZ, respectively). Results are expressed as mean ± SEM of 8–10 individual experiments. $^*P$ values ⩽ 0.05 vs control group.

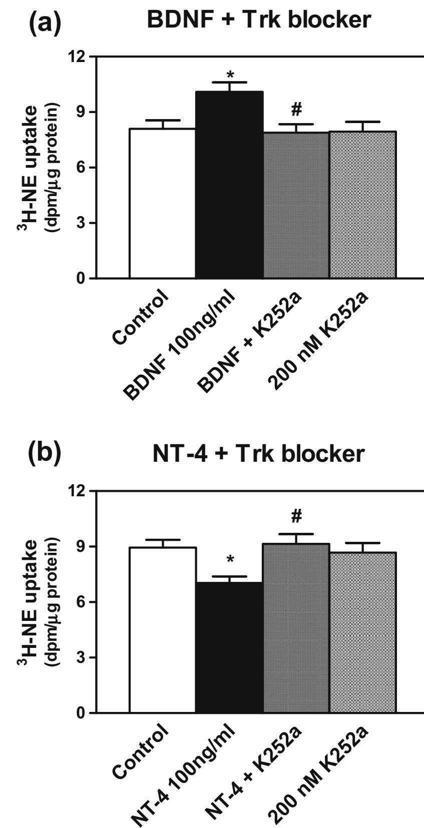

Fig. 3. Effects of 200 nM K252a (Trk receptors blocker) in the presence of (a) 100 ng/ml BDNF or (b) 100 ng/ml NT-4, on neuronal ^{3}H-Norepinephrine (^{3}H-NE) uptake in anterior hypothalamic zone (AHZ). Results are expressed as mean ± SEM of 8–10 individual experiments. Fig. 3a, one way ANOVA F and P values: 4.721 and 0.087, respectively. Fig. 3b, one way ANOVA F and P values: 4.399 and 0.0117, respectively. Bonferroni post test "ad hoc": $^*P$ values < 0.05 vs control group; $^\#P$ values < 0.05 vs BDNF or NT-4.

294002 failed to modify the BDNF-induced decrease of evoked NE release (Fig. 7a); then the PI3K signalling pathway can be discarded in BDNF-stimulating effects on NE release.

On the other hand, NT-4 decreasing effect on evoked NE release was reversed by LY 294002 ($P < 0.01$) but not by U 73122 (Fig. 7b), suggesting that PI3K, but not PLC signalling pathway is related to NT-4-inhibiting effects on NE release.

Thus, although BDNF and NT-4 could stimulate the Trk B receptor to decrease evoked NE release, the intracellular signalling mechanisms involved are different. In this way, BDNF could decrease evoked NE release through PLC activation while NT-4 could reduce it through PI3K stimulation.

4. Discussion

4.1. Neurotrophins and NE uptake

According to present results, 100 ng/ml is the concentration of BDNF and NT-4 that modified neuronal NE uptake in whole hypothalamus as well as in the AHZ. On the other hand, NGF did not modify NE uptake at any concentration or hypothalamic zone explored.

Surprisingly, BDNF and NT-4 exerted opposite effects on NE uptake since BDNF increased the amine uptake while NT-4 decreased it. Few reports have described neurotrophin effects on NE or neurotransmitter uptake. Interestingly, Hyman and co-workers have reported both different and overlapping responses to neurotrophins related to neurotransmitter uptake on mesencephalic dopaminergic neurons. In this way, while BDNF increased dopamine uptake, neither NT-4 nor NGF showed any effects. Although the authors did not investigate the intracellular signalling pathways involved in these effects, they argued that BDNF and NT-4 signal transduction pathways may not be identical (Hyman et al., 1994).

On the other hand, there are controversial reports concerning BDNF, NT-4 and NGF effects on NE and other neurotransmitters uptake. While our results indicate that BDNF increased NE uptake in rat hypothalamus, in cultures of neural crest cells this neurotrophin did not modify the NE uptake (Zhang et al., 1997). Furthermore, BDNF increased GABA uptake in hippocampal neurons through a mechanism which involved Trk B activation and GABA transporter phosphorylation (Law et al., 2000). Finally, in type B lymphoblast cell cultures, BDNF decreased serotonin uptake (Mössner et al., 2000).

Our results showed that NT-4 decreased neuronal NE uptake, though in cultures of dissociated striatal neurons the acute administration of this neurotrophin increased GABA uptake (Widmer and Hefti, 1994).

Finally, NGF increased NE uptake in rat chromaffin cells, while in rat pheochromocytoma cells it decreased the amine uptake (Wakade et al., 1996; Ikeda et al., 2001). Despite the fact that NGF modified NE uptake in cell cultures, our results indicate that NGF did not exert any effect on NE uptake in the hypothalamus.

The different neurotransmitter studied, the diverse types of cells/tissues used and the experimental protocols carried out, could justify the controversial reports showed in the literature concern-

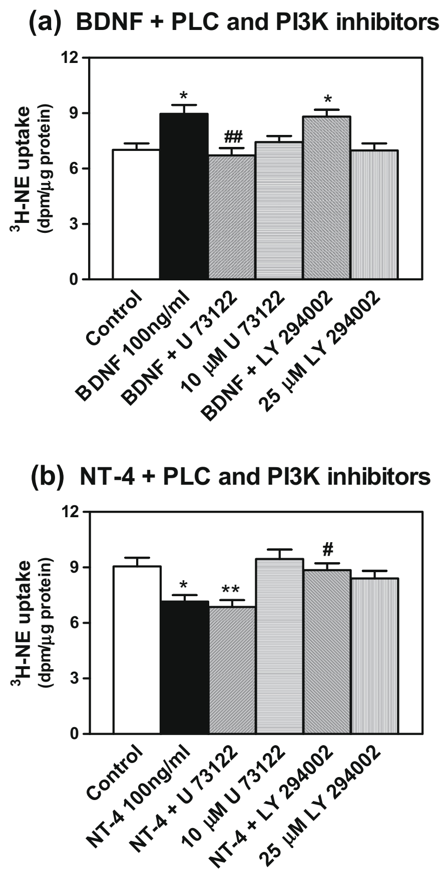

Fig. 4. Effects of 10 µM U 73122 and 25 µM LY 294002 (PLC and PI3 K inhibitors, respectively) in the presence of (a) 100 ng/ml BDNF or (b) 100 ng/ml NT-4 on neuronal ^{3}H-Norepinephrine (^{3}H-NE) uptake in anterior hypothalamic zone (AHZ). Results are expressed as mean ± SEM of 8–10 individual experiments. Fig. 4a, one way ANOVA F and P values: 6.276 and 0.0001, respectively. Bonferroni post test "ad hoc": $^{*}P < 0.05$ vs control group; $^{\#\#}P < 0.01$ vs BDNF. Fig. 4b, one way ANOVA F and P values: 6.828 and 0.0001, respectively. Bonferroni post test "ad hoc": $^{*}P < 0.05$ vs control group; $^{**}P < 0.01$ vs control group; $^{\#}P < 0.05$ vs NT-4.

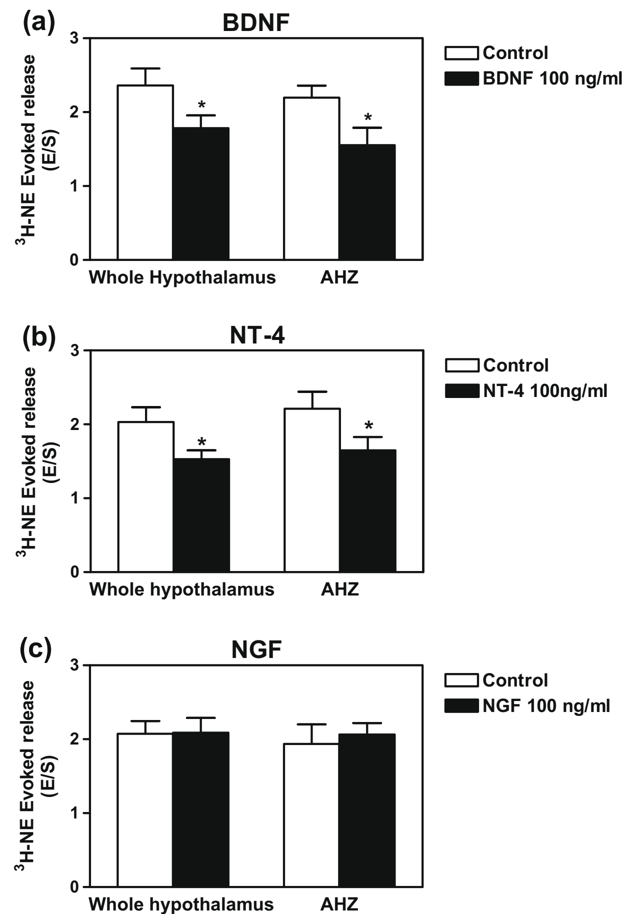

Fig. 5. Effects of 100 ng/ml neurotrophins (a) BDNF, (b) NT-4 and (c) NGF, on evoked ^{3}H-Norepinephrine (^{3}H-NE) release in whole hypothalamus and anterior hypothalamic zone (AHZ). Results are expressed as mean ± SEM of 8–10 individual experiments. $^{*}P$ values ⩽ 0.05 vs control group.

ing neurotrophins contrasting effects on neurotransmitter uptake. For example, in our conditions, hypothalamic tissues were incubated with neurotrophins and ^{3}H-NE simultaneously for 5 min, while in other reports, neurotransmitter uptake assays were carried out after incubation with neurotrophins for periods lasting from 24 to 48 h (Wakade et al., 1996; Ikeda et al., 2001).

The intracellular signalling pathways involved in neurotrophin effects on neurotransmitter uptake remain scarcely investigated. In the present work we showed that Trk receptors blocker, K252a, totally prevented BDNF and NT-4 effects. Although this inhibitor is non-specific and blocks all Trk subtypes, the fact that BDNF and NT-4 bind to Trk B enables us to assume that both neurotrophins exert their effects through the interaction with this receptor. Furthermore, the PLC inhibitor U 73122, completely reversed BDNF effect on neuronal NE uptake, while PI3K inhibitor LY 294002 did not exert any effect. In consequence, we concluded that BDNF increases NE uptake through Trk B receptor followed by PLC, but not PI3K, activation. Moreover, NT-4-induced decrease of NE uptake was inhibited by LY 294002 but not by U 73122, indicating that PI3K is the intracellular messenger involved in the NT-4 effect on NE uptake elicited after Trk B stimulation.

On the other hand, $p75^{NTR}$ involvement on neurotrophin effects of NE uptake could be discarded since $p75^{NTR}$ binds to all neurotrophins with identical affinity. In our experiments BDNF and NT-4, but not NGF, modified NE uptake. In addition, the Trk inhibitor, K252a blocked this effect. Then we suggest that Trk B and not $p75^{NTR}$, is involved in neurotrophin regulatory effects on NE uptake.

NE uptake is a process mediated by the specific transporter NET, localized at the presynaptic membrane of sympathetic neurons and the main final effector which can be implied on NE uptake regulator actions. The NET activity is regulated by several mechanisms such as allosteric modulation, temperature, sodium concentration and phosphorylation by several protein kinases related to PLC, PKC, PI3K and MAPK (Apparsundaram et al., 1998a,b; Yang and Raizada, 1999; Bryan-Lluka et al., 2001; Bönisch and Brüss, 2006; Mandela and Ordway, 2006). In addition, NET is also modulated by hormones, neuropeptides and growth factors such as natriuretic peptides, angiotensin II, angiotensin III, bradikinine and NGF (Vatta et al., 1992, 1996; Lu et al., 1996; Papouchado et al., 1995; Wakade et al., 1996; Ikeda et al., 2001).

Considering that NET is the specific transporter of NE in neurons and that non neuronal uptake was inhibited in our experiments by the addition of hydrocortisone (a non neuronal blocker), the NE uptake must be only carried out by NET. Thus, we suggest that BDNF and NT-4 through the interaction with Trk B, stimulate PLC and PI3K respectively, and these messengers could be the responsible for the modulation of NET activity elicited by BDNF and NT-4.

4.2. Neurotrophins and evoked NE release

Different effects on neurotransmitter release have been reported for neurotrophins (a similar situation to NE uptake men-

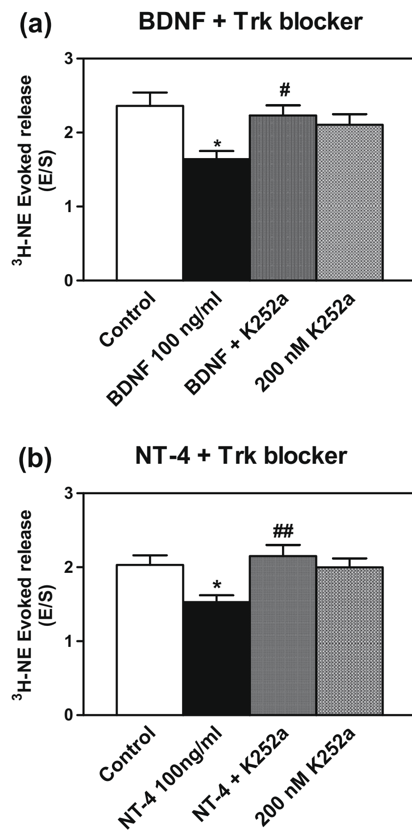

Fig. 6. Effects of 200 nM K252a (Trk receptor blocker) in the presence of (a) 100 ng/ml BDNF or (b) 100 ng/ml NT-4, on evoked ^{3}H-Norepinephrine (^{3}H-NE) release in anterior hypothalamic zone (AHZ). Results are expressed as mean ± SEM of 8–10 individual experiments. Fig. 6a, one way ANOVA *F* and *P* values: 4.794 and 0.009, respectively. Bonferroni post test "ad hoc": $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs BDNF. Fig. 6b, one way ANOVA *F* and *P* values: 4.811 and 0.008, respectively. Bonferroni post test "ad hoc": $^{*}P < 0.05$ vs control group; $^{\#\#}P < 0.01$ vs NT-4.

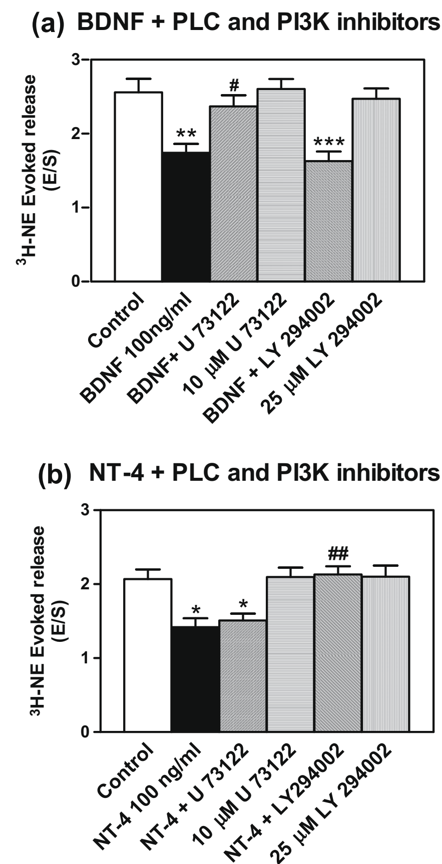

Fig. 7. Effects of 10 μM U 73122 and 25 μM LY 294002 (PLC and PI3 K inhibitors, respectively) in the presence of (a) 100 ng/ml BDNF or (b) 100 ng/ml NT-4 on evoked ^{3}H-Norepinephrine (^{3}H-NE) release in anterior hypothalamic zone (AHZ). Results are expressed as mean ± SEM of 8–10 individual experiments. Fig. 7a, one way ANOVA *F* and *P* values: 9.050 and 0.0001 respectively. Bonferroni post test "ad hoc": $^{**}P < 0.01$ vs control group; $^{***}P < 0.001$ vs control group; $^{\#}P < 0.05$ vs BDNF. Fig. 7b, one way ANOVA *F* and *P* values: 6.897 and 0.0001, respectively. Bonferroni post test "ad hoc": $^{*}P < 0.05$ vs control group; $^{\#\#}P < 0.01$ vs NT-4.

tioned above). In this way, in brain striatal slices, the interaction between BDNF and Trk B increased the evoked release of dopamine and GABA through the stimulation of PI3K and MAPK activities (Goggi et al., 2002, 2003). However, in rabbit retina, BDNF also increased dopamine release through Trk B receptor but activating PLC and not PI3K (Neal et al., 2003). On the other hand, in contrast to Goggi et al. report, Canas et al. demonstrated that this neurotrophin inhibited GABA evoked release in hippocampal synaptosomes (Canas et al., 2004).

Finally, it has been demonstrated that BDNF and NT-3 increased somatostatin release in primary hypothalamic cell cultures (Marmigère et al., 2001; Tapia-Arancibia et al., 2004).

Our results indicate that both BDNF and NT-4 decreased KCl-induced NE release in whole hypothalamus as well as in AHZ. On the other hand, NGF was not able to modify the evoked NE release in the rat hypothalamus. Surprisingly, although both neurotrophins BDNF and NT-4 exerted opposite effects on NE uptake, they produced the same response on KCl-evoked NE release. In addition, K252a reversed the decrease of evoked NE release carried out by BDNF and NT-4, suggesting that these neurotrophins could elicit their effects by coupling to Trk B receptor. Moreover, it must be pointed out that intracellular signalling pathways activated were different, i.e. while BDNF activated PLC to decrease evoked NE release; NT-4 stimulated the PI3K pathway.

It is well established that neurotransmitter release is a complex process that involves a great number of protein-regulated steps and also requires increased cytoplasmatic calcium levels. However, to evoke NE release it is necessary that the increase in calcium levels take place as near as possible to the active zone (Zerbes et al., 2001; Burgoyne and Morgan, 2003; Barclay et al., 2005). Thus, the increase in cytoplasmatic calcium levels subsequent to its release from intracellular storage is not enough to stimulate the exocytotic NE release. Moreover, it has been described that an increase in intracellular calcium concentration decreases the exocytotic NE release through a mechanism involving calpain activation and a posterior cleavage of synaptosomal-associated protein of 25 kDa (Ando et al., 2005). According to this, it is possible that BDNF, through its interaction with Trk B and PLC activation could mediate the decrease of NE release.

4.3. Neurotrophins and noradrenergic neurotransmission

It has been shown that neurotrophins modulate multiple processes (i.e. neurotransmitter release, synaptic plasticity, development and maintenance of nervous tissue) through the activation of different signalling pathways downstream the activation of the same receptor (Huang and Reichardt, 2001, 2003; Goggi et al., 2003; Neal et al., 2003). Moreover, several reports based on the study of knock out animals, have demonstrated differences between BDNF and NT-4 effects (Conover et al., 1995; Erickson et al., 1996; Heppenstall and Lewin, 2001; Gonzalez-Martinez et al., 2005). For instance, severe deficiencies in coordination and

balance were observed in mice lacking BDNF (Ernfors et al., 1994, 1995). In addition, these mice died during the first weeks of life (Jones et al., 1994). On the other hand, NT-4-deficient mice were viable but exhibited a loss of sensory neurons in the nodose-petrosal and geniculate ganglia, while the maintenance of vestibular and trigeminal sensory neurons required BDNF but not NT-4 (Conover and Yancopoulos, 1997). Although the authors of these studies suggested that the different effects could be explained by the activation of different intracellular signalling pathways, the second messengers system involved was not studied in depth (Lopez and Krimm, 2006). According to present results, we show for the first time that BDNF and NT-4 activate two different intracellular signalling pathways to modulate hypothalamic noradrenergic neurotransmission.

The increase in NE uptake together with the decrease in its evoked release elicited by BDNF could diminish NE availability at the synaptic cleft leading to a decrease in noradrenergic activity in the anterior hypothalamic area. Thus, BDNF could act as an inhibitory modulator of noradrenergic neurotransmission like angiotensin 1–7 and natriuretic peptides type A, B and C, considering that they exert similar effects on noradrenergic neurotransmission in the hypothalamus than those of BDNF, and moreover, they are well known inhibitors of sympathetic activity at this level (Vatta et al., 1992, 1996, 1999; Gironacci et al., 2000; Rodríguez Fermepin et al., 2000). On the other hand, although NT-4 decreased NE uptake as well as its evoked release, it would be also an inhibitory modulator of sympathetic neurotransmission, reducing NE availability at the synaptic gap. Likewise, this behaviour was observed in cardiac tissues, where endothelin 1 decreased NE uptake and attenuated exocytotic NE release (Backs et al., 2005).

Considering that the hypothalamus is a heterogeneous area that displays numerous effects, we considered that it is relevant to discriminate which is the hypothalamic zone involved in neurotrophins effects on sympathetic neurotransmission. The fact that BDNF and NT-4 effects were only displayed in the AHZ, suggests that these neurotrophins could be involved in the modulation of several physiological processes related to blood pressure and circadian rhythm regulation and vasopressin and oxytocin secretion. In addition, it has been reported that BDNF and NT-4 modulate different hypothalamic functions. For example, BDNF and Trk B stimulation modified the circadian sensitivity in the hypothalamic suprachiasmatic nucleus (Liang et al., 2000; Allen et al., 2005). Moreover a single intrahypothalamic application of NT-4 or a Trk B agonist antibody reduced food intake and body weight in mice (Tsao et al., 2008).

In conclusion, present findings support the hypothesis that BDNF and NT-4 (but not NGF) acutely applied, modulate noradrenergic neurotransmission in the AHZ through a mechanism involving Trk B receptor, and PLC or PI3K respectively, as intracellular messengers.

BDNF and NT-4, besides its well known properties on neuronal growth and differentiation, could regulate important steps of catecholamine metabolism in neurons of adult rats, like NE uptake and release which are closely involved in sympathetic activity modulation. Considering that the rapid and acute responses elicited by the nervous system implied the presence of noradrenergic networks, further studies might evidence the interaction between BDNF and NT-4 with NET and the exocytotic mechanism involved in NE uptake and release control respectively, as well as the real significance of these neurotrophins in the control of sympathetic activities in the hypothalamus.

Conflict of interest statement

The authors state no conflict of interest.

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References

Allen, G.C., Qu, X., Earnest, D.J., 2005. TrkB-deficient mice show diminished phase shifts of the circadian activity rhythm in response to light. Neurosci. Lett. 378, 150–155.

Ando, K., Kudo, Y., Takahashi, M., 2005. Negative regulation of neurotransmitter release by calpain: a possible involvement of specific SNAP-25 cleavage. J. Neurochem. 94, 651–658.

Apparsundaram, S., Galli, A., DeFelice, L.J., Hartzell, H.C., Blakely, R.D., 1998a. Acute regulation of norepinephrine transport: I. Protein kinase C-linked muscarinic receptors influence transport capacity and transporter density in SK-N-SH cells. J. Pharmacol. Exp. Ther. 287, 733–743.

Apparsundaram, S., Schroeter, S., Giovanetti, E., Blakely, R.D., 1998b. Acute regulation of norepinephrine transport: II. PKC-modulated surface expression of human norepinephrine transporter proteins. J. Pharmacol. Exp. Ther. 287, 744–751.

Backs, J., Bresch, E., Lutz, M., Kristen, A.V., Haass, M., 2005. Endothelin-1 inhibits the neuronal norepinephrine transporter in hearts of male rats. Cardiovasc. Res. 67, 283–290.

Barclay, J.W., Morgan, A., Burgoyne, R.D., 2005. Calcium-dependent regulation of exocytosis. Cell Calcium 38, 343–353.

Barry, S.P., Davidson, S.M., Townsend, P.A., 2008. Molecular regulation of cardiac hypertrophy. Int. J. Biochem. Cell Biol. 40, 2023–2039.

Blum, R., Konnerth, A., 2005. Neurotrophin-mediated rapid signaling in the central nervous system: mechanisms and functions. Physiology (Bethesda) 20, 70–78.

Bönisch, H., Brüss, M., 2006. The norepinephrine transporter in physiology and disease. Handb. Exp. Pharmacol. 175, 485–524.

Bryan-Lluka, L.J., Paczkowski, F.A., Bönisch, H., 2001. Effects of short- and long-term exposure to c-AMP and c-GMP on the noradrenaline transporter. Neuropharmacology 40, 607–617.

Burgoyne, R.D., Morgan, A., 2003. Secretory granule exocytosis. Physiol. Rev. 83, 581–632.

Busche, S., Gallinat, S., Fleegal, M.A., Raizada, M.K., Sumners, C., 2001. Novel role of macrophage migration inhibitory factor in angiotensin II regulation of neuromodulation in rat brain. Endocrinology 142, 4623–4630.

Canas, N., Pereira, I.T., Ribeiro, J.A., Sebastião, A.M., 2004. Brain-derived neurotrophic factor facilitates glutamate and inhibits GABA release from hippocampal synaptosomes through different mechanisms. Brain Res. 1016, 72–78.

Chao, M.V., Rajagopal, R., Lee, F.S., 2006. Neurotrophin signalling in health and disease. Clin. Sci. Lond. 110, 167–173.

Conover, J.C., Yancopoulos, G.D., 1997. Neurotrophin regulation of the developing nervous system: analyses of knockout mice. Rev. Neurosci. 8, 13–27.

Conover, J.C., Erickson, J.T., Katz, D.M., Bianchi, L.M., Poueymirou, W.T., McClain, J., Pan, L., Helgren, M., Ip, N.Y., Boland, P., Friedman, B., Wiegand, S., Vejsada, R., Kato, A.C., DeChiara, T.M., Yancopoulos, G.D., 1995. Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. Nature 375, 235–238.

Eisenhofer, G., 2001. The role of neuronal and extraneuronal plasma membrane transporters in the inactivation of peripheral catecholamines. Pharmacol. Ther. 91, 35–62.

Erickson, J.T., Conover, J.C., Borday, V., Champagnat, J., Barbacid, M., Yancopoulos, G., Katz, D.M., 1996. Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. J. Neurosci. 16, 5361–5371.

Ernfors, P., Lee, K.F., Jaenisch, R., 1994. Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature 368, 147–150.

Ernfors, P., Kucera, J., Lee, K.F., Loring, J., Jaenisch, R., 1995. Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice. Int. J. Dev. Biol. 39, 799–807.

Gironacci, M.M., Vatta, M., Rodríguez Fermepín, M., Fernández, B.E., Peña, C., 2000. Angiotensin-(1–7) reduces norepinephrine release through a nitric oxide mechanism in rat hypothalamus. Hypertension 35, 1248–1252.

Goggi, J., Pullar, I.A., Carney, S.L., Bradford, H.F., 2002. Modulation of neurotransmitter release induced by brain-derived neurotrophic factor in rat brain striatal slices in vitro. Brain Res. 941, 34–42.

Goggi, J., Pullar, I.A., Carney, S.L., Bradford, H.F., 2003. Signalling pathways involved in the short-term potentiation of dopamine release by BDNF. Brain Res. 968, 156–161.

Gonzalez-Martinez, T., Farinas, I., Del Valle, M.E., Feito, J., Germana, G., Cobo, J., Vega, J.A., 2005. BDNF, but not NT-4, is necessary for normal development of Meissner corpuscles. Neurosci. Lett. 377, 12–15.

Helke, C.J., Verdier-Pinard, D., 2000. Neurotrophins alter the numbers of neurotransmitter-ir mature vagal/glossopharyngeal visceral afferent neurons in vitro. Brain Res. 884, 206–212.
Hempstead, B., 2002. The many faces of p75NTR. Curr. Opin. Neurobiol. 12, 260–267.
Heppenstall, P.A., Lewin, G.R., 2001. BDNF but not NT-4 is required for normal flexion reflex plasticity and function. Proc. Natl. Acad. Sci. USA 98, 8107–8112.
Huang, E.J., Reichardt, L.F., 2001. Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. 24, 677–736.
Huang, E.J., Reichardt, L.F., 2003. Trk receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. 72, 609–642.
Hyman, C., Juhasz, M., Jackson, C., Wright, P., Ip, N.Y., Lindsay, R.M., 1994. Overlapping and distinct actions of the neurotrophins BDNF, NT-3, and NT-4/5 on cultured dopaminergic and GABAergic neurons of the ventral mesencephalon. J. Neurosci. 14, 335–347.
Ikeda, T., Kitayama, S., Morita, K., Dohi, T., 2001. Nerve growth factor down-regulates the expression of norepinephrine transporter in rat pheochromocytoma (PC12) cells. Brain Res. Mol. Brain Res. 86, 90–100.
Jones, K.R., Fariñas, I., Backus, C., Reichardt, L.F., 1994. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell 76, 989–999.
Katovich, M.J., Grobe, J.L., Raizada, M.K., 2008. Angiotensin-(1–7) as an antihypertensive, antifibrotic target. Curr. Hypertens. Rep. 10, 227–232.
Kawamoto, Y., Nakamura, S., Nakano, S., Oka, N., Akiguchi, I., Kimura, J., 1996. Immunohistochemical localization of brain-derived neurotrophic factor in adult rat brain. Neuroscience 74, 1209–1226.
Kubista, H., Boehm, S., 2006. Molecular mechanisms underlying the modulation of exocytotic noradrenaline release via presynaptic receptors. Pharmacol. Ther. 112, 213–242.
Kumar, V.M., Vetrivelan, R., Mallick, H.N., 2007. Noradrenergic afferents and receptors in the medial preoptic area: neuroanatomical and neurochemical links between the regulation of sleep and body temperature. Neurochem. Int. 50, 783–790.
Kumar, R., Singh, V.P., Baker, K.M., 2008. The intracellular renin–angiotensin system: implications in cardiovascular remodeling. Curr. Opin. Nephrol. Hypertens. 17, 168–173.
Law, R.M., Stafford, A., Quick, M.W., 2000. Functional regulation of gamma-aminobutyric acid transporters by direct tyrosine phosphorylation. J. Biol. Chem. 275, 23986–239991.
Li, W., Keifer, J., 2008. Coordinate action of pre- and postsynaptic brain-derived neurotrophic factor is required for AMPAR trafficking and acquisition of in vitro classical conditioning. Neuroscience 155, 686–697.
Liang, F.Q., Allen, G., Earnest, D., 2000. Role of brain-derived neurotrophic factor in the circadian regulation of the suprachiasmatic pacemaker by light. J. Neurosci. 20, 2978–2987.
Lohof, A.M., Ip, N.Y., Poo, M.M., 1993. Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. Nature 363, 350–353.
Lopez, G.F., Krimm, R.F., 2006. Epithelial overexpression of BDNF and NT4 produces distinct gustatory axon morphologies that disrupt initial targeting. Dev. Biol. 292, 457–468.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.
Lu, B., 2004. Acute and long-term synaptic modulation by neurotrophins. Prog. Brain Res. 146, 137–150.
Lu, D., Yu, K., Paddy, M.R., Rowland, N.E., Raizada, M.K., 1996. Regulation of norepinephrine transport system by angiotensin II in neuronal cultures of normotensive and spontaneously hypertensive rat brains. Endocrinology 137, 763–772.
Lu, B., Pang, P.T., Woo, N.H., 2005. The yin and yang of neurotrophin action. Nat. Rev. Neurosci. 6, 603–614.
Mandela, P., Ordway, G.A., 2006. The norepinephrine transporter and its regulation. J. Neurochem. 97, 310–333.
Marmigère, F., Choby, C., Rage, F., Richard, S., Tapia-Arancibia, L., 2001. Rapid stimulatory effects of brain-derived neurotrophic factor and neurotrophin-3 on somatostatin release and intracellular calcium rise in primary hypothalamic cell cultures. Neuroendocrinology 74, 43–54.
Mössner, R., Daniel, S., Albert, D., Heils, A., Okladnova, O., Schmitt, A., Lesch, K.P., 2000. Serotonin transporter function is modulated by brain-derived neurotrophic factor (BDNF) but not nerve growth factor (NGF). Neurochem. Int. 36, 197–202.
Neal, M., Cunningham, J., Lever, I., Pezet, S., Malcangio, M., 2003. Mechanism by which brain-derived neurotrophic factor increases dopamine release from the rabbit retina. Invest. Ophthalmol. Vis. Sci. 44, 791–798.
Nishio, T., Furukawa, S., Akiguchi, I., Oka, N., Ohnishi, K., Tomimoto, H., Nakamura, S., Kimura, J., 1994. Cellular localization of nerve growth factor-like immunoreactivity in adult rat brain: quantitative and immunohistochemical study. Neuroscience 60, 67–84.
Oparil, S., Chen, Y.F., Berecek, H., Calhoun, D.A., Wyss, J.M., 1995. The role of the central nervous system in hypertension. In: Laragh, J.H., Brenner, R.H. (Eds.), Hypertension: Pathophysiology, Diagnosis and Management. Raven Press, New York, pp. 713–740.
Palkovits, M., Brownstein, M.J., 1988. Maps and Guide to Microdissection of the Rat Brain. Elsevier, New York.
Papouchado, M.L., Vatta, M.S., Escalada, A., Bianciotti, L.G., Fernández, B.E., 1995. Angiotensin III modulates noradrenaline uptake and release in the rat hypothalamus. J. Auton. Pharmacol. 15, 1–8.
Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates. Academic Press, Australia.
Poo, M.M., 2001. Neurotrophins as synaptic modulators. Nat. Rev. Neurosci. 2, 24–32.
Reichardt, L.F., 2006. Neurotrophin-regulated signalling pathways. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 361, 1545–1564.
Renner, K.J., Gerhardt, G.A., Quadagno, D.M., 1984. Brain catecholamine content during the estrous cycle and in steroid-primed rats. Brain Res. Bull. 12, 363–368.
Rodríguez Fermepin, M., Vatta, M.S., Bianciotti, L.G., Wolovich, T.J., Fernández, B.E., 2000. B-Type and C-type natriuretic peptides modify norepinephrine uptake in discrete encephalic nuclei of the rat. Cell. Mol. Neurobiol. 20, 763–771.
Schuman, E.M., 1999. Neurotrophin regulation of synaptic transmission. Curr. Opin. Neurobiol. 9, 105–109.
Secondo, A., Sirabella, R., Formisano, L., D'Alessio, A., Castaldo, P., Amoroso, S., Ingleton, P., Di Renzo, G., Annunziato, L., 2003. Involvement of PI3'-K, mitogen-activated protein kinase and protein kinase B in the up-regulation of the expression of nNOS alpha and nNOS beta splicing variants induced by PRL-receptor activation in GH3 cells. J. Neurochem. 84, 1367–1377.
Stoop, R., Poo, M.M., 1995. Potentiation of transmitter release by ciliary neurotrophic factor requires somatic signaling. Science 267, 695–699.
Stoop, R., Poo, M.M., 1996. Synaptic modulation by neurotrophic factors: differential and synergistic effects of brain-derived neurotrophic factor and ciliary neurotrophic factor. J. Neurosci. 16, 3256–3264.
Tapia-Arancibia, L., Rage, F., Givalois, L., Arancibia, S., 2004. Physiology of BDNF: focus on hypothalamic function. Front. Neuroendocrinol. 25, 77–107.
Tsao, D., Thomsen, H.K., Chou, J., Stratton, J., Hagen, M., Loo, C., Garcia, C., Sloane, D.L., Rosenthal, A., Lin, J.C., 2008. Trk B agonists ameliorate obesity and associated metabolic conditions in mice. Endocrinology 149, 1038–1048.
Vatta, M.S., Bianciotti, L.G., Locatelli, A.S., Papouchado, M.L., Fernandez, B.E., 1992. Monophasic and biphasic effects of angiotensin II and III on norepinephrine uptake and release in rat adrenal medulla. Can. J. Physiol. Pharmacol. 70, 821–825.
Vatta, M.S., Presas, M., Bianciotti, L.G., Zarrabeitia, V., Fernandez, B.E., 1996. B and C types natriuretic peptides modulate norepinephrine uptake and release in the rat hypothalamus. Regul. Pept. 65, 175–184.
Vatta, M.S., Rodríguez Fermepin, M., Durante, G., Bianciotti, L.G., Fernández, B.E., 1999. Atrial natriuretic factor inhibits noradrenaline biosynthesis and turn-over in the rat hypothalamus. Regul. Pept. 85, 101–177.
Vaz, S.H., Cristóvão-Ferreira, S., Ribeiro, J.A., Sebastião, A.M., 2008. Brain-derived neurotrophic factor inhibits GABA uptake by the rat hippocampal nerve terminals. Brain Res. 1219, 19–25.
Wakade, A.R., Wakade, T.D., Poosch, M., Bannon, M.J., 1996. Noradrenaline transport and transporter mRNA of rat chromaffin cells are controlled by dexamethasone and nerve growth factor. J. Physiol. 494, 67–75.
Widmer, H.R., Hefti, F., 1994. NT-4/5 promotes survival and differentiation of rat striatal neurons developing in culture. Eur. J. Neurosci. 6, 1669–1679.
Yan, Q., Rosenfeld, R.D., Matheson, C.R., Hawkins, N., Lopez, O.T., Bennett, L., Welcher, A.A., 1997. Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. Neuroscience 78, 431–448.
Yang, H., Raizada, M.K., 1999. Role of phosphatidylinositol 3-kinase in angiotensin II regulation of norepinephrine neuromodulation in brain neurons of the spontaneously hypertensive rat. J. Neurosci. 19, 2413–2423.
Zerbes, M., Clark, C.L., Powis, D.A., 2001. Neurotransmitter release from bovine adrenal chromaffin cells is modulated by capacitative Ca(2+)entry driven by depleted internal Ca(2+)stores. Cell Calcium 29, 49–58.
Zhang, J.M., Dix, J., Langtimm-Sedlak, C.J., Trusk, T., Schroeder, B., Hoffmann, R., Strosberg, A.D., Winslow, J.W., Sieber-Blum, M., 1997. Neurotrophin-3- and norepinephrine-mediated adrenergic differentiation and the inhibitory action of desipramine and cocaine. J. Neurobiol. 32, 262–280.