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Substance P, Neurokinins A and B, and Synthetic Tachykinin Peptides Protect Mesencephalic Dopaminergic Neurons in Culture via an Activity-Dependent Mechanism

Bénédicte Salthun-Lassalle, Sabine Traver, Etienne C. Hirsch and Patrick P. Michel

Institut National de la Santé et de la Recherche Médicale Unité 679, Experimental Neurology and Therapeutics, Hôpital de la Salpêtrière, Paris, France

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Running title : Rescue of Dopaminergic Neurons by Tachykinins

Corresponding Author : Patrick P. Michel, INSERM 679, Bâtiment Pharmacie, Hôpital de la Salpêtrière, 47 bd de l'hôpital, 75013, Paris, France. e-mail: ppmichel@ccr.jussieu.fr Tel: 33142162217; Fax: 33144243658

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Abbreviations: araC, cytosine arabinoside; Boc-D-FMK, Boc-D-fluoromethylketone; DA, dopaminergic; DIV, day *in vitro*; GABA, gamma-aminobutyric acid; GDNF, Glial Cell Line-Derived Neurotrophic Factor; 5-HT, serotonin; MAP2, Microtubule associated protein-2; NK, neurokinin; PBS, phosphate-buffered saline; SP, substance P; TH, tyrosine hydroxylase; TTX, tetrodotoxin.

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Abstract

We evaluated the neuroprotective potential of tachykinin peptides using a model system in which mesencephalic dopaminergic (DA) neurons die spontaneously and selectively as they mature. The three native tachykinins, substance P (SP), neurokinin (NK) A and NKB afforded substantial protection against DA cell demise. The selective NK_1 receptor antagonist GR71251 was sufficient in itself to suppress the effect of SP whereas a co-treatment with GR71251 and the NK_3 receptor antagonist SB218795 was required to prevent the effects of both NKA and NKB. Consistent with these results, GR73632, a selective agonist of NK_1 receptors and [pro7]-NKB, a selective agonist of NK_3 receptors, conferred protection to DA neurons whereas GR64349, which activates specifically NK_2 receptors, did not. DA neurons rescued by tachykinins accumulated [^{3}H]-DA efficiently which suggests that they were also totally functional. Neuroprotection by tachykinins was highly selective for DA neurons, rapidly reversed upon treatment withdrawal and reproduced by, but independent of glial cell line-derived neurotrophic factor. Survival promotion by tachykinins was abolished by blocking voltage-gated Na^+ channels with tetrodotoxin or N-type voltage-gated Ca^{2+} channels with ω-conotoxin-MVIIA, which indicates that an increase in neuronal excitability was crucially involved in this effect. Together, these data further support the notion that the survival of mesencephalic DA neurons during development depends largely on excitatory inputs, which may be provided in part by tachykinins.

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Tachykinins belong to a family of neuropeptides that share a common C-terminal sequence Phe-Xaa-Gly-Leu-Met-NH_2 that is crucial for interaction with their receptors (Severini et al., 2002; Pennefather et al., 2004). The mammalian tachykinins include substance P (SP), neurokinin (NK) A and NKB, the effects of which are mediated by the receptors NK_1, NK_2 and NK_3. SP is the most potent tachykinin at the NK_1 receptor site, whereas NKA exhibits the highest affinity for the NK_2 receptor and NKB for the NK_3 receptor (Pennefather et al., 2004). The effects through NK receptors are generally coupled to a G protein (G_q/G_{11}) regulated phosphoinositide pathway (Khawaja and Rogers, 1996), but G protein-independent coupling has also been described (Yang et al., 2003).

NK receptors are implicated in various biological effects that include smooth muscle contraction, inflammatory processes, hypotensive effects and stimulation of gland secretion (Severini et al., 2002). In the nervous system, tachykinins also operate as neuromodulators/neurotransmitters via mechanisms that are generally excitatory (Stacey et al., 2002). In addition, there is evidence that tachykinins also have intrinsic neuroprotective properties. They were found to reverse β-amyloid-induced toxicity (Kowall et al., 1991), to protect against excitotoxic cell death (Calvo et al., 1996) and to limit neurodegeneration caused by trophic factor deprivation (Lallemend et al., 2003).

The loss of nigrostriatal dopaminergic (DA) neurons in Parkinson disease and related disorders leads to profound motor impairment (Agid, 1991). The identification of signals or factors that control the survival and function of DA neurons is therefore of interest as it might not only provide a better understanding of the pathophysiological mechanisms of these diseases, but also foster the development of new therapeutical strategies to halt their progression (Dawson and Dawson, 2002). There is indirect evidence that tachykinins may influence the survival of mesencephalic DA neurons; (1) in the brain, the substantia nigra is the richest area in SP and SP-containing axon terminals (Hokfelt et al., 1991); (2) striatal DA

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depletion in Parkinson disease and in animal models of the disease results in profound changes in SP expression (Mauborgne et al., 1983; Betarbet and Greenamyre, 2004); (3) excitatory stimuli, which are believed to play a key role in the survival of DA neurons during development and possibly in the adult brain (Douhou et al., 2001; Katsuki et al., 2001; Salthun-Lassalle et al., 2004), can be evoked by direct application of NK_1 or NK_3 agonists to mesencephalic DA neurons (Nalivaiko et al., 1997).

To examine the potential of tachykinins to promote the survival of DA neurons, we have used a model system in which these neurons die spontaneously and progressively as a function of time (Michel and Agid, 1996). More specifically, we wished to: 1) evaluate the neuroprotective potential of native and synthetic tachykinins for DA neurons; 2) determine what receptor subtypes are involved; 3) establish whether these mechanisms are shared with or related to that of GDNF, a prototypical neuroprotective factor for DA neurons (Choi-Lundberg et al., 1997); 4) explore the mechanisms underlying neuroprotection.

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Materials and methods

Peptide agonists/antagonists of tachykinin receptors and other pharmacological agents

Native tachykinins SP, NKA, NKB and synthetic peptide agonists/antagonists (GR73632, GR64349, [pro7]-NKB, GR71251, R396) of tachykinin receptors were from NeoMPS (Strasbourg, France). The non-peptide receptor antagonist SB218795 was from Bioblock Scientific (Strasbourg, France). Glial Cell Line-Derived Neurotrophic Factor (GDNF) was purchased from AbCys (Paris, France) and the anti-GDNF neutralizing antibody (#AB 212 NA) from RD systems Europe (Lille, France). The pancaspase inhibitor Boc-D-fluoromethylketone (Boc-D-FMK) was obtained from Calbiochem (Darmstadt, Germany). Others pharmacological reagents were from Sigma-RBI-Aldrich. Tritiated neurotransmitters and tritiated methyl-thymidine were from Amersham Biosciences (Orsay, France).

Mesencephalic cell cultures

Animals were treated in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996), the European Directive N°86/609, and the guidelines of the local institutional animal care and use committee. Cultures were prepared from the ventral mesencephalon of embryonic day 15.5 Wistar rat embryos (Janvier Breeding Center, Le Genest St Isle, France) as described previously (Michel and Agid, 1996). After dissection, pieces of mesencephalic tissue were dissociated mechanically with no enzymatic treatment using a Gilson Pipetman set to 900µl, and plated onto polyethylenimine (1mg/mL; Sigma-Aldrich, St Quentin Fallavier, France)-coated 24-well culture plates. Cells plated at a density of 1.5-2.0x10^5 /cm^2 were then maintained at 37°C in a humidified incubator with a 5% CO_2 atmosphere using 500µL of N5 culture medium (Salthun-Lassalle et al., 2004) supplemented with 5mM glucose, 5% horse serum and 0.5% fetal bovine serum, except for the first 3 days when 2.5% fetal bovine serum was used to enhance cell attachment.

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The cultures were fed daily by replacing 350µl of the culture medium. They were then supplemented with appropriate pharmacological treatments including native tachykinins and agonists/antagonists of tachykinin receptors.

Quantification of neuronal survival

The survival of DA neurons was quantified by counting the number of cells labeled with an antibody against tyrosine hydroxylase (TH), as previously described. Briefly, the cultures were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 15 min. Cells were washed three times with PBS and then incubated 24 hours at 4°C with a monoclonal anti-TH antibody (Diasorin, Stillwater, Min, USA) diluted 1:5000 in PBS containing 0.2% Triton X-100. The TH antibody was then revealed with an anti-mouse IgG Cy3 conjugate (1:500; Sigma-RBI-Aldrich) for 2 hours at room temperature. Mesencephalic cultures contained between 1-2% TH^+ cells at the time of plating. All neurons, regardless of their neurotransmitter phenotype, were identified by labeling microtubule-associated protein-2 (MAP2) with a monoclonal antibody (AP-20, Sigma) diluted 1:100 in PBS revealed with a anti-mouse Alexa Fluor 488 conjugate.

Measurement of neurotransmitter uptake

The functional integrity of DA neurons was evaluated by their ability to take up dopamine (DA) by active transport (Douhou et al., 2001). Uptake was initiated by addition of 50nM [^{3}H]-DA (40Ci/mmol) to cultures pre-incubated for 10 min in 500µl PBS containing 5mM glucose and 100µM ascorbic acid. It was terminated after 15 min by two rapid washes with cold PBS. Cells were scraped off the culture wells and the accumulated tritium counted by liquid scintillation spectrometry. The uptake of [^{3}H]-DA was also visualized by microautoradiography (Troadec et al., 2002). In this case, the incubation time was prolonged

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to 30 min and [^{3}H]-DA was used at 100nM to improve detection. The cultures were then fixed with a mixture of glutaraldehyde/formaldehyde (0.5%:4%) and dehydrated with ethanol. Incorporation of [^{3}H]-DA was detected with the Hypercoat LM-1 emulsion (Amersham Biosciences, Orsay, France) after an exposure of 10 days in the dark at 4°C. In both paradigms, blank values were obtained in the presence of 0.5µM GBR-12909 (Sigma Aldrich). GABA and serotonin (5-HT) uptake was measured, as described using 50nM [^{3}H]-GABA and 20nM [^{3}H]-5-HT, respectively (Salthun-Lassalle et al., 2004). Note that the concentrations of the three neurotransmitters were below the K_m values of their transporters to be within the linear portion of the uptake time course.

Quantification and identification of proliferating cells

[^{3}H]-methyl-thymidine, a marker of DNA synthesis, was used to label and quantify proliferating cells as described (Mourlevat et al., 2003). Mesencephalic cultures maintained up to 7 DIV in the presence of test treatments were exposed to 1µCi [^{3}H]-methyl-thymidine (Amersham Biosciences; 40Ci/mmol) for 3h at 37°C in serum-free N5 medium supplemented with 5mM glucose. After three rapid washes, the cells were allowed to recover for 1h in the same culture medium to remove unincorporated radioactivity. The cultures were fixed in 4% formaldehyde for 15 min and when necessary processed for TH immunofluorescence detection subsequently. They were then dehydrated in ethanol and exposed to Hypercoat LM-1 emulsion (Amersham Biosciences) for 4 days at 4°C to detect the tritiated label.

Quantification of intracellular calcium levels

Cytoplasmic-free calcium levels were measured in individual neurons using Calcium Green-1-AM (Molecular Probes, Eugene, OR) as described previously (Douhou et al., 2001; Salthun-Lassalle et al., 2004).

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Reverse Transcription and Polymerase Chain Reaction

Total RNA from mesencephalic cultures or from rat uterus tissue was isolated with RNABle® solution (Eurobio, Les Ulis, France). Three types of cultures were used: mixed cultures containing neurons and glial cells, pure neuronal cultures where glial cells were eliminated by treatment with 3µM araC and pure astrocyte cultures. First strand cDNA was synthesized from 2µg of total RNA using the "RT" kit (Qiagen, Courtaboeuf, France). To analyze the expression of NK_1, NK_2 and NK_3 receptors, transcripts were amplified by PCR under the following conditions: 35 cycles of 94°C for 30s, 58°C for 30s, and 72°C for 30s with 3µL of the reverse transcription mixture. The primers used for tachykinin receptors were: NK_1, forward (5'-CCTCCTGCCCTACATCAACCC-3') and reverse (5'-CTGTGTCTGGAGGTATCGGG-3'); NK_2, forward (5'-CATCACTGTGGACGAGGGGG-3') and reverse (5'-TGTCTTCCTCAGTTGGTGTC-3'); NK_3, forward (5'-CATTCTCACTGCGATCTACC-3') and reverse (5'-CTTCTTGCGGCTGGATTTGG-3') (Pinto et al., 1999). The PCR products were visualized by electrophoresis in a 2% agarose gel containing 0.1mg/mL ethidium bromide.

Statistical analysis

Simple comparisons between two groups were performed with Student's t-test. Multiple comparisons against a single reference group were made by one-way analysis of variance (ANOVA) followed by Dunnett's test. When all pairwise comparisons were made, the Student-Newman-Keuls test was used. SEM values were derived from at least three independent experiments.

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Results

Native tachykinins SP, NKA and NKB increase the number of DA neurons in mesencephalic cultures

The native tachykinins SP, NKA and NKB increased the number of TH^+ DA neurons in mesencephalic cultures (Fig. 1A-C, E). The effects of SP and NKB on the number of TH^+ cells were restricted to a narrow range of concentrations between 1-50nM and 1-5nM, respectively, with peak effects at 10nM and 1nM (Fig. 1A, C). At higher concentrations, no effect of SP or NKB on the number of DA neurons was observed. NKA treatment increased the number of DA cells as well, but at much higher concentrations (100-1000nM; Fig. 1B, E).

Synthetic peptide agonists of NK_1 and NK_3 but not NK_2 receptors mimic the effects of native tachykinins

SP, NKA and NKB are believed to act preferentially through NK_1, NK_2 and NK_3 receptors, respectively (Pennefather et al., 2004). To determine whether selective agonists of these receptors could reproduce the effects of native tachykinins on DA neurons, we treated mesencephalic cultures with the NK_1 agonist GR73632, the NK_2 agonist GR64349 and the NK_3 agonist [pro7]-NKB. Consistent with the results obtained with SP and NKB, the number of DA neurons increased in the presence of GR73632 and [pro7]-NKB (Fig. 1D), in a narrow range of concentrations. The peak effect was estimated at 10nM for GR73632 and 1nM for [pro7]-NKB (Fig. 1D). Unexpectedly, however, the NK_2 receptor agonist GR64349 did not affect the number of TH^+ cells, indicating that the action of NKA was probably mediated by its non-preferential receptors.

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Selective antagonists of NK_1 and NK_3 receptors prevent the effects of tachykinins on DA neurons

We wished first to address the specificity of the interaction between synthetic tachykinin agonists and NK receptors. Therefore, specific antagonists of NK_1, NK_2 and NK_3 receptors, i.e., GR71251, R396 and SB218795 respectively, were added (all at 1µM) to mesencephalic cultures in the presence of the NK_1 receptor agonist GR73632 or the NK_3 receptor agonist [pro7]-NKB (Fig. 2A). As expected, the NK_1 receptor antagonist GR71251 prevented GR73632-mediated increase in DA cells whereas the NK_2 antagonist R396 and the NK_3 antagonist SB218795 did not (Fig. 2A). Similarly, the effect of the selective NK_3 agonist [pro7]-NKB on DA neurons was abolished by the corresponding antagonist SB218795, but not by the antagonists of NK_1 or NK_2 receptors (Fig. 2A). Note that the number of DA neurons was not further increased when we combined the NK_1 and NK_3 agonists at optimal concentrations (Fig. 2A).

We then intended to determine what receptor mediated the effects of native tachykinins (Fig. 2B-D). The NK_1 antagonist GR71251 was sufficient in itself to suppress the effect of SP, indicating that this tachykinin increased the number of DA neurons only through its preferential receptor (Fig. 2B). Surprisingly, however, both NK_3 and NK_1 antagonists were required to completely suppress the effect of NKB. Separately, each of these antagonists had only partial effects (Fig. 2D). This indicates that the effect of NKB was mediated by the concomitant activation of NK_1 and NK_3 receptors. The effects of NKA that acts preferentially through NK_2 receptors, were resistant to the selective NK_2 receptor antagonist R396 (Fig. 2C), suggesting the probable involvement of non-preferential NK receptors. Accordingly, the effect of NKA was prevented by a treatment combining NK_1 and NK_3 receptor antagonists. Consistent with these different results, mRNA transcripts for NK_1 and NK_3 receptors were detected in mesencephalic cultures using RT-PCR amplification (Fig. 2E) whereas mRNA

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transcripts for NK_2 receptors were not. Note that all three receptors were amplified from rat uterus mRNA used as a positive control (Pinto et al., 1999). Since the three native tachykinins SP, NKA and NKB exerted their effects on DA cells through NK_1 or NK_3 receptors, subsequent experiments except when indicated were performed using the two selective NK_1 and NK_3 receptor agonists GR73632 and [pro7]-NKB.

The increase in TH^+ cells numbers following stimulation of NK_1 and NK_3 receptors is due to neuroprotection

From previous studies, we know that this culture model is characterized by a spontaneous and progressive loss of DA neurons (Michel and Agid, 1996; Salthun-Lassalle et al., 2004). Accordingly, we found that ~50% of TH^+ neurons had disappeared by 5 DIV and more than 70% at 10 DIV (Fig. 3A). We demonstrate, here, that the pancaspase inhibitor Boc-D-fluoromethylketone (Boc-D-FMK) prevented DA cell loss efficiently (Fig. 3A), which confirms indirectly that a slowly occurring apoptotic process was affecting these neurons (Michel and Agid, 1996; Salthun-Lassalle et al., 2004). A chronic treatment with GR73632 (10nM) or [pro7]-NKB (1nM) also reduced TH^+ cell loss substantially indicating that tachykinins were probably acting by preventing the apoptotic mechanism as well. However, to exclude the possibility that NK receptor activation increased the number of TH^+ neurons by stimulating cell division, we treated the cultures with the NK_1 receptor agonist GR73632 in the presence of [^{3}H]-methyl-thymidine. Nuclei positive for the tritiated label were never found in association with TH^+ neurons indicating that NK_1 receptor stimulation did not induce the proliferation of DA neuroblasts or their precursor cells (Fig. 3B). Note that similar results were obtained with [pro7]-NKB (data not shown).

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Tachykinin-dependent survival of DA neurons is a function of the duration of treatment

To determine whether shorter treatments also prevented the death of TH^+ neurons, the addition of the NK_3 receptor agonist [pro7]-NKB (1nM) was delayed after plating. Under these conditions, the number of TH^+ neurons that could be rescued by the treatment diminished progressively (Fig. 4A). Cultures treated continuously for 10 d (0-10 DIV) with 1nM [pro7]-NKB had ~120% more TH^+ neurons than untreated cultures, whereas cultures only treated from 4-10 DIV had only ~40% more. Conversely, the protective effect of [pro7]-NKB was rapidly reversible if the treatment was stopped prematurely and the cultures maintained in control medium up to 10 DIV (Fig. 4B). Characteristically, if [pro7]-NKB was withdrawn at day 8, only 40% of the TH^+ were rescued 2 d later; more strikingly, if withdrawn at 6 DIV, [pro7]-NKB produced no net increase in the number of TH^+ neurons at 10 DIV (Fig. 4B). Similar results were obtained with the NK_1 agonist GR73632 (data not shown).

TH^+ neurons rescued by activation of NK_1 and NK_3 receptors possess a functional DA transporter

To assess the function of the rescued DA neurons, we quantified the uptake of [^{3}H]-DA and combined microautoradiographic detection of the tritiated neurotransmitter with immunofluorescent labeling of TH. Treatments with either the NK_1 agonist GR73632 or the NK_3 agonist [pro7]-NKB increased the number of TH^+ neurons and the number of cells accumulating [^{3}H]-DA, in the same proportion (Fig. 5A, Fig. 6). Under these conditions, virtually all TH^+ neurons contained the tritiated label (Fig. 6), which indicates that both peptide agonists saved a population of neurons that was equipped with a functional DA transporter. Interestingly, the neurites of TH^+ neurons treated with GR73632 and [pro7]-NKB were more intensely labeled with [^{3}H]-DA than in control cultures suggesting that the uptake

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was also more efficient in these neurons. Confirming this observation, the accumulation rate of [^{3}H]-DA per TH$^+$ neuron was augmented by approximately twofold in the presence of the test compounds compared to untreated cultures (Fig. 5B). In contrast, the agonist of voltage-gated Na^+ channels, veratridine, which also provided robust neuroprotection to DA neurons in this model system (Salthun-Lassalle et al., 2004), had no effect on the accumulation rate of DA by these neurons (Figs. 5B and 6). Note the virtual absence of cells accumulating [^{3}H]-DA in cultures treated with the inhibitor of the DA transporter GBR-12909 (0.5µM) prior to the uptake (Fig. 6).

Trophic effects mediated by NK_1 and NK_3 receptors are selective for DA neurons in mesencephalic cultures

TH$^+$ cells represent approximately 1-2% of all neurons at the time of plating. To determine whether NK_1 (GR73632) and NK_3 ([pro7]-NKB) receptor agonists also affected the survival of non-DA neurons, which are predominantly GABAergic and to a lesser extent serotoninergic, we labeled the entire population of mesencephalic neurons with an antibody against MAP2. The number of MAP2$^+$ neurons remained unchanged following chronic treatment with GR73632 or [pro7]-NKB (Fig. 7A), as illustrated in figure 7B. Similar results were obtained when the uptake of tritiated GABA or 5-HT was used to assess the function of GABAergic and serotoninergic neurons, respectively (Fig. 7C).

Survival promotion by stimulation of NK_1 and NK_3 receptors does not result from an antiproliferative effect on glial cells

DA neurons can be rescued efficiently in this culture model by reducing the proliferation of astrocytes or their precursor cells (Mourlevat et al., 2003). To determine whether stimulation of NK_1 and NK_3 receptors affected the proliferation of glial cells, we

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assessed the incorporation of [^{3}H]-methyl-thymidine in cultures treated chronically for 7 DIV with GR73632 or [pro7]-NKB (Fig. 8). Neither GR73632 nor [pro7]-NKB had an influence on the number of thymidine$^+$ nuclei in mesencephalic cultures (Fig. 8B-C), which indicates that NK_1 and NK_3 agonists promoted the survival of DA neurons in spite of the glial proliferation. As expected, however, the synthetic deoxynucleoside cytosine (araC, 1μM) caused an almost complete loss of [^{3}H]-methyl-thymidine nuclei and a robust increase in the number of TH^+ neurons (Fig. 8).

The rescue of DA neurons caused by activation of NK receptors does not depend upon the secretion of a trophic factor

We wished to determine whether the effect mediated by NK_1 and NK_3 receptors was possibly mediated by GDNF, a prototypical trophic factor for DA neurons. Therefore, we tested the effects of GR73632 (10nM) and [pro7]-NKB (1nM) in the presence of an anti-GDNF antibody (AB 212-NA; 5 μg/ml) that neutralizes the biological activity of the trophic peptide (Fig. 9A). Whereas the antibody blocked the increase in DA cell survival produced by GDNF (10ng/mL), it had no effect on neuronal survival promoted by tachykinins. This indicates that the effect of tachykinins was not dependent upon the secretion of GDNF in the culture medium.

To exclude the effect of another putative factor secreted by glial or neuronal cells, we assessed the survival of TH^+ cells maintained in a culture medium that had been conditioned previously by mesencephalic cultures exposed continuously to GR73632 (10nM) or [pro7]-NKB (1nM). The two conditioned media protected the DA neurons but only because they contained GR73632 or [pro7]-NKB, the effects of which were blocked by the corresponding NK_1 and NK_3 receptor antagonists, GR71251 and SB218795 (Fig. 9B).

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Neuroprotection of DA neurons by tachykinins: role of voltage-gated ionic channels

We have shown previously that voltage-gated ionic channels are crucially involved in the survival of DA neurons in this model system (Douhou et al., 2001; Salthun-Lassalle et al., 2004). In particular, we showed that DA neurons can be rescued from death by low-level activation of voltage-gated Na^+ channels using the alkaloid veratridine (Salthun-Lassalle et al., 2004), the effect of which were blocked by the Na^+ channel antagonist TTX. The effects of the agonists of NK_1 (GR73632) and NK_3 ([pro7]-NKB) receptors on DA neurons were prevented by TTX (100nM) indicating that Na^+ channels were also implicated in the effects of tachykinins (Fig. 10A, C). This finding and the fact that suboptimal amounts of veratridine (0.2µM) were able to improve the protective effect of optimal concentrations of [pro7]-NKB (Fig. 10A) and GR73632 (not shown) suggested that the alkaloid and tachykinins promoted DA cell survival by a mechanism that was common. This was apparently not the case since the T-type calcium channel blocker flunarizine (5µM), which prevented the survival promoting effect of the alkaloid (Salthun-Lassalle et al., 2004), was unable to inhibit the effects of [pro7]-NKB or GR73632 (Fig. 10B). The neuroprotective effects of GR73632 and [pro7]-NKB were also resistant to nimodipine (10µM), an inhibitor of the L-type calcium channels. They were reduced, however, by the snail toxin ω-conotoxin MVIIA (0.5µM; Fig. 10B, C), indicating that inward calcium currents through N-type calcium channels played probably a key role in the survival of DA neurons mediated by NK_1 and NK_3 receptors. As expected, the protective effects of native tachykinins, SP and NKB, were also blocked by treatment with TTX and ω-conotoxin MVIIA (Fig. 10A-B). Interestingly, the rescuing effect of veratridine, which was prevented by TTX, remained unaffected by ω-conotoxin MVIIA (Fig. 10B), confirming that the veratridine and tachykinins acted by mechanisms, which were partly related but not identical.

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To confirm that tachykinin-mediated neuroprotection was linked to activation of inward Ca^{2+} currents, we measured intracellular calcium levels in the presence of the various test treatments (Fig. 10D). At concentrations of NK_1 and NK_3 agonists that promote optimal survival of DA neurons, we observed an increase in $[Ca^{2+}]_i$ of ~60-80% above control levels. The capacity of tachykinins to raise $[Ca^{2+}]_i$ was abolished by TTX (0.1µM) or ω-conotoxin MVIIA (0.5µM) which indicates that voltage-gated Na^+ channels and N-type calcium channels played a key role in this effect (Fig. 10D). Unlike ω-conotoxin MVIIA, however, the L-type calcium channel antagonist nimodipine (10µM) and the T-type calcium channel antagonist flunarizine (5µM) failed to reduce the rise in $[Ca^{2+}]_i$ induced by tachykinins (data not shown). Note that veratridine-mediated increase in intracellular calcium was blocked by TTX as expected but not by ω-conotoxin MVIIA (Fig. 10D).

Discussion

We demonstrate here that stimulation of NK_1 and NK_3 but not NK_2 receptors by native tachykinins or synthetic peptide agonists provides robust and selective neuroprotection against DA cell death in mesencephalic cultures. Neuroprotection was mimicked by, but independent of GDNF. It resulted from a depolarizing effect mediated through the activation of TTX-sensitive sodium channels and N-type voltage-gated calcium channels.

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Native and synthetic tachykinins increase the number of DA neurons through activation of NK_1 and NK_3 receptors

We observed that the native tachykinins SP, NKA and NKB substantially increased the number of TH^+ neurons when applied chronically to mesencephalic cultures. SP, NKA and NKB are believed to act preferentially at the NK_1, NK_2 and NK_3 receptor sites, respectively. We wished to determine precisely, however, which of these receptors were responsible for the effects of the native tachykinins on DA neurons. The specific NK_1 receptor antagonist GR71251 suppressed entirely the effect of SP on DA neurons whereas a co-treatment combining GR71251 and the specific NK_3 receptor antagonist SB218795 was required to prevent the action of NKB. This suggests, therefore, that the effect of SP occurred via NK_1 receptors whereas that of NKB required activation of both NK_1 and NK_3 receptors. The effect of NKA appeared mediated entirely by its non-preferential receptors since its action on DA neurons was resistant to the selective NK_2 receptor antagonist R396 and suppressed by a treatment combining the NK_1 and NK_3 receptor antagonists. Confirming that NK_1 and NK_3 receptors were crucial for the effects of tachykinins on DA neurons, the selective NK_1 agonist GR73632 and the selective NK_3 agonist [pro7]-NKB increased the number of DA neurons whereas the NK_2 agonist GR64349 had no effect. Accordingly, only mRNA transcripts encoding the NK_1 and NK_3 receptors were detected in mesencephalic cultures.

The activation of NK_1 and NK_3 receptors is truly neuroprotective for DA neurons

The increase in the number of TH^+ neurons produced by stimulation of NK_1 or NK_3 receptors may result from several mechanisms. The treatments may induce the proliferation of putative DA precursor cells, but this is unlikely since all TH^+ neuroblasts have exited the cell cycle in the mesencephalon of E15.5 embryonic brains used to generate the cultures

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(Rothman et al., 1980). Furthermore, the number of TH^+ cells in treated cultures never exceeded that of TH^+ neuroblasts detected just after plating, and [^{3}H]-methyl-thymidine, a marker of DNA synthesis, was never associated with TH^+ cells in tachykinin-treated cultures. The effect of the tachykinin agonists might be simply to restore the expression of TH within neurons that contain undetectable amounts of the enzyme as they entered a pre-morbid state (Hirsch et al., 1988; Michel and Agid, 1996). Even though tachykinins were reported to stimulate TH protein expression (Friedman et al., 1988), this possibility is doubtful since the tachykinin agonists failed to rescue the fraction of TH^+ neurons that had already disappeared when initiation of the treatments was delayed. The observation that the pancaspase inhibitor BOC-D-FMK was also able to increase the number of DA neurons in this model system indicates that the tachykinins probably reversed an ongoing apoptotic process that is occurring spontaneously (Salthun-Lassalle et al., 2004). The underlying mechanism was, however, not related to that described for the neurotransmitter adenosine and certain antimitotics, which were effective in this culture model through the repression of glial cell proliferation (Michel et al., 1999). Finally, it is worth noting that DA neurons treated with NK_1 and NK_3 receptor agonists efficiently accumulated DA by active transport, which indicates that these neurons were perfectly functional when protected by tachykinins.

TTX-sensitive sodium channels and N-type voltage-gated calcium channels are implicated in tachykinin-mediated neuroprotection

Two observations indicate that inward sodium currents were critical for tachykinin-mediated neuroprotection. The effect of NK_1 and NK_3 receptor agonists was mimicked by veratridine, an agonist of the voltage-gated Na^+ channels (Salthun-Lassalle et al., 2004) and prevented by TTX, an irreversible antagonist of these channels (Catterall, 1980). This result is consistent with electrophysiological data showing that stimulation of NK_3 receptors evokes

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excitatory responses in DA neurons of the substantia nigra (Nalivaiko et al., 1997). We have previously established that low-level activation of voltage-gated Na^+ channels by the alkaloid veratridine protects DA neurons via a mechanism that requires calcium influx through low-threshold activated T-type calcium channels (Salthun-Lassalle et al., 2004). T-type calcium channels are reported to be activated in response to SP in certain types of neurons (Ikeda et al., 2003). In our study, however, the effects of the tachykinin agonists were resistant to flunarizine, an inhibitor of these channels (Santi et al., 2002). High-threshold activated L- and N-type calcium channels which have been shown to contribute to the effects of tachykinins in several model systems (Bayguinov et al., 2003; Sculptoreanu and de Groat, 2003) might also participate to tachykinin-mediated neuroprotection in mesencephalic cultures. L-type calcium currents are implicated in the rescue of mesencephalic DA neurons by depolarizing concentrations of K^+ (Douhou et al., 2001) but a selective antagonist of these channels nimodipine failed to inhibit the action of tachykinins on these neurons. In contrast, N-type calcium channels were probably essential for the survival promoting effect of tachykinins. Indeed, blockade of these channels by the snail toxin ω-conotoxin MVIIA (Hirata et al., 1997) abolished neuroprotection by tachykinins. Consistent with this observation, the moderate rise in $[Ca^{2+}]_i$ elicited by tachykinins was also reversed by ω-conotoxin MVIIA. This elevation was also prevented by blocking Na^+ channels with TTX, which provides further support to the idea that intracellular calcium levels were crucially involved in the survival of these neurons. Finally, the efficacy of TTX to prevent the rise in calcium produced by tachykinins is an indication that Na^+ inward currents may precede the activation of N-type Ca^{2+} channels.

The present results demonstrate that N-type calcium channels possess in common with L-, and T-type calcium channels (Douhou et al., 2001; Salthun-Lassalle et al., 2004), the ability to modulate DA cell survival. These channels have different voltage ranges as well as

rates of activation and inactivation (Hammond, 2001) which probably explains why their recruitment depends largely upon the nature and intensity of the excitatory stimuli applied on DA neurons. Overall, the crucial need of DA neurons for electrical stimulation suggests (1) that they lack adequate excitatory inputs in culture and (2) that tachykinins could act as substitutes. It is interesting to note that only DA neurons in this model system appear dependent on such input. Finally, the rapid reversal of the effects of NK_1 and NK_3 receptor agonists when they are withdrawn indicates that tachykinin-dependent excitatory signals were a permanent requirement for long-term survival of DA neurons.

Is there a link between the survival promotion mediated by tachykinins and GDNF?

We wished to determine whether NK_1 and NK_3 receptor agonists could operate through a mechanism involving GDNF, a major neurotrophic factor for DA neurons (Choi-Lundberg et al., 1997). This was excluded since a neutralizing antibody that prevented the rescue of DA neurons by GDNF did not interfere with the effects of the NK_1 or NK_3 receptor agonists. Other autocrine or paracrine trophic factors were probably not implicated either, as shown by our experiments with conditioned media. These experiments support the idea that tachykinins and synthetic peptide agonists acted directly on DA neurons which express both NK_1 (Futami et al., 1998) and NK_3 receptors (Chen et al., 1998; Friedman et al., 2002). A doubt subsists, however since the presence of NK_1 receptors on DA neurons is still a question of debate (Futami et al., 1998) and the experiments using conditioned culture media might have failed to detect molecules having a short-range diffusion or a limited lifespan. Finally, GDNF being known to stimulate the synthesis of substance P (Ogun-Muyiwa et al., 1999), in particular in the striatum (Humpel et al., 1996), we envisaged the possibility that some of its effects might be mediated by tachykinins. This was unlikely since TTX which prevented the rescuing effect of tachykinins on DA neurons failed to reduce that of GDNF on the same

neurons (Salthun-Lassalle et al., 2004). Accordingly, the antagonists of NK_1 and NK_3 receptors failed to reduce the survival promoting activity of GDNF on DA neurons as well (not shown).

To conclude, we have shown that stimulation of the NK_1 and NK_3 receptors by native or synthetic agonists exerts neuroprotective effects that are highly selective for DA neurons in mesencephalic cultures. This indicates that these neurons may depend in part on tachykinins for their survival during development. Interestingly, survival promotion mediated by tachykinins in mesencephalic cultures was mimicked by GDNF, a trophic factor that prevents the death of DA neurons during development (Oo et al., 2003). Because GDNF is still effective when applied exogenously to injured DA neurons in the adult brain (Choi-Lundberg et al., 1997; Dauer and Przedborski, 2003), it is reasonable to ask whether tachykinins could be neuroprotective in neurodegenerative conditions where DA neurons die selectively such as Parkinson's disease and related disorders.

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FOOTNOTES

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Address correspondence to : Dr Patrick Michel, INSERM U679 (former INSERM U289), Experimental Neurology and Therapeutics, Bâtiment Pharmacie, Hôpital de la Salpêtrière, 47 bd de l'Hôpital, 75013 Paris, France.

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FIGURE LEGENDS

Figure 1. Native tachykinins and selective agonists of NK_1 and NK_3 receptors increase the number of TH^+ cells in mesencephalic cultures. Number of TH^+ neurons as a function of the concentrations of SP (***A***), NKA (***B***) and NKB (***C***). ***D,*** Number of TH^+ neurons in cultures exposed chronically to synthetic agonists of NK_1 (GR73632), NK_2 (GR64349) and NK_3 ([pro7]-NKB) receptors. All of the test compounds, except GR64349, increased TH^+ cell number. *$p<0.05$, different from corresponding control cultures. ***E,*** Illustration of the effects of SP, NKA and NKB on the number of TH^+ cells. Cell counts and illustrations were made using cultures at 10 DIV. Scale bar: 50µm.

Figure 2. NK_1 and NK_3 receptors mediate the increase in TH^+ cells produced by native and synthetic tachykinins. ***A,*** Increase in the number of TH^+ neurons produced by selective synthetic agonists of NK_1 (GR73632, 10nM), NK_2 (GR64349, 100nM) and NK_3 ([pro7]-NKB, 1nM) receptors. Inhibitory effects of selective antagonists of the NK_1, NK_2 and NK_3 receptors, GR71251, R396 and SB218795, respectively. Note that the effects of GR73632 and [pro7]-NKB were not additive. Inhibition of the effects provided by optimal concentrations of SP (***B,*** 10nM), NKA (***C,*** 100nM) and NKB (***D,*** 1nM) in the presence of selective NK receptor antagonists used at 1µM. *$p<0.05$, higher than corresponding control cultures. **$p<0.05$, significant inhibition by NK receptor antagonists. ***E,*** RT-PCR products of the NK_1, NK_2 and NK_3 receptors were resolved by gel electrophoresis in parallel with a 100bp ladder as molecular weight marker. Template mRNA was from mixed glial/neuronal (1), pure neuronal (2) and pure astrocyte (3) cultures. The positive control for the three NK receptors was mRNA from uterus (4); RT-PCR performed without RNA (5). Lower bands correspond to excess primers.

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Figure 3. The activation of NK_1 or NK_3 receptors provides true neuroprotection for mesencephalic DA neurons. ***A,*** Number of TH^+ neurons as a function of the age of the cultures following a chronic treatment with GR73632 (10nM), [pro7]-NKB (1nM), or the broad spectrum and cell-permeable caspase inhibitor Boc-D-FMK (60µM). *p<0.05, different from corresponding age-matched control cultures. ***B,*** Illustration showing that TH^+ cells do not incorporate [^{3}H]-methyl-thymidine following a treatment with GR73632. Similar results were obtained in the presence of [pro7]-NKB. Scale bar: 50µm.

Figure 4. The effects of NK_1 and NK_3 receptor agonists depend upon the duration of treatment and the time at which it is initiated. Number of TH^+ neurons in mesencephalic cultures exposed to 1nM [pro7]-NKB, at various time intervals between 0 and 10 DIV. ***A,*** [pro7]-NKB still increased survival when the treatment was postponed after plating. ***B,*** the effects of [pro7]-NKB were short-lived after withdrawal of the treatment. *p<0.05, different from corresponding control cultures. Similar results were obtained with the NK_1 receptor agonist GR73632.

Figure 5. DA neurons rescued by NK_1 and NK_3 agonists accumulate [^{3}H]-DA efficiently. ***A,*** Number of TH^+ neurons (filled bars) and neurons accumulating [^{3}H]-DA (open bars) in 10 DIV mesencephalic cultures treated chronically with the NK_1 agonist (GR73632, 10nM) or the NK_3 agonist ([pro7]-NKB, 1nM), or with the agonist of voltage-gated sodium channels veratridine (ver, 0.8µM). All three treatments increased the number of neurons that express TH and incorporate [^{3}H]-DA to the same extent. ***B,*** GR73632 and [pro7]-NKB, but not veratridine, substantially increased the uptake of [^{3}H]-DA per TH^+ neuron. *p<0.05, different from corresponding control cultures at 10 DIV. In control cultures, the number of TH^+

neurons was estimated at 880, and the accumulation rates of [^{3}H]-DA at 25 fmol/min/16mm culture well and 2.8x10^{-2} fmol/min/TH$^+$ neuron, respectively.

Figure 6. Illustration of the effects of NK_1 and NK_3 agonists on DA cell numbers and DA uptake. DA neurons from 10 DIV mesencephalic cultures identified by TH immunofluorescent cytochemistry (***left panel***) and subsequently by microautoradiographic detection of [^{3}H]-DA (***right panel***). Cultures were treated between 0-10 DIV with 10nM GR73632, 1nM [pro7]-NKB or 0.8µM veratridine as described above. Control cultures in which [^{3}H]-DA uptake was performed in the presence of 0.5µM of the DA uptake inhibitor GBR-12909 (***bottom panel***). White arrows point to cell bodies of neurons labeled with [^{3}H]-DA. Scale bar: 50µm.

Figure 7. The survival and trophic effects of NK_1 and NK_3 agonists are restricted to DA neurons. ***A,*** Number of TH$^+$ and MAP2$^+$ neurons in mesencephalic cultures exposed for 10 days to selective NK_1 (GR73632, 10nM) or NK_3 ([pro7]-NKB, 1nM) receptor agonists. ***B,*** Illustration showing that neither GR73632 nor [pro7]-NKB affected the number of MAP2$^+$ cells. ***C,*** Treatments with GR73632 and [pro7]-NKB strongly promoted the uptake of [^{3}H]-DA but had no effect on the uptake of [^{3}H]-GABA or [^{3}H]-5-HT. *p<0.05, different from corresponding control cultures. Control values for the number of TH$^+$ and MAP2$^+$ neurons per 16mm culture well at 10 DIV were 1150 and 420x10^3. Control values at 10 DIV for the uptakes of [^{3}H]-DA, [^{3}H]-GABA, and [^{3}H]-5-HT were 19 fmol/min/well, 3.8 pmol/min/well, and 8.8 fmol/min/well, respectively.

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Figure 8. Treatment with NK_1 and NK_3 tachykinin receptor agonists do not affect glial cell proliferation in mesencephalic cultures. The number of TH^+ neurons at 10 DIV (***A***), and the number of thymidine$^+$ nuclei at 7 DIV (***B***) in mesencephalic cultures maintained in the presence of the NK_1 agonist GR73632 (10nM), the NK_2 agonist GR64349 (100nM) and the NK_3 agonist [pro7]-NKB (1nM). AraC was used at 1µM. *$p<0.05$, different from corresponding control cultures. ***C,*** Illustration of the effects of [pro7]-NKB and araC on the number of thymidine$^+$ cells.

Figure 9. Role of GDNF or other trophic factors in the neuroprotective effects of NK_1 and NK_3 receptor agonists. ***A,*** Number of TH^+ neurons in mesencephalic cultures treated with GDNF (10ng/mL), NK_1 (GR73632) and NK_3 ([pro7]-NKB) receptor agonists in the presence, or not, of a neutralizing anti-GDNF antibody (AB 212 NA; 5µg/mL). ***B,*** The culture media conditioned by GR73632 or [pro7]-NKB-treated cultures (CM) increased the survival of DA neurons when transferred to receiving cultures. The effects of these conditioned media were completely inhibited by antagonists of NK_1 (GR71251) and NK_3 (SB218795) receptors, respectively. Note that the NK_2 receptor antagonist failed to inhibit the effect of the CM. *$p<0.05$, lower than corresponding cultures treated with GDNF; **$p<0.05$, lower than cultures maintained with conditioned medium (CM), alone. ( ) Signifies that GR73632 and [pro7]-NKB were not added directly to the cultures but transferred with the CM.

Figure 10. Role of voltage-gated Na^+ and Ca^{2+} channels in the neuroprotective effect of tachykinins. ***A,*** Number of TH^+ neurons at 10 DIV, in cultures that were exposed to veratridine (ver), GR73632 and [pro7]-NKB in the presence or not of TTX, an antagonist of the voltage-gated sodium channels. ***B,*** Number of TH^+ neurons at 10 DIV, in cultures that were exposed to veratridine, GR73632 and [pro7]-NKB in the presence or not of flunarizine

(flun), nimodipine (nimo) and ω-conotoxin MVIIA (ω-conot), antagonists of T-type, L-type and N-type voltage-gated calcium channels, respectively. Veratridine (0.2 or 0.8μM as indicated above); GR73632 (10nM); [pro7]-NKB (1nM); TTX (100nM); flunarizine (5μM); ω-conotoxin MVIIA (0.5μM). ***C,*** Immunofluorescent detection of TH^+ neurons in control cultures and in cultures treated chronically for 10 d with [pro7]-NKB in the presence or not of TTX or ω-conotoxin (ω-con). Scale bar: 50μm. ***D,*** Calcium-Green-1 fluorescence levels in 7 DIV cultures. Treatments with TTX or ω-conotoxin were used to modulate the effects of veratridine, GR73632 and [pro7]-NKB. *$p<0.05$, different from corresponding control cultures. **$p<0.05$, significant inhibition by the channel antagonist.

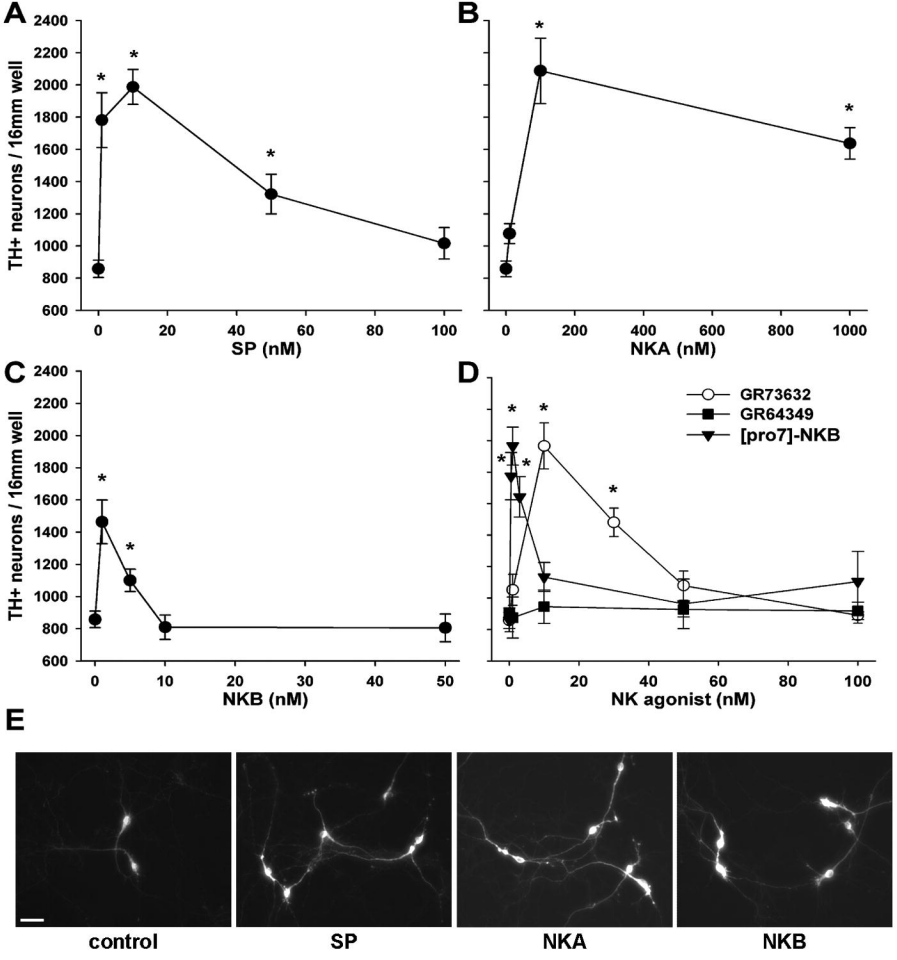
A
B
C
D
E
TH+ neurons / 16mm well
SP (nM)
NKA (nM)
NKB (nM)
NK agonist (nM)
GR73632
GR64349
[pro7]-NKB
control
SP
NKA
NKB

Figure 1

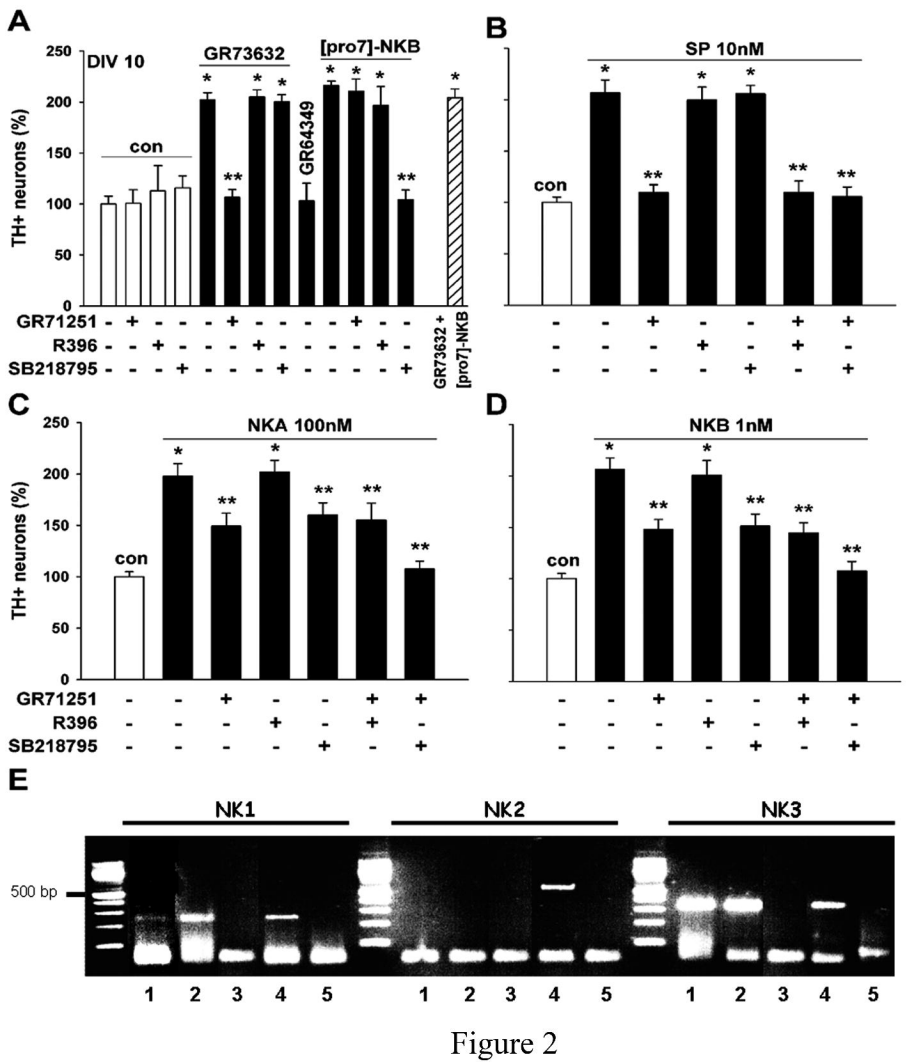
A
DIV 10
GR73632
[pro7]-NKB
GR64349
con
TH+ neurons (%)
250
200
150
100
50
0
GR71251
R396
SB218795
GR73632 + [pro7]-NKB
B
SP 10nM
con
C
NKA 100nM
D
NKB 1nM
E
NK1
NK2
NK3
500 bp
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

Figure 2

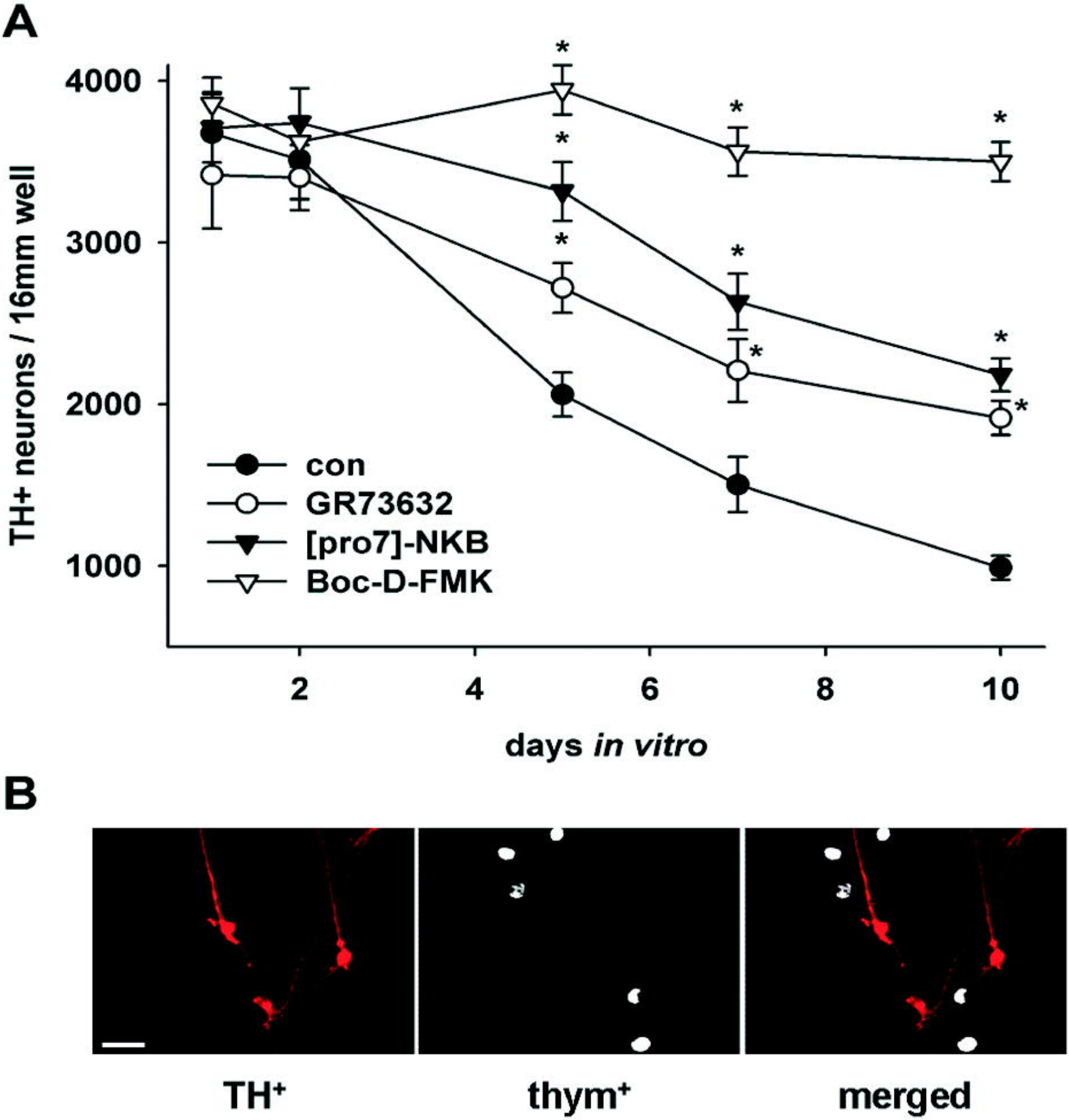
A
4000
3000
2000
1000
TH+ neurons / 16mm well
con
GR73632
[pro7]-NKB
Boc-D-FMK
2
4
6
8
10
days in vitro
B
TH+
thym+
merged

Figure 3

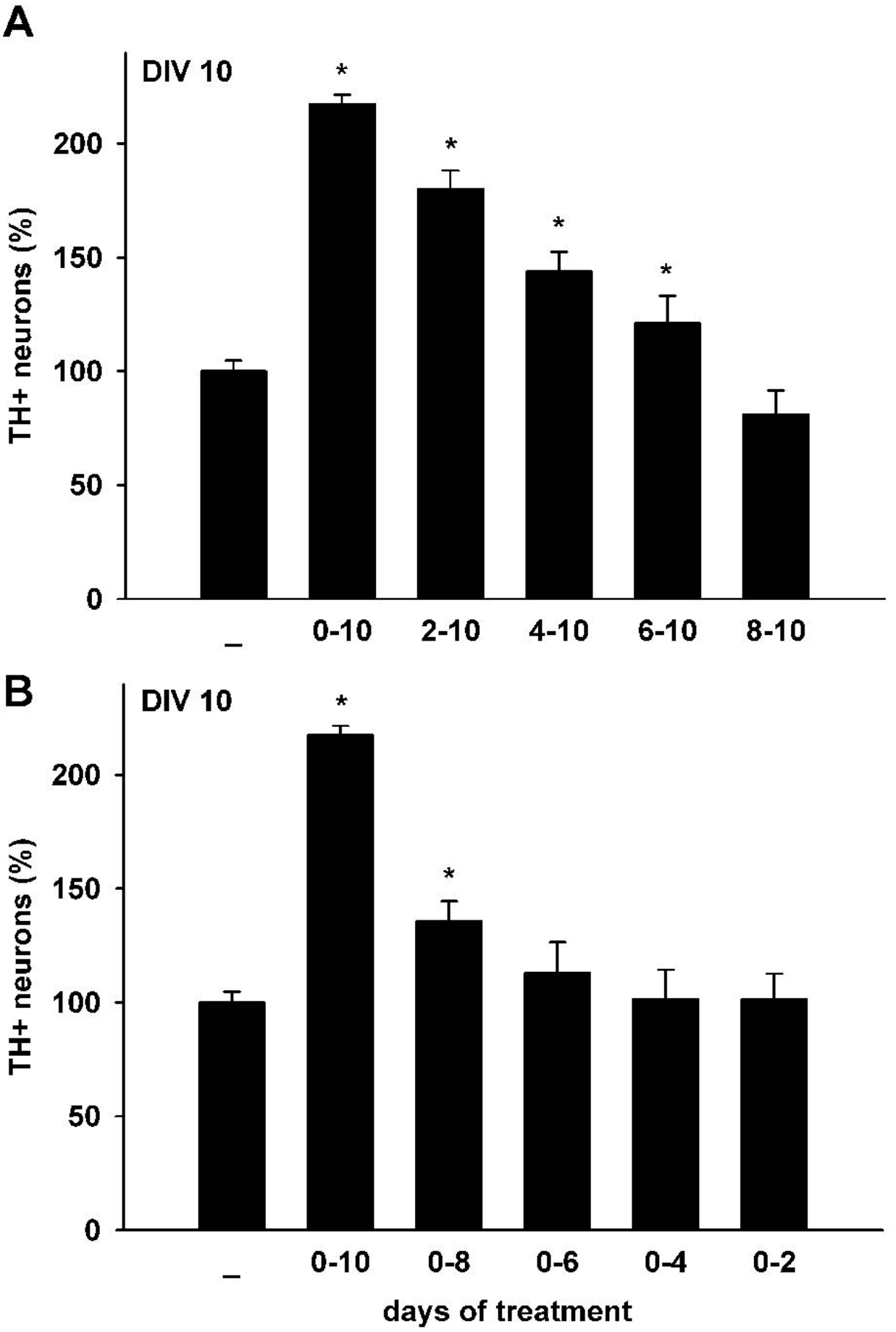
A
DIV 10
TH+ neurons (%)
200
150
100
50
0
*
*
*
*
–
0-10
2-10
4-10
6-10
8-10
B
DIV 10
TH+ neurons (%)
200
150
100
50
0
*
*
–
0-10
0-8
0-6
0-4
0-2
days of treatment

Figure 4

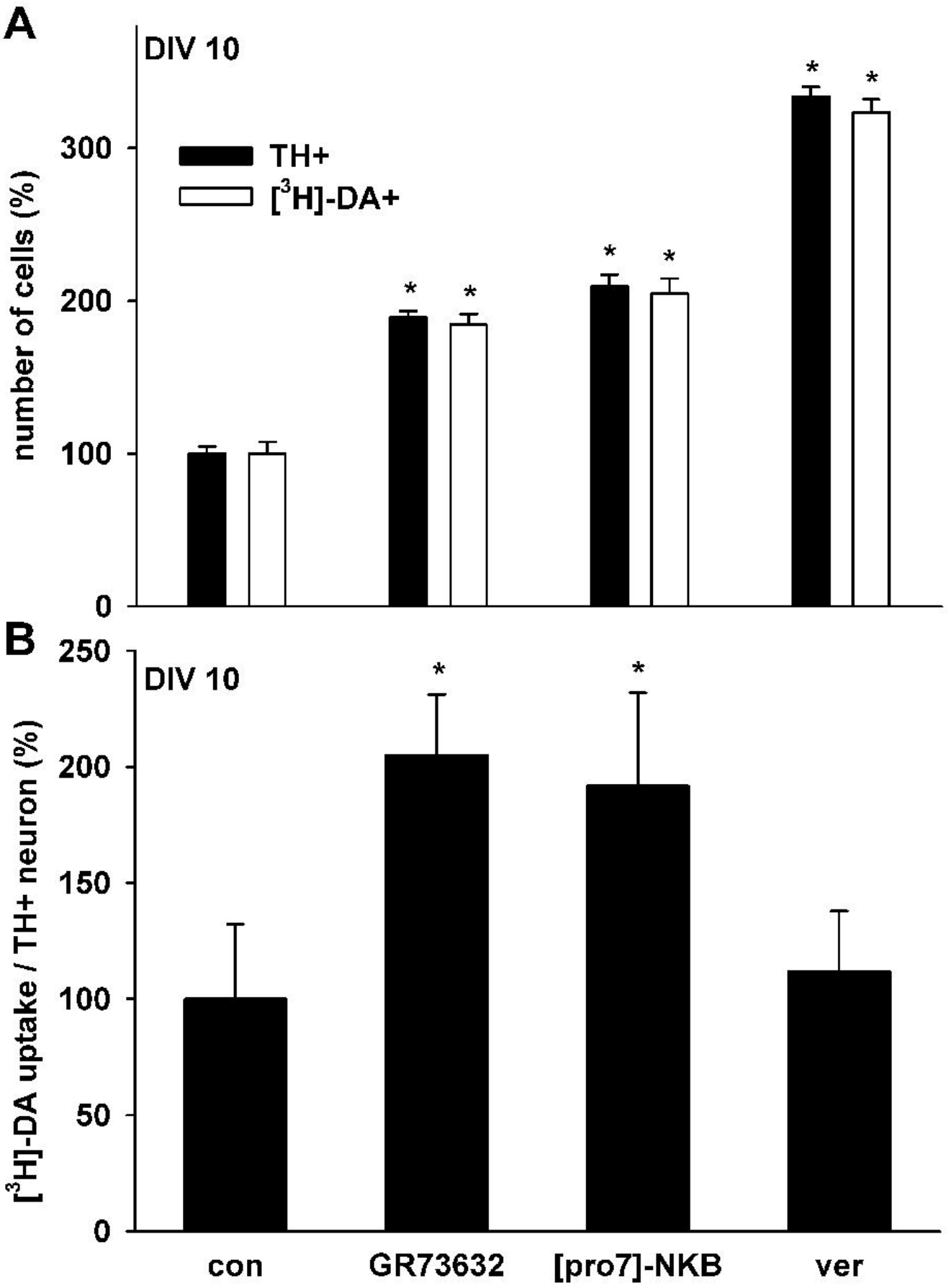
A
DIV 10
TH+
[3H]-DA+
number of cells (%)
0
100
200
300
*
B
DIV 10
[3H]-DA uptake / TH+ neuron (%)
0
50
100
150
200
250
con
GR73632
[pro7]-NKB
ver

Figure 5

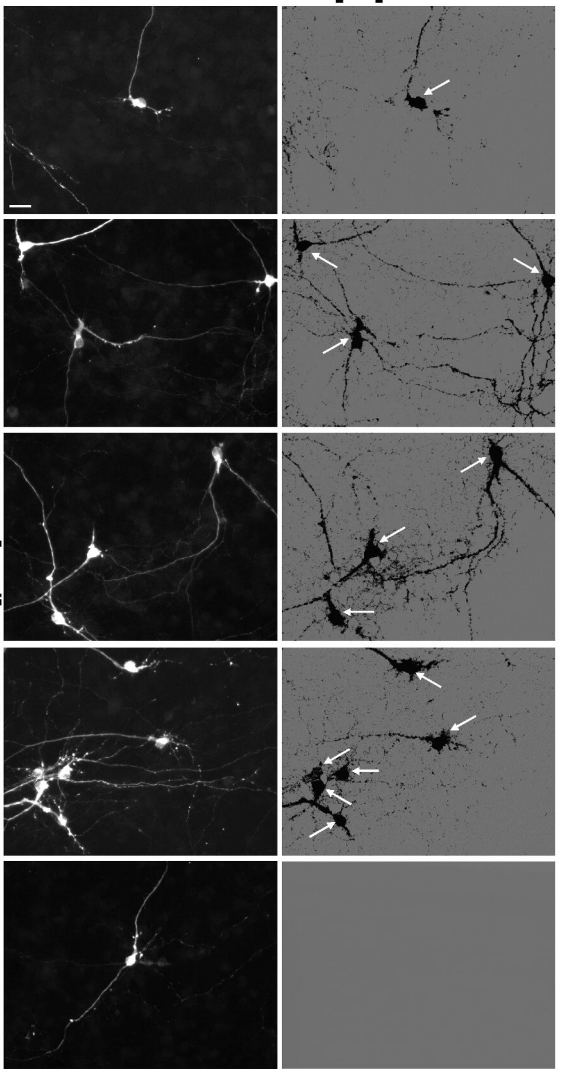
TH+ cells
[3H]-DA+ cells
control
GR73632
[pro7]-NKB
veratridine
GBR-12909

Figure 6

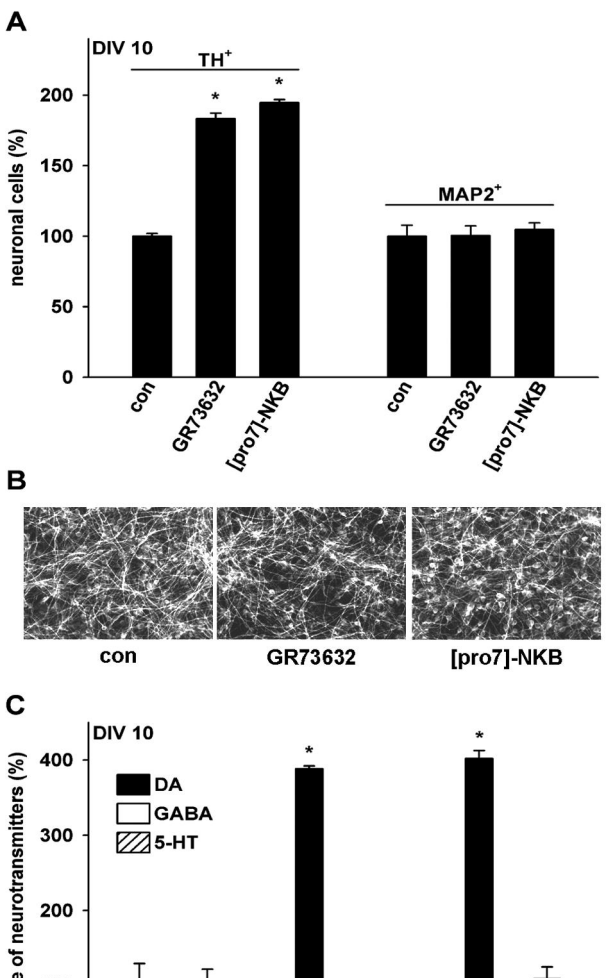
A
DIV 10
TH+
MAP2+
neuronal cells (%)
0
50
100
150
200
*
*
con
GR73632
[pro7]-NKB
con
GR73632
[pro7]-NKB
B
con
GR73632
[pro7]-NKB
C
DIV 10
DA
GABA
5-HT
uptake of neurotransmitters (%)
0
100
200
300
400
*
*
Con
GR73632
[pro7]-NKB

Figure 7

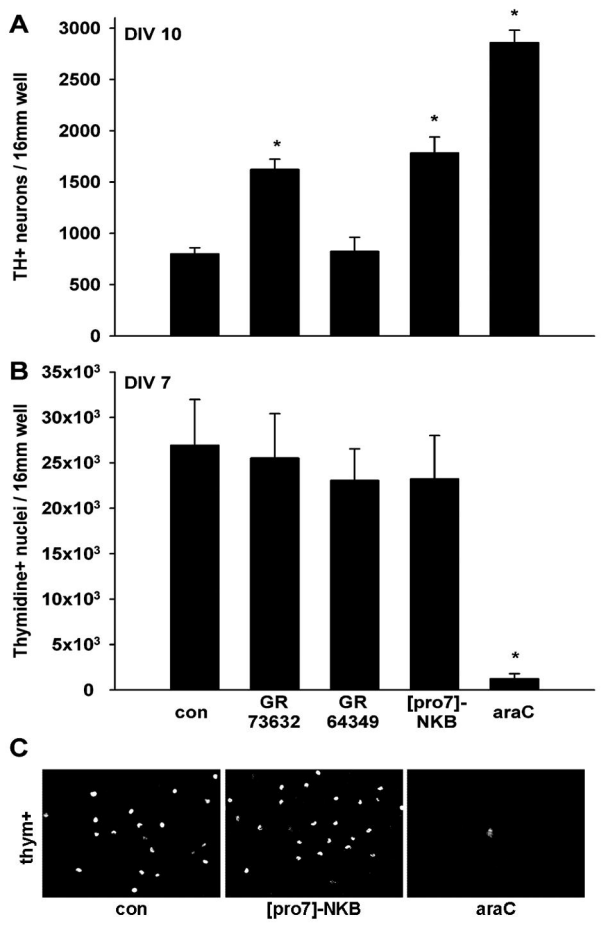
A
DIV 10
TH+ neurons / 16mm well
3000
2500
2000
1500
1000
500
0
*
*
*
B
DIV 7
Thymidine+ nuclei / 16mm well
35x10³
30x10³
25x10³
20x10³
15x10³
10x10³
5x10³
0
*
con
GR 73632
GR 64349
[pro7]-NKB
araC
C
thym+
con
[pro7]-NKB
araC

Figure 8

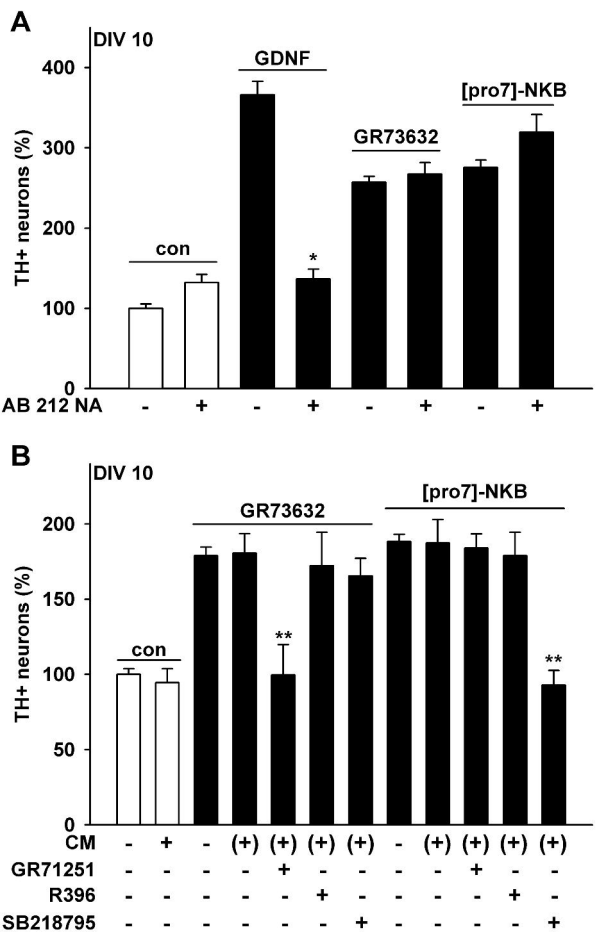
A
DIV 10
GDNF
[pro7]-NKB
GR73632
con
TH+ neurons (%)
400
300
200
100
0
*
AB 212 NA - + - + - + - +
B
DIV 10
GR73632
[pro7]-NKB
con
TH+ neurons (%)
200
150
100
50
0
**
**
CM - + - (+) (+) (+) (+) - (+) (+) (+) (+)
GR71251 - - - - + - - - - - + - -
R396 - - - - - + - - - - - + -
SB218795 - - - - - - + - - - - - +

Figure 9

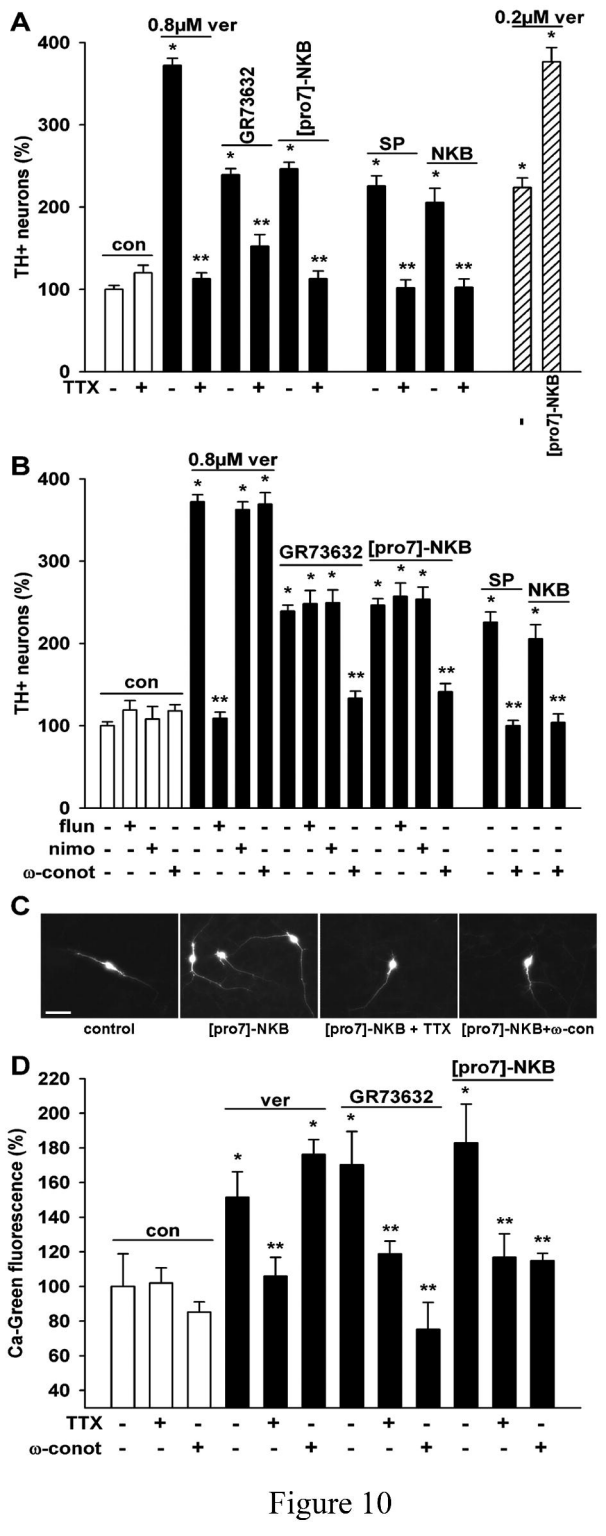
A
0.8μM ver
0.2μM ver
GR73632
[pro7]-NKB
SP
NKB
con
TH+ neurons (%)
TTX
[pro7]-NKB
B
0.8μM ver
GR73632
[pro7]-NKB
SP
NKB
con
TH+ neurons (%)
flun
nimo
ω-conot
C
control
[pro7]-NKB
[pro7]-NKB + TTX
[pro7]-NKB+ω-con
D
[pro7]-NKB
ver
GR73632
con
Ca-Green fluorescence (%)
TTX
ω-conot

Figure 10