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The Sleep Modulating Peptide Orexin-B Protects Midbrain Dopamine Neurons from Degeneration, Alone or in Cooperation with Nicotine

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Running title: Survival promotion of dopamine neurons by orexin

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ABBREVIATIONS: 2-APB: 2-aminoethoxydiphenyl borate; α BgTx: α -bungarotoxin; Ca^{2+}_{cyt} : cytosolic calcium; DA: dopamine; DANT: dantrolene; DIV: day in vitro; EMPA: N-ethyl-2-[(6-methoxy-3-pyridinyl)(2-methylphenyl)sulfonyl]amino]-N-(3-pyridinylmethyl)-acetamide; ER: endoplasmic reticulum; GDNF: Glial cell line-derived neurotrophic factor; IP3: inositol-1,4,5-triphosphate; IP3R: IP3 receptor; MAP-2: microtubule-associated protein-2; MECA: mecamylamine; nAChR: nicotinic acetylcholine receptor; NIC: nicotine; OX: orexin; OX1R: orexin1 receptor; OX2R: orexin2 receptor; PBS: phosphate-buffered saline; PD: Parkinson disease; RBD: Rapid eye movement sleep Behavior Disorder; SB408124: N-(6,8-Difluoro-2-methyl-4-quinolonyl)-N'-[4-(dimethylamino) phenyl]-urea; SN: substantia nigra; TCS1102: N-[1,1'-Biphenyl]-2-yl-1-[2-[(1-methyl-1H-benzimidazol-2-yl)thio]acetyl-2-pyrrolidine dicarboxamide; TH: tyrosine hydroxylase. VDDC: voltage-dependent calcium channels.

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Abstract

To determine whether orexinergic hypothalamic peptides can influence the survival of brainstem dopamine (DA) neurons, we used a model system of rat midbrain cultures in which DA neurons degenerate spontaneously and progressively as they mature. We established that orexin (OX) B provides partial but significant protection to spontaneously dying DA neurons whereas the homologous peptide OXA has only marginal effects. Importantly, DA neurons rescued by OXB accumulated DA efficiently by active transport suggesting that they were functional. G-protein-coupled OX1 and OX2 receptors were both present on DA neurons but the protective effect of OXB was attributable solely to OX2 receptors; a selective inhibitor of this receptor subtype EMPA, suppressed this effect whereas a selective agonist [Ala11,D-Leu15]-OXB reproduced it. Survival promotion by OXB required intracellular calcium mobilization via IP3 and ryanodine receptors. Nicotine, a well-known neuroprotective molecule for DA neurons improved OXB-mediated rescue through the activation of α -bungarotoxin-sensitive (presumably $\alpha 7$) nicotinic receptors, although nicotine had no effect on its own. Altogether, our data suggest that the loss of hypothalamic orexinergic neurons that is occurring in Parkinson disease might confer an increased vulnerability to midbrain DA neurons in this disorder.

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Introduction

Orexin-(OX) A and OXB also known as hypocretin-1 and -2 are small hypothalamic neuropeptides derived from a common prepro-OX precursor through proteolytic processing (Sakurai et al., 1998; de Lecea et al., 1998). The two OX peptides which present high sequence homology in their C-terminal half regions (Takai et al., 2006), are highly conserved between humans and mice, with identical OXA sequences and just two amino acid substitutions in OXB (Sakurai et al., 1998). After being presumably synaptically released (de Lecea et al., 1998), OXA and OXB cause the excitation of target neurons through the activation of G-protein-coupled receptors, namely OX1 receptors (OX1R) and OX2 receptors (OX2R). Whereas OXA signals through both OX1R and OX2R, OXB is thought to operate mainly through OX2R (Scammell and Winrow, 2011; Gotter et al., 2012). The effects of OX peptides on their cognate receptors are mediated intracellularly through a rise in cytosolic calcium ($\text{Ca}^{2+}_{\text{cyt}}$) occurring primarily through intracellular calcium release channels even if other mechanisms implicating voltage-dependent calcium channels (VDCC) and transient receptor potential channels have been also described (Kukkonen et al., 2001; Kohlmeier et al., 2008; Nakamura et al., 2010).

The orexin system is involved in feeding behaviors and reward processes and it also operates as a critical regulator of sleep/wake states (Haynes et al., 2002; Boutrel et al., 2010; Perez-Leighton et al., 2013; Tsujino and Sakurai, 2013). Lack of orexin neurotransmission produces narcolepsy, a disabling sleep disorder associated with daytime sleep attacks, nocturnal insomnia, hallucinations and Rapid eye movement sleep Behaviour Disorder (RBD) (Lin et 1999; Peyron et al., 2000). OX neurons also partially degenerate in Parkinson disease (PD) (Fronczek et al., 2007; Thannickal et al., 2007), a motor disorder which is primarily caused by the loss of substantia nigra (SN) dopamine (DA) neurons in the brainstem (Dauer and Przedborski 2003; Schultz, 2007). This probably explains why some of the symptoms

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encountered in narcolepsy and more specifically RBD, are also frequently associated with PD (Thannickal et al., 2007; De Cock et al, 2008).

Interestingly, prospective studies have estimated that at least 40-65% of patients with idiopathic RBD will eventually develop a defined neurodegenerative phenotype, almost always a 'synucleinopathy' and most frequently PD (Boeve et al., 2001; Postuma et al., 2012; Arnulf, 2012). Therefore, one may assume that the molecular mechanism producing sleep dysregulation in PD patients may be linked to some extent to that causing DA cell demise and that OX peptides may be involved, directly or indirectly, in this process. Supporting this possibility, OXA was reported recently to attenuate the effects of the dopaminergic toxin 1-methyl-4-phenylpyridinium in SHSY-5Y cells (Feng et al, 2014). However, there is presently no study addressing the effect of OX peptides on primary DA neurons.

In the present study, we tested the survival promoting effects of OXA and OXB in a model system of midbrain cultures in which DA neurons degenerate progressively and selectively as they mature (Guerreiro et al., 2008; Toulorge et al., 2011). We were also interested in determining whether nicotine (NIC), a well-known neuroprotective molecule for DA neurons (Quik et al., 2007; Srinivasan et al, 2014), had an impact on the effect of OX peptides. Our data demonstrate that OXB promotes DA cell survival in midbrain cultures through the activation of OX2R and that NIC amplifies this effect through the activation of $\alpha 7$ nicotinic acetylcholine receptors (nAChRs). OXA appears, however, to have marginal effects on DA cell survival.

Material and methods

Pharmacological reagents

OXB, the modified OXB peptide [Ala11, D-Leu15]-OXB, the dual OXR antagonist N- [1,1'-Biphenyl]-2-yl-1-[2-[(1-methyl-1H-benzimidazol-2-yl)thio]acetyl-2-pyrrolidine

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dicarboxamide (TCS1102), the selective OX1R antagonist N-(6,8-Difluoro-2-methyl-4-quinoliny)-N'-[4-(dimethylamino)-phenyl]urea (SB408124), the OX2R antagonist N-Ethyl-2-[(6-methoxy-3-pyridinyl)[(2-methylphenyl)sulfonyl]amino]-N-(3-pyridinylmethyl)-acetamide (EMPA), the α 7nAChR antagonist α -bungarotoxin (α BgTx) and the inositol-1,4,5-triphosphate receptor (IP3R) antagonist 2-aminoethoxydiphenyl borate (2-APB) were all from Tocris Bioscience (RD Systems Europe, Lille, France). OXA was purchased either from Tocris Bioscience or Enzo Life Sciences (Villeurbanne, France). The blockers of VDCC nifedipine, flunarizine and ω -conotoxin MVIIA, NIC, the non-specific nAChR antagonist mecamylamine (MECA) and the ryanodine receptor (RyR) blocker dantrolene (DANT) were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Midbrain cultures

Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), European Directive 86/609, and the guidelines of the local institutional animal care and use committee. Mixed cultures were prepared from the ventral midbrain of gestational age 15.5 days Wistar rat embryos (Janvier LABS, Le Genest-St-Isle, France). Dissociated cells in suspension obtained by mechanical trituration of midbrain tissue were seeded at a density of $1.2-1.5 \times 10^5$ cells/cm² onto Nunc 48-well multi-dish plates or, when appropriate, onto Nunc Lab-Tek glass chamber slides (Thermo Scientific, Villebon-sur-Yvette, France) pre-coated with 1 mg/mL polyethylenimine diluted in borate buffer pH 8.3 as previously described (Toulonge et al., 2011). Cultured cells were maintained in N5 culture medium (Kaufman and Barrett, 1983) supplemented with 5 mM glucose, 5% horse serum, and 0.5% fetal calf serum, except for the first 3 days in vitro (DIV), when the concentration of fetal calf serum was set at 2.5% to favor initial maturation of the cultures (Toulonge et al., 2011). DA neurons detected by tyrosine hydroxylase (TH)

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immunofluorescence staining, represented approximately 2-3% of the total number of neuronal cells present in these cultures, just after plating. These cultures did not contain noradrenergic neurons from the locus coeruleus (Traver et al., 2006). Culture medium replacements (350 μ l / 500 μ l) and pharmacological treatments were carried out every day. Stock solutions of OX peptides were made in distilled water and stored at 4 °C for up to 5 days for optimal results.

Protein detection by immunofluorescence

The cultures, fixed for 12 min using 4% formaldehyde in Dulbecco's phosphate-buffered saline (PBS), were washed twice with PBS before an incubation step at 4°C for 24-72 h with primary antibodies. A monoclonal anti-TH antibody diluted 1/5000 (ImmunoStar Inc, WI, USA) or a polyclonal anti-TH antibody diluted 1/1000 (US Biologicals, Salem, MA, USA) were used to assess the survival of DA neurons whereas all neurons, regardless of their neurotransmitter phenotype, were identified on the basis of their content in microtubule-associated protein (MAP-2) using a monoclonal antibody diluted 1/50 (clone AP-20, Sigma-Aldrich). Astrocytes and more generally glial cells were characterized using a rabbit glial fibrillary acidic protein antibody (GFAP) (1/100; Dako, Trappes, France) and a monoclonal vimentin antibody (1/100; Clone V9, Sigma Aldrich), respectively. OX1R was detected with a rabbit polyclonal antibody (# ab68718; 1/100) and OXR2 with a goat polyclonal antibody (# ab115024; 1/100) both obtained from Abcam (Cambridge, UK). All primary antibodies were diluted in PBS containing 0.2 % Triton X-100, with the exception of the two OXR antibodies which were diluted in PBS only. Detection of the primary antibodies was performed with appropriate Alexa Fluor conjugates of anti-mouse, anti-rabbit or anti-goat secondary antibodies (Invitrogen, Carlsbad, CA). Cell counting was performed at 200x magnification using a 20x objective matched with a 10x ocular. The number of TH⁺ neurons in each culture

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well was estimated after counting 20 visual fields distributed along the X- and Y-axes. Counts of MAP-2⁺ neurons were performed in 6 visual fields randomly distributed in the whole surface area of each culture well.

Western blotting analysis

Cultures were washed once with cold PBS and cells were scraped off with 50 μ l of Laemmli buffer supplemented with 0.5 M dithiothreitol. Proteins extracts (40 μ g of protein per lane) were resolved on NuPAGE Novex precast 10% Bis-Tris gels (Life Technologies, Grand Island, NY) and then transferred onto 0.2- μ m nitrocellulose membranes before incubation overnight at 4°C with OX1R (1/500) and OX2R (1/1000) antibodies. Membranes were then probed with infrared fluorescence-conjugated secondary antibodies, a goat anti-rabbit IRDye 800CW and a donkey anti-goat IRDye 680RD, both diluted 1/10000 in PBS-Tween 0,05% (LI-COR Biosciences, Lincoln, NE). Proteins were visualized by scanning the membranes with an Odyssey infrared imaging system (LI-COR Biosciences).

Measurement of DA uptake

The functional integrity and synaptic function of DA neurons were evaluated by their ability to accumulate tritiated DA (50 nM; 40 Ci/mmol, Perkin Elmer, Waltham, MA, USA), as previously described (Salthun-Lassalle et al., 2005).

Quantification of proliferating cells

[Methyl-³H]-thymidine (specific activity, 70-90Ci/mmol; Perkin Elmer) a marker of DNA synthesis was used to label and quantify proliferating cells as described previously (Guerreiro et al., 2008).

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Quantification of cytosolic calcium levels

Changes in free cytosolic ($\text{Ca}^{2+}_{\text{cyt}}$) calcium levels were monitored in individual neurons using the cell-permeant calcium probe fluo-4-acetomethyl-ester (Fluo-4 Direct kit; Invitrogen, Cergy Pontoise, France). Pre-loading times with fluo-4 were 20 min and calcium traces were followed as a function of time to assess $\text{Ca}^{2+}_{\text{cyt}}$ at steady state levels. Results are expressed as fractional change in fluorescence relative to baseline (F/F_0). Data acquisition was performed using the HCI software (Hamamatsu Corp., Bridgewater, NJ, USA) and a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan) equipped with an ORCA-D2 digital camera (Hamamatsu). Fluorescent images of randomly chosen fields were acquired with a 20x fluorescent objective. Note that because of technical limitations imposed by our culture model, calcium data were extrapolated from the whole population of neuronal cells to the small number of DA neurons present in midbrain cultures. This extrapolation appears acceptable since the receptor that mediates the effects of OX is also expressed by a majority of non-dopaminergic mesencephalic neurons.

Statistical analysis

Multiple comparisons against a single reference group were made by one-way analysis of variance followed by Dunnett's test. When all pairwise comparisons were required, the Student-Newman-Keuls test was used. SEM values were derived from at least five independent experiments.

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Results

OXB exerts trophic effects for DA neurons in midbrain cultures

We found that OXB produces a concentration-dependent increase in the number of DA (TH⁺) neurons when applied chronically for 10 DIV to rat midbrain cultures (Fig. 1A). The effect of OXB plateaued between 10-30 nM. In this range of concentrations, the increase in TH⁺ cell numbers was approximately two-fold. The concentration of OXB causing half of the maximal increase in TH⁺ cell numbers was estimated to be 1 nM. The peptide OXA which is structurally related to OXB exerted limited effects and only at the highest concentrations tested (Fig. 1A). The trophic factor GDNF (20 ng/ml) used as reference neuroprotective peptide for DA neurons (Pascual et al., 2008) was slightly more efficacious than OXB in this experimental setting. An antibody with the capacity to neutralize the trophic effect of GDNF was unable to reduce OXB-mediated neuroprotection, which indicates that the effect of OXB was unrelated to that of GDNF (unshown data). Of interest, the increase in TH⁺ cell numbers elicited by OXB was associated with a proportional elevation in [³H]-DA uptake, a biochemical parameter taken as an index of the survival and function of DA neurons (Fig. 1B). The effect of OXB for DA neurons is illustrated in Fig. 1C.

OXB provides selective protection to vulnerable DA neurons

When comparing TH⁺ cell numbers at 0 DIV (i.e., at the time of plating) and at 10 DIV, we found that more than 70% of these neurons had died in the course of maturation of the cultures (Fig. 2A), in good agreement with previous reports (Salthun-Lassalle et al., 2004; Toulorge et al., 2011). This indicates that the increase in TH⁺ cell numbers observed in the presence of OXB is most likely due to a reduction of DA cell demise that is spontaneous in this model system. Note that the effect of OXB was specific to DA neurons, as other midbrain

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neurons characterized by their content in microtubule-associated protein-2 (MAP-2) remained unaffected by OXB in the same cultures (Fig. 2B).

OXB does not affect glial cell proliferation

Precursor cells of astrocytes have been shown to participate actively to DA cell demise in this culture system (Mourlevat et al, 2003; Rousseau et al, 2013) which suggests that they may represent a possible target for the action of OXB. Also in favor of this possibility, it was reported that OXB can exert anti-proliferative effects on some cell types, including rat calvarial osteoblast-like cells (Ziolkowska et al., 2008) and rat adrenocortical cells (Spinazzi et al., 2005). The assessment of proliferation by a measure of the incorporation of thymidine revealed that OXB (10 nM) had, however, no significant impact on glial cell division in this culture model (astrocytes and their precursor cells represent more than 95% of all dividing glial cells in mature midbrain cultures) (Fig. 3A,B). At variance a treatment with ARA-C (1 μ M), an anti-mitotic drug protective for DA neurons in this experimental setting when added to the cultures in the presence of MK-801 (1 μ M) to prevent secondary excitotoxic stress associated with culture medium changes (Mourlevat et al., 2003), resulted in a strong reduction of thymidine accumulation. Figure 3C confirms that neither astrocytic precursor cells that only express vimentin (red fluorescence) nor astrocytes that express both GFAP and vimentin (yellowish-green fluorescence) were affected by a treatment with OXB. In comparison ARA-C had a drastic repressive effect on all types of glial cells. Thus, we may assume that OXB was not protective via a direct effect on the astrocytes or their precursor cells.

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Both OX receptor subtypes are present on DA neurons

Using polyclonal antibodies for each OXR subtype, we established that OX1R and OX2R were present in a large fraction of neuronal cells in midbrain cultures and in particular in the subpopulation of DA neurons characterized on the basis of their TH content (Fig. 4A). The specificity of OX1R and OX2R antibodies was confirmed by western immunoblotting analysis (Fig. 4B) as the gel-resolved proteins had expected sizes of about 50 and 40 kDa, respectively (Karteris et al., 2005).

The effects of OXB are mediated through OX2R

We wished to determine which receptor subtype was involved in the protective effects of OXB for DA neurons (Fig. 4C). Using a pharmacological approach, we found that both the dual orexin receptor antagonist TCS1102 (1 nM) and the selective OX2R antagonist EMPA (100 nM) suppressed the survival promoting effects of OXB for DA neurons while neither TCS1102 nor EMPA had an impact *per se* on the survival of these neurons. The selective OX1R antagonist SB408124 (1 μ M) failed, however, to reduce DA cell rescue by OXB, indicating that only the OX2R subtype was involved in the peptide effect. Consistent with these observations, the selective agonist of OX2R [Ala11, D-Leu15]-OXB (10 nM) [a modified peptide where the L-leucine residues at positions of 11 and 15 of OXB are replaced by L-alanine and D-Leucine (Asahi et al., 2003)] mimicked the rescuing effect of OXB for DA neurons and this protective effect was prevented by EMPA. Note that the small protective effect of OXA for DA neurons was also abolished by blockade of OX2R with EMPA. Finally, we established that OXA (10 nM) was not able to significantly improve the effect of OXB (10 nM).

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Role of VDCC and ER calcium release channels in the effects of OXB

Because we found previously that intracellular calcium plays a key role in the control of DA cell survival (Salthun-Lassalle et al., 2004; Guerreiro et al., 2008), we wished to determine whether blockers of VDCC or endoplasmic reticulum (ER) calcium release channels were capable to reduce OXB-mediated neuroprotection. The protective effect of OXB was resistant to the blockade of L-type, T-type or N-Type VDCC by nifedipine (20 μ M), flunarizine (5 μ M) and ω -conotoxin MVIIA (1 μ M), respectively (Fig. 5). The effects of OXB were, however, abolished by blockade of IP3Rs with 2-APB (10 μ M) (Fig 5). Blockade of RyR channels with DANT (30 μ M) also reduced substantially but only partially OXB-mediated DA cell rescue. Note that neither 2-APB nor DANT had an impact alone on the survival of DA neurons.

NIC can improve the neuroprotective action of OXB for DA neurons via the activation of α 7 nAChRs

We then established that the partial rescue of DA neurons by OXB was improved by the alkaloid NIC (1 μ M) (Fig. 6), which is protective in experimental situations that mimic DA cell death in PD (Quik et al., 2007; Toulorge et al., 2011). A non-specific blocker of nAChRs, MECA (50 μ M) (Papke et al, 2001; Rabenstein et al, 2006) and a specific blocker of brain α 7 nAChRs α BgTx (1 μ M) (Dickinson et al, 2008) prevented the effect of NIC but not that of OXB. MECA and BgTx also failed to reduce DA cell survival when applied alone to the cultures. TCS1102 (1 nM), the dual OX receptor antagonist that prevented the protective effect of OXB, also abolished that of NIC. As expected from previous studies (Toulorge et al., 2011), NIC afforded no protection by itself in this experimental setting. Interestingly, a selective α 7 nicotinic receptor agonist PNU 282987 (Bodnar et al., 2005) also improved the

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action of OXB although with lesser efficacy than NIC. Like NIC, PNU 282987 failed to protect DA neurons in the absence of OXB.

Modulation of $\text{Ca}^{2+}_{\text{cyt}}$ by OXB and NIC

We then used the fluorescent dye fluo-4 for imaging $\text{Ca}^{2+}_{\text{cyt}}$ in cultures exposed to OXB and other treatments of interest. We failed to detect a significant rise in basal $\text{Ca}^{2+}_{\text{cyt}}$ levels in cultures receiving OXB, at concentrations that were neuroprotective (Fig. 7). As expected from previous studies (Toulorge et al., 2011), NIC alone also failed to elevate basal calcium levels. Yet, NIC caused a significant and persistent increase in $\text{Ca}^{2+}_{\text{cyt}}$ when applied to cultures exposed chronically to OXB. Confirming that the rise in calcium elicited by NIC depended on the activation of OX2R by OXB, it was suppressed by blocking OX2R with EMPA.

Discussion

Present data show that the hypothalamic peptide OXB protects vulnerable midbrain DA neurons from degeneration and preserves their function while the homologous peptide OXA has only limited effects. DA cell rescue by OXB was independent of glial cells. Instead it occurred through the activation of OX2R on DA neurons, leading subsequently to intracellular calcium mobilization via stimulation of ER calcium release channels. Of interest, NIC enhanced the survival promoting action of OXB, although it had no effect on its own.

OXB exerts protective effects for DA neurons

We established that the number of TH^+ neurons was increased selectively and in a concentration-dependent manner in midbrain cultures chronically exposed to OXB whereas the homologous peptide OXA had only limited effects. The effect of OXB could possibly be explained by the induction of the TH enzyme in midbrain neurons not expressing this protein

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originally, as reported previously by us (Traver et al., 2006) and by others (He et al., 2011) in other brain catecholaminergic culture systems. The number of TH⁺ neurons in 10 DIV midbrain cultures treated chronically with OXB being approximately twice higher than in control cultures but still lower than in sister cultures taken for evaluation just after plating, one may reasonably assume that OXB operated by reducing DA cell demise that occurs spontaneously and selectively in this culture system by apoptosis (Salthun-Lassalle et al., 2004).

OXB exerts protective effects for DA neurons through the activation of OX2R

The presence of mRNA encoding the two orexin receptors, OX1R and OX2R has been previously reported in midbrain SN DA neurons (Marcus et al., 2001) and direct orexinergic inputs have been described onto these neurons in both rodents and humans (Peyron et al., 1998, Hrabovszky et al., 2013). In line with these observations, we demonstrated that OX1R and OX2R were expressed by a majority of DA neurons in midbrain cultures, letting us to assume that the protective action of OXB resulted from a direct effect on these neurons.

We next wished to identify the receptor subtype involved in OXB-mediated DA cell rescue. We first showed that the dual OX receptor antagonist TCS1102 and the selective OX2R antagonist EMPA (Malherbe et al., 2009) suppressed the protection provided by OXB, thus clearly implicating OX2R in this effect. In line with this interpretation, the effect of OXB was resistant to the OX1R antagonist SB408124 (Langmead et al., 2004) and it was mimicked by [Ala11, D-Leu15]-OXB, a modified OXB neuropeptide showing a 400-fold selectivity for OX2R over OX1R (Asahi et al., 2003). This conclusion is also in agreement with various reports showing that OXB signals principally through OX2R (Sakurai et al., 1998; Gotter et al., 2012). Note that the small survival promoting action of OXA was also blocked by EMPA, suggesting the implication of OX2R in this effect. OXA and OXB having an equal affinity for

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OX2R (Gotter et al., 2012), one may assume that OXB was more effective than OXA because it provided a more sustained response through this receptor in a context of chronic neurodegeneration. Coherent with this view, OXA and OXB were reported to be equally neuroprotective in a model of cortical cultures wherein neuronal cells undergo acute insults (Sokołowska et al., 2014) whereas efficacy of OXA decreased over time in a culture system mimicking mitochondrial dysfunction in PD (Feng et al, 2014).

OXB exerts protective effects for DA neurons by stimulating ER calcium release channels

OX peptides being able to modulate intracellular calcium levels in target neurons (Kukkonen et al., 2001; Ishibashi et al., 2005; Nakamura et al., 2010), we wished to determine whether calcium ions intervened in the protective effects of OXB for DA neurons. Blockade of VDCC failed to reduce the survival promoting effects of OXB whereas 2-APB which selectively blocks endoplasmic IP3R totally abolished the protective effect of OXB. This indicates that OXB probably stimulated IP3 production in DA neurons, presumably through a phospholipase C (PLC)-inositol IP3 pathway and as a result, favored the mobilization of ER calcium through IP3R. This is consistent with the known coupling of OX2Rs to PLC via Gq (Karteris et al., 2005; Zawilska et al., 2013). Note that 2-APB did not cause by itself a reduction in DA cell survival, indicating that the need for IP3R activation in survival promotion emerged only in the population of DA neurons the most susceptible to degeneration and responsive to OXB treatment. It is also worth noting that blocking RyR channels with DANT also partially inhibited the rescue of DA neurons by OXB suggesting that ER calcium released via IP3R might also serve to activate RyR through a calcium-induced calcium release mechanism. Consistent with this possibility, a cross talk between IP3R and RyR has been already described in skeletal muscle cells (Tjondrokoesoemo et al.,

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2013). Present data are also globally consistent with previous studies showing that ER calcium pools may be crucial for the control of DA cell survival in a state of chronic neurodegeneration (Cali et al., 2012; Michel et al., 2013).

OXB can unmask the protective effects of NIC for DA neurons

Our data show that OXB protects only a fraction of DA neurons committed to die in the present paradigm. Hence, we wished to determine whether it was possible to improve the action of OXB with NIC, an alkaloid known to exert protective effects for DA neurons in a number of experimental models of PD (Quik et al., 2007; Michel et al., 2013).

NIC was indeed able to improve the rescuing effects of OXB for DA neurons via a mechanism blocked by α BgTx which demonstrates the key role played by $\alpha 7$ -nAChRs in this effect in line with previous findings (Toulorge et al., 2011). Likewise PNU 282987, a selective $\alpha 7$ nicotinic receptor agonist (Bodnar et al., 2005) improved the action of OXB although with lesser efficacy than NIC. Most interestingly, the dual OX receptor antagonist TCS1102 reversed both OXB- and NIC-mediated protection whereas α BgTx solely prevented the effect of NIC. This suggested that the protective effect of the alkaloid for DA neurons depended entirely on OXB-mediated signaling events. This is also in agreement with the fact that NIC provides no protection by itself for DA neurons in this experimental paradigm (Toulorge et al., 2011).

Modulation of $\text{Ca}^{2+}_{\text{cyt}}$ in OXB-mediated neuroprotection

Finally, considering the likely involvement of ER calcium release channels in the protective effect of OXB, we made attempts to correlate neuronal rescue to changes in $\text{Ca}^{2+}_{\text{cyt}}$ levels. To our surprise, we failed to detect any significant rise in basal $\text{Ca}^{2+}_{\text{cyt}}$ levels when OXB was applied alone to the cultures, at concentrations that were protective for DA neurons.

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This is rather unexpected as OXB was reported to elicit robust calcium elevations and to cause sustain excitation of various neuronal cell types (Ishibashi et al., 2005; Nakamura et al., 2010) in particular through OX2R (Huang et al., 2010). Besides, the neuroprotective effect of NIC for DA neurons which was unmasked here by OXB, was reported previously to be gated by $\text{Ca}^{2+}_{\text{cyt}}$ (Toulorge et al., 2011). One may therefore speculate that the activation of ER calcium release channels by OXB did not result in a calcium elevation within the bulk cytosol but rather in sub-plasma membrane microdomains located presumably near $\alpha 7$ nicotinic receptors. This would explain why we failed to detect any calcium changes after OXB treatment using a conventional imaging technique. Consistent with this interpretation, Ca^{2+} microdomains have been reported in the vicinity of the plasma membrane following IP_3 -induced Ca^{2+} release from intracellular stores (Rizzuto and Pozzan, 2006). NIC itself caused a small but sustained elevation in basal $\text{Ca}^{2+}_{\text{cyt}}$ but only in the situation where OXB was present in the cultures. The OX2R blocker EMPA prevented both the rise in calcium and the protective action of NIC, indicating that the calcium elevation produced by NIC (i) strictly relied on OXB-dependent signaling events and (ii) also possibly contributed *per se* to survival promotion. We cannot totally exclude, however, that the nicotinic component of neuroprotection could proceed independently of calcium. Indeed, NIC was reported to stimulate intracellular pharmacological chaperoning of nicotinic receptors, an effect causing a reduction in ER stress and unfolded protein response (Srinivasan et al, 2012), i.e., cellular events possibly implicated in PD-mediated neurodegenerative changes (Hoozemans et al., 2007).

Although any extrapolation from our *in vitro* work to a pathological situation must be regarded as speculative, our data raise the intriguing possibility that an early loss of hypothalamic orexinergic neurons may render DA neurons particularly prone to degeneration

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in the context of PD. This concept would be also coherent with the fact that a large fraction of patients with RBD presents a high risk of developing PD (Boeve et al., 2001; Postuma et al., 2012; Arnulf, 2012), presumably because they have already a reduced number of hypothalamic OX containing neurons (Fronczek et al., 2007; Thannickal et al., 2007). The cooperative effects between OXB and NIC also suggest that the loss of brainstem cholinergic neurons that is also observed in PD patients and the ensuing deficit in nicotinic excitatory inputs onto SN DA neurons (Karachi et al., 2010) might further enhance the vulnerability of DA neurons.

Authorship contributions

Participated in research design: Michel

Conducted experiments: Florence, Guerreiro, Hamadat, Rousseau

Contributed new reagents or analytic tools: -

Performed data analysis: Florence, Guerreiro, Hamadat, Michel, Rousseau

Wrote or contributed to writing of the manuscript: Michel, Hirsch

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Footnotes

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Figure legends

Figure 1: OXB increases TH⁺ cell numbers in midbrain cultures. *A*, Number of TH⁺ neurons in 10 DIV cultures as a function of the concentrations of OXA and OXB (0.01-30 nM). The effects of OXA and OXB are compared with the reference peptide GDNF used at an optimal concentration of 20 ng/ml. *B*, Uptake of [³H]-DA in cultures chronically treated with OXB. *C*, Illustration of the effects of OXB for DA neurons. Scale bar = 20 μm. * P<0.05 vs. corresponding 10 DIV control cultures.

Figure 2: OXB partially rescues spontaneously dying midbrain DA neurons. *A*, Number of TH⁺ neurons in 10 DIV cultures exposed or not chronically to OXA or OXB (both at 10 nM) in comparison to the number of TH⁺ neurons present just after plating (0 DIV). *B*, Number of MAP-2⁺ neurons in 10 DIV cultures exposed or not chronically to OXA or OXB in comparison to the number of MAP-2⁺ neurons present just after plating (0 DIV). * P<0.05 vs. corresponding 10 DIV control cultures.

Figure 3: OXB does not affect glial cell proliferation. *A*, Counts of TH⁺ neurons at 10 DIV in midbrain cultures maintained in the presence of OXB (10 nM) or ARA-C (1 μM). *B*, measure of the incorporation of [³H]-thymidine at 7 DIV in midbrain cultures maintained with the same treatments as before. *P < 0.05 vs. corresponding controls. *C*, Illustration of the effects of previous treatments on astroglial cells and their precursor cells following double immunofluorescent staining with GFAP and vimentin. Neither astrocytic precursor cells that only express vimentin (red fluorescence) nor astrocytes that express both GFAP and vimentin (yellowish-green fluorescence) are affected by OXB. Note in comparison the drastic effect of ARA-C on all types of glial cells. Scale bar: 50 μm.

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Figure 4: Role of OXR in the survival promoting effects of OXB for midbrain DA neurons. **A**, Immunodetection of OX1R and OX2R (green labels) in DA (TH⁺) neurons (red label). White arrows point to TH⁺ neurons containing OXR. Note that both receptors were also found in a large fraction of non-dopaminergic neurons. Scale bar = 10 μm. **B**, Western immunoblotting analysis of OX1R and OX2R using total protein extracts from midbrain cultures. **C**, Counts of TH⁺ neurons in 10 DIV midbrain cultures chronically exposed to OXB (10 nM) in the presence or not of the dual orexin receptor antagonist TCS1102 (1 nM), the selective OX2R antagonist EMPA (100 nM), the selective OX1R antagonist SB408124 (1 μM) or OXA (10 nM). Some cultures were also treated with the selective OX2R agonist [Ala¹¹, D-Leu¹⁵]-OXB or with OXA (10 nM), applied in the absence or presence of EMPA. None of the OXR antagonists had an impact on its own on DA cell survival.* P<0.05 vs. corresponding untreated- culture. # P<0.05 vs. corresponding cultures exposed to OXB, [Ala¹¹, D-Leu¹⁵]-OXB or OXA.

Figure 5: Neuroprotection by OXB requires the mobilization of ER calcium. Number of TH⁺ neurons in 10 DIV cultures chronically exposed or not to OXB (10 nM) in the presence or not of blockers of VDCC, nifedipine (NIF; 20 μM), flunarizine (FLU; 5 μM), or ω-conotoxin MVIIA (ωCONO; 1 μM) or of blockers of ER calcium release channels, 2-APB (10 μM) or DANT (30 μM). TH⁺ cell numbers were also assessed in cultures exposed to blockers of VDCC or blockers of calcium release channels, only.*P < 0.05 vs. corresponding untreated cultures; # P < 0.05 vs corresponding cultures exposed to OXB, alone.

Figure 6: NIC can improve OXB-mediated protection of DA neurons via activation of α7nAChR. Number of TH⁺ neurons in 10 DIV cultures chronically exposed to OXB (3 nM) only, or to OXB and NIC (1 μM) in the absence or presence of the non-selective nAChR

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antagonist MECA (50 μ M), the α 7-nAChR antagonist BgTx (1 μ M) or the dual orexin receptor antagonist TCS1102 (1 nM). Some sets of cultures were also exposed to PNU 282987 (1 μ M), a selective α 7nAChR agonist in the presence of OXB. NIC, PNU 282987, MECA, BgTx or TCS1102 had no effect on their own on TH⁺ cell numbers. The trophic peptide GDNF (20 ng/ml) was used as reference protective molecule for DA neurons. * P <0.05 vs. control. ** P <0.05 vs. OXB-treated cultures. # P <0.05 vs. OXB + NIC-treated cultures.

Figure 7: Modulation of Ca²⁺_{cyt} by OXB. Changes in steady state calcium levels in 8 DIV cultures acutely exposed to OXB (10nM) or NIC (1 μ M), each separately or in combination. Some of the cultures receiving OXB + NIC were also pretreated with the OX2R blocker EMPA (100 nM). Note that in case of combined treatments, OXB was applied before NIC. * P<0.05 vs. control. #P<0.05 vs. OXB + NIC-treated cultures.

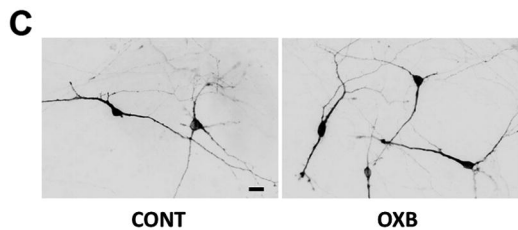
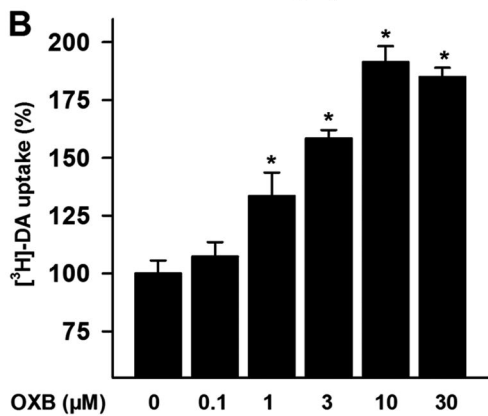
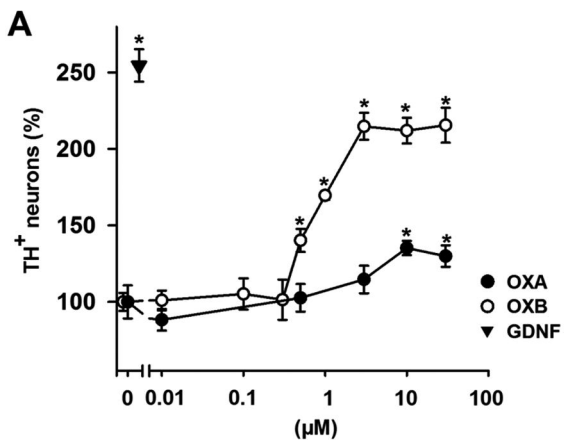


Figure 1

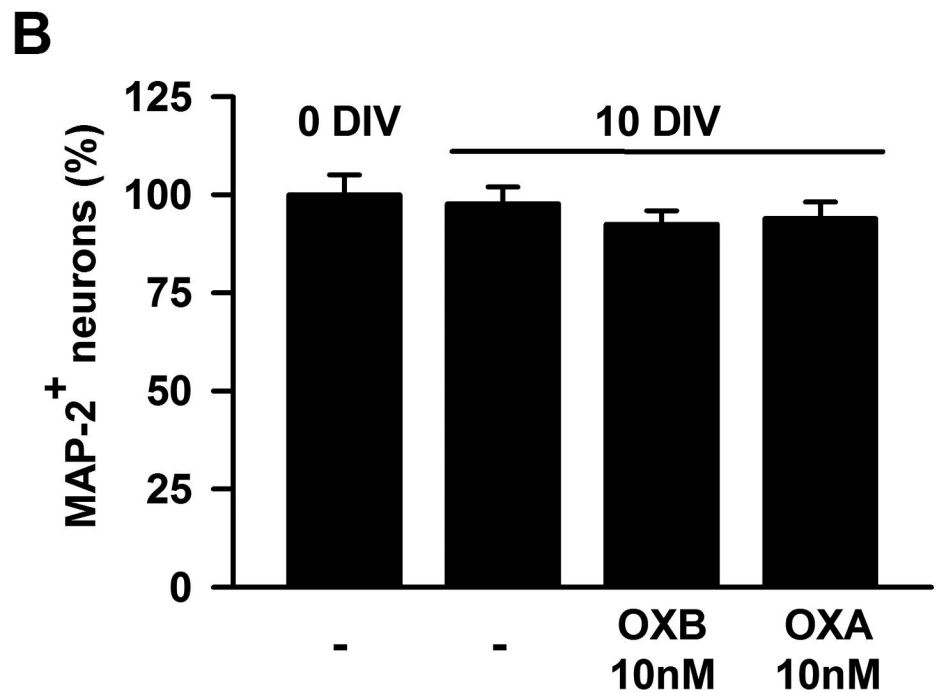
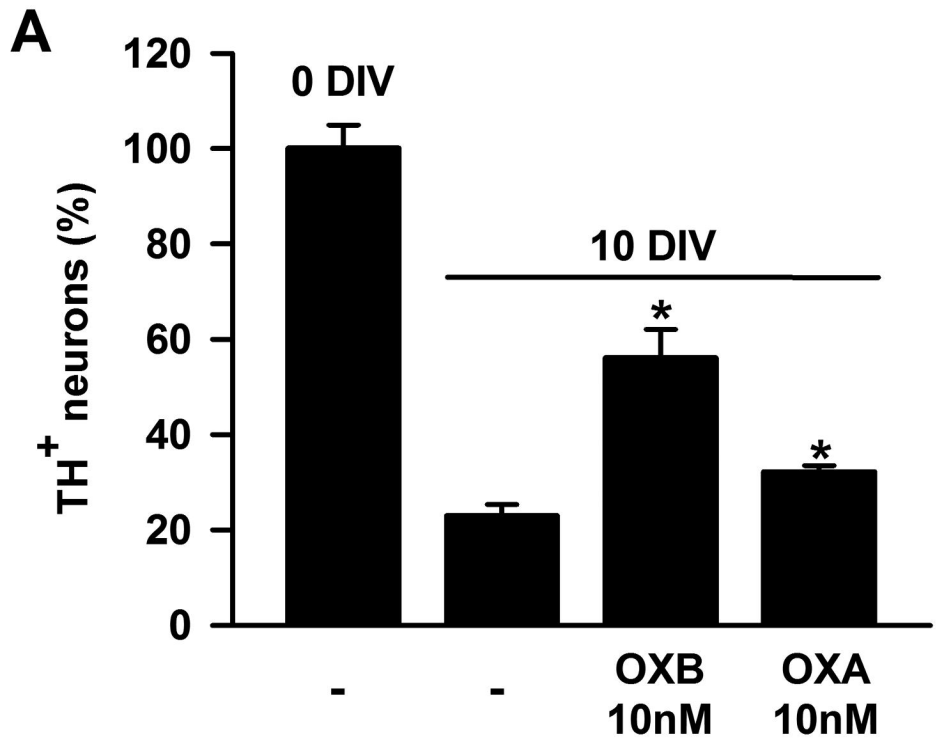


Figure 2

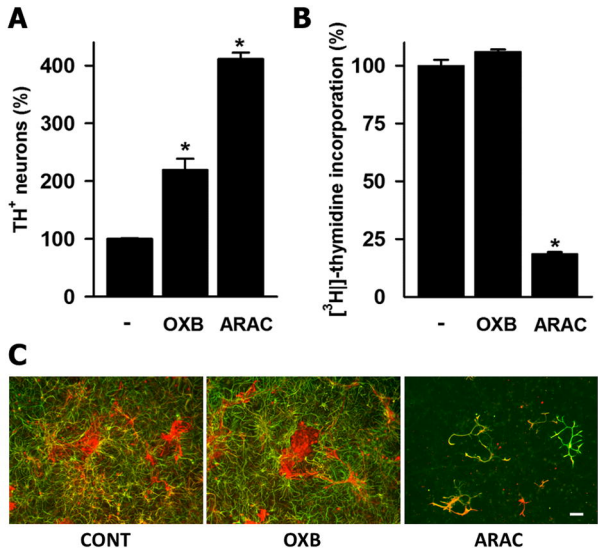


Figure 3

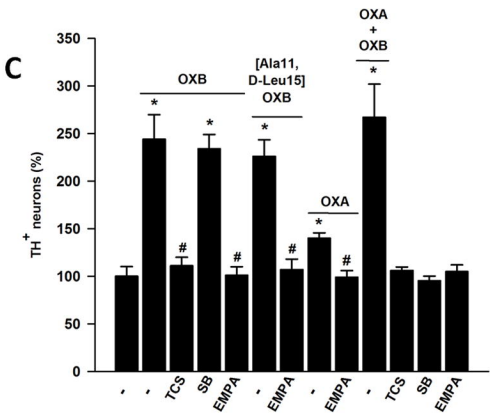
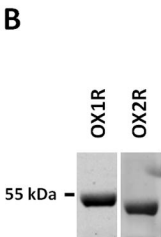
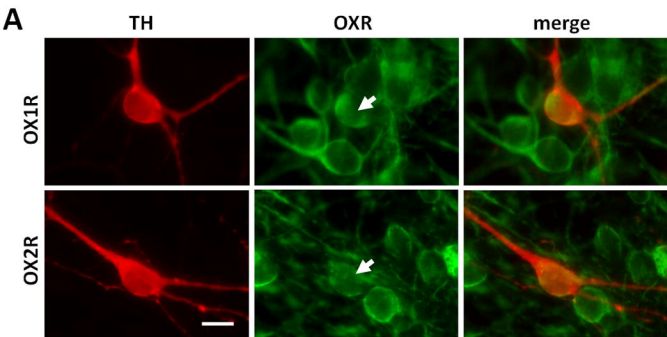


Figure 4

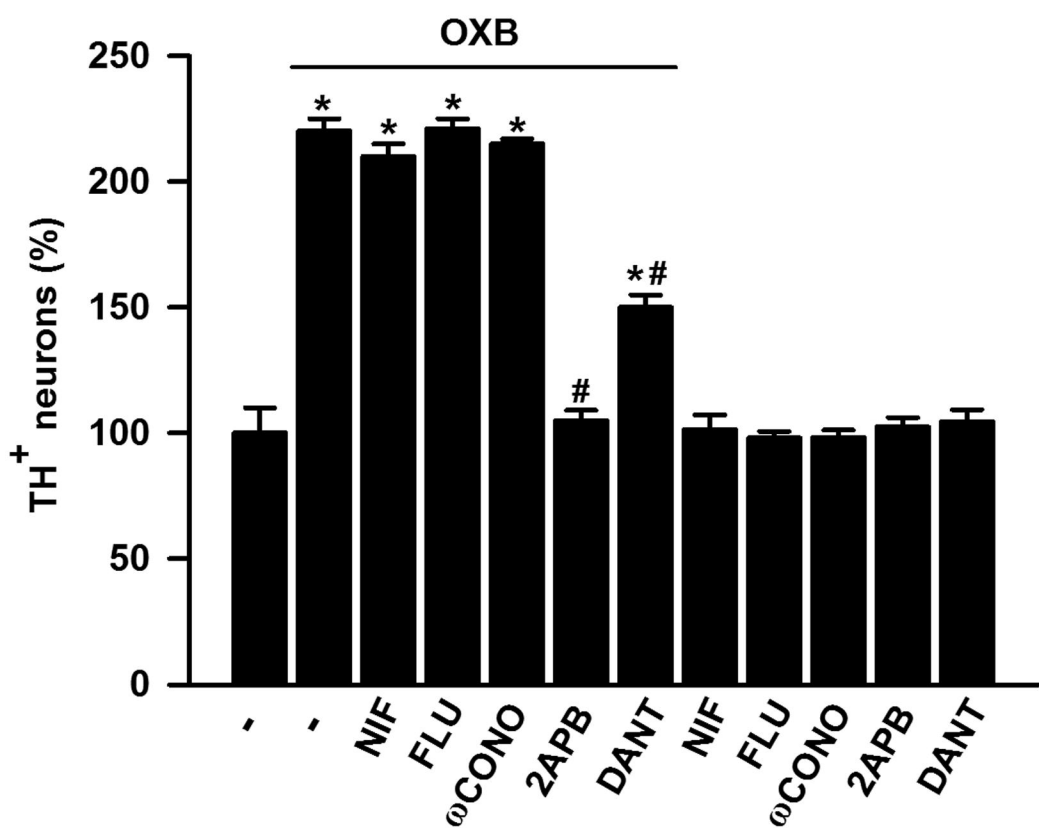


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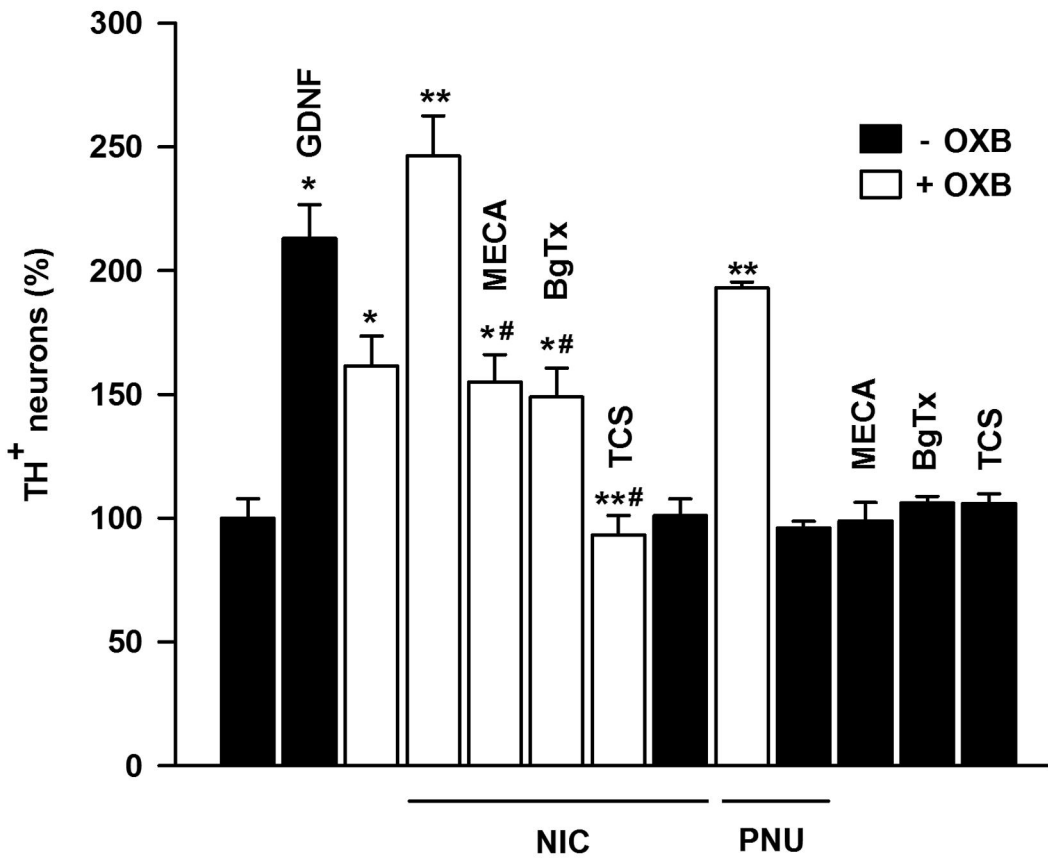


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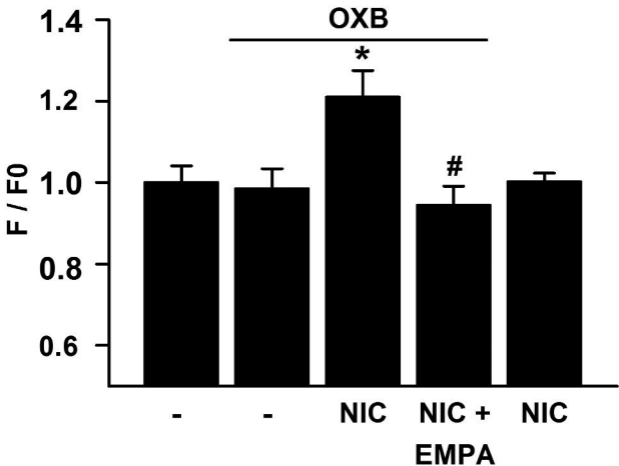


Figure 7