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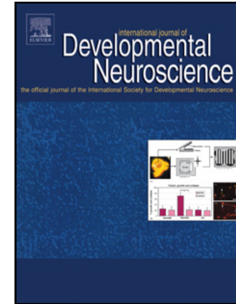

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NGF, TrkA-P and neuroprotection after a hypoxic event in the developing central nervous system

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Highlights

- Programmed cell death involves the neurotrophin–neurotrophin receptor system
- An hypoxic event changes the expression level of ligand NGF and the p75 receptor
- An hypoxic event produces a bimodal profile in the activation of the TrkA receptor
- NGF possesses a neuroprotective effect in the developing central nervous system
- NGF neuroprotection requires the activation of receptor TrkA

ABSTRACT

A decrease in the concentration of oxygen in the blood and tissues (hypoxia) produces important, sometimes irreversible, damages in the central nervous system (CNS) both during development and also postnatally. The present work aims at analyzing the expression of nerve growth factor (NGF) and p75 and the activation of TrkA in response to an acute normobaric hypoxic event and to evaluate the possible protective role of exogenous NGF. The developing chick optic tectum (OT), a recognized model of corticogenesis, was used as experimental system by means of in vivo and in vitro studies.

Based on identification of the period of highest sensitivity of developmental programmed cell death (ED15) we show that hypoxia has a mild but reproducible effect that consist of a temporal increase of cell death 6 h after the end of a hypoxic treatment. Cell death was preceded by a significant early increase in the expression of Nerve Growth Factor (NGF) and its membrane receptor p75. In addition, we found a biphasic response of TrkA activation: a decrease during hypoxia followed by an increase -4 hours later- that temporally coincide with the interval of NGF

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overexpression. To test the NGF - NGF receptors role in hypoxic cell death, we quantified, in primary neuronal cultures derived from ED15 OT, the levels of TrkA activation after an acute hypoxic treatment. A significant decline in the level of TrkA activation was observed during hypoxia followed, 24 h later, by significant cell death. Interestingly, this cell death can be reverted if TrkA inactivation during hypoxia is suppressed by the addition of NGF.

Our results suggest that TrkA activation may play an important role in the survival of OT neurons subjected to acute hypoxia. The role of TrkA in neuronal survival after injury may be advantageously used for the generation of neuroprotective strategies to improve prenatal insult outcomes.

Keywords acute hypoxia, optic tectum, neurotrophin, cell death, neuroprotection

1. INTRODUCTION

Hypoxia is defined as a low availability of oxygen (O_2) due to different causes (suffocation, anemia, poisoning, decrease in O_2 partial pressure and others conditions). The brain is particularly vulnerable to hypoxia since neurons are amongst the most sensitive cells to a reduction in O_2 partial pressure (Buck et al, 2006; Haddad, 1994; Jonas et al., 2005; Larson et al, 2014). Any decrease in the availability of O_2 either in the fully differentiated or developing central nervous system (CNS) leads to brain damage mediated by cytotoxic agents and the activation of the cell death pathways (Barks & Silverstein, 1992; Martin et al., 1997; Johnston 2001). It is known that there is a characteristic delay period between a hypoxic event and the appearance of the first manifestation of cell damage (Balduini et al., 2004). Several reports show that a lower ATP level as a result of the hypoxia correlates with an earlier appearance of signs of cell damage (Lipton, 1999). However, the molecular mechanisms that occur during that period are not fully elucidated (LaManna, 2007; Taoufik & Probert, 2008). A clear comprehension of this process could help in developing of new therapeutic strategies.

Several experimental models, implemented in different species, can be found in the literature on the effects of hypoxia on neurodevelopment. In the present work we analyze the avian optic tectum (OT) corticogenesis, a widely used standardized model in developmental neurobiology (De Long y Coulombre, 1965; Itasaki y Nakamura, 1996; Luksch, 2003; Mey and

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Thanos, 2000; Puelles and Bendala, 1978; Rapacioli et al, 2011, 2016; Scicolone et al, 1995). The temporal patterns of the different steps involves in corticogenesis (such as neuroprogenitors proliferation, neuronal determination, migration, stratification and differentiation) coincide with the sequence of developmental events in mammals and thus can be appropriately extrapolated and compared with the same processes in mammalian species. Several neuronal types, neuronal circuits, neurotransmitters and neuromodulators are commons to avians and mammals. An experimental advantage of the avian model reside in the fact that the system can be analyzed free of maternal variables and influences during the hypoxic event. An additional advantage derives from the fact that the structural changes involved in the OT corticogenesis are known in detail and, for that reason, their alterations by any insult can be easily detected and characterized.

<InlineShape1>The number of each kind of neuron in each area of the CNS is the net result of a proliferation-mediated overproduction of neurons and the subsequent removal of redundant elements by means of a finely regulated process of programmed cell death-dependent (PCD) (Nowakowski et al, 2002). There are two distinct periods of PCD In the developing optic tectum (OT). The first period takes place between ED 7.5-8 but the molecular mechanisms that regulate this period of neuronal death are still not clear (Zhang and Galileo, 1998). This event occurs in the ventricular zone, the zone where newly-born pre-migratory neurons accumulate before their radial migration towards their definitive site of residence.

The second period of PCD corresponds to post-migratory neurons that have already arrived at their sites of final residence. The appropriate number of this neurons is later adjusted by a regulated process of PCD (Miller & Kaplan, 2001; Yamaguchi and Miura, 2015). This second phase of PCD takes place during the neuronal differentiation, a process characterized by the differentiation of the neuronal body, the production of neurites and establishment of synaptic contacts with their potential neuronal targets. It is considered that only those neurons that have successfully established their synaptic connections remain as terminally differentiated neurons integrated within stabilized neuronal circuits.

It is considered that neurotrophins (NTs) play a key role in regulating the second period of PCD and that the availability of appropriate amount of NTs would be crucial for neuronal survival (Oppenheim, 1991; Yamaguchi and Miura, 2015, Kristiansen and Ham, 2014, Ichim et al, 2012).

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NTs are a family of structurally related proteins involved in processes of cell survival, cell death, axonal growth, synaptogenesis and neural plasticity in the developing and mature CNS (Rydén et al., 1997; Mitre et al, 2017). Four members of this family have been characterized in mammals: Nerve Growth Factor (NGF), *brain-derived neurotrophic factor* (*BDNF*), NT-3 and NT-4. Each of them acts on different neuronal populations in both the central and the peripheral nervous systems. NTs exert their functions by activating two kinds of membrane receptors: (a) the tyrosine kinase receptors TrkA, TrkB and TrkC that bind with high affinity to NGF, BDNF, NT-3 and NT-4, respectively and (b) the p75 receptor which lacks intrinsic tyrosine kinase activity and displays variable affinity for different NTs. Classically, p75 has been considered to trigger an apoptotic pathway while Trk receptors have been linked to the transduction of “positive” signals such as cell proliferation and survival. This dual system of signal transduction allows different modes of NT signaling in developing neurons. In neurons populations that co-express both kinds of NTs receptors, the p75 receptor increases the affinity and activation of Trk receptors even at low levels of NT expression (Barker, 2004). In this way, the activation of the Trk receptors abolishes or modulates the apoptotic effect of p75 and the final result varies significantly depending on the cell contexts in which these receptors are expressed. As a rule, it is considered that NT induces PCD, through p75, in cells that do not express Trk receptors (Bibel & Barde, 2000). As an example, during the early developmental stages NGF causes PCD of retinal neurons that express p75 and do not express TrkA (Frade et al., 1996).

Neurotrophins, particularly NGF and BDNF, have a neuroprotective effect against excitotoxicity (Nguyen, 2010), glucose deprivation (Cheng, 1991), ischemia (Shigeno et al., 1991), hypoxia (Zhu et al., 2007; Ishitsuka et al., 2012) and several other kinds of neuronal injury. The molecular mechanisms involved in the neuroprotective effects of NGF and its receptors have not yet been completely elucidated. In order to test whether the level of expression and/or activation of the different components of the NGF-NGF receptor (NGF-NGFR) system are modified by an acute hypoxic event, and whether this changes have an impact on neuronal survival, we developed two experimental strategies. We analyzed *in vivo*, by means of a validated avian hypoxic model (Vacotto et al, 2008; Giusti et al, 2012; Pozo Devoto et al, 2013), the correlation between the progression of cell death after hypoxia and the expression and activation

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of components of the NGF-NGFR system. Using primary neuronal cultures derived from the developing OT we developed an *in vitro* hypoxic model to analyze the role of NGF-NGFR system in neuronal survival after hypoxia. The present work aims at analyzing the expression of NGF and p75 and the activation of TrkA in response to an acute normobaric hypoxic event and to evaluate the possible protective role of exogenous NGF. Our results show that the cell death induced by hypoxia is preceded by a period of NGF upregulation with a concomitant activation of TrkA. Furthermore, the NGF neuroprotective effect detected *in vitro* is hindered by the inhibition of TrkA activation, suggesting a protective role of NGF-TrkA again hypoxia during development.

2. RESULTS

2.1 The programmed cell death in the developing OT reaches a maximum at ED15

In order to determine the temporal window of the second wave of PCD during the normal development, the density of neurons undergoing spontaneous apoptosis was quantified on successive days from ED12 to ED18. The TUNEL technique was performed on histological sections of OT and the number of TUNEL+ cells was quantified. Figure 1 shows that the number of neurons undergoing PCD increased significantly between ED13 and ED16, reaching a maximum on ED15. In view of these results the ED15 was chosen as the most appropriate developmental stage to evaluate the amount of PCD cell death during the neurotrophic period.

2.2 Hypoxia causes a significant increase in cell death 6 h after injury

It is known that there is a characteristic delay between a hypoxic event and the appearance of the first signs of cell damage (Balduini et al., 2004). For that reason, prior to any analysis of the susceptibility of a tissue to a hypoxic injury, the delay period between the onset of the hypoxia and the time of appearance of PCD must be precisely determined. To this end, the TUNEL technique was carried out on histological sections of OT obtained from ED15 embryos subjected to an acute hypoxic event and analyzed after different periods of reoxigenation from 0 to 12 h. A significant increase (around 60 %) in the density of TUNEL+ cells was observed after 6 h of reoxigenation (Figure 2). The percentage of TUNEL+ cell returns to normal levels 12 h after reoxigenation.

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2.3. Hypoxia promotes changes in the levels of expression of NGF, p75 and in the activation of TrkA.

Three sets of immunohistochemical studies were performed in order to analyze the changes in the expression of NGF and p75 and the activation of TrkA in response to hypoxia. Immunolabeling with anti-NGF, anti-p75, anti-TrkA and anti-P-TrkA antibodies were performed on histological sections of both control and hypoxic developing OT on ED15. The levels of expression of these proteins were quantified in terms of the mean optical density (MOD) of the immunoreactive cells as a function of the time after the hypoxic treatment during a period of 12 hours.

Figure 3 A shows that the MOD of the NGF+ neurons increase sharply after 2 h of hypoxia and then declines. The MOD remains over the normal values up to the 6 h and then returns to the normal values after 12 h of reoxigenation. Figure 3 B shows that the expression of TrkA receptor remains unmodified during the entire period of observation. Figure 3 C, however, shows that the level of activation of this receptor (P-TrkA) displays a typical bimodal profile with three phases: a) during the hypoxic treatment there was a significant decrease in the activated receptor; b) the level of P-TrkA recovered the normal values after 2 h of reoxigenation and, by 4 h, the level of activation significantly exceeded the control value, c) afterwards, between 4 and 12 h of reoxigenation the immunolabeling decreased to the normal values. Figure 3 C shows that the expression of p75 remains ummofidied during the hypoxia but increased significantly during a period of 2 - 4 hours of reoxigenation. After this interval of overexpression it returns to normal values. It must be noted that there is a period of at least 2 hours of reoxigenation along which the level of p75 is over the normal values while the value of P-TrkA is not.

2.4 Hypoxic treatment decreases cell survival in primary neuronal cultures

In order to characterize the effect of a hypoxic treatment on neuronal survival *in vitro*, primary neuronal cultures prepared from dissociated OT obtained from ED15 embryos were incubated for 4 h in a reduced oxygen atmosphere. The density of morphologically normal neurons was quantified in control cultures, at the end of the hypoxic treatment (0 h) and after a period of 24 and 48 hours of reoxigenation. The hypoxic treatment produces a decrease in

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neuronal survival that accentuates as a function of the time (60% of control levels after 48 hs) (Fig.4D).

2.5. NGF protects cultured neurons from acute hypoxia

In order to evaluate a possible neuroprotective effect of NGF *in vitro*, primary neuronal cultures were pre-treated for 30 min with 50 ng/ml of NGF and then subjected to the standardized acute hypoxic treatment. Neuronal density was quantified at the end of the hypoxic period and after 24 and 48 hs of reoxigenation. The presence of NGF in the culture protects the neurons from the hypoxic damage. In fact, while a striking decrease in the neuronal density occurs in the absence of NGF no significant neuronal loss occurs in those cultures pre-treated with NGF (Fig. 5).

2.6. Pre-incubation with NGF prevents the TrkA inactivation promoted by the hypoxic event

The experimental design described in the preceding paragraph was used to evaluate whether P-TrkA could be involved in the process of neuroprotection induced by a pre-incubation with NGF. Control cultures (C), hypoxic cultures (H) and hypoxic cultures pre-incubated with NGF (H+NGF) were used in order to analyze immunocytochemically the changes in the level activation of TrkA (P-TrkA) in the presence or absence of NGF. The immune-staining of this receptor is easily recognized as punctuate (granular) labeling distributed in neurites, perikarion and nuclei (Fig. 6 A–C). Figure 6 D shows that the number of granules / cell body, taken as quantitative parameter of TrkA activation, significantly decreases during the hypoxic treatment (0 h) and that the pre-incubation of the cultured neurons with NGF prevents the hypoxia-mediated decrease in this receptor activation at 0 h. This result, together with the protective effect of NGF against apoptosis, suggests that NGF may exert its neuroprotection effect through the TrkA receptor.

2.7. The neuroprotective effect of NGF is mediated by TrkA activation

In order to test whether the NGF-promoted neuroprotection is mediated by the activation of TrkA, its activity was inhibited by the specific inhibitor k252a. The above described experimental design was used to analyze cell survival and TrkA activation in control cultures, hypoxic cultures and hypoxic cultures pre-incubated with NGF. Cell survival was analyzed by means of the nuclear staining with Hoechst and TrkA activation by means of immunofluorescence against P-TrkA. Figure

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7 A shows that the k252a inhibitor produced a remarkable inhibitory effect on the neuroprotection exerted by NGF at 24 and 48 h post-hypoxia. Coherently, the k252a inhibitor also produces a significant decrease in the activation of TrkA (Fig. 7 B).

3. DISCUSSION

The present study analyzes the role of three essential components of the NT– NT receptors system - NGF, TrkA and p75 - as regulators of the second wave of PCD in the developing OT and the potential protective role of NGF and P-TrkA receptor after an acute hypoxic event.

Our results show that, in the developing OT, the peak of the "neurotrophic phase" of PCD takes place at ED15 and that, during this period, the differentiating neurons are sensitive to acute hypoxia both *in vivo* and *in vitro*. We also observed *in vivo* that the delayed PCD induced by the hypoxic event is preceded by several earlier molecular changes that can be associated to the NT–NT receptors system: (**a**) an early decrease in the basal level of TrkA activation during the hypoxic event which followed by (**b**) an increase in the expression of NGF, and a later (**c**) increase in the expression of the p75 receptor 2 hs after hypoxia. By 4 hs after hypoxia the activation of TrkA is significantly increased and 2 hs later all these changes are rapidly reversed to the basal levels. However, as judged by the extensive cell loss observed both *in vivo* and *in vitro*, the early changes in the NT-NT receptors system seem to trigger a cascade of events that irreversibly leads to PCD several hours later.

Neuronal cell death is a developmental cell behavior with specific morpho- and histogenetic effect. The amount of each type of neurons in every area of the CNS depends on a balance between neural stem cells and neuroprogenitors proliferation and PCD (Nowakowski et al, 2002; Yeo and Gautier, 2004; Stiles and Jernigan, 2010; Arya and White, 2015). Two main periods of PCD occurs in the developing OT (Zhang and Galileo, 1998; Pozo Devoto et al, 2006). The first one that takes place during the proliferative phase of the neuroepithelium (between ED 7.5-8), removes redundant newly-born premigratory neurons located at the subventricular zone. The second phase, usually called as "neurotrophic PCD", takes place during the period of neuronal differentiation (ED 14–16) and reduces subpopulations of differentiating post-migratory neurons that have already arrived at their definitive position in the OT cortex. Experimental evidence

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indicates that this second phase depends on the establishment of appropriate synaptic contacts (Miura, 2015). These contacts are essential for neuronal survival since they provide appropriate reciprocal supply of trophic signal or survival signal between pre- and postsynaptic neurons (Barde, 2013).

Trophic signals do not only participate during normal development. There is evidence indicating that the NTs and their receptors play important roles in different neural and non-neural tissues subjected to hypoxia and/or other noxa.

Studies performed in different species have demonstrated that both NGF and NGF m-RNA increase after several injuries. In fact, these changes were reported to occur in the rat following hippocampal lesions (Gasser et al., 1986), bilateral decortication (Lorez et al., 1988), cerebral hypoxia (Lorez et al., 1989) and post-axotomy regeneration of the vagus nerve (Lee et al., 2001). The same changes have been found to occur in mice during reinnervation of the area damaged by a myocardial injury (Oh et al., 2006). Similarly, an increase in NGF has been observed after damage by refractive corneal surgery in rabbits (Wu et al., 2009).

It must be mentioned that some authors also reported a decrease in the levels of NGF and NGF-mRNA, but not of the rest of the NTs, in several areas of the post-natal rat brain after different noxa. These differences were interpreted in terms of a differential sensitivity of the mechanisms regulating NTs expression to the hypoxic injury (Popic et al., 2012). With regards to the TrkA receptor, several examples of decreased expression, associated to neuronal loss and/or dysfunction following hypoxic injuries can be found in the literature. As an example, spinal cord contusion in rats produces an immediate decrease in the expression of TrkA and others Trks m-RNA. These changes are followed by a progressive neuronal loss in the lesion and perilesional area (Liebl et al., 2001).

With regards to the possible therapeutic relevance of our results, it can be mentioned that there is a vast literature demonstrating that hypoxic preconditioning provides tolerance to subsequent hypoxic insults. Hypoxic preconditioning cannot be applied during human pregnancy but there are several experimental approaches, in other species, aiming at elucidating the biological bases of the tolerance provided by preconditioning. It was demonstrated that

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preconditioning involves transcriptional changes. As examples, an ischemic preconditioning upregulates transcription of NGF and BDNF (Truettner et al., 2002; Matsushima et al., 1998) and produces a reinforcing effect on the transcription factor HIF-1 accumulation during a subsequent hypoxic injury (Giusti and Fiszer de Plazas, 2012). It was also shown that a mild hypobaric hypoxia preconditioning induces persistent up-regulation of various transcription factors (c-Fos, NGFI-A, pCREB, NF-kB) both before and after a severe hypoxic event (Rybnikova et al, 2002; 2009). It is interesting mentioning that, in the field of medicine, experimental and clinical studies have shown that NGF up-regulation plays a pivotal role in protecting neurons against ischemia (Lindvall et al, 1994). Besides, it has been reported that intraventricular NGF administration leads to significant improvement of children with hypoxic-ischemic brain injuries (Fantacci et al, 2013). The neuroprotective role of NGF is also illustrated by the fact that the precocious NGF up-regulation after a trauma correlates with improved outcome in children with severe traumatic brain injury (Chiaretti et al, 2008). Interestingly it was also reported that intranasal NGF administration improved cerebral functions in patients with severe traumatic brain injury (Chiaretti et al, 2017).

In vivo and *in vitro* studies indicate that, when acting individually, the Trk receptor activation functions as a positive signal leading to cell survival and growth while the p75 receptor activation functions as an apoptotic signal leading to PCD (Frade et al., 1996; Bibel & Barde, 2000). However, the final result of the activation of these receptors by NTs depends on the context in which they are activated. This is due to the fact that the co-expression of both receptors leads to interactions between both signaling pathways. In fact, p75 activation increases the affinity of Trk for NTs (Barker, 2004) and, subsequently, the Trk activation neutralizes the apoptotic effect of the p75 activation. As a rule, NTs induce PCD through p75 in cells that do not express Trk receptors (Bibel, 2000)(Kume et al., 2000).

Our results allows postulating that an acute hypoxic event produces a dysregulation in the mechanisms involved in neuronal survival and that this phenomenon includes, apart from other potential factors, an imbalance in the NGF - NT receptors system, i.e., an imbalance between (**a**) the expression of NGF, (**b**) the expression of p75 and (**c**) the activation of TrkA. This complex response can be decomposed into two phases. There is an early phase (0 – 2 hs) along which the protective effect of the TrkA activation transiently decreases while the NGF and p75 apoptotic

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receptor expression increases: an ideal condition for the activation of the apoptotic pathway. The second phase is characterized by changes that critically reverts the situation described in the first one: (**a**) 2 hours after hypoxia the NGF expression increases and (**b**) two hours later TrkA activation increases abruptly and, by 4 hs, exceeds the control level. It is plausible that this situation could counteract the apoptotic condition created during the first phase and can be considered as a delayed protective response to recover those cells that were not severely lesioned during the hypoxia insult. This proposal is supported by the *in vitro* experiments. The addition of NGF to the culture medium before the hypoxia prevented the decrease in the activation of TrkA and also the increase in neuronal death, thus confirming the neuroprotective effect. When TrkA phosphorylation was inhibited using k252a, the effects on neuronal survival were similar to those produced by hypoxia. Thus, the final result of a hypoxic event seems to depend on an appropriate balance between the level of expression and activation of NGF, p75 and TrkA.

Our results on the effects of the hypoxia on the TrkA activation contribute to better understand the survival pathway initiated by this receptor. The decrease in the level of P-TrkA observed immediately after hypoxia *in vivo* could be due to an imbalance between the tyrosine kinases and the tyrosine phosphatases. It is known that these enzymes dynamically regulate the P-TrkA level and that cell damages alter this balance (Pei et al., 2000). Marsh et al. (2003) identified a protein that regulates the P-TrkA dephosphorylation and showed that when this receptor is steadily dephosphorylated, i.e., inactive, the survival of rat sympathetic neurons in culture decreases. This effect is seen even in the presence of NGF.

In conclusion, the results show that (**a**) the PCD that normally occur in the developing OT involved changes in the NT - NTR system, and that (**b**) an hypoxic event installed during this brief period produces significant changes in the level of ligand NGF and the p75 receptor and (**c**) a bimodal profile in the level of activation of the TrkA receptor. This study confirms the neuroprotective effect of NGF in the developing CNS. This effect requires the activation of receptor TrkA.

4. MATERIALS Y METHODS

4.1. Animals and hypoxic treatment in vivo

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Fertile chicken (Gallus gallus domesticus) specific pathogen free eggs from White Leghorn were obtained from a local hatchery and incubated at 38 °C and 60% relative humidity. At ED15 or stage 41 according to the criteria of Hamburger and Hamilton (1951), embryos were subjected to a global hypoxic treatment as previously described by Rodriguez Gil et al. (2000). Briefly, eggs were vertically placed in a 10 l plastic chamber inside the incubator (in the same conditions of temperature, pressure and humidity as the control eggs) and subjected to a stream of 8% O_2/92% N_2 for 60 min, at a flow rate of 1 l/min. The chamber contained retention valves to allow escape of gases in excess while avoiding mixing with atmospheric air, and a storage space with calcium hydroxide to absorb CO_2 formed during hypoxic treatment. After normobaric hypoxic treatment, eggs were immediately processed or returned to normoxic conditions in the incubator, and allowed to recover for 2, 4, 6, 12, 24 and 48 h. Control animals were those embryos not subjected to the hypoxic treatment. In normal development analysis, they were killed at same hours of the ED12-18. All procedures described were performed in accordance with guidelines approved by Buenos Aires University's Animal Care and Use Committee and the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All efforts were made to minimize the number of animals utilized and their suffering.

ED15 embryos were subjected to a normobaric hypoxic treatment as previously described by Rodríguez Gil et al. (2000). Briefly, eggs were vertically placed in a 10 l plastic chamber inside the incubator (in the same conditions of temperature, pressure and humidity as the control eggs) and subjected to a stream of 8% O_2 / 92% N_2 for 60 min, at a flow rate of 1 l / min. The chamber contained retention valves to allow the escape of gases in excess while avoiding mixing with atmospheric air, and a storage space with calcium hydroxide to absorb the CO_2 produced during hypoxic treatment. After the normobaric hypoxic treatment, some embryos were immediately processed and others were returned to normoxic conditions in the incubator, and allowed to recover for different times. Control embryos were not subjected to the hypoxic treatment; they were processed at the same time as the treated embryos.

4.3. Tissue preparation

Embryos were anesthetized by hypothermia, removed from the eggs, staged according to the HH stages. Brains were dissected out in ice-cold 0.1 mol l^{-1} sodium phosphate buffer (pH 7.4),

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plus 0.9% w/v NaCl (PBS) and then fixed by immersion in 4% paraformaldehyde in PBS at 4ºC for 12 hours. After fixation, optic lobes were dissected, washed in PBS, dehydrated in graded ethanols, cleared in xylene and embedded in Paraplast at 56°C (Product No. 8889-501006. Pellet form). Serial 5 µm thick sections oriented parallel to the longitudinal anatomical axis were obtained and stored at 4°C.

4.4. Antibodies

Sections were immunolabeled with the primary antibodies listed in Table 1.

4.5. Immunofluorescence

Sections were deparaffinized, rehydrated, rinsed in PBS and processed for antigen retrieval by treatment with sodium citrate (10mM / PBS), pH 6 for 40 minutes at 95°C. Nonspecific binding was blocked by preincubating the sections in 5% normal goat serum (NGS) in PBS with Triton X-100 (PBS/Tx: PBS 0.1M pH 7.4/Triton X-100, 0.03%) for 30 minutes at RT in humidity chamber. The immunolabeling was performed with primary antibodies diluted in PBS/TX containing 0.5% NGS. Sections were incubated with the primary antibodies for 20 hours at 4°C in humidity chamber.

After several rinses in PBS, sections were incubated with secondary antibodies Cy2 conjugated for 4 hours at RT in a dark humidity chamber. Sections were then rinsed in PBS and counterstained with Hoechst. After rinsing, slides were mounted with polyvinyl alcohol mounting medium with DABCO, antifading (10981, Fluka).

4.6. Immunohistochemistry

This protocol replicates the steps for immunofluorescence until the incubation with the primary antibody. After the incubation with the primary antibody, endogenous peroxidase activity was quenched with 3% H2O2 in PBS for 10 minutes in the dark. Sections were incubated with biotinylated secondary antibody diluted with PBS/TX for 2 hours at 37ºC in a humidity chamber. Slides were then washed for 5 minutes in PBS/TX and incubated for 30 minutes with streptavidine/HRP (1:400). After washing, slides were incubated for 2 minutes with DAB (3,3′-

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diaminobenzidine tetrahydrochloride) (Sigma) and immediately washed under tap water. Slides were then dehydrated and mounted with Canada balsam.

4.7. TdT-mediated dUTP nick-end labeling (TUNEL)

Detection of DNA double-strand breaks by TUNEL was performed according to the instructions of the manufacturer (Promega). Sec-tions were rehydrated and then incubated in citrate buffer as described above. The sections were then incubated in equilibrium buffer for 10 min and followed by reaction buffer (equilibrium buffer, nucleotide mix with fluorescein conjugated dUTPs and terminal deoxynucleotidyl transferase) for 60 min. The reaction was stopped with SSC 2X for 15 min, washed with PBS-T and counterstained with Hoechst for 10 min.

4.8. Cell quantification

For histological sections cell quantification was performed on equatorial sections (halfway between the dorsal and ventral surfaces) and transversal sections (halfway between the rostral and the caudal poles). Images were obtained along the complete radial axis with an epifluorescent microscope (Zeiss Axiophot) coupled with a CCD camera. Quantifications were performed using ImageJ (NIH, Bethesda, MD, USA).

For primary neuronal cultures cell quantification was performed in 10 areas selected at random. Images were obtained with epifluorescent microscope (Zeiss Axiophot) coupled with a CCD camera. Quantifications were performed using ImageJ (NIH, Bethesda, MD, USA).

4.8.1. Quantification of the TUNEL+ cells density

TUNEL+ cells were manually counted using ImageJ in assembled radial axis images. The density of positive cells was expressed normalized to 1 mm^2 of at least three different experiments. Given the absence of statistically significant differences between the right and the left OTs, both set of data were grouped.

4.8.2. Mean optical density (MOD) of immunoreactive cells

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Images were capture under identical, calibrated exposure conditions. All images were equally background-corrected, and converted to 8-bit grey scale. The MOD was evaluated within ten areas of 10^4 μm^2 in ten different sections per biological sample. The MOD was measure on cells whose borders were traced manually. Data were expressed as MOD / cell ± SEM of at least three different experiments. Because no statistically significant differences were found between right and left OTs, data were grouped.

4.8.3. Neuronal density

Hoechst stained neurons with normal morphology, corroborated by phase contrast, were counted and the density of neurons was normalized to 1 mm^2: [number of neurons / mm^2] ± SEM of at least three different experiments.

4.8.4. Evaluation of P-TrkA expression

The number of P-TrkA+ granules / cell body was quantified in all neurons present in the evaluated images. Data were expressed as number of granules / cell ± SEM of at least three different experiments.

4.9. Primary neuronal culture

Cell cultures were prepared from optic tecta of ED15 embryos. Briefly, optic tecta were dissected, fragmented in Hanks-glucose medium and incubated in trypsin–ethylenediaminetetraacetic acid (EDTA) followed by dissociation with a Pasteur pipette. The dissociated cells were resuspended in culture medium (DMEM, N2, glutamine 2 mM and Penicillin/Streptomycin) and plated onto poly-D-lysine-coated culture dishes. Cultured neurons were kept in incubator at 37°C and 5% CO_2.

4.10. Hypoxic treatment *in vitro*

After two days of culture, plates with the same density of neurons (≈ 140 neurons/mm^2) were selected. Treated plates were placed in a sealed plastic chamber and maintained at 37°C at 0.5% O_2 and 99.5% N_2 for 4 hours. The presence of check valves allows the escape of gases. The CO_2 produced during the hypoxic treatment was absorbed by Calcium hydroxide present inside the chamber. The O_2 concentration was monitored every 30 minutes by means of a digital

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oximeter. After hypoxic treatment, plates were processed or returned to normoxic conditions for different times. Control plates were kept in standard conditions; they were processed at the same time as treated plates.

4.11. Neuroprotective treatment

In order evaluate a possible neuroprotective effect of NGF *in vitro*, primary neuronal cultures were treated for 30 min with 50 ng/ml of NGF and then submitted to the standardized acute hypoxic treatment.

In order to test whether the NGF-promoted neuroprotection is mediated by the P-TrkA receptor, the effect of this receptor was abolished by means of its specific inhibitor k252a at concentrations of 50 nM, 100 nM, 200 nM and 500 nM. Primary neuronal cultures were treated for 30 min with NGF and k252a and then submitted to the standardized acute hypoxic treatment.

4.12. Immunofluorescence of cultured neurons

Cultured cells were fixed with 4% paraformaldehyde and 4% sucrose in 0.1 M PBS. After extensive washes with PBS, cells were soaked in blocking solution (4% normal goat serum [NGS] in 0.1 M PBS), and subsequently incubated with primary antibodies (Table I) for 20 h at 4°C. Then, were incubated with secondary antibodies: Alexa Fluor 594 goat anti-rabbit IgG or Alexa Fluor 488 F(ab′)2 fragment of goat anti-mouse IgG (2 μg/ml; Invitrogen Co.). Cells were then rinsed in PBS and counterstained with Hoechst. After rinsing, slides were mounted with polyvinyl alcohol mounting medium with DABCO, antifading (10981, Fluka).

4.13. Statistical methods

All data are presented as mean ± SEM of at least three different experiments. Statistical analysis was performed using analysis of variance followed by Dunnett's post-hoc test, as indicated in the figure legends. A value of $P < 0.05$ was considered as statistically significant.

5. COMPETING INTERESTS

The authors have no competing interests to declare

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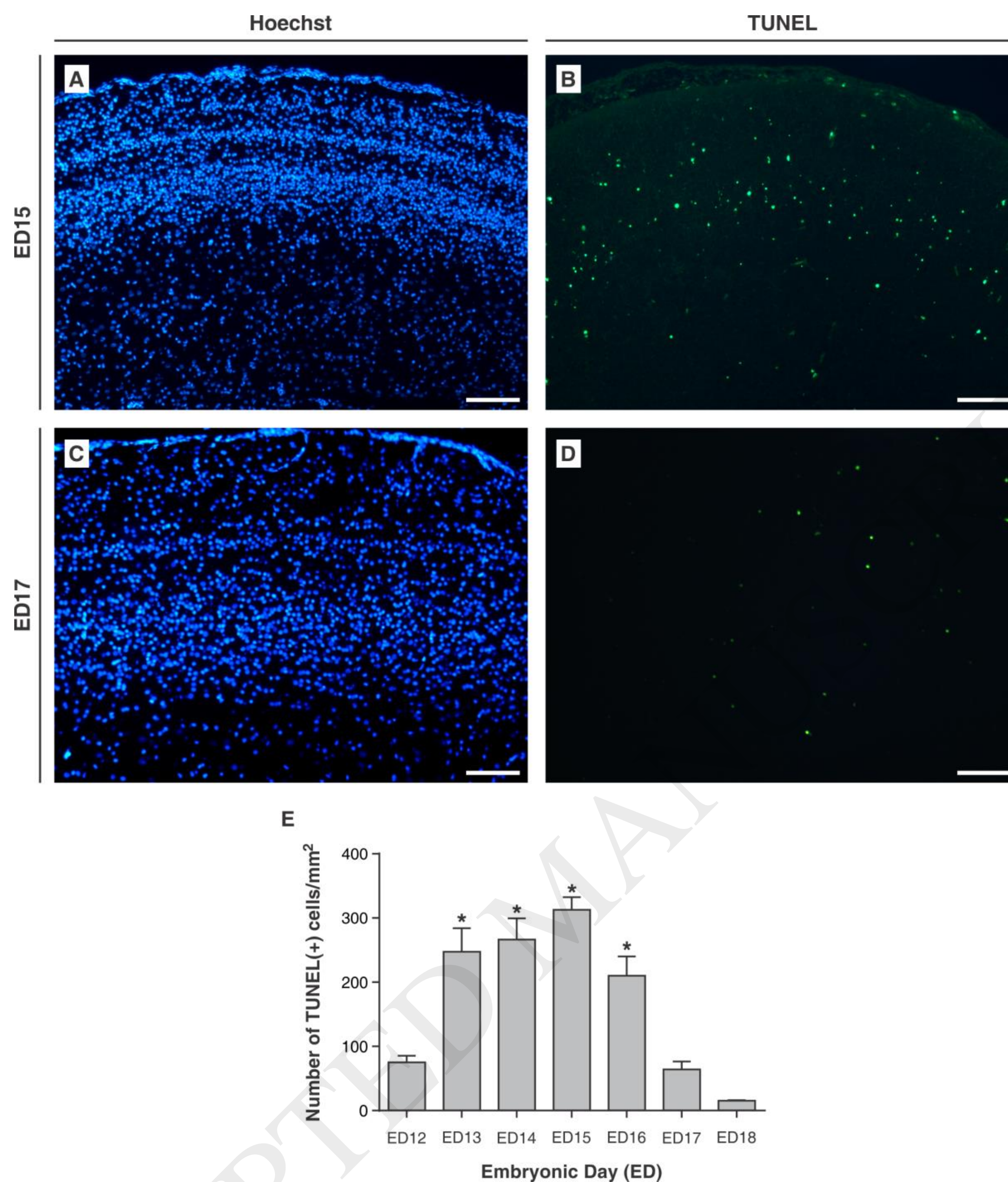

Figure 1. The peak of the second wave of PCD occurs at ED15. A-D. Representative microphotographs showing Hoechst nuclear staining (A and C) and TUNEL+ (B and D) cells in histological sections of OT obtained from ED15 and ED17 embryos. **E.** Bar graph of the density of TUNEL+ cells, normalized to 1 mm^2 , as a function of the ED. Values are expressed as means ± SEM of four independent experiments. A one way ANOVA followed by a Dunnett's post-test comparing each ED versus ED12 was performed. Scale Bar = 100 um. *: $p<0.05$.

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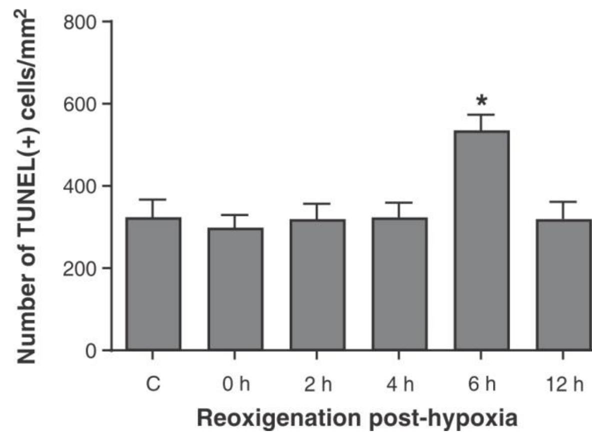

Figure 2. The first signs of cell death appear 6 hours after the hypoxic treatment. ED15 embryos were subjected to hypoxia (8% O_2; 60 min) and returned to normoxic conditions for different time periods (0, 2, 4, 6 and 12 h). TUNEL reaction was performed on OT sections and TUNEL+ cells were counted and normalized to 1 mm^2. An increase in TUNEL+ cells is observed at 6 h post-hypoxia returning to control levels at 12 h. Values are expressed as means ±SEM of three independent experiments. A one way ANOVA was performed, followed by a Dunnett's post-test comparing each post-hypoxia time versus control (normoxic) treatment. *: $p<0.05$.

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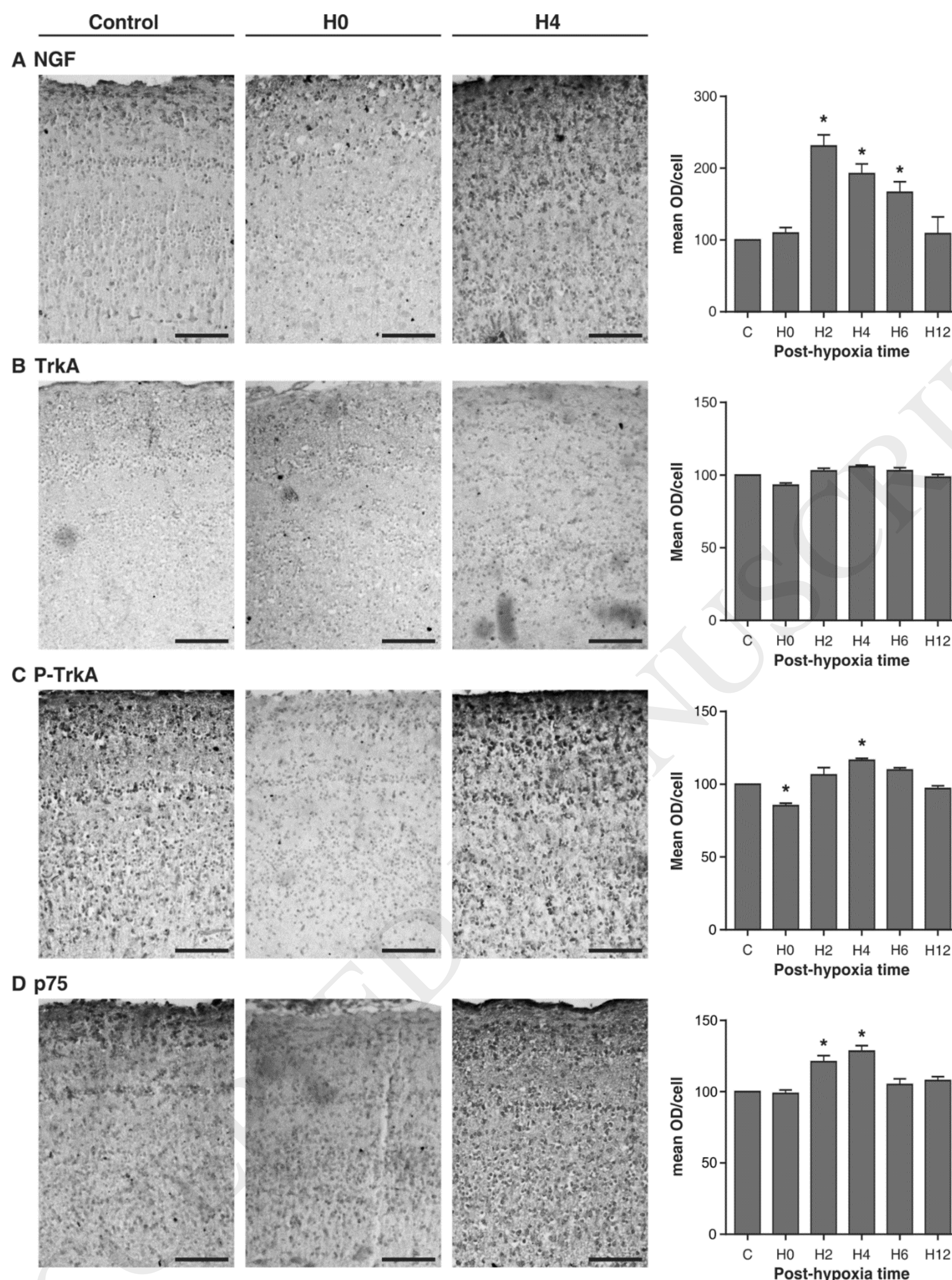

Figure 3. Hypoxia increases NGF, p75 expression and changes the level of TrkA activation. ED15 embryos were subjected to hypoxia and OT sections were immunolabeled for NGF (**A**), Total TrkA (**B**), p-TrkA (**C**) and p75 (**D**) Each panel shows the immunolabeling for each marker in normoxic, immediately after hypoxia (H0) and 4 hs post-hypoxia (H4). Bar graphs show the cellular mean optic density present in each treatment for each marker normalized to normoxic levels. **A.** NGF expression significantly increases at H2 and is maintained up to H6. **B,C.** Total TrkA expression remained unchanged while its activation decreases immediately after hypoxia (H0) and significantly increases at H4. **D.** p75 shows an increase in expression at H2 and H4. Values are expressed as means ±SEM of three independent experiments. A one way ANOVA followed by a Dunnett's post-test was performed for each marker. Scale Bars = 100 um. *: $p<0.05$.

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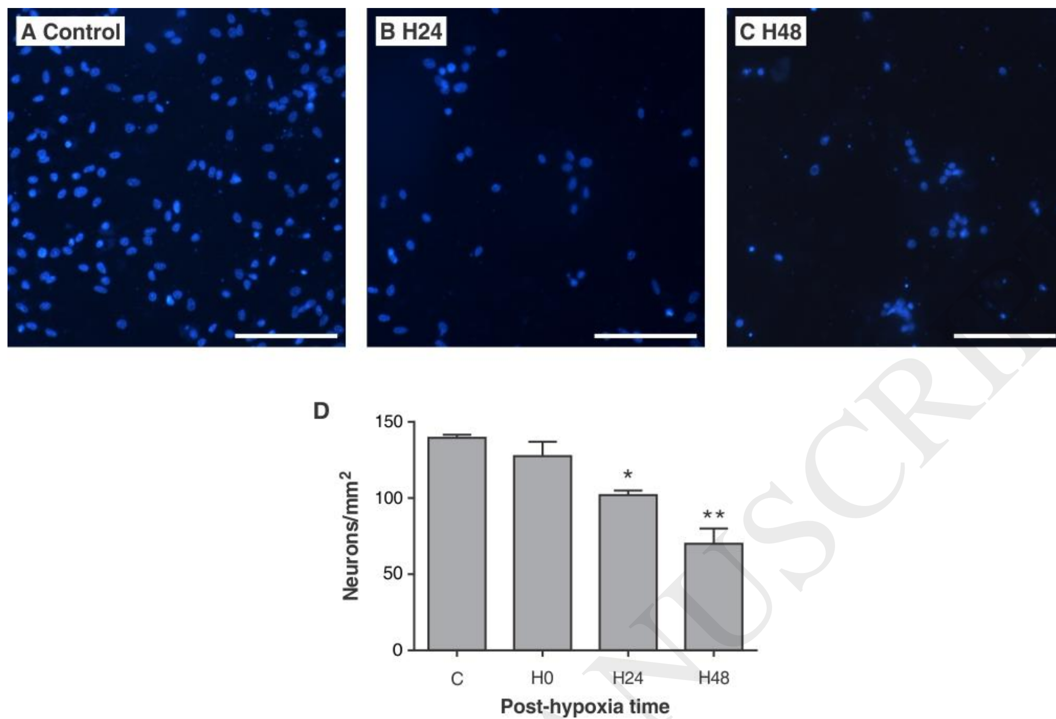

Figure 4. Hypoxic treatment *in vitro* increases neuronal death after 24 hs of recovery. Primary neuronal cultures from ED15 OT were subjected to hypoxia (0.5% O2, 4 hs) and returned to normoxic conditions for different times. **A-C.** Hoechst stained neurons with normal morphology were counted and normalized to 1 mm^2 for normoxic (**A**), 24 hs (**B**) and 48 hs post-hypoxia (**C**). **D.** Bar graphs show a significant decrease in neuronal density observed at 24 and 48 h post-hypoxia. Values are expressed as means ± SEM of four independent experiments. A one way ANOVA followed by a Dunnett's post-test was performed. Scale Bars = 100 um. *: $p<0.05$.

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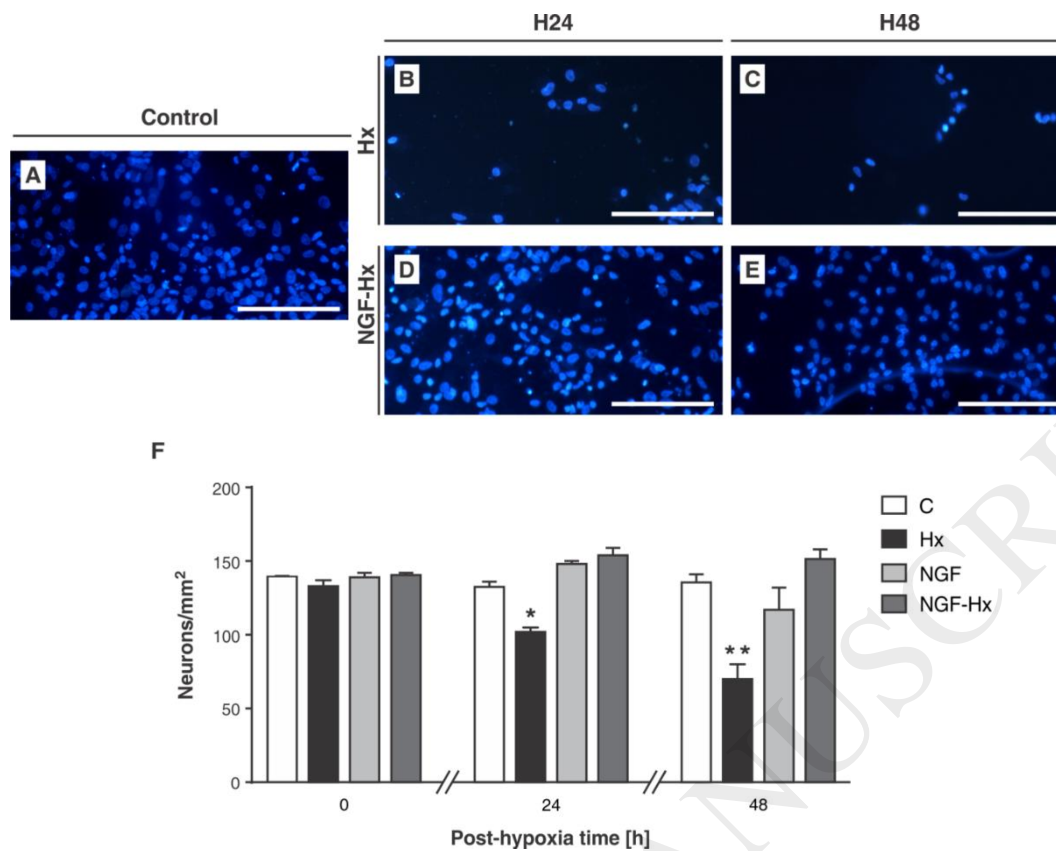

Fig.5. NGF prevents the hypoxia-induced neuronal death. Primary neuronal cultures were subjected to hypoxia in the presence or absence of NGF (50 ng/ul, 30 min before hypoxia). **A-E.** Representative microphotographs of Hoechst-stained neurons showing cellular density from normoxic (**A**), hypoxic (**B,C**) and hypoxic pre-incubated with NGF (**D,E**) cultures. **F.** Graph bars of neuronal density. The neuron density quantification reveals that pre-incubating neurons with NGF protects the neurons against the hypoxia. Values are expressed as means ± SEM of four independent experiments. A two way ANOVA followed by a Dunnett's post-test was performed. Scale Bars = 100 um. *: $p<0.05$.

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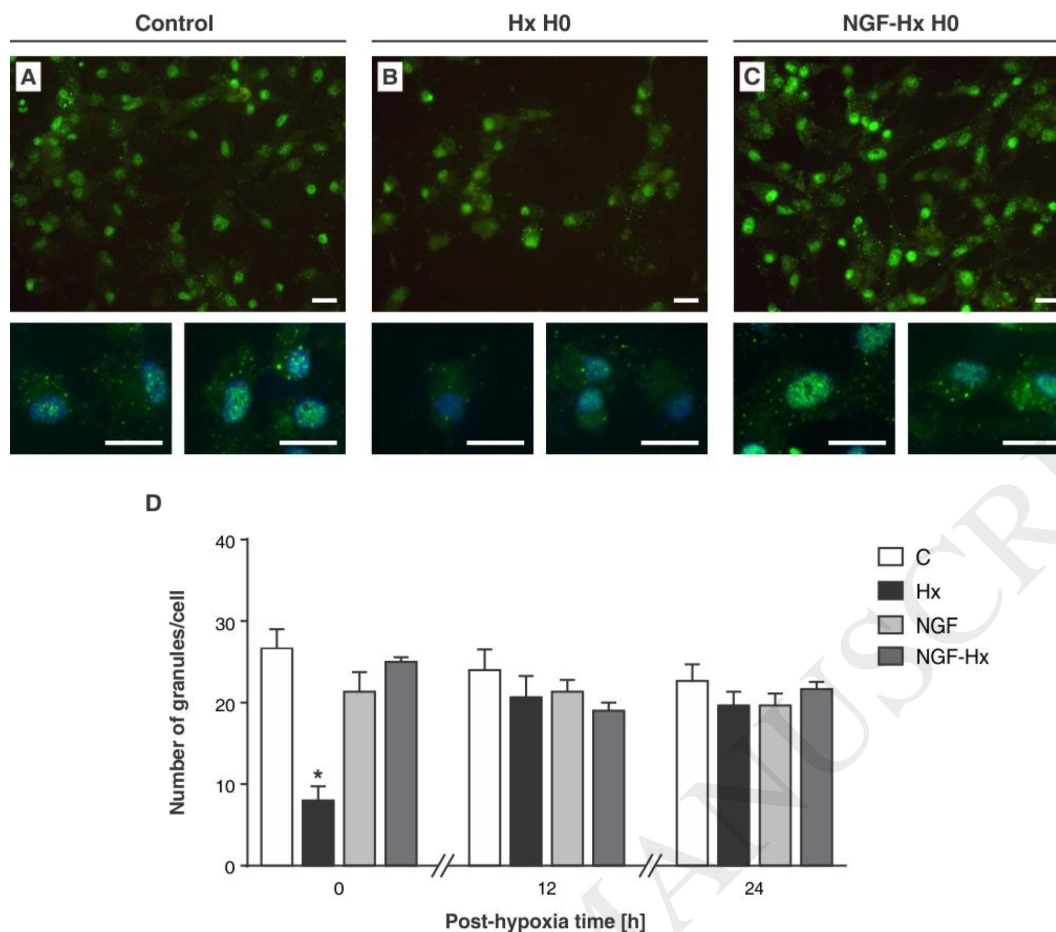

Figure 6. NGF prevents the decrease in P-TrkA level induced by acute hypoxia. P-TrkA immunolabeling (fluorescence) in primary neuronal cultures subjected to hypoxia in presence or absence of NGF. **A-C.** Merged images of Hoechst (nuclear, blue) and P-TrkA (green) stained granules from normoxic (**A**), hypoxic (**B**) and NGF-incubated hypoxic cultures (**C**). **D.** The number of granules/neuron was quantified for each treatment at 0, 12 and 24 h post-hypoxia. Hypoxia significantly reduces P-TrkA receptor but returns to normoxic values by 12 h. The presence of NGF prevents the inactivation of P-TrkA induced by hypoxia (**B** vs **C**). Values are expressed as means ± SEM of four independent experiments. A two way ANOVA followed by a Dunnett's post-test was performed. Scale Bars = 25 um. *: $p<0.05$.

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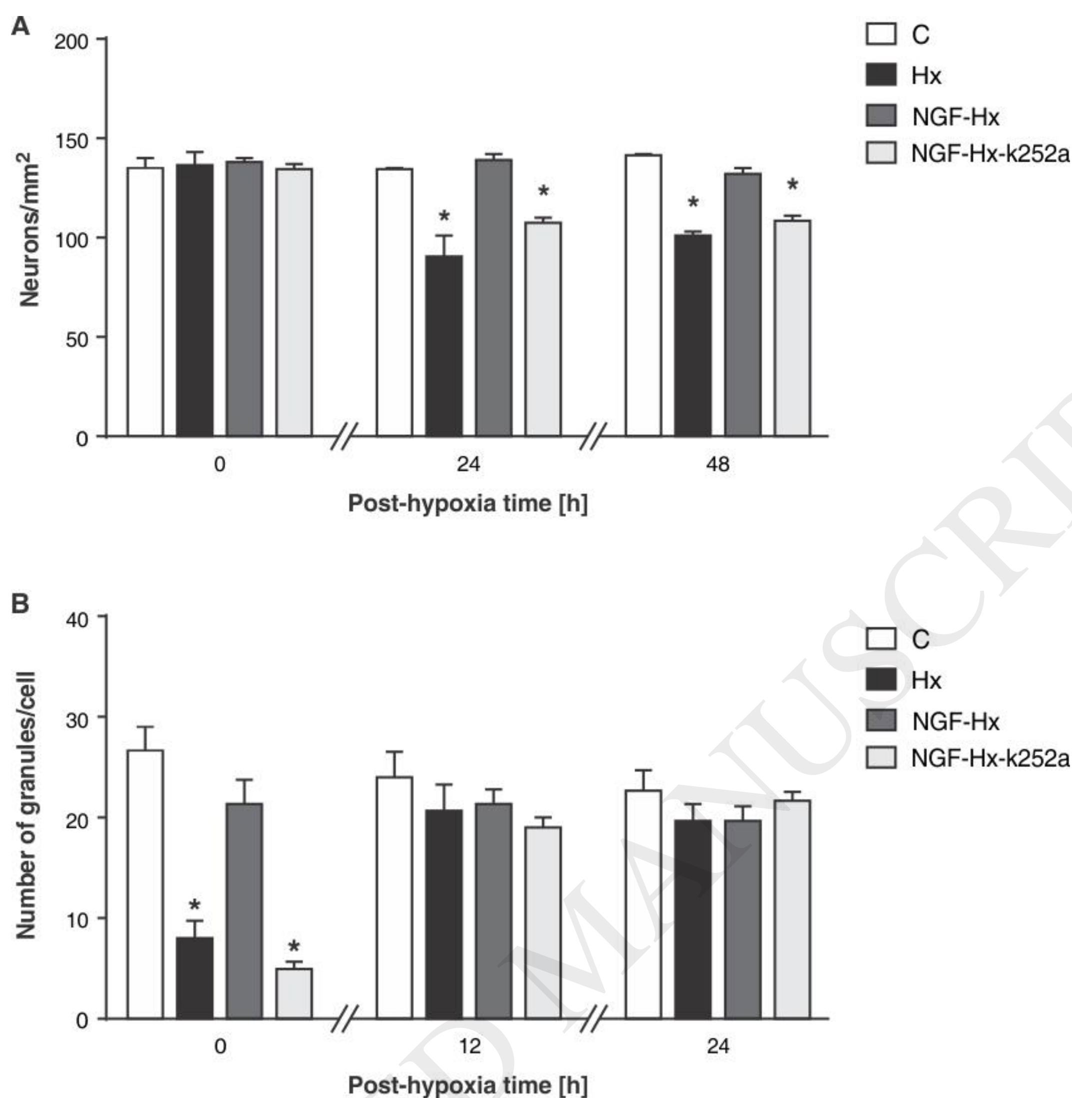

Fig.7. The specific NGF inhibitor k252a neutralizes the NGF-mediated neuroprotection by inhibiting TrkA phosphorylation. Primary neuronal cultures were subjected to hypoxia and incubated with NGF or NGF plus k252a (100 nM). **A.** The neuronal density quantification reveals that k252a neutralized the NGF-mediated neuroprotective effect at 24 and 48 h post-hypoxia. There is a significant decrease in neuronal density at 24 and 48 h in hypoxic cultures and hypoxic cultures incubated with NGF plus k252a. **B.** Number of P-TrkA+ granules/neuron for each treatment at 0, 12 and 24 hours post-hypoxia. The inhibitor k252a inhibits TrkA phosphorylation induced by NGF under hypoxic conditions. The mean number of granules/neuron decreased immediately after reoxygenation (H0), as in the absence of NGF treatment. Values are expressed as means ± SEM of three independent experiments. A two way ANOVA followed by a Dunnett's post-test was performed. Scale Bars = 25 um. *: $p<0.05$.

Table 1. Primary antibodies characteristics

Antibody	Supplier/code	Immunogen	Dilution

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Rabbit polyclonal Anti-NGF	Millipore #AB5583	Synthetic corresponding to amino acids 84-104 of rat proNGF (Accession # P25427)	1:500
Rabbit polyclonal Anti-TrkA	Cell signaling #AF1056	Mouse myeloma cell line NS0-derived recombinant rat TrkA Ala33-Pro418 (Accession # P35739)	1:1000
Rabbit polyclonal Anti-Phospho-TrkA (Tyr490)	Cell signaling #9141	Synthetic phosphopeptide corresponding to residues surrounding Tyr490 of human TrkA.	1:500
Rabbit polyclonal Anti-p75 NTR (Neurotrophin Receptor)	Millipore #07-476	GST fusion protein corresponding to the intracellular domain (residues 274-425) of rat p75 neurotrophin receptor.	1:500