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Review

Cell non-autonomous functions of homeoproteins in neuroprotection in the brain

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ABSTRACT

Homeoproteins transcription factors can transfer between cells and play important roles in development. However, some of these homeoproteins are expressed in the adult, but their function is unknown. The loss of mesencephalic dopaminergic (mDA) neurons is the cause of Parkinson's disease. In mice lacking a functional allele for the Engrailed 1 homeoprotein, mDA neurons progressively die starting about 6 weeks after birth. Infusion of recombinant Engrailed stops the death of these neurons demonstrating that homeoproteins can be neuroprotective. This has been extended to retinal ganglion cell neurons (RGCs), which die in glaucoma and optic neuropathies. The homeoprotein Otx2 promotes the survival of injured adult RGCs both in vitro and in vivo. These examples raise the possibility that homeoproteins may provide neuroprotection to neurons vulnerable in other neurodegenerative diseases.

1. Introduction

Homeoproteins are homeodomain-containing transcription factors that play a crucial role in patterning, neurogenesis and neuronal differentiation during brain development [1–5]. Some of these factors continue to be expressed in the adult brain but their adult functions are not well documented. A unique property shared by a number of homeoproteins is that they can be secreted and internalized by live cells [6,7]. Homeoprotein non-cell-autonomous activity represents a novel signaling mechanism with several established functions. In the case of the visual system, for example, Pax6 intercellular transfer regulates the size of the eye anlagen [8], Engrailed secretion by the optic tectum participates in retino-tectal patterning [9,10] and Otx2 internalization by paravalbumin interneurons in the cortex opens and closes the critical period for binocular vision [11].

Another interesting feature of certain homeoproteins is their ability to regulate protein translation through interaction with the translation initiation factor eIF4E [12]. The idea that Engrailed might regulate local translation is also supported by the observation that Engrailed 1 (En1) is present in the dendrites of midbrain neurons and that Engrailed growth cone guidance activity requires local protein translation at the growth cone [9,13]. Beyond their physiological and developmental interest, the transduction properties of homeoproteins are particularly attractive in a therapeutic context. This review addresses the issue of the potential therapeutic use of exogenous Engrailed and Otx2 proteins in animal models of Parkinson disease and glaucoma. The potential use of cell penetrating peptides for the transduction of small peptides or proteins for neuroprotection or cell replacement therapies is also briefly discussed.

2. Engrailed and Parkinson's disease

2.1. Hallmarks and pathogenesis of Parkinson's disease

Dopamine (DA) plays important functions in several neurological and psychiatric disorders [14,15]. Mesencephalic dopaminergic (mDA) neurons in the ventral midbrain represent the main source

Abbreviations: CPP, cell penetrating peptide; DA, dopamine; GCL, ganglion cell layer; INL, inner nuclear layer; iPSCs, inducible pluripotent stem cells; mDA, mesencephalic dopaminergic; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridium; NMDA, *N*-methyl-D-aspartic acid; PD, Parkinson's disease; ONL, outer nuclear layer; RGC, retinal ganglion cell; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VTA, ventral tegmental area

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of dopamine in the mammalian brain. The mDA neurons in the substantia nigra pars compacta (SNpc) innervate the dorsal striatum and form the nigrostriatal pathway that is involved in the control of voluntary movements. mDA neurons of the ventral tegmental area (VTA) project to various areas such as nucleus accumbens, cortex, septum, amygdala and olfactory tubercle to form the meso-limbic and meso-cortical pathways involved in emotion-based behaviors such as motivation and reward, as well in some specific learning tasks (Fig. 1A).

The main hallmark of PD, the second most common neurodegenerative disorder, is a slow and progressive death of mDA neurons in the SNpc and to a lesser degree in the VTA. mDA cell loss is associated with the presence of intraneuronal inclusions, called Lewy bodies, containing mainly α-synuclein, neurofilaments and ubiquitin [16,17]. This loss ultimately results in severe dopamine deficiency in the striatum responsible for typical PD-associated motor symptoms such as akinesia, bradykinesia, tremor, rigidity and loss of postural control [16]. As mentioned above, mDA neurons of the VTA and other non-nigral areas are relatively spared but their milder loss nonetheless leads to psychiatric disorders and dementia associated with PD in about 30% of all PD cases [16,17]. Unfortunately, PD progresses asymptomatically for several years before the first motor symptoms appear when almost 80% of mDA neurons are lost. Currently available therapies (i.e., L-DOPA administration, or subthalamic stimulation) are not preventive and do not slow mDA cell loss.

Apart from familial monogenic forms of PD in which certain mutated genes have been identified (i.e., α-synuclein, Pink1, Parkin, DJ-1 and Lrrk2), the molecular basis of sporadic idiopathic PD remains largely unknown. However, it has emerged from several studies that protein misfolding and dysfunction of the ubiquitin-proteasome pathway, as well as oxidative stress and mitochondrial dysfunction play pivotal roles in mDA neurodegeneration [18]. Studies following the discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridium (MPTP) could cause PD in humans have pointed to mitochondrial complex I impairment as a major culprit in PD pathogenesis [19]. Most of the mutations found in human PD genes also affect mitochondrial integrity and/or function, directly or indirectly. More recently, findings showing transfer of α-synuclein between glia and neurons have brought new insights into the mechanisms of disease progression [20–22].

2.2. Engrailed as a survival factor for mDA neurons in the adult

A detailed knowledge of the genes and mechanisms that govern mDA neuron development and survival could provide new therapeutic strategies to prevent or halt the loss of mDA neurons and/or restore their function. In this respect, tremendous progress has been made in the recent years in dissecting the genetic networks and signaling pathways involved in mDA neuron development. These studies have established that induction, specification, maturation, maintenance and survival of these neurons are regulated by the concerted action of signaling molecules of the Shh, Fgf8, Tgf-ß and Wnt pathways and a number of transcription factors including En-1, Otx2, Pitx3 or Nurr1 [23]. Of particular interest is the fact that many of these transcription factors remain expressed throughout adulthood, although their adult functions are not well characterized. In this context, we were interested in examining the role of Engrailed in adult mDA neurons.

Loss of function studies have established that Engrailed genes are required for the survival of embryonic mDA neurons during development and that pro-survival activity of the genes is dose-dependent [24–27]. *En1* continues to be expressed in adult mDA neurons of the SNpc and VTA. To examine whether En1 is a survival factor for adult mDA neurons, *En1* heterozygous mice (carrying only one functional *En1* allele; *En1*+/−; *En2*+/+) were analyzed at various ages for mDA survival. We found (Fig. 1B) that the number of tyrosine hydroxylase (TH)-positive neurons in the SNpc of *En1* heterozygous mice started to decline progressively at 6 weeks of age reaching a 38% reduction (compared to wild-type mice) at 48 weeks [28]. Intriguingly and similarly to PD in humans, progressive mDA cell death was also observed in the VTA but to a lesser degree (20% at 48 weeks).

The number of mDA neurons in *En1* heterozygous mice did not further decrease in older animals, suggesting that some mDA neuron populations are less dependent upon En1 activity for their survival. The lower death of VTA mDA cells and the role demonstrated for Otx2 in the survival of VTA mDA neurons [29,30] suggest that Otx2 might be contributing to the greater VTA mDA neuronal survival observed in the *En1* heterozygous mice. This latter hypothesis is also supported by recent data on the pro-survival role of Otx2 in animal models of glaucoma [31]. This does not eliminate other candidates, including other transcription factors. For instance conditional ablation of *Nurr1* in the adult using a Cre/loxP strategy leads to the slow and progressive loss of mDA neurons [32]. Thus, the study of transcription factors that control various steps of mDA cell development should be extended to a possible role in mDA neuron maintenance and thus PD pathogenesis in the adult. It is noteworthy that *En1* has recently been identified as a possible susceptibility gene for sporadic PD in humans [33].

2.3. Engrailed heterozygous mice as a valuable animal model for PD

A number of transgenic and knockout mouse lines have been generated using human PD genes to examine the function of these genes in mDA cell physiology and to develop animal models for PD. These mice have been and still are very useful to gain insight into various aspects of PD pathogenesis. However, most of these animal models do not exhibit key features of PD such as the slow progressive demise of mDA neurons in the SNpc and a decreased sensitivity of VTA cells. In this respect *En1* heterozygous mice may provide the most faithful model of PD. In support of this possibility are PD-like motor symptoms (Fig. 1C) that include decreased spontaneous locomotor activity (distance travelled, rearing), increased amphetamine sensitization or decreased motor coordination and sensorimotor learning (rotarod test) observed in these mice [28]. In addition, *En1* heterozygous mice present non-motor behavioral symptoms such as an increased depression-like behavior (forced swimming test), anhedonic-like behavior (decreased saccharine preference) and poor social interactions that are similar to symptoms in patients. These phenotypes suggest that the mesolimbic system is also affected, probably a consequence of mDA cell death in the VTA.

We are currently further characterizing this model to unravel the mechanisms responsible for mDA neuron degeneration resulting from *En1* haplo-deficiency. We are particularly interested in determining whether degeneration is anterograde or retrograde. A retrograde degeneration would further tighten the link with the human pathology.

2.4. Therapeutic potential of Engrailed or Otx2 to prevent mDA cell loss

Homeoprotein internalization and addressing to the cytoplasm and the nucleus of live cells in vitro and in vivo makes it tempting to consider these proteins as putative therapeutic molecules. This property was exploited in a gain of function approach to demonstrate that En1/2 proteins can function as survival factors for adult mDA neurons. Engrailed infused dorsal to the SNpc in 6 week-old *En1* heterozygous mice was able to diffuse into the SNpc and to completely halt mDA cell loss (Fig. 1B) [28]. We are currently evaluating the ability of En1/2 to rescue mDA neurons in other neurotoxic and genetic PD models. As mentioned above, mDA

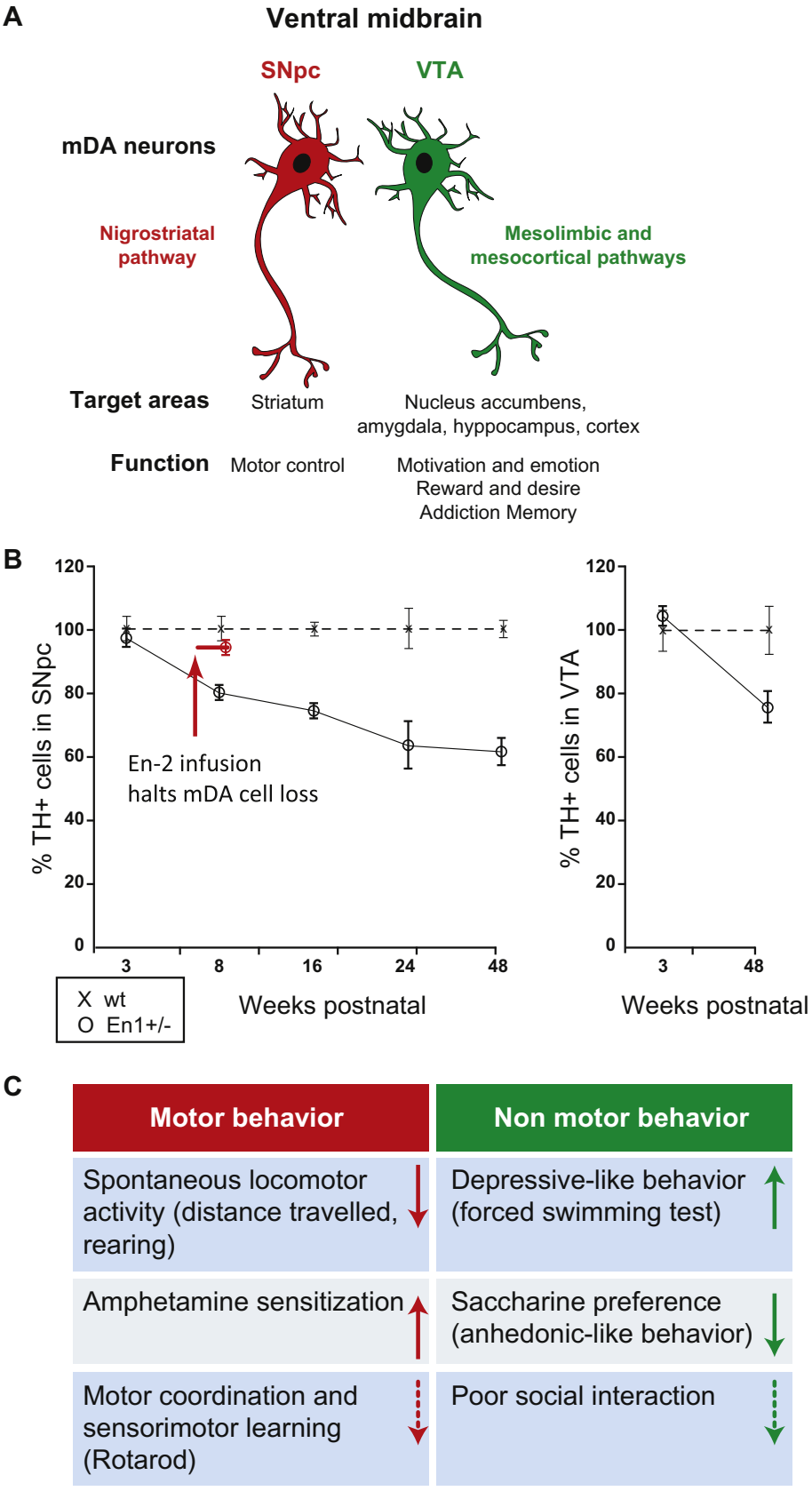

Fig. 1. (A) mDA neurons in the SNpc form the nigrostriatal pathway while those in the VTA contribute to mesolimbic and mesocortical pathways. Loss of mDA neurons in the SNpc results in motor symptoms of PD while loss of VTA mDA neurons leads to cognitive/psychiatric changes. B, left. *En1+/−* mice show a progressive loss of TH-positive mDA cells of the SNpc that starts at about 6 weeks after birth. Infusing recombinant Engrailed starting at 6 weeks (red line) arrests the loss of mDA cells measured at 9 weeks (red circle). (B) Right. The loss of mDA neurons in VTA occurs later and is less pronounced as compared to the SNpc. (C) *En1+/−* mice manifest both motor and non-motor PD-like symptoms. For details see [28].

neurons in the VTA are relatively spared in PD and it is suspected that this is due to a greater dependence on Otx2 than En1/2. Conditional knockout of *Otx2* in the *En1* expression domain in the midbrain results in a dramatic reduction of TH-positive projections in all target areas of VTA mDA neurons whereas projections to the dorsolateral striatum, a target of mDA neurons of SNpc, are relatively spared [30,34]. In line with the idea that Otx2 is a survival factor for these neurons in the adult, is the demonstration that Otx2 gain of function (using lentiviral vectors) protects mDA neurons against MPTP intoxication in both VTA and SNpc [35]. Like many homeoproteins, Otx2 is also able to transduce cells and has been used to protect retinal ganglion cells in an excitotoxic model of glaucoma as described below or to manipulate the critical period plasticity in the visual cortex [11]. It would be interesting to examine to what extent Otx2 transduction can protect mDA neurons in different PD models.

3. Otx2 and glaucoma

3.1. The retina and glaucoma

The eye is a window onto the brain with the retina being a part of the CNS accessible outside of the brain case and the brain blood barrier. The retina is a sheet of identified neuronal cells in stereotypic layers and has been used as a model in neuroscience (Fig. 2A). Light is transduced by cone and rod photoreceptors in the outer nuclear layer that synapse onto bipolar cells in the inner nuclear layer. Bipolar cells pass on visual information to retinal ganglion cells (RGCs) that provide the only output from the retina to the brain via the optic nerve. In addition to this vertical organization of information transfer, the organization of the retina also permits horizontal information processing by the horizontal cells in the inner nuclear layer that modulate the synapses between photoreceptors and bipolar cells and by amacrine cells that modulate the information transfer between bipolar cells and the RGCs. Loss of RGCs due to glaucoma or optic neuropathies leads to irreversible and profound blindness.

Glaucoma is the second leading cause of blindness in people over 50 years of age and there are an estimated 67 million cases in the world today. Although the specific cause of glaucoma is generally unknown important risk factors include age, ethnic group, ocular trauma, diabetes and hypertension. A number of cases are associated with elevated intraocular pressure, however, not all patients with elevated intraocular pressure respond to treatment.

Otx2 is a mammalian ortholog of the orthodenticle homeogene of drosophila [36]. In situ hybridization studies of retina have shown *Otx2* mRNA to be expressed in photoreceptors and bipolar cells (Fig. 2A), while immunolocalization studies have reported Otx2 protein in photoreceptors, bipolar cells and RGCs [37,38]. The presence of Otx2 in RGCs in the absence of detectable mRNA can be explained by the capture by RGCs of the protein released by photoreceptors and/or bipolar cells [11,39]. The ability of RGCs to take up extracellular Otx2 was directly shown in experiments in which recombinant Otx2 was injected into the eye and the protein was detected in cells of RGC axon targets, the lateral geniculate nucleus and the superior colliculus [11,40]. Based on the ability of RGCs to take up Otx2 as shown for Engrailed and mDA cells, and that Engrailed promotes survival of mDA cells (see above) we hypothesized that Otx2 would stimulate the survival of injured adult RGCs.

3.2. Otx2 and RGC survival

A first series of experiments was conducted using cells cultured from adult mouse retina. In these cultures 80–90% of injured RGCs die within 24–48 h. Otx2 in the nM range increased the number of RGCs 2- to almost 5-fold at 6 days in vitro (Fig. 2B and [31]). In this type of mixed culture all retinal cell types are put in culture. Therefore, it is not clear from these results if Otx2 acts directly on the RGCs or whether Otx2 stimulates the expression of a survival factor by other cell types. To answer this, RGCs were purified from adult rat retina and cultured with or without Otx2. Again, Otx2 in the nM range stimulated RGC survival in a dose-dependent manner, demonstrating that Otx2 acts directly on injured adult RGCs to promote their survival.

3.3. Otx2 and sparing of visual function

N-Methyl-D-aspartic acid (NMDA) kills RGCs in vivo and has been proposed as a rapid model of glaucoma [41,42]. Two millimolar NMDA typically reduced the number of RGCs by 33–60% as assessed by counting cyano fluorescent protein-expressing RGCs or by qPCR for Brn3A, a surrogate marker for RGCs [31]. 30 ng of Otx2 injected into the eye offered 100% protection against NMDA-induced RGC excitotoxicity (Fig 2C). This quantity of Otx2 corresponds to an initial dose of about 100 nM before the protein is degraded and/or taken up by the general circulation. To evaluate whether the surviving RGCs remain functional, visual acuity was assessed with an optomotor test. Intraocular NMDA significantly reduced the number of head turns in response to rotating pattern of black and white bars at a spatial frequency of 0.375c/d demonstrating a loss of visual acuity. The number of head turns in mice injected with NMDA and Otx2 was significantly greater than in mice injected with NMDA alone and was not different from the number before treatment [31]. Thus, Otx2 not only promotes the survival of RGCs in vivo, but it also prevents the loss of visual function.

3.4. Mechanism of Otx2 RGC survival activity

Otx2 injected into the eye accumulates in retina [31]. At low therapeutic doses Otx2 is primarily in the ganglion cell layer, however at higher doses the protein accumulates in cell bodies in all retinal layers. In the ganglion cell layer exogenous Otx2 can be observed in nuclei and cytoplasm. Previous work on Engrailed showed that two amino acids in the third helix of the homeodomain are essential for protein internalization by cells [43]. By analogy we mutated the corresponding amino acids in Otx2 and predicted that the mutated protein would be internalization deficient. Whereas wild type Otx2 accumulated in cell nuclei and cytoplasm of cells in the retina, the mutant protein did not [31]. Interestingly, internalization deficient Otx2 did not have any RGC survival-promoting activity in vitro or in vivo. Thus Otx2 internalization is necessary for Otx2 survival activity.

Additional experiments showed that dissociated adult RGCs in culture die by apoptosis and that Otx2 survival-promoting activity can be at least partially attributed to anti-apoptotic action. In vivo, NMDA causes RGC death by apoptosis and perhaps by other mechanisms. The fact that Otx2 protects 100% in vivo is consistent with an anti-apoptotic activity of the protein [31]. It is currently unknown if Otx2 blocks an apoptotic pathway or stimulates a pro-survival pathway by transcriptional and/or translational control of proteins such as BAD, Bax or Bcl2. Alternatively, but not mutually exclusive, Otx2 may stimulate rapid production of small effector molecules.

4. Protein transduction for neurodegeneratives diseases

Protein transduction represents an attractive wider approach for the delivery of therapeutic proteins or peptides to confer neuroprotection in the brain or for generating various neuronal types

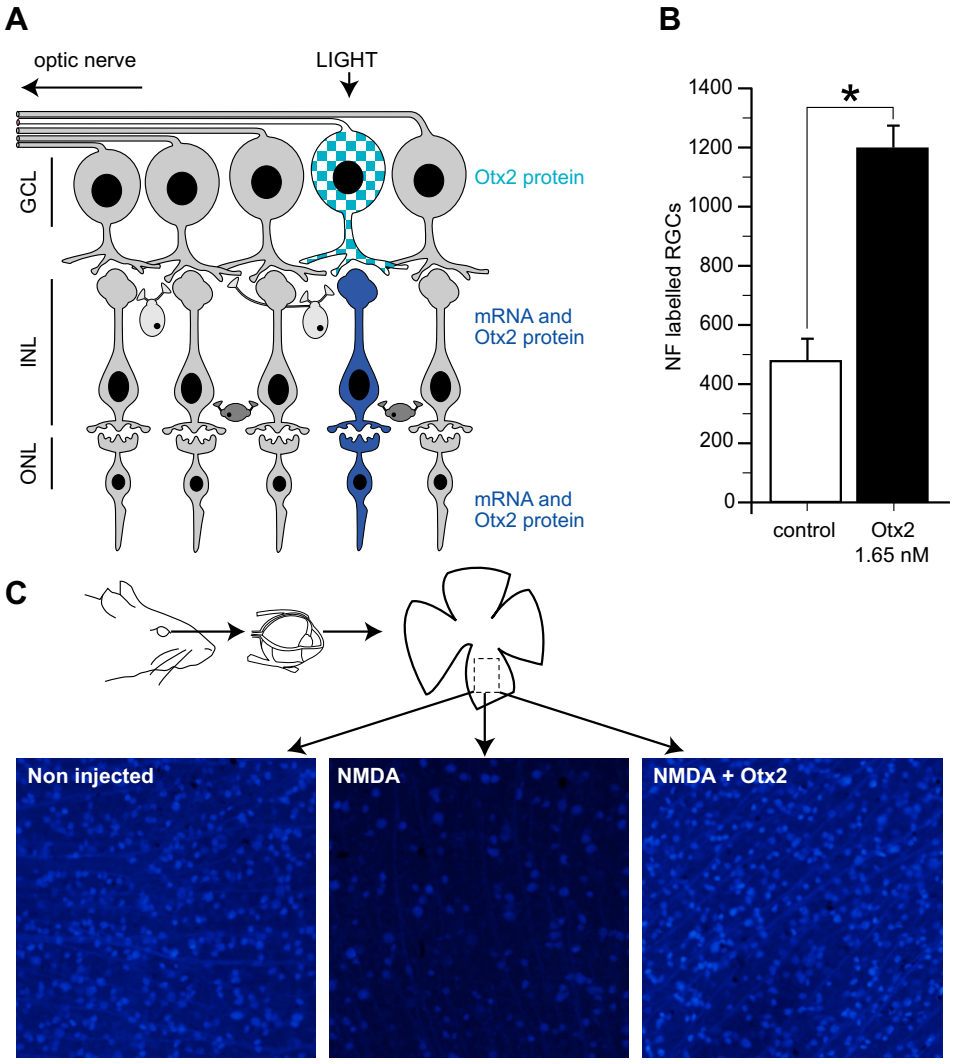

Fig. 2. (A) Schematic of the mammalian retina. GCL: Ganglion cell layer; INL: Inner nuclear layer; ONL: outer nuclear layer. (B) Dissociated adult mouse retinal cells were cultured in defined conditions. RGCs identified by neurofilament immunofluorescence and the number surviving at 6 days in culture were significantly increased when cells were treated with Otx2. (C) Otx2 protects against the NMDA-induced loss of RGCs in vivo. Thy1/CFP mice were injected with NMDA or NMDA + Otx2 in the right eye and 4 days later the retinae were flat mounted (upper). NMDA greatly reduces CFP-positive RGCs (middle) compared to the non injected eye (left). Otx2 prevents the NMDA-induced loss of CFP-positive RGCs (right). For details see [31].

towards cell replacement therapies for neurodegeneratives diseases. For instance, cell-permeable tat-HSP70 or tat-Bcl2 proteins have been used to protect mDA neurons against MPTP in vitro or in vivo [44,45]. The delivery of small peptides using cell penetrating peptides (CPPs) can also be an original strategy to disrupt protein-protein interactions in order to interfere with signaling pathways that are dysregulated in neurodegenerative conditions. Interestingly, a CPP designed to inhibit c-Jun N-terminal kinase in this manner was reported to be highly effective in models of neuronal degeneration following ischemia or against excitotoxic retinal ganglion cell death [46,47].

Besides potential applications for neuroprotection, protein transduction has also been used to generate inducible pluripotent stem cells (iPSCs) for cell replacement therapies [48]. Transfection of the transcription factors Oct4, Sox2, Klf4 and c-Myc can induce pluripotency in mouse fibroblasts. Neurons derived from such reprogrammed fibroblasts can functionally integrate into fetal brain and improve symptoms of rats with PD-like symptoms. Interestingly, human iPSCs can also be generated by direct transduction of fibroblasts with these four transcription factors using CPPs [49]. However, an important drawback in the potential use of cell replacement strategies might arise from the recent observations that α-synuclein from affected cells can propagate into grafted dopaminergic neurons, and perhaps into other grafted cells eventually neutralizing their therapeutic activity [50].

Finally, protein transduction was recently used to generate new animals models of PD. Tat-mediated delivery of mutated α-synuclein (A30P or A53T) or wt nitrated α-synuclein to the SNpc can lead to rapid death of mDA neurons and degeneration of the nigrostriatal pathway [51,52]. This approach might be very useful to generate gain of toxic function models of PD in a relatively short time-scale that would be useful for testing novel therapeutic approaches.

5. Concluding remarks

Here we presented two examples of homeoprotein neuroprotection in the adult brain relevant to human neurodegenerative diseases. The effectiveness of Engrailed and Otx2 homeoproteins as potential therapeutic molecules in PD and glaucoma derives largely from their ability to readily cross cell membranes. While this ability is due to their homeodomains, it is possible that other regions of these proteins are important or perhaps even necessary

for their neuroprotective effects. Homeoproteins can act on several levels such as gene transcription, protein translation and possibly epigenetically, and it will be interesting to identify the targets of their neuroprotective action. Finally, the expression of other homeoproteins in other brain regions provides a glimmer of hope that if neuroprotective activity were present in the other proteins of the family, homeoproteins might provide interesting reagents for the treatment of other neurodegenerative diseases such as Alzheimer's and Huntington's.

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