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The Role of Otx2 in Adult Mesencephalic–Diencephalic Dopaminergic Neurons

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Abstract Mesencephalic and diencephalic dopaminergic (mdDA) progenitors generate two major groups of neurons corresponding to the A9 neurons of the substantia nigra pars compacta (SNpc) and the A10 neurons of the ventral tegmental area (VTA). MdDA neurons control motor, sensorimotor and motivated behaviour and their degeneration or abnormal functioning is associated to Parkinson's disease and psychiatric disorders. Although relevant advances have been made, the molecular basis controlling identity, survival and vulnerability to neurodegeneration of SNpc and VTA neurons remains poorly understood. Here, we will review recent findings on the role exerted by the transcription factor Otx2 in adult mdDA neurons. Otx2 expression is restricted to a relevant fraction of VTA neurons and absent in the SNpc. In particular, Otx2 is prevalently excluded from neurons of the dorsal–lateral VTA, which expressed Girk2 and high level of the dopamine transporter (Dat). Loss and gain of function mouse models revealed that Otx2 controls neuron subtype identity by antagonizing molecular and functional features of the dorsal–lateral VTA such as Girk2 and *Dat* expression as well as vulnerability to the parkinsonian MPTP toxin. Furthermore, when ectopically expressed in the SNpc, Otx2 suppresses *Dat* expression and confers efficient neuroprotection to MPTP toxicity by suppressing efficient DA uptake.

Keywords Otx2 · mdDA · Dopamine transporter · Ventral tegmental area · Substantia nigra pars compacta

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Introduction

Dopamine, one of the neurotransmitters of the vertebrate central nervous system, is synthesized by specialized populations of neurons distributed in the telencephalon, diencephalon and mesencephalon. Mesencephalic and diencephalic dopaminergic (mdDA) neurons are located in stereotypic positions corresponding to the ventral tegmental area (VTA), the substantia nigra pars compacta (SNpc) and the retrorubral field (RRF). MdDA neurons of the VTA project to the ventromedial striatum, nucleus accumbens, temporal lobe and olfactory tubercle, while those of the SNpc innervate the dorsolateral striatum [1–3]. The clinical relevance of mdDA neurons is high because of their regulatory and modulating functions in motor, sensorimotor and motivated behaviours. Indeed, impairment in survival and/or development of mdDA neurons is responsible for abnormal control of voluntary movement and cognition [3–9]. Degeneration of dopamine (DA) neurons in the SNpc and concomitant loss of DA innervation to the striatum leads to the characteristic symptoms of Parkinson's disease (PD) while dysregulation of VTA DA neurons may contribute to psychiatric disorders such as drug addiction, anhedonia and schizophrenia. These pathologies highlight the relevance of this population of neurons and the enormous effort to understand the molecular basis controlling identity and fate of mdDA progenitors as well as

survival, functioning and sensitivity to neurodegeneration of mature mdDA neurons. Relevant advances have been made in the last years in the comprehension of the molecular mechanism controlling neurogenesis and survival of mdDA neurons [10–13]. In this context, several transcription factors including Pitx3, Lmx1a, Lmx1b, En1/2, Msx1, Foxa2, Ngn2 and Otx2, the orphan nuclear receptor Nurr1, the Wnt1 and Wnt5a members of the Wnt family and the retinoic acid play a relevant role in the neurogenesis of mdDA neurons [14–36]. However, despite the advancement on mdDA neurogenesis, little is known about the molecular mechanism controlling phenotypic identity of VTA and SNpc neurons as well as functional diversity between and within SNpc and VTA neurons. For example and strictly related to PD disease, the molecular basis determining for SNpc neurons a higher sensitivity to neurodegeneration as compared to VTA neurons is virtually unknown. In this context, DA uptake, which is one of the crucial steps for determining the cytosolic level of DA may be differentially regulated in SNpc and VTA neurons by the cellular level of the glycosylated active form of the dopamine transporter (glyco-Dat), whose distribution is not uniform, but preferentially restricted to SNpc and dorsal–lateral VTA neurons [37]. Interestingly, the distribution of glyco-Dat correlates with the neurodegeneration map caused by PD and 1-*methyl*-4-phenil-1, 2, 3, 6-tetrahydropyridine-HC1 (MPTP) toxin [37–40]. Furthermore, the fact that several gene markers are expressed in specific subsets of VTA and SNpc neurons reinforces the idea that these two groups of neurons may include multiple neuronal subtypes, potentially determining a complex source of functional diversity [41, 42]. In this context, calbindin D28K (Calb) is expressed in a relevant fraction of VTA neurons proposed to be more resistant to neurodegeneration and in a small subset of SNpc neurons; conversely, the G-protein-gated inwardly rectifying K^+ channel (Girk2) is expressed in a relevant proportion of SNpc neurons and in the dorsal–lateral VTA together with glyco-Dat; similarly, the aldehyde dehydrogenase family 1, subfamily A1 gene, also known as Ahd2, or Aldh1a1 or Raldh1, is expressed in the SNpc and in the ventral–medial VTA [10, 25, 26, 43–49]. Altogether these findings are reinforcing the idea that VTA and SNpc neurons may require during post-mitotic maturation and adult life further genetic instructions regulating maintenance, survival, natural cell-death and sensitivity to degeneration. Here, we have reviewed recent findings indicating that the transcription factor Otx2, which plays a relevant role in mdDA neurogenesis [33–36] is also required in the VTA to control neuron subtype identity and may be involved in DA metabolism by antagonizing efficient DA uptake. Through this control Otx2 may provide mdDA neurons with resistance to the parkinsonian MPTP neurotoxin [49, 50]

Otx2 Expression is Restricted to VTA Neurons in the Adult Brain

Based on the fact that Otx2 is required in mdDA progenitors only for neurogenesis of mesencephalic DA (mesDA) neurons, it was investigated whether Otx2 is also expressed in mature mdDA neurons. A detailed analysis of Otx2 expression has revealed that in mouse at the embryonic day (E) 12.5 Otx2 is abundantly expressed in mdDA progenitors located in the floor plate region of the posterior diencephalon and mesencephalon, while in differentiated TH^+ neurons is detected only in a fraction of those originating from mesDA progenitors and fated to populate the VTA [41, 49]. This finding, which correlates with a similar specificity previously reported for Otx2 in the control of proliferation and differentiation of mesDA progenitors [35], has suggested the existence of a fine mechanism preventing Otx2 expression in TH^+ neurons of the SNpc. This mechanism should operate prevalently during post-mitotic transition of progenitors fated to generate SNpc neurons and should be extremely efficient. In contrast Otx2 is a stable molecular correlate of a large fraction of VTA neurons even during post-natal life [49]. Despite the great effort lavished to identify selective markers of the SNpc and VTA neurons, very few gene functions, if any, have been shown to be exclusively expressed in the VTA or SNpc. Nevertheless, several genes (e.g. Girk2, Ahd2, Calb) are preferentially but not exclusively expressed in SNpc (Girk2, Ahd2) or VTA (Calb) neurons [26, 41, 42, 44, 46, 49, 51]. Alternatively, gene functions such as Pitx3, Nurr1, Lmx1b, En1 and Foxa2, which play a crucial role in specification and survival of mdDA neurons, are ubiquitously expressed in post-mitotic embryonic and adult VTA and SNpc neurons [10–13]. In this context, Otx2 represents the first transcription factor with a crucial role in mesDA neurogenesis that is expressed in adult VTA neurons and excluded from those of the SNpc. A further observation is that, although restricted to VTA neurons, Otx2 is expressed only in a subset of them thus reinforcing the idea that VTA neurons are differentially patterned and, on the basis of their expression code and position, may include neuronal subpopulations with different functional properties (Fig. 1) [49]. This possibility is also supported by the restricted expression exhibited by Calb, Girk2 and Ahd2, and the phenotypic analysis of Pitx3 mutants. Indeed the combinatorial analysis of Calb, Girk2 and Ahd2 expression patterns in the VTA suggests the presence of several neuronal subpopulations [25, 44, 46, 49] and lack of Pitx3, which is expressed in virtually all mdDa neurons, generates the selective loss of SNpc neurons [10–12]. Otx2 expression is prevalently excluded from neurons of the dorsal–lateral corner of the VTA, which exhibit Girk2

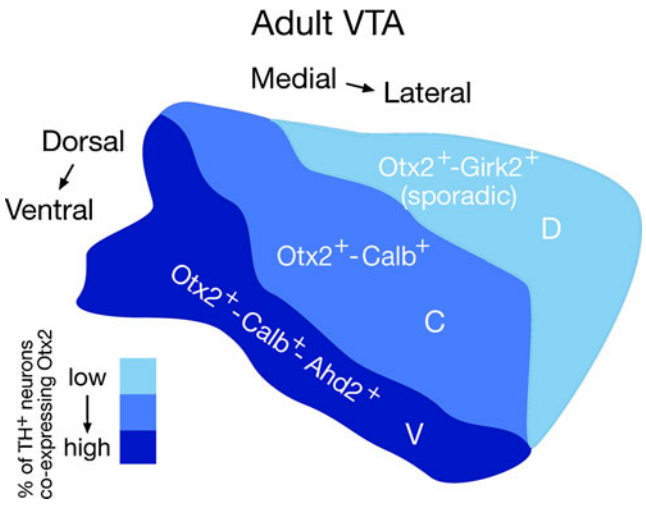

Fig. 1 Schematic representation summarizing Otx2 expression in the VTA of adult mice. Otx2 is prevalently detected in TH^+ neurons located in the central and ventral VTA with a graded increase along the dorsal–ventral axis. In particular, Otx2 is co-expressed with TH in most of the ventral–medial VTA neurons, and this distribution is gradually decreased in the central and dorsal–lateral VTA. Marker analysis has also shown that Otx2 is sporadically co-expressed with Girk2, while most if not all of the $Otx2^+$ neurons are $Calb^+$, and those located in the ventral VTA are also $Ahd2^+$. Abbreviations: *VTA* ventral tegmental area, *D* dorsal, *C* central, *V* ventral

expression and high level of glyco-Dat; while Otx2 is co-expressed with Calb in the central VTA and with both Calb and Ahd2 in the medial and ventral VTA (Fig. 1). Noteworthy, the percentage of TH^+ neurons co-expressing Otx2 exhibits a graded increase along the dorsal–ventral (D-V) axis of the VTA (Fig. 1). This finding suggests that VTA neurons may be regionalized in subpopulations of differentially specified neurons. In this context it has been suggested that Calb is expressed in VTA neurons more resistant to neurodegeneration, while Girk2 and glyco-Dat in SNpc and VTA neurons more vulnerable to neurodegeneration [37, 46–48].

Otx2 Requirement for Neuron Subtype Identity in the VTA

Conditional loss and gain of function mouse models have been generated and studied to investigate whether Otx2 is required in adult VTA neurons to control their identity and functional features [50]. To this aim, Otx2 has selectively been ablated from the VTA or ubiquitously activated in SNpc and VTA neurons by using a mouse mutant carrying a post-mitotic mdDA-specific Cre recombinase inserted into the 3′ untranslated region of the Dat gene ($Dat^{ICre/+}$) [52], which, thereby, is not affected in Dat synthesis. Moreover, to provide a detailed analysis of the Otx2 contribution in VTA neurons and investigate selectively $Otx2^+$ neurons even in the absence of the Otx2 protein, the $Otx2^{flox}$ allele has been coupled with the $Otx2^{GFP}$ allele [50]. To study the consequence of Otx2 ubiquitous activation in SNpc and VTA neurons, the $Dat^{ICre/+}$ mouse model has been employed also to generate an allelic series of mouse mutants expressing increasing dosages of Otx2. To this aim it has been employed the $tOtx2^{ov/+}$ transgenic mouse model [35] and a second mutant carrying an Otx2 conditionally activable allele in the *Rosa26* (*R26*) locus ($R26^{Otx2/+}$). Thus the inactivation of Otx2 has been studied in the VTA of $Dat^{ICre/+}$; $Otx2^{GFP/+}$ control and $Dat^{ICre/+}$; $Otx2^{GFP/flox}$ mutant mice and the activation in the SNpc and VTA of $Dat^{ICre/+}$; $tOtx2^{ov/+}$, $Dat^{ICre/+}$; $R26^{Otx2/+}$, $Dat^{ICre/+}$; $tOtx2^{ov/ov}$, $Dat^{ICre/+}$; $R26^{Otx2/Otx2}$ and $Dat^{ICre/+}$; $tOtx2^{ov/ov}$; $R26^{Otx2/Otx2}$ mutants [50]. This analysis has revealed no relevant difference in the number of GFP^-–TH^+ and GFP^+–TH^+ neurons between $Dat^{ICre/+}$; $Otx2^{GFP/+}$ control and $Dat^{ICre/+}$; $Otx2^{GFP/flox}$ mutant brains suggesting that Otx2 is apparently not required for survival of VTA neurons. The identity of GFP^+ neurons has been analyzed by determining whether the percentage of GFP^+ neurons co-expressing Calb, Ahd2 or Girk2 is altered in the absence of Otx2. While a similar percentage of GFP^+–$Calb^+$ and GFP^+–$Ahd2^+$ neuronal subsets has been detected in control and mutant mice, in contrast, the percentage of GFP^+–$Girk2^+$ neurons has shown a remarkable increase in mice lacking Otx2. This increase is particularly evident in the central VTA. Apparently no abnormality has been detected in the expression of Pitx3, Foxa2 and Nurr1, which play crucial roles in the establishment and survival of mdDA neurons [10, 31, 53]. Thus, these findings suggest that Otx2 is required to antagonize prevalently in neurons of the central VTA the expression of Girk2, a marker of the dorsal–lateral VTA. A similar analysis performed in mouse mutants expressing high dosage of Otx2 ($Dat^{ICre/+}$; $R26^{Otx2/Otx2}$ and $Dat^{ICre/+}$; $R26^{Otx2/Otx2}$; $tOtx2^{ov/ov}$) has revealed that the TH^+ subpopulations expressing Calb or Ahd2 or Girk2 exhibit no relevant difference in the SNpc, while in the VTA of these mutants a significant reduction in the percentage of $Girk2^+$–TH^+ neurons has been detected. Therefore, lack of Otx2 induces in neurons of the central VTA the expression of Girk2, which is prevalently confined to $Otx2^-$ neurons of the dorsal–lateral VTA (Fig. 2), and consistent with this finding, robust activation of Otx2 also in the $Otx2^-$ fraction of VTA neurons, generates a relevant reduction in the number of TH^+ neurons expressing high level of Girk2 (Fig. 2) [50]. This suggests that Otx2 is required in neurons of the central VTA to antagonize identity features of the dorsal–lateral VTA. Noteworthy, a major role for Otx2 in embryogenesis is to define the border between adjacent territories or compartments as in the case of the midbrain–hindbrain border or in the ventral

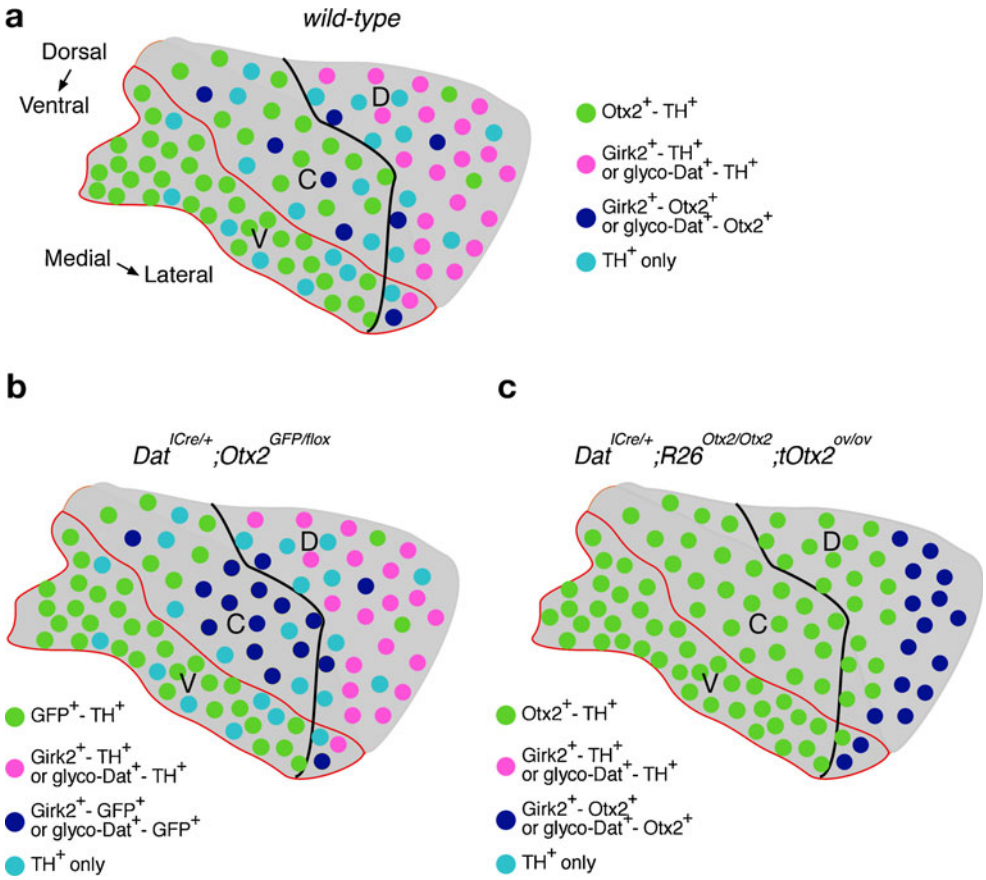

Fig. 2 Schematic summary of the phenotypic impairments detected in the VTA of mouse mutants lacking or ubiquitously expressing extra-copies of Otx2. **a** Otx2 is normally expressed in a relevant fraction of VTA neurons prevalently confined to the central and medial–ventral region of the VTA, even though few $Otx2^+$ neurons may be detected also in dorsal–lateral neurons which in large majority are $Girk2^+$ and glyco-Dat^+. **b** In the absence of Otx2, a relevant increase in the number of GFP^+ neurons expressing Girk2 and glyco-Dat is detected in the central region of the VTA. This increase is reminiscent of a medial–ventral expansion of the dorsal–lateral identity. **c** Conversely, ectopic expression of Otx2 in the dorsal–lateral VTA generates a repression of the dorsal–lateral identity, as revealed by decrease in the number of neurons expressing Girk2 and high level of glyco-Dat. Together these abnormalities lead us to hypothesize a presumptive boundary running between the two subpopulations of VTA neurons expressing or not Otx2 (*black line*). Neurons are represented as *filled circles* and the number of those expressing the different combinations of markers (*filled circles in pink*, *blue*, *green* and *pale blue*) approximately reflects to the experimental percentages. Abbreviations as in the previous figure

midbrain between progenitors of the mesDA and red nucleus compartments [33, 34]. In these processes, Otx2 operates as a repressing factor by interacting with co-repressing molecules such as Grg4 [33, 34, 54, 55]. The expression profile and the phenotype observed in mutants lacking or activating Otx2 in VTA neurons support the possibility that VTA neurons are distributed in compartment-like subregions whose identity is maintained by a molecular code of transcription factors together with positive and negative interacting co-factors. In this context Otx2 would represent one of these factors whose competence appears limited to define the identity of central VTA and/or the presumptive border between central and dorsal–lateral VTA (Fig. 2).

Otx2 Antagonizes Efficient DA Uptake in $Otx2^+$ Neurons of the VTA

Proper DA uptake by mdDA neurons is a crucial step for DA signalling. This event is controlled by the distribution and membrane concentration of glyco-Dat [37]. Glyco-Dat is differentially expressed in SNpc and VTA neurons being prevalently detected at high level in nigrostriatal SNpc neurons and at a lower level in the majority of mesolimbic VTA neurons [37, 56, 57]. In the VTA, the expression of Otx2 and glyco-Dat is largely complementary, thus indicating that Otx2 is prevalently expressed in neurons with a reduced DA uptake. Interestingly, high level of glyco-Dat co-segregates with Girk2 expression in VTA and SNpc.

Importantly, loss or ectopic expression of Otx2 influences the cellular level and distribution of glyco-Dat. Indeed, the percentage of GFP^+ neurons co-expressing high level of glyco-Dat is significantly increased in the central VTA of mutants lacking Otx2, and, consistently, the striatal amount of glyco-Dat as well as the percentage of SNpc and VTA TH^+ neurons expressing high level of glyco-Dat is remarkably diminished in mutants activating Otx2 [50]. These data thus suggest that Otx2 is required to confine VTA neurons expressing high level of glyco-Dat to the dorsal–lateral VTA, and, through this antagonism, by limiting the number of VTA neurons with efficient DA uptake, Otx2 may be required to negatively modulate DA signalling in neurons of the central region of the VTA (Fig. 2). Moreover, through this control Otx2 might likely contribute to define the VTA map of DA uptake. Consistent with this interpretation, the analysis of mouse mutants ectopically expressing Otx2 in dorsal VTA and SNpc has revealed a severe reduction in the number of neurons expressing high level of glyco-Dat. At the molecular level, Otx2 modulates glyco-Dat levels by controlling negatively (directly or indirectly) the level of *Dat* mRNA rather than the glycosylation process [50]. In this context, an attracting possibility is that Otx2 might control *Dat* expression in antagonism with Nurr1, which is in turn required to activate *Dat* expression [58, 59]. Thus, in the central VTA but not in the dorsal–lateral VTA and SNpc, the Nurr1 promoting activity on *Dat* expression would be counteracted by the Otx2 antagonistic effect. However, so far no experimental evidence has been collected supporting this hypothesis.

Otx2 is a Potent Neuroprotective Factor Responsible for SNpc and VTA Differential Sensitivity to MPTP

Glyco-Dat efficiently binds the DA analogue neurotoxin MPTP [37, 38, 60, 61] and its expression, as above mentioned, has recently been shown to be high in SNpc neurons more sensitive to degeneration in PD patients and MPTP-treated mice, and low in more resistant VTA neurons [37]. On this basis it has been studied whether Otx2 depletion in the VTA or its ectopic expression in SNpc and in all the VTA neurons may alter the vulnerability of these neurons to MPTP-induced neurodegeneration. To this aim the number of TH^+, GFP^+–TH^+ and GFP^-–TH^+ neurons has been determined in the VTA of MPTP-treated and untreated control and mutant mice lacking Otx2. In this context, the GFP^-–TH^+ neuronal subset represents a sort of internal control, since, in principle, it should not be additionally affected by the lack of Otx2. Interestingly, a significant reduction in the number of total TH^+ neurons and GFP^+–TH^+ neurons has been detected in MPTP-treated mutant mice, while the GFP^--TH^+ subpopulation is similarly affected by the MPTP treatment in control and mutant mice. Furthermore, when TH^+ neurons of MPTP-treated control mice were subdivided in those expressing Otx2 (GFP^+–TH^+) or not (GFP^-–TH^+), the vulnerability to MPTP of the GFP^-–TH^+ neuronal subset was remarkably higher of that expressing Otx2 (GFP^+–TH^+). Noteworthy, the sensitivity to MPTP of GFP^-–TH^+ VTA neurons is similar to that observed for SNpc neurons in wild-type mice. When this analysis has been performed in mutants lacking Otx2, the neuronal loss of the GFP^+-TH^+ subpopulation is increased, resulting similar to that of the SNpc or the GFP^-–TH^+ VTA neuronal subset. Conversely, ubiquitous activation of Otx2 is sufficient to provide SNpc and VTA neurons with high resistance to MPTP. In particular, mice activating the highest level of Otx2 are almost completely insensitive to the MPTP treatment. In summary these findings indicate that (1) Otx2 may selectively provide $Otx2^+$ (GFP^+)–TH^+ VTA neurons with reduced vulnerability to MPTP thus suggesting that Otx2 may act in the VTA as a physiological neuroprotective factor; (2) in control mice the $Otx2^-$ (GFP^-)–TH^+ VTA subpopulation, which is prevalently composed by $Girk2^+$ neurons expressing high level of glyco-Dat, exhibits a sensitivity to MPTP similar to that of SNpc neurons; (3) in the absence of Otx2 the vulnerability of $GFP^+(Otx2^-)$–TH^+ VTA neurons to MPTP increases and is similar to that of the SNpc; and (4) the Otx2-dependent neuroprotection may be conferred to SNpc neurons and to the $Otx2^-$ VTA neuronal fraction [50]. Together, these findings suggest that Otx2 may remarkably contribute to the SNpc and VTA differential vulnerability to MPTP through modulation of glyco-Dat level, which in turn, may favour or antagonize MPTP internalization with consequent reduction or increase of resistance to MPTP neurotoxicity. The molecular basis controlling SNpc and VTA differential vulnerability to MPTP and Parkinsonian neurodegeneration represents a yet unsolved key issue. Interestingly, the MPTP vulnerability exhibited by VTA neurons of mice lacking Otx2 is comparable to that of normal SNpc neurons, and robust ectopic expression of Otx2 in SNpc generates high resistance of these neurons to MPTP. These findings suggest that Otx2 is responsible, at least in part, for SNpc and VTA differential vulnerability to MPTP-induced neurodegeneration and may confer this property also to SNpc neurons [50]. Collectively, these findings suggest that the Otx2 properties might be exploited for potential design of future therapeutic strategies in regenerative medicine of PD.

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