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Otx2 selectively controls the neurogenesis of specific neuronal subtypes of the ventral tegmental area and compensates En1-dependent neuronal loss and MPTP vulnerability

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ABSTRACT

Understanding the molecular basis underlying the neurogenesis of mesencephalic–diencephalic Dopaminergic (mdDA) neurons is a major task fueled by their relevance in controlling locomotor activity and emotion and their involvement in neurodegenerative and psychiatric diseases. Increasing evidence suggests that mdDA neurons of the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) represent two main distinct neuronal populations, which, in turn, include specific neuronal subsets. Relevant studies provided important results on mdDA neurogenesis, but, nevertheless, have not yet clarified how the identity of mdDA neuronal subtypes is established and, in particular, whether neurogenic factors may direct progenitors towards the differentiation of specific mdDA neuronal subclasses. The transcription factor Otx2 is required for the neurogenesis of mesencephalic DA (mesDA) neurons and to control neuron subtype identity and sensitivity to the MPTP neurotoxin in the adult VTA. Here we studied whether Otx2 is required in mdDA progenitors for the generation of specific mdDA neuronal subtypes. We found that although expressed in virtually all mdDA progenitors, Otx2 is required selectively for the differentiation of VTA neuronal subtypes expressing Ahd2 and/or Calb but not for those co-expressing Girk2 and glyco-Dat. Moreover, mild over-expression of Otx2 in SNpc progenitors and neurons is sufficient to rescue *En1* haploinsufficiency-dependent defects, such as progressive loss and increased MPTP sensitivity of SNpc neurons. Collectively, these data suggest that mdDA progenitors exhibit differential sensitivity to Otx2, which selectively influences the generation of a large and specific subset of VTA neurons. In addition, these data suggest that Otx2 and En1 may share similar properties and control survival and vulnerability to MPTP neurotoxin respectively in VTA and SNpc.

Introduction

Mesencephalic and diencephalic dopaminergic (mdDA) neurons differentiate from progenitors located in the floor plate region of caudal prosomeres and mesencephalon and give rise to the A9 neurons of the substantia nigra pars compacta (SNpc) and the A10 neurons of the ventral tegmental area (VTA) (Björklund and Isacson, 2002; Smidt and Burbach, 2007; Marín et al., 2005; Fu et al., 2012). MdDA neurons play a crucial role in the control of motor, sensorimotor and motivated behaviors (Jellinger, 2001). Degeneration of SNpc neurons is associated to the characteristic symptoms of Parkinson's disease (PD) whereas abnormal functioning of VTA neurons is involved in psychiatric disorders. Relevant advances have been made in the comprehension of the molecular mechanism controlling the neurogenesis of mdDA neurons (Smidt and Burbach, 2007; Prakash and Wurst, 2006; Simeone et al., 2011; Andersson et al., 2006a,b; Kele et al., 2006; Omodei et al., 2008; Ono et al., 2007). However, despite these advancements, it is virtually unknown whether the identity of

Abbreviations: IF, Interfascicular nucleus; PN, Paranigral nucleus; PBP, Parabrachial pigmented nucleus; SNpc, Substantia nigra pars compacta; VTA, Ventral tegmental area

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SNpc and VTA neurons as well as the neuronal subtypes they include are selectively programmed in mdDA progenitors by specific neurogenic factors. Indeed, the fact that several gene markers are expressed in specific subsets of VTA and SNpc neurons reinforces the idea that these two different groups of neurons may include multiple neuronal subtypes (Chung et al., 2005; Greene et al., 2005; Thompson et al., 2005; Di Salvio et al., 2010a,b). We have previously concentrated our efforts on the role exerted by the transcription factor Otx2 in mdDA neurogenesis and adult mdDA neurons. Otx2 is expressed in both diencephalic and mesencephalic DA progenitors but is prevalently required for neurogenesis of mesDA neurons by controlling identity and proliferating activity of mesDA progenitors (Puelles et al., 2003, 2004; Prakash et al., 2006; Omodei et al., 2008). On the other hand, Otx2 expression in adult mice is restricted to a relevant subset of VTA neurons and excluded from those of the SNpc (Chung et al., 2005; Di Salvio et al., 2010b). In VTA neurons Otx2 is prevalently co-expressed with Calbindin D28K (Calb) and aldehyde dehydrogenase family 1, subfamily A1 gene (Ahd2) and only marginally with those located in the lateral-dorsal corner of the VTA and expressing the G-protein-gated inwardly rectifying K^+ channel (Girk2) and a high level of the glycosylated active form of the Dopamine Transporter (glyco-Dat). Functional studies showed that Otx2 is post-mitotically required to control VTA neuronal subtype identity and to provide $Otx2^+$ VTA neurons with resistance to the 1-methyl-4-phenil-1,2,3,6-tetrahydropyridine-HCl (MPTP) parkinsonian neurotoxin (Di Salvio et al., 2010a). Here, we have investigated whether Otx2 controls in mesDA progenitors the generation of specific neuronal subtypes or that of all VTA neuronal subsets regardless of their molecular identity. Our data show that Otx2 is selectively required in mdDA progenitors for the neurogenesis of $Otx2^+$ VTA neurons, which include the subtypes expressing Calb and/or Ahd2. These findings suggest that mdDA progenitors may exhibit differential responsiveness to the same transcription factor and provide evidence that this differential responsiveness may be functionally relevant for programming the generation of specific neuronal subsets. Noteworthy, the VTA neuronal subtypes whose neurogenesis is sensitive to Otx2, correspond to those more resistant to MPTP and less vulnerable to Parkinsonian neurodegeneration (Di Salvio et al., 2010a; Afonso-Oramas et al., 2009). In this context, we find that mild over-expression of Otx2 in SNpc progenitors and neurons significantly compensates vulnerability to MPTP and progressive SNpc neuronal loss caused by *En1* haploinsufficiency (Sgadò et al., 2006; Sonnier et al., 2007; Alvarez-Fischer et al., 2011).

Materials and methods

Mouse strains

The generation of the $Otx2^{GFP/+}$, $Otx2^{flox/+}$, $En1^{Cre/+}$ and $tOtx2^{ov/+}$ mouse strains has previously been described (Puelles et al., 2003; Omodei et al., 2008; Di Salvio et al., 2010a). As for the $Otx2^{CreER/+}$ mouse model, the targeting molecule was obtained by replacing the genomic region spanning the entire Otx2 coding sequence with the tamoxifen-inducible $CreER^{T2}$ recombinase followed by the *Otx2* 3′UTR and polyadenylation signal. The CreER activity was correctly and efficiently driven by Otx2 as deduced by β-Gal staining of $Otx2^{CreER/+}$; *R26R*/+ embryos administered with Tamoxifen (TX) (data not shown).

In situ hybridization and immunohistochemistry

Immunohistochemistry was performed on PFA fixed, wax-included embryos and brains as previously described (Omodei et al., 2008). The rabbit antibodies were directed against Ahd2 (Abcam, 1:1000), Girk2 (Alomone Labs, 1:80), Calb (Swant, 1:400), Lmx1a (Abcam 1:100); Pitx3 (Zymed Invitrogen, 1:300), cleaved Caspase3 (Cell Signaling, 1:100) and TH (Chemicon, 1:600); the goat antibodies against Ahd2 (Abcam, 1:100), Calb (R&D System, 1:30), Otx2 (R&D System, 1:50), Nurr1 (R&D System, 1:20), and GFP (Abcam, 1:100); a second TH antibody was generated in mouse (Chemicon, 1:600); the antibody against glyco-Dat was raised in rat (Abcam, 1:150); the β-Gal antibody was raised in chicken (Abcam, 1:700); the Lmx1b antibody was raised in guinea pig and kindly provided by Prof. C. Birchmeier.

MPTP treatment

Twelve to fourteen week old mice ($n=7$) received a total of 4 intraperitoneal injections of MPTP (25 mg/Kg/injection) at 2 h interval and were sacrificed 7 days after the last injection. Control mice of the same age ($n=7$) were injected with saline solution only. This protocol was previously determined (Di Salvio et al., 2010a) and adopted because of less variability and a better survival of mice.

Cell fate analysis

Pregnant females were intraperitoneally injected with a single dose of TX (50 mg/Kg/injection) at embryonic day (E) 7.7, E8.5, E9.5 and sacrificed at E18.5 or at post-natal day 30 (p30) for immunohistochemistry experiments. At p30 approximately 35–40% of SNpc and VTA TH^+ neurons co-expressed β-Gal. In these mice CreER was activated by a single TX administration at E8.5.

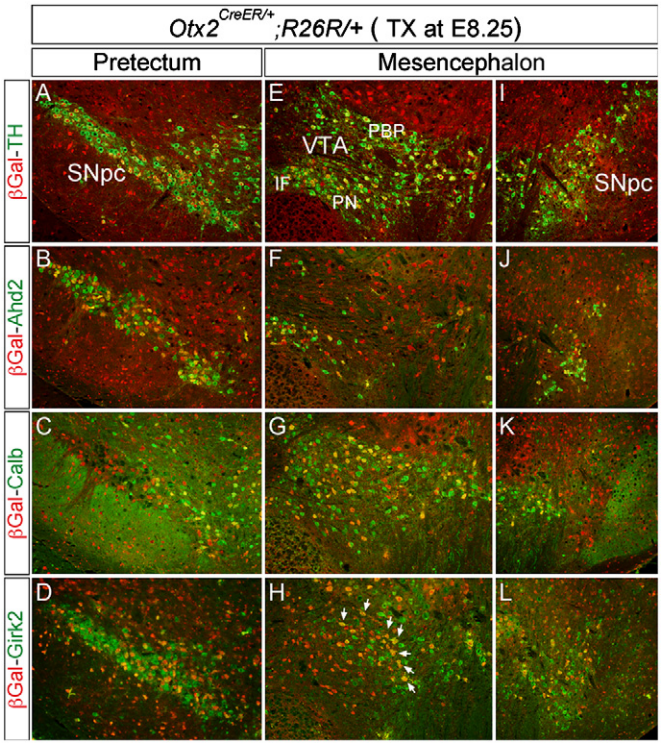

Fig. 1. Cell-fate of mdDA progenitors expressing Otx2. (A–L) Immunohistochemistry experiments with β-Gal and TH (A, E and I), β-Gal and Ahd2 (B, F and J), β-Gal and Calb (C, G and K), and β-Gal and Girk2 (D, H and L) on p30 $Otx2^{CreER/+}$; *R26R*/+ brain administered with TX at E8.5 show that Otx2-driven CreER activates β-Gal expression in mdDA progenitors fated to generated SNpc and VTA TH^+ neurons co-expressing Ahd2 or Calb or Girk2.

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Cell-counting analysis

For each strain, cell-counting of TH^+, TH^+–$Calb^+$, TH^+–$Ahd2^+$, TH^+–$Girk2^+$ and TH^+–glyco-Dat^+ neurons was performed on a number of 7 brains between 12 and 14 weeks of age; 7 brains respectively at 4, 10 and 17 weeks of age were analyzed for *wt*, $En1^{Cre/+}$ and $En1^{Cre/+}$; $tOtx2^{ov/+}$ mouse strains to detect *En1*-dependent survival abnormalities of SNpc neurons.

For all cell-counting experiments, we followed the same procedure previously reported (Di Salvio et al., 2010a). Briefly, brains were systematically sectioned in 8 adjacent series of slides and two series (the 3rd and the 6th) immunostained. Anatomical levels 1 and 6 for both SNpc and VTA were excluded and levels 2–5 selected on both sides of the midline and photographed (Fig. S1). Pictures were printed in A4 format and the number of TH^+ neurons included in the demarcated areas (Fig. S1) was determined. The total cell number ± the standard deviations was shown (Tables S1–S3).

Statistical analysis

Statistical analysis was performed as previously reported (Di Salvio et al., 2010a). To determine the numerical size of the different subsets of TH^+ neurons, the number of TH^+–$Calb^+$, TH^+–$Ahd2^+$, TH^+–$Girk2^+$ and TH^+–glyco-Dat^+ neurons detected in each brain was first normalized to the mean of total TH^+ neurons. For MPTP experiments, TH^+ neurons counted in each of the MPTP-treated brains were compared to those detected in each of the MPTP-untreated brains of the same genotype. The *P* value was calculated by using the *one tail Student t* test.

Results and discussion

Otx2 is ubiquitously expressed in mdDA progenitors fated to generate SNpc and VTA neurons

We have previously reported that in adult mdDA neurons, Otx2 expression is restricted to a large fraction of VTA neurons co-expressing Calb and/or Ahd2. In particular Otx2 is excluded from SNpc neurons and from those of the VTA co-expressing Girk2 and high level of glyco-Dat (Di Salvio et al., 2010a,b). Here, we have investigated the possibility that Otx2 is required in mdDA progenitors to regulate the differentiation of specific VTA and/or SNpc neuronal subtypes, an aspect never addressed before. To this aim we first revisited the Otx2 expression in mdDA progenitors and, then, analyzed whether $Otx2^+$ mdDA progenitors were fated to differentiate in SNpc and VTA neuronal subtypes that we could identify by using Calb, Ahd2 and Girk2 as markers (Di Salvio et al., 2010b). In E9.2, E10.5, E11.5 and E12.5 *wt* embryos Otx2 was fully co-expressed with Sox2, Lmx1b and Lmx1a in mdDA progenitors of pretectum, anterior mesencephalon and posterior mesencephalon (Fig. S2 and data not shown). Noteworthy, at E12.5 Otx2 was detected also in post-mitotic mesDA neurons of the posterior mesencephalon (arrow in Fig. S2W and X). Then to determine the neuronal fate of $Otx2^+$ mdDA progenitors, we employed a mouse model which expressed the $CreER^{T2}$ gene under the transcriptional control of Otx2. In this experiment $CreER^{T2}$ activity was induced in $Otx2^{CreER/+}$; *R26R*/+ mice by a single TX administration at E7.7 or E8.5 or E9.5. Mice injected with TX at E.8.5 were analyzed at p30 and, those injected at E7.7 or E9.5, at E18.5. In the pretectum a relevant fraction of TH^+ SNpc neurons co-expressing Ahd2, Girk2 and Calb were also β-Gal^+ (Fig. 1A–D). Similarly, in mesDA neurons of the VTA and SNpc, β-Gal was co-expressed with Calb or Ahd2 or Girk2 in numerous TH^+ neurons (Fig. 1E–L). For E18.5 embryos administered with TX at E7.7 or E9.5, the cell fate analysis showed similar results (Fig. S3). However, since at E18.5 Girk2 expression was not yet restricted to the dorsal-lateral VTA, presumptive $Girk2^+$ neurons of the dorsal-lateral VTA were indirectly assigned as those not expressing Calb and positive for TH and β-Gal (arrows in Fig. S3I, J, S and T) by performing TH, β-Gal, Calb triple immunohistochemistry assays. These data thus indicate that Otx2 is expressed between E7.7 and E9.5 in mdDA progenitors fated to generate SNpc and VTA neuronal subtypes expressing Calb, Ahd2 or Girk2, which together cover the vast majority of mdDA neurons. These findings clearly show that Otx2 is expressed in mdDA progenitors fated to generate both

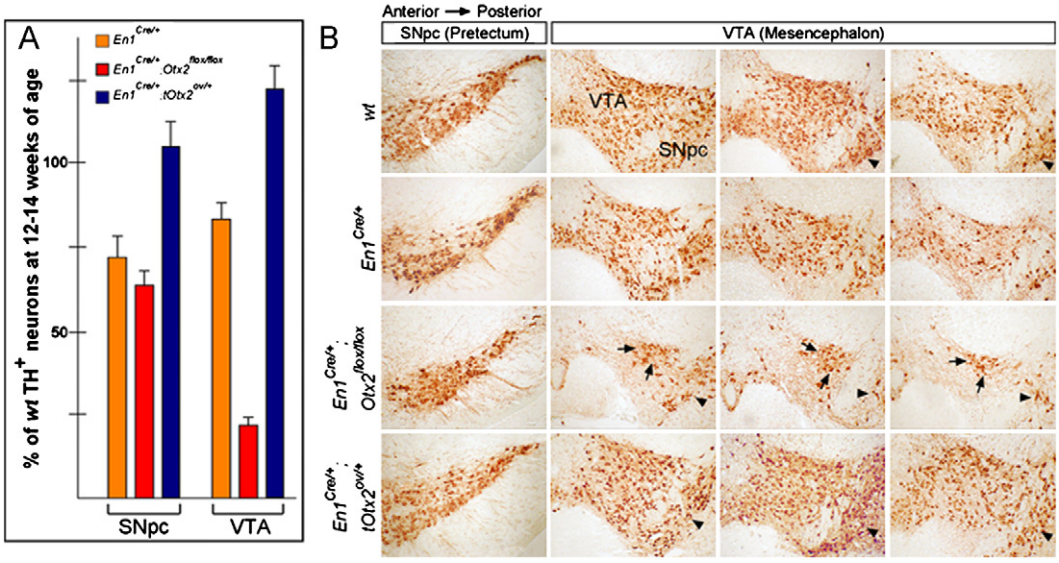

Fig. 2. Lack or mild over-expression of Otx2 in mdDA progenitors affects the generation of VTA neurons. (A) Graphic representation showing the percentage of *wt* TH^+ SNpc and VTA neurons detected in $En1^{Cre/+}$, $En1^{Cre/+}$; $Otx2^{flox/flox}$ and $En1^{Cre/+}$; $tOtx2^{ov/+}$ mutant mice at 12–14 post-natal weeks. (B) TH immunostaining of sequential sections along the pretectum and mesencephalon of *wt*, $En1^{Cre/+}$, $En1^{Cre/+}$; $Otx2^{flox/flox}$ and $En1^{Cre/+}$; $tOtx2^{ov/+}$ mice shows that while the SNpc generated from pretectal progenitors is not severely affected, the VTA exhibits a severe reduction of TH^+ neurons in $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice and a remarkable increase in $En1^{Cre/+}$; $tOtx2^{ov/+}$ mutants. Note that, compared to *wt* mice, also the number of SNpc neurons of mesencephalic origin is diminished in $En1^{Cre/+}$; $Otx2^{flox/flox}$ and increased in $En1^{Cre/+}$; $tOtx2^{ov/+}$ mutants (arrowhead in B). The arrows in (B) point to VTA neurons concentrated in the presumptive lateral-dorsal corner of the VTA. Abbreviations: as in previous Figure.

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TH^+–$Otx2^-$ and TH^+–$Otx2^+$ neurons. TH^+–$Otx2^-$ neurons correspond to those of the SNpc and the VTA subset expressing Girk2; while TH^+–$Otx2^+$ neurons correspond to the VTA neuronal subtypes expressing Calb and/or Ahd2.

Otx2 is required in mdDA progenitors for the selective generation of VTA neuronal subtypes expressing Calb and Ahd2

We previously reported that Otx2 plays a remarkable role in mesDA progenitors by controlling their proliferating activity and differentiation cascade (Omodei et al., 2008). Nevertheless, this analysis did not address whether lack of Otx2 affected the neurogenesis of specific SNpc and VTA neuronal subsets. Since cell-fate experiments showed that Otx2 is expressed in mdDA progenitors fated to generate SNpc and VTA neuronal subtypes that we could identify by using Calb, Ahd2 and Girk2 as markers, we have investigated whether Otx2 is required for the neurogenesis of these mdDA neuronal subtypes. To this aim we analyzed mdDA neuronal subclasses in adult mice lacking or over-expressing mild dosage of Otx2 in mdDA progenitors by *En1*-driven Cre recombinase ($En1^{Cre/+}$; $Otx2^{flox/flox}$ and $En1^{Cre/+}$; $tOtx2^{ov/+}$) (Omodei et al., 2008), and compared them to *wt* and $En1^{Cre/+}$ mice. TH^+ cell-counting showed that, compared to 12–14 week old *wt* mice ($n=7$), the percentage of pretectum-derived SNpc neurons was decreased in $En1^{Cre/+}$; $Otx2^{flox/flox}$ ($n=7$) and $En1^{Cre/+}$ ($n=7$) mutant mice (for $En1^{Cre/+}$ $71 \pm 6\%$ of *wt*; for $En1^{Cre/+}$; $Otx2^{flox/flox}$ $63 \pm 4\%$ of *wt*) (Sonnier et al., 2007; Sgadò et al., 2006), and fairly unaffected in mutant mice over-expressing Otx2 ($n=7$) (Fig. 2A and B). In contrast, compared to *wt* and $En1^{Cre/+}$ mice, the percentage of TH^+ neurons detected in the VTA was remarkably reduced in $En1^{Cre/+}$; $Otx2^{flox/flox}$ ($21 \pm 2\%$ of *wt*) mice and moderately increased in those over-expressing Otx2 ($122 \pm 7\%$ of *wt*) (Fig. 2A and B). Similar abnormalities in the number of TH^+ neurons were also observed in mesencephalic-derived SNpc of both $En1^{Cre/+}$; $Otx2^{flox/flox}$ and $En1^{Cre/+}$; $tOtx2^{ov/+}$ mice (arrowhead in Fig. 2B). Noteworthy, in the VTA of $En1^{Cre/+}$;

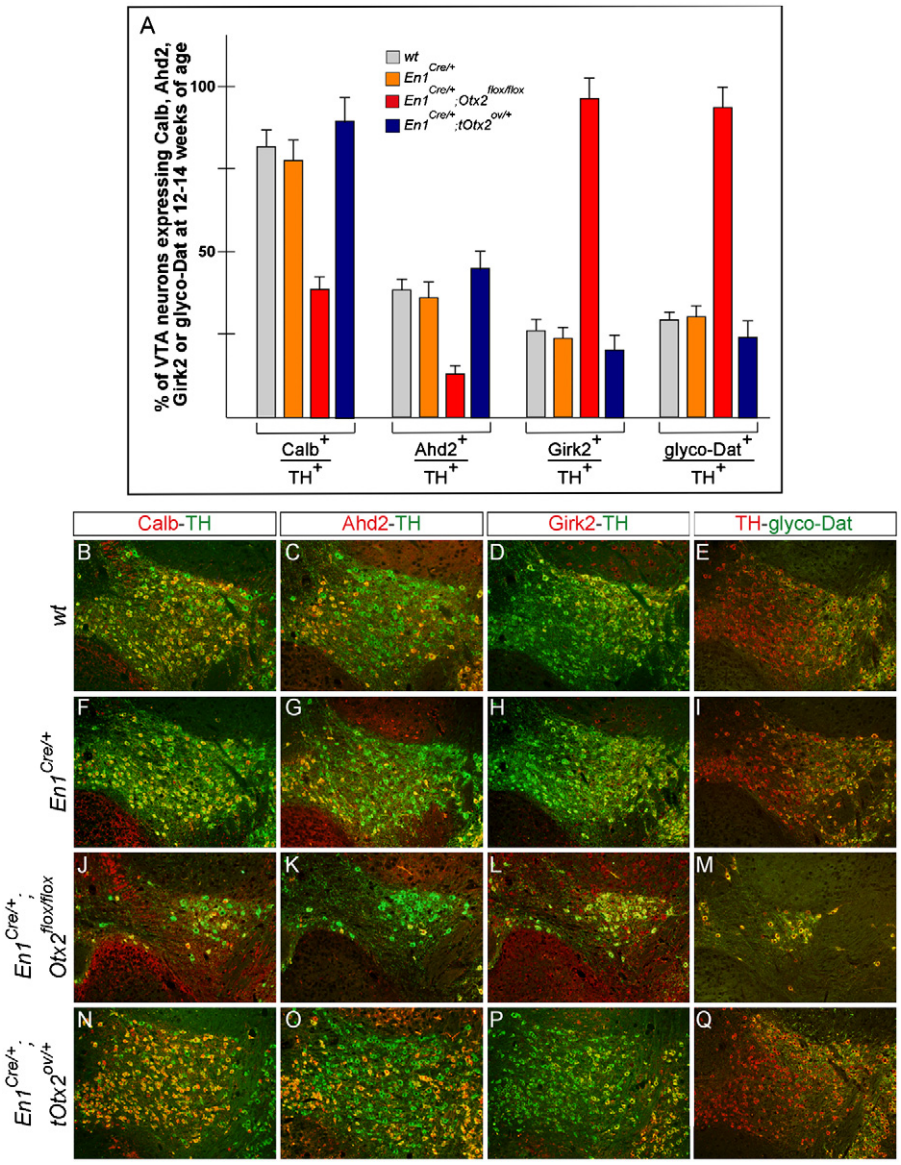

Fig. 3. Loss of Otx2 in mdDA progenitors severely affects the generation of specific VTA neuronal subsets. (A) Graphic representation showing that, compared to 12–14 week old *wt* and $En1^{Cre/+}$ mice, in $En1^{Cre/+}$; $Otx2^{flox/flox}$ mutants virtually all VTA neurons are $Girk2^+$ and exhibit high level of glyco-Dat, while a small percentage of them is $Calb^+$ or $Ahd2^+$; conversely, in $En1^{Cre/+}$; $tOtx2^{ov/+}$ mice a mild reduction in the number of $Girk2^+$ and glyco-Dat^+ neurons and a moderate increase in the percentage of those expressing Calb or Ahd2 was observed. (B–Q) Representative VTA sections of *wt*, $En1^{Cre/+}$, $En1^{Cre/+}$; $Otx2^{flox/flox}$ and $En1^{Cre/+}$; $tOtx2^{ov/+}$ mice immunostained with Calb and TH (B, F, J and N), Ahd2 and TH (C, G, K and O), Girk2 and TH (D, H, L and P) and glyco-Dat and TH (E, I, M and Q).

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Otx2$^{flox/flox}$ mutants, most of the TH$^+$ neurons were concentrated in the presumptive lateral-dorsal corner where Otx2$^-$–TH$^+$ neurons co-expressing Girk2 and high level of glyco-Dat were located in *wt* mice (arrows in Fig. 2B). Then, we analyzed in the same brains the number of SNpc and VTA neurons belonging to the subsets expressing Calb or Ahd2 or Girk2 and glyco-Dat. No significant difference among the different genotypes was detected in the SNpc (Fig. S4 and Table S1). Conversely, in the VTA of mice lacking Otx2, the number of neurons belonging to these subtypes appeared remarkably and selectively affected (Fig. 3 and Table S1). Indeed, compared to *wt* (Fig. 3A–E) and *En1*$^{Cre/+}$ mutants (Fig. 3A and F–I), in *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mice the percentage of VTA TH$^+$ neurons expressing Calb ($38\pm4\%$ vs $82\pm5\%$ of *wt*, $P\ll0.001$) and Ahd2 ($13\pm2\%$ vs $38\pm3\%$ of *wt*, $P\ll0.001$) was dramatically diminished, while virtually all VTA TH$^+$ neurons were Girk2$^+$ ($96\pm7\%$ vs $26\pm3\%$ of *wt*, $P\ll0.001$) or expressed high level of glyco-Dat ($93\pm6\%$ vs $29\pm2\%$ of *wt*, $P\ll0.001$) (Fig. 3A, J–M). Conversely, mice over-expressing Otx2, although not dramatically affected as in the previous case, exhibited an increase in the percentage of VTA neurons expressing Calb ($89\pm7\%$ vs $82\pm5\%$ of *wt*, $P=0.02$) and Ahd2 ($44\pm5\%$ vs $38\pm3\%$ of *wt*, $P=0.012$) and a reduction in the percentage of Girk2$^+$ ($21\pm4\%$ vs $26\pm3\%$ of *wt*, $P=0.02$) and glyco-Dat$^+$ ($24\pm5\%$ vs $29\pm2\%$ of *wt*, $P=0.03$) neurons (Fig. 3A and N–Q). Although these findings on *En1*$^{Cre/+}$; *tOtx2*$^{ov/+}$ would lead us to investigate mouse mutants over-expressing a higher dosage of Otx2 in mdDA progenitors, this analysis was prevented by the perinatal lethality of *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ mice. Nevertheless, the analysis of *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ embryos at E18.5 showed that, compared to *wt* and *En1*$^{Cre/+}$; *tOtx2*$^{ov/+}$ mutants, these mutants exhibited a remarkable increase of TH$^+$–Calb$^+$ and TH$^+$–Ahd2$^+$ neurons (Fig. 4A–F), thus suggesting that high dosage of Otx2 in mesDA progenitors promotes the generation of VTA neuronal subtypes affected by loss of Otx2. Although the abnormalities in the total number of mesDA neurons exhibited by mutants lacking or over-expressing Otx2 are at least in part contributed by the Otx2 effect on proliferation of mesDA progenitors (Omodei et al., 2008), our data suggest that the altered proportion of VTA neuronal subsets, particularly evident in *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mice, should be caused by a selective effect of Otx2 on the differentiation of VTA progenitors fated to generate Calb$^+$ and Ahd2$^+$ subtypes. This is also in agreement with the fact that inactivation or strong over-expression of Otx2 in post-mitotic VTA neurons do not affect the survival of Calb$^+$ and Ahd2$^+$ neurons, whose identity is perturbed only in a fraction of those located in the central VTA (Di Salvio et al., 2010a). Nevertheless, a selective cell death of Otx2$^+$ neurons could not be excluded *a priori*. To assess whether the depletion in Calb$^+$ and/or Ahd2$^+$ VTA subsets was due to impairment in their neurogenesis or selective cell-death, we analyzed E13.5, E15.5 and E18.5 embryos lacking Otx2 in combination with the *Otx2*^{GFP} null allele (*En1*$^{Cre/+}$; *Otx2*$^{GFP/flox}$), which allowed us the detection of Otx2$^+$ neurons and progenitors in the absence of the Otx2 protein. Since *En1*$^{Cre/+}$; *Otx2*$^{GFP/flox}$ mutants were lethal at birth, this analysis could not be extended to post-natal stages. Compared to E13.5 *En1*$^{Cre/+}$; *Otx2*$^{GFP/+}$ control embryos (Fig. 5A–C), in the mesencephalon of *En1*$^{Cre/+}$; *Otx2*$^{GFP/flox}$ mutants most of the GFP$^+$ cells stalled in the ventricular zone, where they exhibited Nurr1 expression (Fig. 5F) and only a few of them exited the ventricular zone and co-expressed TH or Pitx3 or Nurr1 in the mantle zone (arrows in Fig. 5D–F). Indeed, the majority of the cells expressing TH or Pitx3 or Nurr1 in the mantle zone were GFP$^-$. This suggests that in the absence of Otx2 most of the mesDA progenitors exhibited a severe impairment in both terminating the differentiation process and exiting the ventricular zone. Noteworthy, the finding that Nurr1 was not activated in the ventricular zone at E12.5 (Omodei et al., 2008), suggests that progenitors lacking Otx2, although in delay, tried to accomplish the differentiation program. In this context, at E15.5, the number of Pitx3$^+$ or Nurr1$^+$ or TH$^+$ neurons detected in the mesencephalic mantle zone of *En1*$^{Cre/+}$; *Otx2*$^{GFP/flox}$ mutants remained remarkably lower of those detected in control embryos (Fig. 5G–L), but increased when compared to E13.5 *En1*$^{Cre/+}$; *Otx2*$^{GFP/flox}$ embryos. Nevertheless, in both cases the fraction of mesDA neurons co-expressing GFP appeared remarkably small (arrows in Fig. 5J–L). At E18.5, compared to control embryos (Fig. 5M–P), the number of TH or Pitx3 neurons co-expressing GFP (arrows in Fig. 5Q and R) or the proportion of TH$^+$ neurons co-expressing Calb or Ahd2 were very low in *En1*$^{Cre/+}$; *Otx2*$^{GFP/flox}$ mutants (Fig. 5S and T). The same analysis in the pretectal area revealed that the generation of SNpc neurons was not severely affected as it was in the mesencephalon (Fig. S5). Together with the findings reported in adult *En1*$^{Cre/+}$; *Otx2*$^{GFP/flox}$ mice, these data suggest that the large majority of mesDA neurons successfully exiting the ventricular zone, were Otx2$^-$ and post-natally expressed Girk2 and glyco-Dat. Then, to investigate the possibility that loss of GFP$^+$ VTA neurons may be also caused by selective apoptosis of these cells we analyzed at E13.5, E15.5 and E18.5 whether GFP$^+$ cells were positive for cleaved Caspase3. Virtually no cells co-expressing GFP and cleaved Caspase3 were identified (Fig. S6). In sum, these data indicate that Otx2 controls in mesDA progenitors the selective differentiation of Calb$^+$ and Ahd2$^+$ VTA neuronal subtypes, which correspond to those that prevalently express Otx2 post-natally. Nevertheless, it cannot be excluded that the reduction in VTA neurons might be also contributed by an Otx2 requirement in early post-mitotic neurons. Further models selectively ablating Otx2 in early post-mitotic neurons will help at deciphering this aspect.

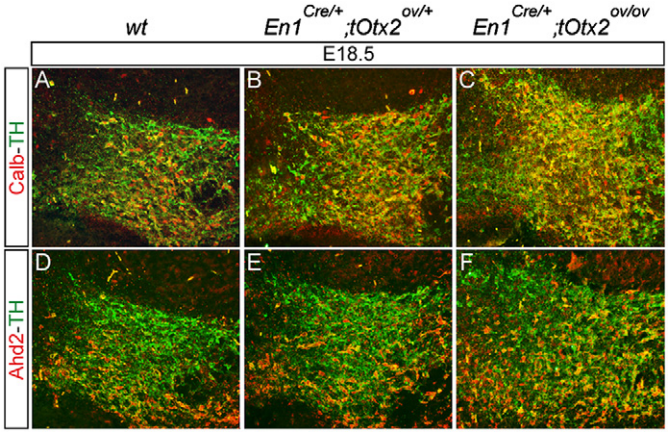

Fig. 4. High dosage of Otx2 correlates with increased generation of VTA neurons expressing Calb and Ahd2. (A–F) Immunohistochemistry experiments performed on E18.5 *wt*, *En1*$^{Cre/+}$; *tOtx2*$^{ov/+}$ and *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ embryos with Calb and TH (A–C) and Ahd2 and TH (D–F) show that the increase in TH neurons co-expressing Calb and/or Ahd2 correlates with increased dosage of Otx2 in mesDA progenitors.

Otx2 mild over-expression rescues survival defects and MPTP vulnerability of SNpc neurons in En1 haploinsufficient mice

We have previously reported that robust activation of Otx2 in SNpc and VTA neurons abolished almost completely their vulnerability to the MPTP neurotoxin, while MPTP sensitivity was not significantly affected by post-mitotic mild activation of Otx2 in SNpc and VTA neurons (Di Salvio et al., 2010a). Moreover, we have also shown that the vulnerability of VTA neurons post-mitotically lacking Otx2 was similar to that of *wt* SNpc neurons or to that of *wt* Otx2$^-$–Girk2$^+$–glyco-Dat$^+$ VTA neurons (Di Salvio et al., 2010a). In this context, it has recently been reported that

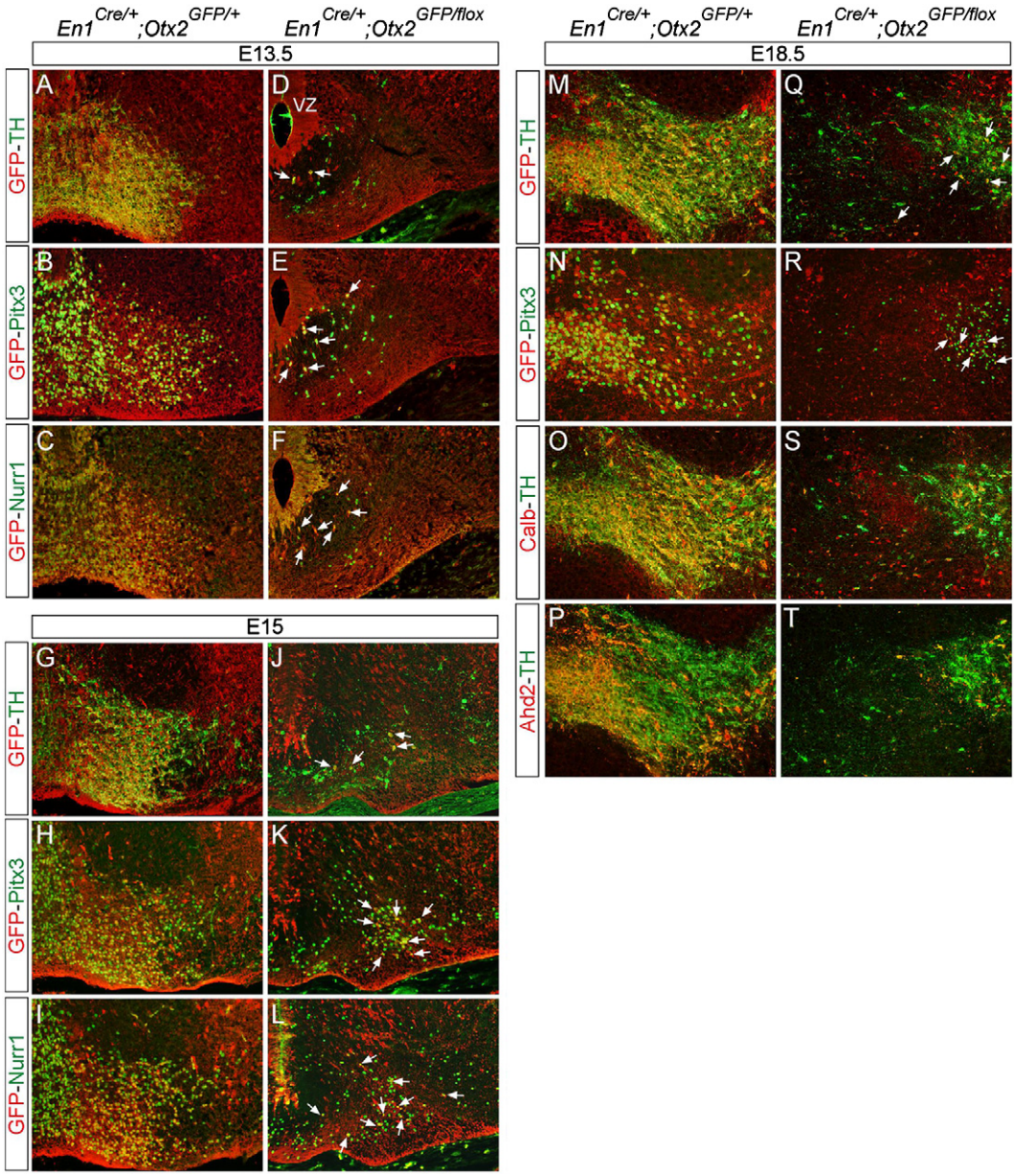

Fig. 5. Lack of Otx2 in mesDA progenitors prevalently affects the differentiation of mesDA neurons expressing Otx2. (A–T) Immunohistochemistry experiments performed on E13.5, E15.5 and E18.5 $En1^{Cre/+}$; $Otx2^{GFP/+}$ and $En1^{Cre/+}$; $Otx2^{GFP/flox}$ embryos with GFP and TH (A, D, G, J, M and Q), GFP and Pitx3 (B, E, H, K, N and R), GFP and Nurr1 (C, F, I and L), Calb and TH (O and S) and Ahd2 and TH (P and T) show that, compared to $En1^{Cre/+}$; $Otx2^{GFP/+}$ control embryos, only few $Pitx3^+$ or TH^+ or $Nurr1^+$ neurons are identified in the mantle zone of $En1^{Cre/+}$; $Otx2^{GFP/flox}$ mutants and very few of them are GFP^+ (arrows in D–F and J–L) or $Calb^+$ (S) or $Ahd2^+$ (T). Note that at E13.5 most of the GFP^+ cells are stalled in the ventricular zone (D,E,F), where they co-expressed Nurr1 (F). Abbreviation: VZ, ventricular zone.

En1 is an important survival factor in SNpc neurons and may provide these neurons with resistance to MPTP neurotoxin and 6-hydroxydopamine by enhancing the translation of two mitochondrial complex 1 proteins (Alvarez-Fischer et al., 2011). On this basis we studied whether $En1^{Cre/+}$; $Otx2^{flox/flox}$ VTA neurons being all positive for Girk2 and high level of glyco-Dat, exhibited a sensitivity to MPTP similar to that of the SNpc neurons; and whether mild over-expression of Otx2 in *En1* haploinsufficient mice attenuated the vulnerability to MPTP of SNpc and VTA neurons. We found that, compared to 12–14 week old *wt* mice, the sensitivity to MPTP tended to increase in $En1^{Cre/+}$ mutants both in SNpc ($69 \pm 6\%$ vs $74 \pm 6\%$ of *wt*, $P=0.04$) and VTA ($78 \pm 7\%$ vs $84 \pm 4\%$ of *wt*, $P=0.05$) (Fig. 6(A); and Table S2), while in $En1^{Cre/+}$; $Otx2^{flox/flox}$ mutants the MPTP vulnerability of the VTA was similar to that of the SNpc ($66 \pm 7\%$ in SNpc and $70 \pm 7\%$ in VTA), and significantly increased when compared to that of *wt* VTA neurons ($70 \pm 7\%$ vs $84 \pm 4\%$ of *wt*, $P=0.001$) (Fig. 6(A); and Table S2). On the other hand, compared to *wt*, $En1^{Cre/+}$; $tOtx2^{ov/+}$ showed a mild increase in MPTP resistance both in SNpc ($80 \pm 4\%$ vs $74 \pm 5\%$ of *wt*, $P=0.03$) and VTA ($88 \pm 6\%$ vs $84 \pm 4\%$ of *wt*, $P=0.1$); conversely, when they were compared to $En1^{Cre/+}$, the MPTP resistance was significantly increased both in SNpc ($80 \pm 4\%$ vs $69 \pm 6\%$ of $En1^{Cre/+}$, $P=0.002$) and VTA ($88 \pm 6\%$ vs $78 \pm 7\%$ of $En1^{Cre/+}$, $P=0.008$). These data confirm that lack of Otx2 in mesDA progenitors selectively affects the generation of VTA neurons with highest resistance to MPTP and indicate that mild activation of Otx2, although not sufficient to counteract efficiently MPTP vulnerability of *wt* mdDA neurons, may significantly compensate MPTP vulnerability caused by *En1* haploinsufficiency in both SNpc and VTA. The observed protection against MPTP might reflect a true survival, or be consequence of a potential repression of Dat expression as previously reported (Di Salvio

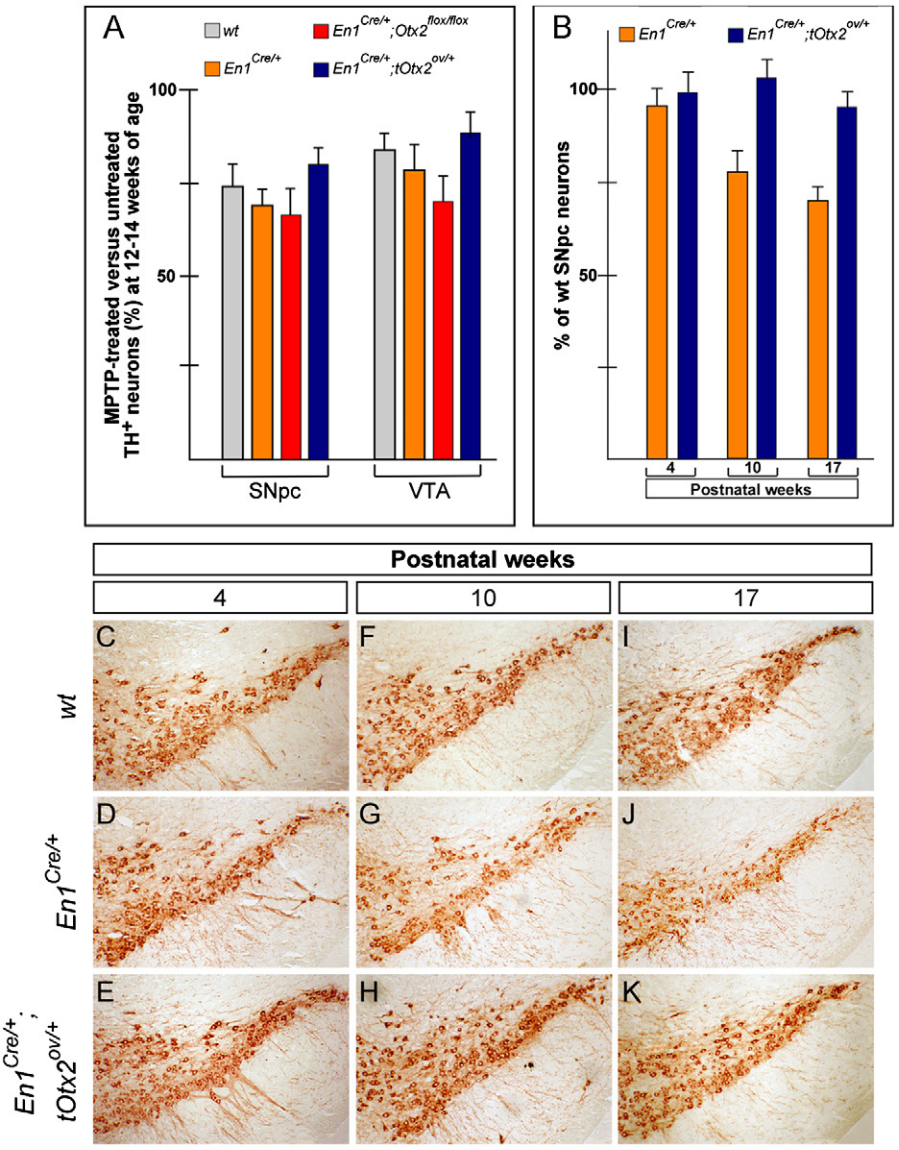

Fig. 6. Mild over-expression of Otx2 rescues MPTP vulnerability and progressive loss of En1 haploinsufficient SNpc neurons. (A) Graphic representation showing in percentage the ratio between MPTP-treated and untreated SNpc and VTA neurons of 12–14 week old *wt*, $En1^{Cre/+}$, $En1^{Cre/+}$; $Otx2^{flox/flox}$ and $En1^{Cre/+}$; $tOtx2^{ov/+}$ mice. Note that compared to *wt*, the MPTP vulnerability of $En1^{Cre/+}$ and $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice shows a mild increase in the SNpc, while in the VTA the MPTP sensitivity of $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice is remarkably higher; instead, compared to $En1^{Cre/+}$ and $En1^{Cre/+}$; $Otx2^{flox/flox}$ mutants, a significant reduction in MPTP vulnerability was detected in the SNpc and VTA of $En1^{Cre/+}$; $tOtx2^{ov/+}$ mice. (B) Compared to $En1^{Cre/+}$, the number of SNpc neurons detected in *wt* and Otx2 mutants at 4, 10 and 17 postnatal weeks shows that, while $En1^{Cre/+}$ mutants exhibit a graded neuronal loss, in Otx2 over-expressing mice the number of SNpc neurons is maintained similar to that of *wt* animals. (C–K) Immunostaining of *wt* (C, F and I), $En1^{Cre/+}$ (D, G and J), and $En1^{Cre/+}$; $tOx2^{ov/+}$ (E, H and K) mice showing that, compared to *wt* SNpc, the number of TH^+ neurons gradually decreases only in $En1^{Cre/+}$ mice.

et al., 2010a). Then, we analyzed whether mild Otx2 over-expression was sufficient to rescue mdDA survival defect of $En1^{Cre/+}$ mice (Sonnier et al., 2007; Sgadò et al., 2006; Alvarez-Fischer et al., 2011). Indeed, previous studies showed that *En1* haploinsufficiency resulted in a progressive post-natal reduction of the number of SNpc neurons. In particular, these previous studies have shown that 4 week old $En1^{+/-}$ mutants exhibited the normal number of SNpc and VTA neurons but, subsequently, they showed a graded loss of mdDA neurons reaching the highest level at around 6 months of age. Cell-counting analysis revealed that, compared to *wt* mice (Fig. 6(B, C, F and I); and Table S3), in $En1^{Cre/+}$ mice the number of SNpc neurons decreased with a temporal profile and in a percentage similar to those reported (Fig. 6(B, D, G and J); and Table S3). Remarkably, this progressive cell-loss was not observed in $En1^{Cre/+}$; $tOtx2^{ov/+}$ mice (Fig. 6(B, E, H and K); and Table S3). Together these findings and those previously reported (Di Salvio et al., 2010a; Alvarez-Fischer et al., 2011; Sonnier et al., 2007; Sgadò et al., 2006) lead us to propose that Otx2 is a survival factor for mdDA neurons and, in this context, it might exert a function similar to that of En1 (Alvarez-Fischer et al., 2011). Interestingly, En1 is expressed in both SNpc and VTA neurons, while Otx2 shows a precise restriction to $Girk2^-$ neurons of the VTA. We speculate that these two factors may define different dosage-dependent thresholds of MPTP vulnerability in SNpc and VTA. Thus, VTA neurons co-expressing both genes should correspond to those with lowest MPTP sensitivity. Supporting this possibility, $Otx2^+$–$En1^+$ VTA neurons are the most resistant to MPTP, but if Otx2 is post-mitotically ablated, the MPTP vulnerability of these neurons is similar to that of *wt* SNpc neurons (Di Salvio et al., 2010a);

accordingly, here we have shown that *En1*-haploinsufficiency defects in survival and MPTP vulnerability of SNpc neurons are efficiently rescued by mild over-expression of Otx2.

4. Conclusions

Previous studies have investigated the role of Otx2 in mdDA neurogenesis and post-mitotic VTA neurons. During neurogenesis Otx2 controls proliferation and identity of mesDA progenitors (Puelles et al., 2004; Prakash et al., 2006; Omodei et al., 2008) and in VTA neurons specifies subtype identity and confers resistance to MPTP (Di Salvio et al., 2010a; Simeone et al., 2011). In this study we have investigated a different aspect of mdDa neurogenesis. Indeed, since we found that Otx2 is expressed in mdDa progenitors fated to generate SNpc and VTA neuronal subsets expressing Calb or Ahd2 or Girk2, we questioned whether Otx2 is required for the differentiation of all these neuronal subsets or it controls the generation of a specific subset of them. Our data indicate that Otx2 controls selectively the generation Otx2$^+$ VTA neuronal subsets expressing Calb and/or Ahd2. This finding suggests that factors other than Otx2 might likely specify in mdDA progenitors the fate of Girk2$^+$ neurons of the SNpc and dorsal-lateral VTA. Our data also suggest that Otx2 defines a presumptive neurogenic "boundary" between two main populations of mdDA neurons: those Otx2$^-$–Girk2$^+$ more vulnerable to MPTP and those Otx2$^+$–Girk2$^-$ less vulnerable to MPTP. Strictly related to this, we have shown that mild over-expression of Otx2 in SNpc progenitors and neurons is sufficient to significantly rescue survival defects and MPTP sensitivity caused by En1 haploinsufficiency (Sonnier et al., 2007; Sgadò et al., 2006; Alvarez-Fischer et al., 2011).

Collectively these findings lead us to propose that the generation of mdDA neuronal subtypes may be controlled by progenitor subsets whose differentiation program is sensitive to specific neurogenic factors among which Otx2.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.10.022.

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