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DYRK1A promotes dopaminergic neuron survival in the developing brain and in a mouse model of Parkinson's disease

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In the brain, programmed cell death (PCD) serves to adjust the numbers of the different types of neurons during development, and its pathological reactivation in the adult leads to neurodegeneration. Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A) is a pleiotropic kinase involved in neural proliferation and cell death, and its role during brain growth is evolutionarily conserved. Human *DYRK1A* lies in the Down syndrome critical region on chromosome 21, and heterozygous mutations in the gene cause microcephaly and neurological dysfunction. The mouse model for *DYRK1A* haploinsufficiency (the *Dyrk1a*$^{+/-}$ mouse) presents neuronal deficits in specific regions of the adult brain, including the substantia nigra (SN), although the mechanisms underlying these pathogenic effects remain unclear. Here we study the effect of *DYRK1A* copy number variation on dopaminergic cell homeostasis. We show that mesencephalic DA (mDA) neurons are generated in the embryo at normal rates in the *Dyrk1a* haploinsufficient model and in a model (the mBACtg*Dyrk1a* mouse) that carries three copies of *Dyrk1a*. We also show that the number of mDA cells diminishes in postnatal *Dyrk1a*$^{+/-}$ mice and increases in mBACtg*Dyrk1a* mice due to an abnormal activity of the mitochondrial caspase9 (Casp9)-dependent apoptotic pathway during the main wave of PCD that affects these neurons. In addition, we show that the cell death induced by 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), a toxin that activates Casp9-dependent apoptosis in mDA neurons, is attenuated in adult mBACtg*Dyrk1a* mice, leading to an increased survival of SN DA neurons 21 days after MPTP intoxication. Finally, we present data indicating that Dyrk1a phosphorylation of Casp9 at the Thr125 residue is the mechanism by which this kinase hinders both physiological and pathological PCD in mDA neurons. These data provide new insight into the mechanisms that control cell death in brain DA neurons and they show that deregulation of developmental apoptosis may contribute to the phenotype of patients with imbalanced *DYRK1A* gene dosage.
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The total number of neurons in the brain, and ultimately the size of this organ, depends both on the number of cells that are produced during neurogenesis and the number of neurons that die due to physiological programmed cell death (PCD). Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A) regulates brain growth in a dose-dependent manner,[1] and indeed, loss-of-function mutations in *DYRK1A* (minibrain in *Drosophila melanogaster*) cause microcephaly and several neurological alterations in humans,[2–5] mice[6] and flies.[7] Accordingly, it has been proposed that haploinsufficiency of *DYRK1A* is the cause of the microcephaly and developmental delay associated to partial monosomy of chromosome 21 involving *DYRK1A*.[8] Moreover, triplication of the gene has been associated to the developmental brain dysfunctions and age-associated neurodegeneration observed in Down syndrome.[9–11]

Anatomical analysis of adult *Dyrk1a* mutant mice that model human diseases involving an imbalance in *DYRK1A* gene dosage (the *Dyrk1a*$^{+/-}$ mouse and the mBACtg*Dyrk1a* mouse, carrying one or three functional copies of *Dyrk1a*, respectively) revealed a positive correlation between *Dyrk1a* gene copy number, the overall size of the brain and the number of neurons in specific regions.[1] DYRK1A regulates several fundamental neurodevelopmental processes, including proliferation, neuron differentiation and PCD.[12] Overexpression of DYRK1A in neural precursors attenuates proliferation and promotes the differentiation of neurons in different model systems.[13–15] Conversely, treatment of neural progenitors with DYRK1A kinase inhibitors increases proliferation.[15] Although these data are consistent with some of the defects in cellularity identified in specific brain regions of *Dyrk1a* gene copy number mutants, they cannot

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Abbreviations: Casp3, caspase3; Casp9, caspase9; DA, dopaminergic; E, embryonic day; mDA, mesencephalic dopaminergic; MPTP, 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine; PCD, programmed cell death; P, postnatal day; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area
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explain the severe microcephaly evident in mice and humans carrying one functional copy of *DYRK1A*, or the overall macrocephaly in the mBACtg*Dyrk1a* model carrying three *Dyrk1a* alleles.[1,5] Thus, deregulation of other DYRK1A functions might also contribute to the defects in brain cellularity in these *Dyrk1a* gene copy number mutants, such as those described in retinal neurons that restrain developmental PCD.[16]

Dopaminergic (DA) neurons in the substantia nigra (SN) and ventral tegmental area (VTA) have an important role in controlling fine motor actions, as well as in motivation and reward behaviours, and their loss is associated with Parkinson's disease.[17] In aged *Dyrk1a*$^{+/-}$ mice the SN is smaller and contains fewer DA neurons than in wild-type mice.[18] These mutant animals are hypoactive, with altered gait dynamics, and as these defects are evident preweaning and in young animals,[6,18,19] as well as in children with heterozygous mutations in *DYRK1A*,[3–5] they might arise during development.

To provide insight into the aetiology of the neurological phenotype caused by *DYRK1A* haploinsufficiency, here we studied the development of mesencephalic DA (mDA) neurons in *Dyrk1a*$^{+/-}$ and mBACtg*Dyrk1a* mouse models. The results obtained show that *Dyrk1a* copy number variation does not affect the generation of DA neurons, but rather it modifies the number of these neurons that undergo physiological PCD due to an inhibitory effect of the Dyrk1a kinase on the apoptotic activity of caspase9 (Casp9), the initiator caspase in the mitochondrial-dependent apoptotic pathway.[20] Thus, deregulation of Casp9-dependent PCD during development may contribute to the brain size defects observed in aneuploidies involving *DYRK1A.*

As inappropriate re-activation of the mitochondrial-dependent apoptotic pathway in mature mDA neurons contributes to the neurodegeneration associated with Parkinson's disease,[21] we used the mBACtg*Dyrk1a* mouse model to assess whether basal Dyrk1a-dependent inhibition of Casp9 apoptotic activity could restrain the neurodegeneration induced *in vivo* by the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Our results show that the apoptotic response to the toxin in mBACtg*Dyrk1a* mice is significantly attenuated, leading to an increase in the number of SN pars compacta DA neurons that resist the pathological insult.

Results

Dyrk1a promotes the survival of mDA neurons during the physiological period of PCD. DA neurons in the SN and VTA originate from progenitors in the ventral neuroepithelium and they subsequently migrate radially to the mantle zone where they differentiate. The distribution of the neurons can be tracked by following that of tyrosine hydroxylase (TH), a marker of DA neurons that is initially detected in mice at around embryonic day (E)10[22,23] (see scheme in Figure 1a). As the distribution of Dyrk1a protein in the developing ventral mesencephalon is unknown, we used a Dyrk1a-specific antibody[16,24] to analyse the expression of the protein in this region at two developmental stages: at E12.5, during the phase of DA neurogenesis, and at postnatal day (P)0, when neurogenesis has been completed and the excess DA neurons generated are removed by PCD[25] (see scheme in Figure 2a). In the embryo, Dyrk1a protein accumulates in the cytoplasm of post-mitotic cells within the intermediate zone and in cells that already express TH in the mantle zone (Figure 1b). By P0, Dyrk1a was also evident in differentiating neurons that express TH in the SN (Figure 1c) and VTA (data not shown). This distribution is compatible with Dyrk1a participating in the embryonic and postnatal development of mDA neurons, supporting the possibility that the deficit in SN DA neurons observed previously in 2-year-old *Dyrk1a*$^{+/-}$ mice arises during development.[18]

To test this possibility, we quantified the number of cells expressing TH in the mesencephalon of *Dyrk1a*$^{+/+}$ and *Dyrk1a*$^{+/-}$ embryos at E14.5, soon after the period of DA neurogenesis in the ventral mesencephalon (Figure 2a). The number and distribution of TH^{+} neurons was similar in both

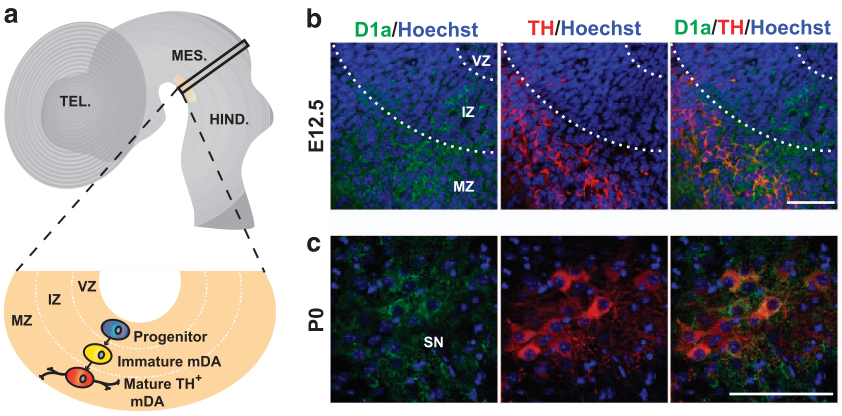

Figure 1 Expression of the Dyrk1a protein in the mouse ventral mesencephalon during development. (**a**) Schematic sagittal view of the mouse embryonic neural tube (E11.5 to E14.5) indicating the site of DA neurogenesis in the mesencephalon (yellow area) and the position occupied by DA progenitors (blue), immature DA neurons (TH^{-} cell in yellow) and mature DA neurons (TH^{+} cells in red) in a transverse section at the level indicated by the black rectangle. (**b** and **c**) Confocal images showing co-localization of Dyrk1a (D1a in green) and TH (red) in the ventral mesencephalon of wild-type E12.5 embryos (**b**) and P0 mice (**c**). HIND, hindbrain; IZ, intermediate zone; MES, mesencephalon; MZ, mantle zone; TEL, telencephalon; VZ, ventricular zone. Bar = 50 μm

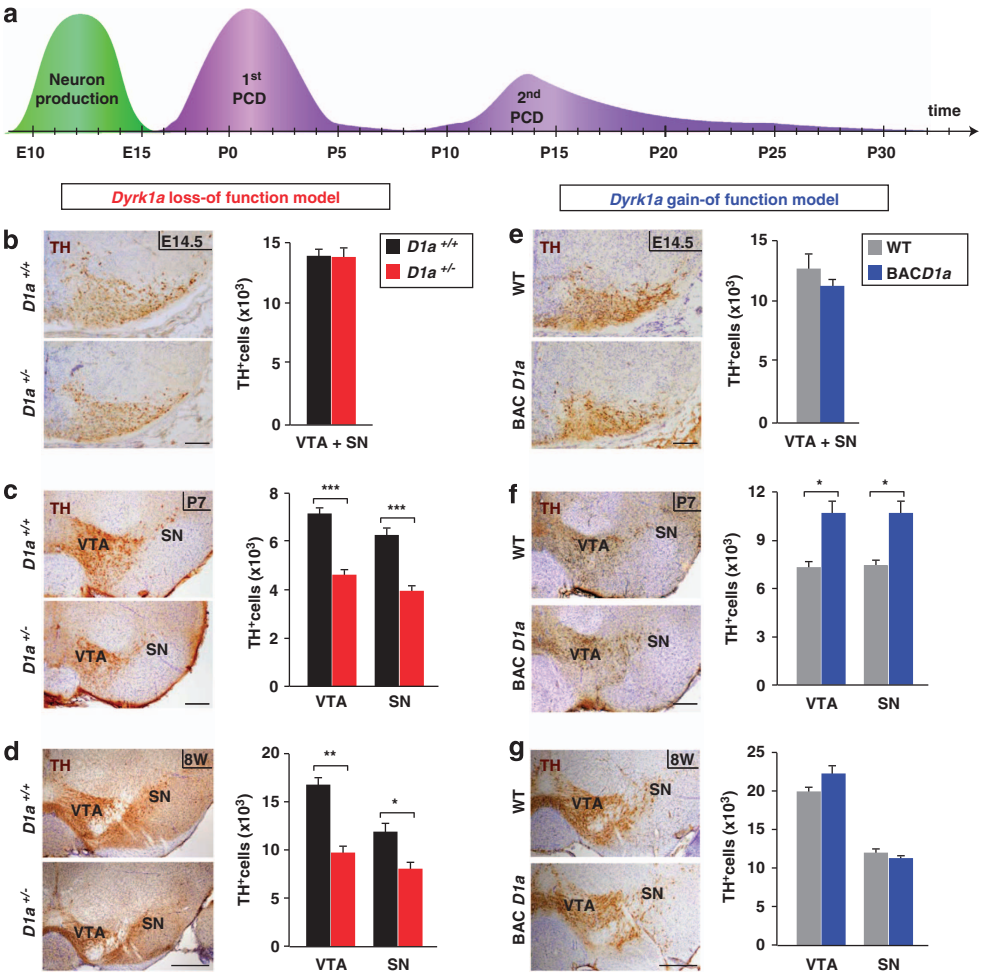

Figure 2 TH$^+$ cell counts in the mesencephalon of *Dyrk1a* mutant mice at different developmental stages. (**a**) Scheme indicating the period of DA neuron production (green) and of PCD (purple) in the mouse ventral mesencephalon.[27] (**b–g**) Representative mesencephalic sections stained for TH from *Dyrk1a*$^{+/+}$ (*D1a*$^{+/+}$) and *Dyrk1a*$^{+/-}$ (*D1a*$^{+/-}$) brains (**b–d**) and from wild-type (WT) and mBACtg*Dyrk1a* (BAC*D1a*) brains (**e–g**) at the developmental stages indicated, and total number of TH$^+$ cells in the whole ventral mesencephalon (VTA + SN; (**b** and **e**), or in the VTA and the SN alone (**c**, **d**, **f** and **g**). Histogram values are the mean ± S.E.M. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; $n = 3$ in (**b**, **e** and **f**); $n = 4$ in (**c** and **g**); $n = 5$ in (**d**). Bar = 100 μm (**b**, **c**, **e** and **f**), 300 μm (**d** and **g**)

these genotypes (Figure 2b), indicating that mDA neurons in *Dyrk1a*$^{+/-}$ embryos are produced normally. Moreover, these neurons seemed to differentiate correctly in the *Dyrk1a*$^{+/-}$ mutants, given that they expressed nuclear-orphan-receptor-1 (Nurr1), a transcription factor that is required for the maintenance of DA neurons,[23] and that they innervated the striatum at E16.5, the correct stage of development[26] (Supplementary Figure S1). Thus, the generation and specification of mDA neurons appear to be unaltered in *Dyrk1a*$^{+/-}$ mice.

The number of mesencephalic TH$^+$ neurons in *Dyrk1a*$^{+/-}$ mutants remained normal at P0 (VTA + SN: *Dyrk1a*$^{+/+}$ = 18 877 ± 2364 and *Dyrk1a*$^{+/-}$ = 18 434 ± 697; $P = 0.86$; $n = 4$). However, when we assessed the number of these neurons in the mesencephalon of *Dyrk1a*$^{+/+}$ and *Dyrk1a*$^{+/-}$ mice at P7, subsequent to the major programmed wave of DA neuron cell death (Figure 2a), fewer TH$^+$ cells were detected in *Dyrk1a* mutant mice (37% less) than in wild-type mice (Figure 2c). Despite the PCD that occurs, the number of mesencephalic TH$^+$ neurons increases during postnatal development,[27] and indeed, TH$^+$ cells were more abundant in young (8-week-old) *Dyrk1a*$^{+/+}$ mice than at P7. A similar age-dependent increase in TH$^+$ neurons was observed in 8-week-old *Dyrk1a*$^{+/-}$ mice, although there were fewer of these neurons in mutant animals (Figures 2c and d). Importantly, the reduction in TH$^+$ cells at this age (38%) was similar to the reduction of SN DA neurons reported previously in aged *Dyrk1a*$^{+/-}$ mice.[18] In both young *Dyrk1a*$^{+/+}$ and *Dyrk1a*$^{+/-}$ mice the mesencephalic neurons that expressed the dopamine active transporter (DAT) also contained TH (Supplementary Figure S2). Moreover, we did not find differences between genotypes in the proportion of Nissl-stained neurons that contained TH (*Dyrk1a*$^{+/+}$: SN = 0.43 ± 0.03; VTA = 0.43 ± 0.03 and *Dyrk1a*$^{+/-}$: SN = 0.46 ± 0.01; VTA = 0.46 ± 0.04; $n = 4$), indicating that *Dyrk1a* haploinsufficiency does not affect TH expression, but rather that it reduces the number of DA cells expressing this enzyme. Together, these results suggest that the loss of DA neurons in *Dyrk1a*$^{+/-}$ mice occurs during the major wave of developmental cell death in these cells.

To further assess how Dyrk1a influences DA neuron development, we performed a similar analysis in the mBACtg*Dyrk1a* model that expresses three copies of the mouse *Dyrk1a* gene.[1] As observed in the *Dyrk1a*$^{+/-}$ model, the number of TH^{+} cells in the mesencephalon of mBACtg-*Dyrk1a* mice was normal at E14.5 (Figure 2e) but not at P7, when there were significantly more DA neurons in the mutant (Figure 2f). However, and in contrast to what happens in the *Dyrk1a*$^{+/-}$ mouse, young mBACtg*Dyrk1a* mice had normal numbers of DA neurons in both the SN and VTA (Figure 2g). The second wave of PCD in mDA neurons takes place during the second and third week of life[25] (Figure 2a), when synaptogenesis is maximal in the developing striatum.[28] Therefore, the excess of DA neurons in P7 mBACtg*Dyrk1a* mice may be offset by the exacerbated wave of cell death during this period, possibly due to limited neurotrophic support of target neurons.[27] This is supported by the fact that P21 mBACtg*Dyrk1a* and wild-type animals have similar numbers of SN TH^{+} neurons (8588 ± 813 *versus* 8388 ± 659; $P = 0.85$; $n = 4$).

Overall, our results indicate that changes in Dyrk1a dosage do not affect the generation and differentiation of mouse mDA neurons but they modify the number of these neurons that die during the main wave of DA PCD. Such cell death is increased in the loss-of-function mutant and decreased in the gain-of-function mutant. This protective effect of Dyrk1a might be mediated through its kinase activity, as harmine, an inhibitor of the enzymatic activity of DYRK1A,[9] induces a dose-dependent reduction of TH^{+} neurons in primary mesencephalic cell cultures (Figure 3a).

Dyrk1a promotes the survival of mDA neurons during development by restricting the activity of the intrinsic apoptotic pathway. The first wave of post-natal PCD in rodents involves the activation of the intrinsic (mitochondrial-dependent) cell death pathway.[25] In this pathway, the release of cytochrome *c* from the mitochondria to the cytosol that is induced by apoptotic stimuli leads to Casp9 cleavage, which promotes cell death by activating downstream executioner caspases.[20,29] There is evidence that phosphorylation of the Thr125 residue in Casp9 prevents its cleavage in these circumstances and, hence, the subsequent activation of the intrinsic apoptotic pathway.[30] DYRK1A is known to phosphorylate Casp9 at Thr125 (Laguna *et al.*[16] and Seifert *et al.*[31]) and this phosphorylation is crucial to protect differentiating retinal neurons from mitochondrial-dependent cell death.[16] To determine whether phosphorylation of Casp9 by Dyrk1a is involved in the protection of DA neurons in the developing ventral mesencephalon, we used a phospho-site-specific antibody and quantified the amount of Thr125-phosphorylated Casp9 in the ventral mesencephalon of *Dyrk1a*$^{+/-}$, mBACtg*Dyrk1a* and wild-type littermate mice during the main wave of developmental DA neuron cell death, by P2 (Figure 2a). In mesencephalic extracts derived from *Dyrk1a*$^{+/-}$ animals, there was significantly less Dyrk1a protein, which was correlated with a significant decrease in the pool of Thr125-phosphorylated Casp9 (Figure 3c).

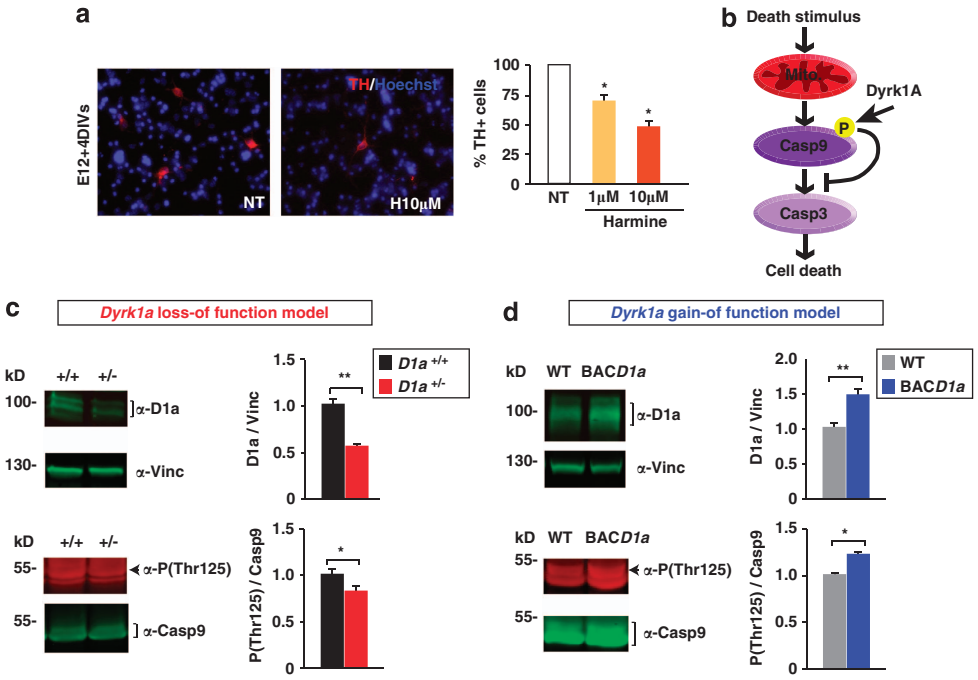

Figure 3 Effect of the DYRK1A inhibitor harmine on DA neuron survival and the levels of phosphorylated Casp9 in the developing ventral mesencephalon of *Dyrk1a* mutant mice. (**a**) Representative images of E12 mesencephalic cells after 4 days in culture (DIV) stained for TH (red), and the percentage of TH^{+} cells in cultures maintained in the presence or absence (NT) of harmine. (**b**) Scheme indicating the effect of Dyrk1a phosphorylation on the intrinsic cell death pathway. (**c** and **d**) Representative western blottings and their quantifications showing the levels of Dyrk1a (D1a) normalized to the amount of Vinculin (Vinc), and the proportion of Casp9 that is Thr125 phosphorylated (P(Thr125)) in total mesencephalic extracts from P2 *Dyrk1a*$^{+/+}$ (*D1a*$^{+/+}$) and *Dyrk1a*$^{+/-}$ (*D1a*$^{+/-}$) mice (**c**), and from wild-type (WT) and mBACtg*Dyrk1a* (BAC*D1a*) mice (**d**). Mito, mitochondria. Histogram values are the mean ± S.E.M. *$P \leq 0.05$; **$P \leq 0.01$ ($n = 3$ in (**a**); $n = 4$ in (**c** and **d**))

Conversely, there was an increase in the amount of Dyrk1a protein in mBACtg*Dyrk1a* extracts that was correlated with an increase in the pool of phosphorylated Casp9 (Figure 3d).

We assessed the activity of the Casp9-dependent apoptotic pathway in the mesencephalon of postnatal *Dyrk1a* mutant mice, studying the distribution of the active (cleaved) forms of Casp9 and its downstream executioner caspase3 (Casp3) in the TH-immunostained mesencephalic region of *Dyrk1a*$^{+/-}$, mBACtg*Dyrk1a* and wild-type littermate mice. In wild-type animals, active Casp9 and Casp3 immunostaining can be observed in apoptotic TH^{+} cells at P0 (Figure 4a). As expected, there were significantly more apoptotic cells expressing active Casp9 in *Dyrk1a*$^{+/-}$ mice (Figure 4b) and fewer in mBACtg*Dyrk1a* mice (Figure 4c). Consistently, there were more apoptotic cells expressing active Casp3 in *Dyrk1a*$^{+/-}$ animals (Figure 4d) and fewer in mBACtg*Dyrk1a* animals (Figure 4e). Together, these results indicate that the defects in DA cellularity observed in P7 *Dyrk1a* mutant mice arise from the aberrant activity of Casp9-mediated apoptosis, and that Thr125 phosphorylation of Casp9 by Dyrk1a can regulate cell death in these neurons.

Dyrk1a overexpression promotes the survival of SN pars compacta DA neurons in the MPTP-induced mouse model of Parkinson disease. There is evidence that the intrinsic apoptotic pathway participates in the neurodegeneration of SN pars compacta DA neurons in Parkinson's disease. Indeed, dying neurons in post-mortem Parkinson's tissue display morphological characteristics of apoptosis, including the activation of Casp9 and Casp3, cell shrinkage, chromatin condensation and DNA fragmentation.[21,32] Moreover, Dyrk1a protein is still found in the cytoplasm of TH^{+} neurons of the SN pars compacta after development (Figure 5a, upper panels). Taking into account the pro-survival action of Dyrk1a on developing DA neurons reported here (Figure 4), we hypothesized that Dyrk1a could restrain the neurodegenerative response in a parkinsonian context. To test this possibility we assessed the effect of the parkinsonian neurotoxin MPTP[33] on adult mBACtg*Dyrk1a* mice, which still contain relatively large amounts of Dyrk1a protein and Thr125-phosphorylated Casp9 in the ventral mesencephalon (Figure 5b), while establishing a normal distribution of Dyrk1a in their SN (Figure 5a).

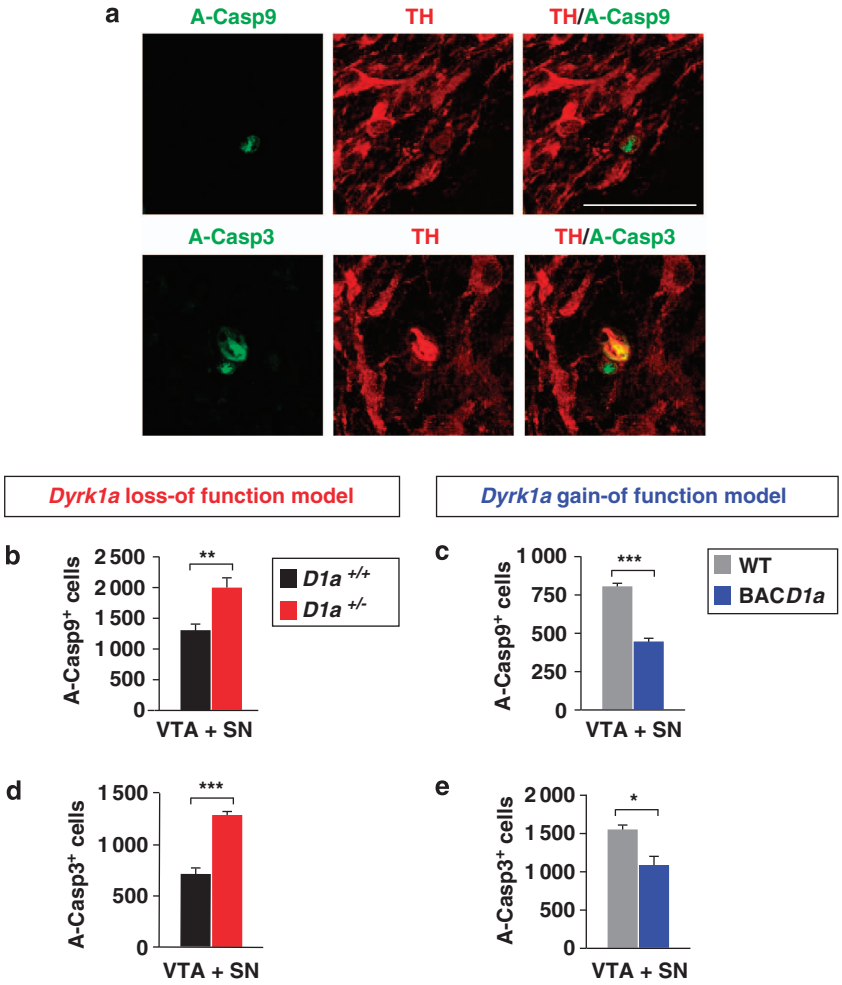

Figure 4 Numbers of apoptotic cells in the ventral mesencephalon of newborn *Dyrk1a* mutant mice. (**a**) Confocal images from brain sections of a P0 wild-type (WT) mouse showing TH expression in mesencephalic neurons that contain active Casp9 (A-Casp9) or active Casp3 (A-Casp3), and total numbers of A-Casp9^{+} cells (**b** and **c**) and A-Casp3^{+} cells (**d** and **e**) in the ventral mesencephalon (VTA + SN) of P0 *Dyrk1a*$^{+/+}$ (*D1a*$^{+/+}$) and *Dyrk1a*$^{+/-}$ (*D1a*$^{+/-}$) mice (**b** and **d**), and of wild-type (WT) and mBACtg*Dyrk1a* (BAC*D1a*) mice (**c** and **e**). Histogram values are the mean ± S.E.M. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ ($n = 4$ in (**b** and **d**); $n = 3$ in (**c** and **e**))

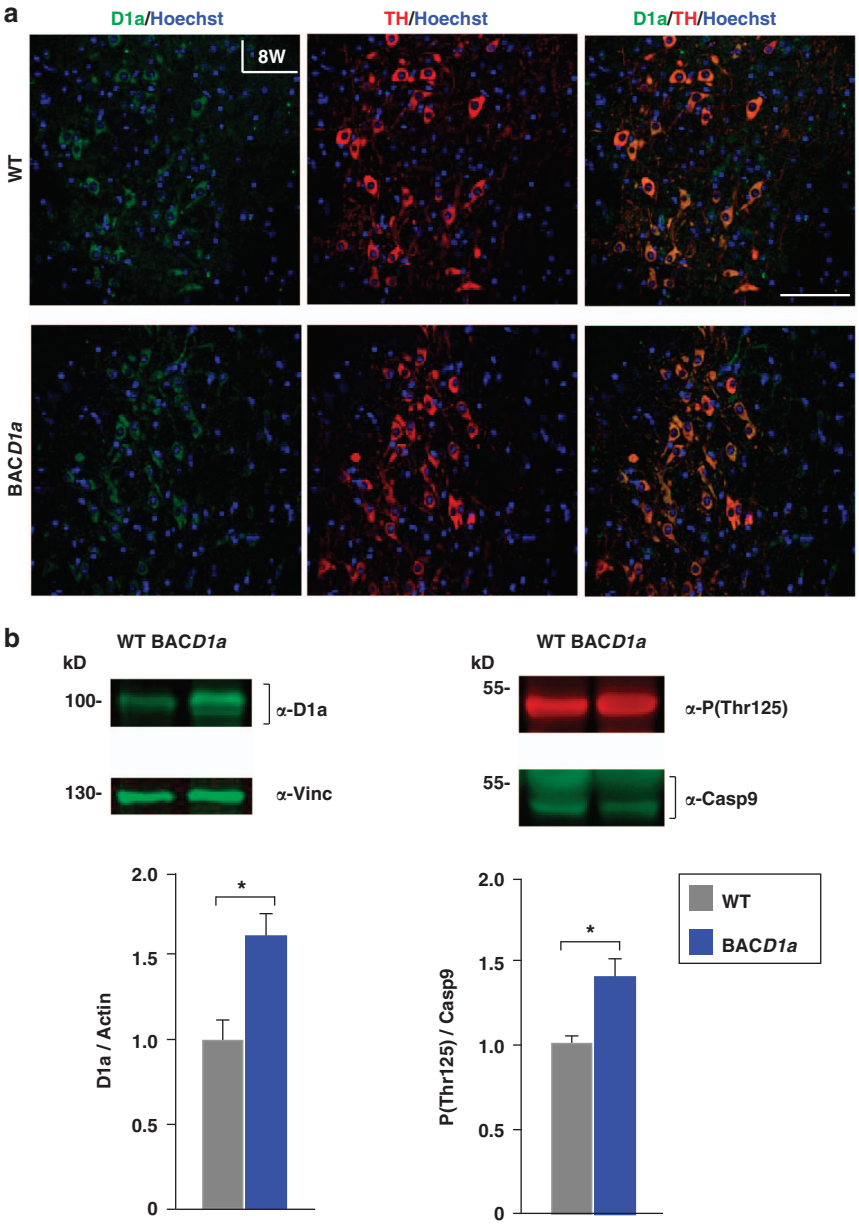

Figure 5 Expression of Dyrk1a and the levels of phosphorylated Casp9 in the ventral mesencephalon of adult mBACtg*Dyrk1a* (BAC*D1a*) mice. (**a**) Confocal images showing a similar Dyrk1a distribution in the SN of 8-week-old (8W) wild-type (WT) and BAC*D1a* mice. Bar = 100 μm. (**b**) Representative western blottings and their quantifications showing the levels of Dyrk1a (D1a) normalized to the amount of Vinculin (Vinc), and the proportion of Casp9 that is Thr125 phosphorylated (P(Thr125)) in total mesencephalic extracts prepared from 12- to 16-week-old WT and BAC*D1a* littermates. Histogram values are the mean ± S.E.M. $^*P \leq 0.05$ ($n = 5$)

Given the importance of the mitochondria in the pathogenesis of Parkinson's disease[32] and the multiple functions of DYRK1A,[34] we assessed the status of the mitochondria in these mice. The relative levels of mitochondrial components, including cytochrome *c* oxidase complex subunits, were similar in the mesencephalon of adult mBACtg*Dyrk1a* and wild-type mice (Supplementary Figure S3). Likewise, there were no differences in oxygen consumption in isolated brain mitochondria from either wild-type or mBACtg*Dyrk1a* mice (Supplementary Figure S4a). With regard to the sensitivity of brain mitochondria to 1-methyl-4-phenylpyridinum ion, the active MPTP metabolite, similar inhibition of ADP-stimulated oxygen consumption supported by complex I substrate glutamate/malate (respiration, state 3) was observed in mBACtg*Dyrk1a* and wild-type mitochondria. In addition, both genotypes produced similar amounts of reactive oxygen species and displayed similar levels of mitochondria antioxidant proteins (Supplementary Figures S4b and c). Together, these data indicate that mitochondrial biogenesis and function is well preserved in mBACtg*Dyrk1a* mice, and that the inhibitory action of the parkinsonian toxin MPTP on the mitochondria is not affected in these animals.

We then challenged mBACtg*Dyrk1a* mice and their wild-type littermates with a sub-acute regime of MPTP

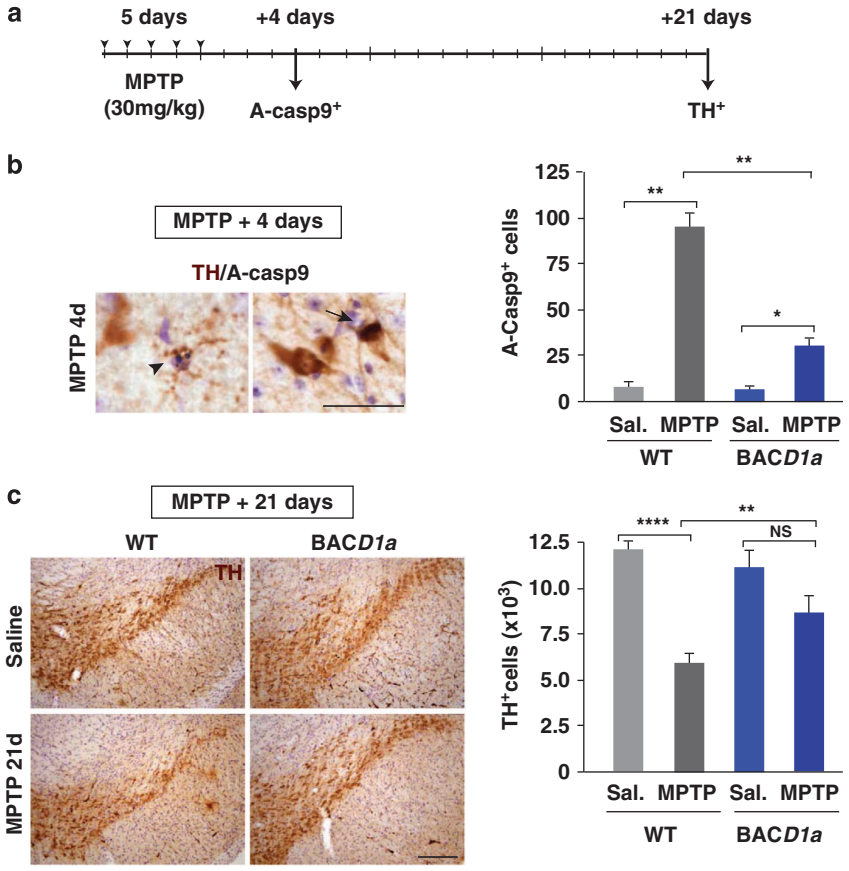

Figure 6 MPTP intoxication of adult mBACtg*Dyrk1a* mice. (**a**) Schedule of MPTP intoxication employed in experiments in (**b** and **c**). (**b**) Dual immunohistochemistry for TH and active Casp9 (A-Casp9) in the SN pars compacta of a wild-type (WT) animal 4 days after MPTP intoxication, showing a pyknotic cell (arrowhead) and an active Casp9$^+$ (black) cell (arrow) expressing TH (brown), and the total numbers of active Casp9$^+$ cells quantified in the SN pars compacta of WT and mBACtg*Dyrk1a* (BAC*D1a*) animals injected with saline (Sal.) or MPTP. (**c**) Representative images showing TH$^+$ cells in the SN pars compacta of saline- or MPTP-treated WT and BAC*D1a* animals 21 days after MPTP intoxication, and the quantification of the total TH$^+$ cells in this region for the four experimental groups. Histogram values are the mean ± S.E.M. N, not significant ($P > 0.05$); *$P \leq 0.05$; **$P \leq 0.01$; ****$P \leq 0.0001$ ($n = 3$; p-ANOVA = 0.001 in (**b**) and $n = 5$; p-ANOVA = 0.01 in (**c**)). Bar = 100 μm (**b** and **c**)

(30 mg/kg/day for five consecutive days: see schedule in Figure 6a), a protocol known to kill SN pars compacta DA neurons by activating mitochondrial-dependent apoptotic pathways in a time-dependent manner, peaking 4 days after the last MPTP injection.[35–37] Basal levels of both Dyrk1a protein and phosphorylated Casp9 in these animals did not change after MPTP treatment (Supplementary Figure S5). Yet, at the peak of MPTP-induced apoptotic cell death, pyknotic cells and cells expressing active Casp9 were clearly detected in wild-type and mBACtg*Dyrk1a* mice (Figure 6b). However, there were significantly fewer apoptotic active Casp9$^+$ cells in mBACtg*Dyrk1a* mice administered MPTP than in the equivalent wild-type mice (Figure 6b). Indeed, mBACtg*Dyrk1a* mice exhibited a marked attenuation of MPTP-induced SN pars compacta DA cell death by day 21 post MPTP (Figure 6c), once the MPTP-induced lesion had been stabilized.[36]

Together, these results show that moderate overexpression of Dyrk1a in mature mDA neurons attenuates the apoptotic response induced by MPTP intoxication, leading to an enhanced survival of SN*pc* DA neurons in this model.

Discussion

In this work we show that DYRK1A regulates Casp9-mediated cell death in mouse mDA neurons *in vivo* and in two different context: during the main wave of developmental PCD in these neurons and in the SN pars compacta of adult animals that have been challenged with the parkinsonian neurotoxin MPTP. In both contexts, the amount of Dyrk1a protein in the ventral mesencephalon is directly correlated to the levels of Casp9 phosphorylated at the inhibitory Thr125, and it is inversely correlated to the number of DA neurons that die by the intrinsic (Casp9-mediated) cell death pathway. There is evidence that basal DYRK1A phosphorylation of pro-Casp9 at Thr125 restrains Casp9-dependent apoptosis in the developing retina[16] and in cultured cells under hyperosmotic stress.[38] DYRK1A is synthesized as a constitutively active kinase[39] and, therefore, it is assumed that its kinase activity is directly related to the amount of protein.[9,34] Our data suggest that Dyrk1a is the kinase that phosphorylates Casp9 in the developing and adult mesencephalon, and that this phosphorylation is not modified by MPTP intoxication. Accordingly, we propose a model in which the amount of Dyrk1a protein in

brain DA neurons establishes the levels of inhibitory Thr125 phosphorylation of Casp9, which in turn defines the threshold of the apoptotic stimulus that is needed to irreversibly activate the caspase cell death cascade, either during development or in a pathological neurodegenerative condition in the adult.

The altered size of certain regions in the brain of *Dyrk1a*$^{+/-}$ and mBACtg*Dyrk1a* mutant mice is prominent in some cases (i.e., the hippocampus and the ventral thalamic postero lateral and postero medial nuclei) but not in others (i.e., the cerebral cortex). The prominently affected regions are smaller and with less neurons in the *Dyrk1a*$^{+/-}$ model, and they are larger and with more neurons in the mBACtg*Dyrk1a* model.[1] Therefore, deregulation of Casp9-dependent developmental cell death could contribute to the defects in cellularity in these brain regions. Nonetheless, alterations in the cytoarchitecture of the brain are complex in *Dyrk1a* mutants, with regions showing higher densities of neurons in the *Dyrk1a*$^{+/-}$ haploinsufficient model and lower densities in the mBACtg*Dyrk1a* triploid model.[1,6] These observations indicate that in addition to PCD, other developmental processes that regulate neural progenitor proliferation and differentiation must also be disturbed in specific domains of the developing brain in *Dyrk1a* mutants.[12]

The excess of DA neurons observed in the mBACtg*Dyrk1a* overexpression model after the main postnatal wave of PCD was not longer evident at P21. As the second wave of DA neuron PCD in the mouse takes place during the second and third week of life,[25] this observation raises the possibility that the second wave of cell death was exacerbated in mBACtg-*Dyrk1a* mice as an attempt to counterbalance the excess of DA neurons and adjust the number of projection neurons to their targets. During the second wave of PCD, at P14, the levels of Thr125-phosphorylated Casp9 in the ventral mesencephalon of mBACtg*Dyrk1a* mice are still higher than in the wild types (Supplementary Figure 6a), and consequently mBACtg*Dyrk1a* mutants at this stage have less cells expressing active Casp9 (Supplementary Figure 6b). However, and contrary to what we have observed in newborn mBACtg*Dyrk1a* mice, the number of apoptotic cells expressing active Casp3 was slightly increased in P14 mBACtg-*Dyrk1a* mice (Supplementary Figure 6b). Together, these observations suggest that the second wave of DA neuron PCD involves, in addition to the Casp9-mediated pathway, another caspase-dependent cell death pathway. Indeed, there is evidence that cell death of DA differentiating neurons induced by deprivation of growth factors is mediated by a non-classical mitochondria-independent cell death pathway that involves the activation of several caspases and the death receptor-caspase8 pathway.[40] The existence of such mechanisms may explain why the defects in brain size and cellularity are more severe in *Dyrk1a*$^{+/-}$ mice than in mBACtg*Dyrk1a* mice.[1]

SN pars compacta DA neurons in adult mBACtg*Dyrk1a* mice were protected against MPTP-induced neurodegeneration, suggesting that therapies aimed at preventing Casp9 cleavage could ameliorate or delay neurodegeneration in Parkinson's disease. This concept is supported by studies showing a certain degree of protection against MPTP intoxication through the administration of the pan-caspase inhibitor Q-VD-OPH,[41] the expression in transgenic mice of the general caspase inhibitor protein baculoviral p35,[42] or by adenoviral gene transfer of the X-chromosome-linked inhibitor of apoptosis.[43] Although neuroprotection in these studies was analysed shortly after MPTP administration, the results shown here indicate that inhibition of Casp9 cleavage can protect DA cells from MPTP-induced cell death over longer periods of time. Unfortunately, the beneficial effect of caspase inhibitors in Parkinson's disease has been challenged by the negative results of clinical trials into the use of caspase inhibitors,[44] which could be due to the difficulties to design clinical trials relevant to neuroprotection, not only given the heterogeneity of the disease but also due to the absence of *in vivo* indicators of neuronal dysfunction to monitor the possible effects of the drug.

In addition to Casp9, DYRK1A was shown to phosphorylate α-synuclein *in vitro* and acute overexpression of both DYRK1A and α-synuclein enhances the formation of intracellular inclusions in immortalized hippocampal H19-7 cells, which is associated to a decreased survival of these cells.[45] The neuroprotection offered against MPTP and the fact that 6-month-old mBACtg*Dyrk1a* mice have a normal complement of DA neurons indicate that triplication of the mouse *Dyrk1a* gene alone does not compromise the viability of SN pars compacta DA neurons, but instead it protects these cells against pathological insult. Based on the effect of DYRK1A on α-synuclein phosphorylation, it has been suggested that DYRK1A might favour the formation of Lewy bodies in Parkinson's disease and in Down syndrome.[10] However, the possible effect of trisomy 21 in Lewy body formation has been challenged by clinical studies showing that parkinsonian signs are less evident in Down syndrome individuals than in patients with advanced Alzheimer's disease[46] and also by pathological observations revealing the absence of Lewy bodies in the SN of adults with Down syndrome.[47] Although the neuroprotection offered by Dyrk1a described here could differ in a context of complete trisomy 21 in humans, the results of this work raise concern about possible deleterious effects of excessive therapeutic DYRK1A inhibition to improve cognitive performance in adults with Down syndrome.[9]

In summary, our data shows that normal levels of DYRK1A kinase activity are necessary for the survival of mDA neurons during development and in the adult brain after re-activation of the intrinsic cell death pathway in a pathological situation. These results could be of interest in the context of Parkinson's disease and challenge the general association of DYRK1A overexpression with neurodegenerative diseases. In addition, our data provide insights into the aetiology of the microcephaly and motor gait dysfunctions in children with heterozygous mutations in the *DYRK1A* gene.

Materials and Methods

Unless otherwise stated, all materials were obtained from Sigma-Aldrich (Tres Cantos, Madrid, Spain).

Animals. The generation of the *Dyrk1a*$^{+/-}$ mouse and the mBACtg*Dyrk1a* mouse was described previously.[1,6] Both *Dyrk1a* mutants were maintained on their original genetic backgrounds and genotyped by PCR:[1,6] *Dyrk1a*$^{+/-}$ mice by repeated backcrossing of *Dyrk1a*$^{+/-}$ males to C57BL/6Jx129S2/SvHsd F1 females (Harlan Laboratories, Bicester, UK) and mBACtg*Dyrk1a* mice by repeated backcrossing of transgenic males to C57BL6/J females (Charles River Laboratories, L'Arbresle, France). Fertility of *Dyrk1a*$^{+/-}$ mice is compromised when the mutants were crossed with C57BL6/J mice, which precluded having both *Dyrk1a* mutants on the same genetic background. The day of the vaginal plug was

defined as E0, and the day of birth was defined as P0. We used wild-type littermates as controls and males for the studies in adult animals, except for the respiratory and reactive oxygen species production assays that were performed on both males and females.

All the experimental procedures were carried out in accordance with the European Union guidelines (Directive 2010/63/EU) and the followed protocols were approved by the ethics committees of the Parc de Recerca Biomèdica de Barcelona (PRBB) and Research Institute-University Hospital Vall d'Hebron (VHIR).

MPTP treatment. To induce sub-acute MPTP toxicity, 14- to 24-week-old wild-type or mBACtg*Dyrk1a* male mice received one daily i.p. injection of MPTP-HCl (30 mg/kg of free base) on five consecutive days, as described previously.[48] MPTP-treated mice and saline-injected control animals were then killed at the time after the last injection indicated (Figure 6a).

Tissue processing for histology and immunostaining. To obtain embryonic and P0 tissue, whole heads were fixed by immersion in 4% paraformaldehyde (PFA) for 24 h at 4 °C, cryoprotected with 30% sucrose in PBS, embedded in Tissue-Tek O.C.T. (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands), frozen in isopentane at −30 °C and sectioned on a cryostat. Cryosections (16 μm) were collected on Starfrost precoated slides (Knittel Glasser, Braunschweig, Germany) and distributed serially. Postnatal and adult (2- to 6-month-old) mice were deeply anaesthetized in a CO_2 chamber and transcardially perfused with 4% PFA. The brains were removed, post-fixed and cryoprotected as indicated above, and cryotome (40 μm) sections were then distributed serially. For Dyrk1a immunostaining, the post-fixed brains were sectioned directly on a vibratome (40 μm).

Immunostainings. Immunohistochemistry on brain sections was performed following the avidin–biotin–peroxidase method (Vectastain ABC kit, Vector Labs, Burlingame, CA, USA). Briefly, sections were incubated in PBS at 37 °C for 15 min and then endogenous peroxidase activity was blocked with 3% H_2O_2 and 10% methanol in PBS for 30 min at room temperature (RT). The sections were then blocked for 2 h at RT in PBS containing 0.2% Triton-X100 (Merck, Madrid, Spain) and 10% fetal bovine serum (FBS; Life Technologies S.A., Alcobendas, Spain) and probed for 12 to 48 h at 4 °C with the primary antibodies diluted in Antibody Buffer (AB: PBS containing 0.2% Triton-X100 and 5% FBS). After washing, the sections were incubated with the corresponding biotinylated secondary antibody diluted in AB, washed and incubated with an avidin–biotin–peroxidase solution according to the manufacturer's indications. Peroxidase activity was visualized with 0.03% diaminobenzidine (DAB) and 0.003% H_2O_2. All samples were counterstained with Nissl (0.1% Cresyl violet acetate) to visualize non-immunopositive cells.

For dual immunohistochemistry with α-active-Casp3 or α-active-Casp9 and α-TH antibodies using the avidin–biotin–peroxidase method, we performed a sequential labelling. Cryosections were first incubated with α-active-Casp3 or α-active-Casp9, and antibody binding was detected using the appropriate biotinylated secondary antibody and the biotin–avidin complex (Vectastain, Vector Labs), which was visualized with 0.03% DAB and 0.2% nickel ammonium sulphate. Sections were then treated for 30 min with 10% methanol and 3% H_2O_2 in PBS to inhibit the residual peroxidase activity and they were incubated with the TH antibody. The following steps were performed as described before.

Secondary Alexa Fluor-conjugated antibodies (Life Technologies S.A.) were used for dual immunofluorescence and Hoechst was used to stain the nuclei following the same protocol as for immunohistochemistry but without blocking endogenous peroxidase activity.

A complete list of the antibodies used in immunohistochemistry and immunofluorescence can be found in Supplementary Table 1.

Cell counts on tissue sections. The TH^+ cells were quantified in brain sections counterstained with Nissl using stereological methods. Sections from animals of different genotypes were processed in parallel to avoid daily variations in immunostaining. The TH^+ cells in E14.5 tissue was estimated by counting the immunopositive cells in dissectors that covered 25% of the reference area in every four serially distributed sections of the ventral mesencephalon. The TH^+ cell number in the SN and VTA of P7, P21 and adult samples was counted in dissectors covering 15% of the reference area in every three serial sections. The proportion of TH^+ cells relative to Nissl stained neurons was quantified by counting both types of cells in dissectors covering the 10% of the reference area every six serial sections. The coefficient of error tolerated was $<10\%$ and the dissectors with the upper right corner outside the sampled area were not included in the total number of dissectors. The nuclei that contacted one of the two predetermined adjacent sides of the rectangular dissector frame were not included in the count. The number of active $Casp3^+$ and active $Casp9^+$ cells were calculated by counting the immunopositive cells in the entire region labelled with the anti-TH staining in one of every six serially distributed sections at P0, and in one every three serially distributed sections from the P14 mice and the adult saline and MPTP-treated animals.

Mesencephalic primary cultures. Mouse primary cultures enriched in DA neurons were prepared as described elsewhere.[49] Briefly, the ventral mesencephalon was isolated from E12.5 C57BL6/J embryos and the mechanically dissociated cells were seeded on cover slides coated with poly-D-lysine and laminin, and cultured in the presence of a mixture of F12/DMEM media (Life Technologies S.A.) supplemented with 0.25% bovine serum albumin, N1 additives and antibiotics (Life Technologies S.A.). After 4 days in culture, the cells were treated with harmine (harmine-HCl 1–10 μM dissolved in H_2O MilliQ; TCI Europe N. V., Zwijndrecht, Belgium) or vehicle (H_2O MilliQ) overnight, fixed and immunostained. The coverslips were sampled systematically, counting the number of TH^+ cells in at least 12 fields per coverslip and in two coverslips per condition. Cell counts were performed in three independent cultures.

Western blotting. Protein extracts (60 μg) were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Little Chalfont, UK) that was probed with antibodies whose binding was detected by infrared fluorescence using the LI-COR Odyssey IR Imaging System V3.0 (LI-COR Biosciences, Cambridge, UK). A complete list of the antibodies used to probe western blottings is provided in Supplementary Table 2.

Statistical analysis. The data were analysed by the two-tailed Student's *t*-test or by ANOVA followed by a two-tailed Student's *t*-test, and they are presented as the mean ± S.E.M. Any differences were considered significant at *P*-values ≤ 0.05. The *n* indicated in the figure legend refers to the minimum number of biological samples (embryos, mice or cell cultures) of the same condition (genotype) that were analysed in each experiment: $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

The methodology followed to isolate brain mitochondria and to perform the experiments with isolated mitochondria are indicated in the Supplementary Material and Methods.

Conflict of Interest

The authors declare no conflict of interest.

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