Differential Impairment of Catecholaminergic Cell Maturation and Survival by Genetic Mitochondrial Complex II Dysfunction

Blanca Díaz-Castro¹,², C. Oscar Pintado¹, Paula García-Flores¹,², José López-Barneo³¹,² and José I. Piruat³¹,²

¹Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla. Sevilla, Spain.
²Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Spain

#Corresponding authors:
José I. Piruat (jpiruat-ibis@us.es) and José López-Barneo (lbarneo@us.es)
Instituto de Biomedicina de Sevilla
Campus Hospital Universitario Virgen del Rocío
Avenida Manuel Siurot s/n
41013 Sevilla, Spain. Phone: +34 955 923001; fax: +34 955 923101

Running title: NEURODEGENERATION IN SdhD DEFICIENCY
The SDHD gene (subunit D of succinate dehydrogenase) has been involved in the generation of paragangliomas and pheochromocytomas. Loss of heterozygosity of the normal allele is necessary for tumor transformation of the affected cells. As complete SdhD deletion is lethal, we have generated mouse models carrying a “floxed” SdhD allele and either an inducible (SDHD-ESR strain) or a catecholaminergic tissue-specific (TH-SDHD strain) Cre recombinase. Ablation of both SdhD alleles in adult SDHD-ESR mice did not result in generation of paragangliomas or pheochromocytomas. In contrast, carotid bodies from these animals showed smaller volume than controls. In accord with these observations, the TH-SDHD mice had decreased cell number in the adrenal medulla, carotid body and superior cervical ganglion. They also manifested inhibited postnatal maturation of mesencephalic dopaminergic neurons and progressive cell loss during the first year of life. These alterations were particularly intense in the substantia nigra, the most affected neuronal population in Parkinson’s disease. Unexpectedly, TH+ neurons in the locus coeruleus and group A13, also lacking the SdhD gene, were unaltered. These data indicate that complete loss of SdhD is not sufficient to induce tumorigenesis in mice. They suggest that substantia nigra neurons are more susceptible to mitochondrial damage respecting other catecholaminergic cells, particularly during a critical postnatal maturation period.
Mitochondrial complex II (MCII; succinate-ubiquinone oxidoreductase [Sdh]) is composed of four nuclear-encoded subunits (A, B, C, and D) that couple oxidation of succinate to fumarate in the Kreb’s cycle to the mitochondrial electron transport chain (ETC). This is achieved by transferring electrons from the flavin moiety in SdhA to iron-sulfur clusters in SdhB and then, to ubiquinone bound to SdhC and SdhD. These last subunits also serve to anchor the whole complex to the inner mitochondrial membrane (21, 58). Genetic defects in MCII generate several human diseases (for a review see 43). Mutations in Sdh subunits, particularly in SdhB, C, and D, commonly produce familial pheochromocytomas and paragangliomas. These are highly vascularized, mostly benign, tumors occurring in the adrenal gland and the carotid body (CB) but also in other catecholaminergic neural crest-derived tissues (3, 34). Cell lines with reduced Sdh activity by mutations in SdhB or SdhC show signs of oxidative damage and apoptosis, although mutant cells escaping apoptosis may undergo tumor transformation (19, 24, 25). Indeed, spontaneous loss of heterozygosity (LOH) in adult humans carrying a mutant $SDHD$ allele ($SDHD^{+/−}$) can induce tumorigenesis in the affected cells (5, 6). The tumorigenic potential of MCII mutations has been suggested to derive from the accumulation of succinate, which inhibits $α$-ketoglutarate-dependent prolyl hydroxylases (PHDs) thus causing a “pseudo-hypoxic” condition with HIF-1$α$ stabilization and nuclear translocation in normoxia (8, 41, 51). This would result in constitutive activation of genes that favor tumor growth (42). Impairment of electron transfer from reduced flavin to ubiquinone in SdhC- or SdhD-deficient cells can lead to excessive production of reactive oxygen species (ROS) (21, 58). Hence, it
has also been proposed that oxidative stress could contribute to tumorigenesis due
to DNA damage (25) and/or ROS-dependent HIF-1α up-regulation (13, 19).

The molecular effects of MCII dysfunction in vivo are practically unknown,
as bi-allelic genetic deletion of any of the Sdh genes tested so far (SdhB and SdhD
knockouts) produce embryonic lethality (4, 31, 40). Moreover, heterozygous SdhD-
deficient aged mice do not present tumors or any other obvious pathology,
although they seem to have subtle CB alterations (4, 40). The objective of the
current research was to develop an SdhD conditional knockout mutant mouse that
could recapitulate the LOH required in humans for tumor formation in peripheral
paraganglia. To this end, we have generated mouse models carrying a “floxed”
SdhD allele and either a ubiquitously expressed tamoxifen-inducible Cre
recombinase (SDHD-ESR mouse), or a Cre recombinase under control of the
tyrosine hydroxylase (TH) promoter (TH-SDHD mouse), the rate-limiting enzyme
for catecholamine synthesis. Our goals were to ascertain whether ablation of the
SdhD gene induces either cell death or tumor transformation in vivo and to
compare the vulnerability of peripheral and central catecholaminergic neurons to
primary mitochondrial ETC dysfunction. In this regard, we were particularly
interested in the analysis of dopaminergic neurons in the substantia nigra pars
compacta (SNpc), the most important neuronal population affected in Parkinson’s
disease (PD), as mitochondrial impairment has long been associated with the
pathogenesis of this neurodegenerative disorder (14, 15, 48). Herein, we report
that deletion of the SdhD “floxed” allele in adult heterozygous (SdhDflx/) mice did
not result in generation of paragangliomas or pheochromocytomas. Moreover,
germ-line deletion of the SdhD allele restricted to TH+ tissues did not induce tumor transformation of the catecholaminergic cells, despite the animals survived for up to a year. In contrast, these last mice showed a selective degeneration of cathecolaminergic cells in the peripheral and central nervous system and a pronounced and progressive parkinsonian phenotype. Interestingly, neuronal loss preferentially affected the SNpc and other structures that reach maturation during early postnatal life. Catecholaminergic nuclei, such as the locus coeruleus, that seem to be matured at birth were unaffected.
MATERIALS AND METHODS

Generation of the SDHD-ESR and TH-SDHD mouse strains

To obtain both the inducible and tissue-specific SdhD mouse mutant strains we engineered a “floxed” allele, SdhD<sup>flox</sup>, which contains two LoxP sites flanking exons 2 to 4 and a NEO cassette for cell selection (Figure 1A). This construct was targeted to the SdhD genomic locus by homologous recombination in 129SvJ background R1 mouse embryonic stem (ES) cells. Proper targeting was tested by southern blot of genomic DNA digested with HindIII and hybridized against an external 5’ probe (Fig. 1C). To test the excision of the SdhD<sup>flox</sup> allele (Fig. 1B), targeted ES clones were electroporated with a plasmid containing the CRE recombinase gene. DNA from these cells was digested with EcoRV and analyzed by southern blot (Figure 1D) against a NEO probe. ES clones carrying the SdhD<sup>flox</sup> allele were used for blastocyst injection and chimera generation. Germ line transmission of the SdhD<sup>flox</sup> allele rendered heterozygous animals, which were subsequently mated with the SdhD<sup>+/−</sup> mouse (40) carrying either the tamoxifen-inducible CRE recombinase (20) or the Th-IRES-Cre transgenes (29) to generate the experimental SDHD-ESR and TH-SDHD mouse lines respectively. Littermates with SdhD<sup>flox/+</sup> and SdhD<sup>flox/-</sup> genotypes lacking CRE recombinase were used as control individuals. Unless otherwise specified, results from both control groups were pooled since no differences between them was found for the phenotypes tested. Routine genotyping was performed for the SdhD alleles by PCR with the following primers: 5’-AATTGTGCAGAAGTGAG-3’, 5’-GCTGCATACGCTTGATC-3’, 5’-CATCAAGGCTCACAGTC-3’.
Mouse husbandry and pharmacological treatments

Mice were housed under temperature-controlled conditions (22 °C) in a 12 h light/dark cycle, provided with food and water ad libitum. Either high (100 µg/g of animal during four days) or low (50 µg/g during two days) doses of tamoxifen dissolved in corn oil were administered by daily i.p. injections. Antioxidant tempol (Sigma) was given to pregnant females in drinking water protected from light at 2 mM, starting 1-2 days before delivery of pups and continued until the sacrifice of littermates (day P15). All experiments were performed in accordance with institutional guidelines approved by the ethics committee of the Hospital Universitario Virgen del Rocio.

SdhD mRNA level

Adrenal medullas or striata were dissected and stored frozen at -80° until processing. Total RNA was prepared by use of RNeasy Micro-kit (QIAGEN) according to manufacturer’s directions. Reverse-transcription of mRNA was performed with the Superscript II Reverse Transcriptase kit (Invitrogen), and SdhD cDNA was amplified by quantitative PCR with the following primers: 5´-CCAGCACATTCACTGTCA-3´ and 5´-ATCAGCCCCAAAGAGCAAA-3´ in presence of SYBRGreen®. The Arbp housekeeping gene was used for normalization.

Mitochondria isolation and mitochondrial complex II activity

Mitochondria isolation from mouse kidney was performed as reported (40). Succinate-ubiquinone oxi-reductase activity was determined according to ref. 40 with slight modifications. Briefly, 30-50 µg of protein were assayed at 30°C.
Samples were diluted 1:4 in the essay reaction buffer (25 mM KH$_2$PO$_4$ pH 7.2, 5 mM MgCl$_2$, 3 mM Potassium cyanide, 2.5 mg/ml bovine serum albumin) and liquid nitrogen frozen-thawed three times before the essay. Enzymatic activity was measured for a period of two minutes as the decrease in the absorbance at 600 nm due to the reduction of 50 µM 2,6-dichlorophenol-indophenol (DCPIP) coupled to reduction of 130 µM ubiquinone-1. The reaction was carried out in presence of 3.6 µM antimycin, 5 µM rotenone and 10 mM succinate.

**ATP level**

Adrenal medullas were dissected and stored frozen at -80º until processing. For ATP determination, total extracts from thawed adrenal medullas were prepared by smashing them in 50 µl of 100mM Tris-HCl; 4 mM EDTA with quartz sand and a plastic pestle followed by boiling for 3 minutes. After ice cooling, samples were spun 1 minute at 10000g. Supernatant was collected for ATP determination with the ATP bioluminescence kit HSII (Roche). Between 1 and 0.01 µg of protein were assayed as in this range the assay was linear. Protein concentration was determined by absorbance at 280 nm.

**Oxidative damage**

Mitochondrial DNA oxidative damage (44) and lipid peroxidation (35) were determined according to published protocols. Protein oxidation was analyzed with the OxyBlot™ Protein Oxidation Detection Kit and OxyIHC™ Oxidative Stress Detection Kit (Millipore), according to manufacturer’s directions.

**Tissue preparation and histochemistry**
Histological analyses and stereological cell counts were done following previously described protocols (23, 32, 38). In brief, mice were anesthetized and killed by decapitation. Dissected tissues were fixed in paraformaldehyde and embedded in OCT (Tissue-Tek), for carotid body and superior cervical ganglion, or paraffin, for adrenal gland and brain, before sectioning. Peripheral tissue sections 10 µm thick were used for tyrosine hydroxylase (TH) immunostaining. Nuclei were detected by DAPI staining. Coronal mouse brain sections 20 µm thick were used for tyrosine hydroxylase (TH), NeuN, parvalbumin or DARP32 immunostaining. Antibodies and the dilution factors used were as follows: polyclonal antibody against TH (Novus Biologicals), 1:5000; monoclonal antibody against NeuN (Chemicon), 1:200; polyclonal antibody against parvalbumin (Swant), 1:5000; polyclonal antibody against DARP32 (Millipore), 1:5000. For 3,3'-Diaminobenzidine based detection the Envision+ kit (DAKO) was used according to the recommended manufacturer’s protocol. For fluorescent detection the alexa-568 anti-rabbit IgG was used. Size of adrenal medulla was estimated with the ImageJ® software on photographs. In brain, unbiased stereological counts of TH and NeuN immunoreactive neurons was performed in one every six brain sections covering the complete structure of interest by using the CAST-Grid® system coupled to a microscope OLYMPUS with automated platform. The two hemispheres were considered.

In situ hybridization

Paraformaldehyde fixed brains were embedded in gelatin and sectioned in 50 µm coronal slices by vibratome. Slices were incubated with an UTP-digoxigenin labeled antisense RNA probe obtained by T7 promoter driven transcription of a
SdhD cDNA containing plasmid (I.M.A.G.E clone 3989833) overnight at 57°C. After extensive washing, slices were incubated with an antidigoxigenin antibody conjugated with alkaline phosphatase (Roche) in a 1:1000 dilution overnight at 4°C, followed by incubation in 6 µl/ml NBT y 4,6 µl/ml BCIP (Roche) for 7 hours at room temperature for detection.

**X-gal staining and confocal microscopy**

Expression of CRE recombinase in catecholaminergic tissues of the TH-SDHD mouse was evaluated by mating these mice with the floxed Rosa26-lacZ (R26R) mouse (52). X-gal staining was done as reported before (37, 56). Images of TH immunostained sections containing X-gal stain precipitates were acquired using TCS SP2 Leica confocal microscope. Lateral projection images were generated from stacks of optical sections spaced 0.8 µm using the Leica software package.

**HPLC**

Striata were dissected from brains in ice-cold PBS and sonicated on ice. Homogenates were filtered by ultrafree-MC centrifuge filter units (Millipore) and kept at -80° until use. For HPLC analysis of dopamine and related metabolites, samples were passed through a chromatographic ALB-215 column (ANTEC Leyden) following manufacturer’s directions.

**Open field tests**

To assess the motor dysfunction phenotype, mice were subjected to open-field test in a 22.5 cm by 22.5 cm arena for 1 hour. Individual animals were monitored using an automatic tracking system (SMART, Panlab). Traveled distance and resting time parameters were calculated using the SMART software (version 2.5.14)(38)
Statistical analysis

Data are presented as mean ± standard error (SEM). Statistical significance is assessed by either Student’s t-test with Levene test for homogeneity of variances in case of normal distribution, or the non-parametric U-Mann Witney test in case of non-normal distributions. The PASW18 software was used for statistical analysis.
RESULTS

Survival of SdhD-deficient mice and loss of peripheral catecholaminergic cells

Heterozygous SdhD<sup>flox/−</sup> animals with complete germ-line ablation of SdhD in catecholaminergic cells (TH-SDHD mice) appeared healthy at birth and, although smaller in size, they seemed to develop normally during the first postnatal months. However, most mutant mice died before the first year of life (Fig. 1E) probably due to the progressive and extensive cell loss occurring in some catecholaminergic organs and brain nuclei (see below).

TH-SDHD mice, even those that survived for up to one year, did not show any indication of tumor transformation in the peripheral catecholaminergic tissues. On the contrary, quantitative histological analyses showed a marked decrease of catecholaminergic cells. We studied in detail the adrenal medulla (AM), a relatively large organ almost exclusively formed by compacted strands of chromaffin cells that allowed us to carry out biochemical analyses. AM volume was approximately similar in newborn wild type and SdhD-deficient animals; however, maturation and growth of the chromaffin tissue occurring during the first postnatal weeks, was markedly altered in TH-SDHD mice (Fig. 2A and B). AM cells also showed a significant decrease of intracellular ATP content indicating an alteration of ETC (Fig. 2C). We have used several methodologies (see “Material and Methods”) to ascertain whether SdhD deletion induced an oxidative stress in the surviving AM tissue with inconclusive results. However we did see some evidence of selective increase of lipid peroxidation in chromaffin cells of mutant animals (Fig. 2D). As the
AM, other TH+ structures such as the carotid body (CB) and neighboring superior cervical ganglion (SCG) of juvenile animals also presented a clear decrease in the number of catecholaminergic cells (Fig. 2E-H).

As it could be possible that complete maturation of the catecholaminergic organs is a prerequisite for tumor induction after SdhD deletion, we also generated a time-inducible SdhD mutant model by breeding the SdhD “floxed” mouse line with a strain containing a tamoxifen-inducible CRE (20). These animals (denoted as SDHD-ESR) carried a paternally inherited SdhD null allele and a maternal SdhD “floxed” allele to be excised after CRE activation, thus mimicking the human scenario for familiar paraganglioma. Measurement of SdhD mRNA levels in adrenal medulla and kidney soon after tamoxifen treatment confirmed deletion of the maternally-inherited SdhD allele in adult mutant mice (Fig 3A). In accord with these data, a parallel decrease of succinate-ubiquinone oxi-reductase activity was measured in isolated kidney mitochondria (Fig. 3B). Analyses of AM and CB in the SDHD-ESR strain performed before the death of the mice (occurring ~3 weeks after tamoxifen treatment) indicated the absence of tumorigenesis in these organs and a clear trend towards degeneration of the carotid body (Fig. 3C and D). SDHD-ESR animals treated with lower doses of tamoxifen (see “Material and Methods”) survived longer (~8-9 weeks) but showed no signs of tumor transformation or hypertrophy in either AM or CB (data not shown).

Impaired SNpc postnatal maturation and maintenance in TH-SDHD mouse

In parallel with the catecholaminergic cell loss in the peripheral nervous system, TH-SDHD animals showed a marked and progressive postnatal reduction in the
number of ventral mesencephalic TH+ neurons (Figure 4A). Vulnerability of
dopaminergic neurons of the substantia nigra pars compacta (SNpc) and ventral
tegmental area (VTA) to mitochondrial SdhD deficiency was evaluated by
comparative cell count analyses in control and in TH-SDHD mice during the first
year of life (Figure 4B-D). Although neonatal animals already had a small
population of TH+ ventral mesencephalic cells, most SNpc and approximately half
of VTA dopaminergic neurons in normal mice acquired their post-mitotic TH+
phenotype during the first postnatal month and, after reaching a maximum, their
number remained constant in adulthood (9, 26). This pattern of ontogenic
development was altered in TH-SDHD animals. They were born with a non-
significant, slight decrease of TH+ cell number in SNpc and similar cell number in
VTA in comparison with their respective controls, but showed a characteristic
inhibition of postnatal neuron maturation and progressive death of TH+ cells upon
reaching adulthood. These processes, impaired maturation and accelerated
degeneration of dopaminergic cells, were quite aggressive in the SNpc, thus
resulting in an almost total disappearance of TH+ cells in this structure between 6-
12 months of animal age (Figure 4B). In the same time period, ~30% of TH+ cells
in VTA remained (Figure 4C). These observations indicate that, as it occurs in PD
patients (14, 15), SNpc neurons are far more sensitive to mitochondrial dysfunction
than VTA. Staining of ventral mesencephalic neurons with NeuN, a pan-neuronal
marker, in 3.5 months-old animals further demonstrated the differential neuronal
death in SNpc and VTA of TH-SDHD mice rather than a loss of TH expression
(Figure 4D). The molecular mechanism of dopaminergic mesencephalic neuronal
death in the TH-SDHD mice is unknown, as we could not demonstrate any indication of either caspase-3 activation or nucleus fragmentation in tissue samples from these animals.

Ablation of the SdhD gene in catecholaminergic cells of the TH-SDHD mouse is expected to increase the production of ROS in mitochondria, as mutations in Sdh proteins have been demonstrated to increase oxidative stress in both in vivo and in vitro systems (19, 25). In accord with this concept we have also observed an increase of lipid peroxidation in AM tissue from TH-SDHD animals (see Fig. 2D above). Hence, we tested to see the effect of the superoxide dismutase mimetic tempol (57) upon the postnatal maturation of SNpc neurons. To this end, we added the antioxidant to the drinking water at late stages of gestation and until sacrifice of littermates 15 days after birth. Tempol induced a 48% increase in the number of TH+ SNpc neurons in TH-SDHD animals (4998±261 neurons in treated versus 3370±486 neurons in non-treated animals; mean±SEM, n=7 and 4 respectively, P=0.01). However, the effect of tempol in control animals was smaller (13%) and non-significant (8740±647 neurons in treated versus 7743±349 in non-treated mice; n=5, P=0.21).

**Brain catecholaminergic neurons matured at birth were unaffected by SdhD ablation**

The analysis of catecholaminergic cell death in the TH-SDHD mouse was extended to brainstem nuclei, such as the noradrenergic locus coeruleus (LC) or the dopaminergic group A13, in which most of the neurons had already acquired the catecholaminergic phenotype at birth. In contrast with the accentuated
neuronal loss seen in SNpc, and to a lesser extent in VTA, of TH-SDHD animals, neuron numbers in LC and group A13 were unaltered by the mutation during the first year of life (Figure 5A-D). As no antibodies for SdhD protein immunodetection are available, we attempted in situ histochemical determination of succinate-ubiquinone oxi-reductase activity in brain sections. This technique did not provide conclusive results probably because affected neurons (without oxi-reductase activity) were intermingled with unaffected cells.

Therefore, to confirm that CRE-mediated recombination was taking place in the unaffected LC and A13 neurons, we checked the expression of functional CRE recombinase, as well as the deletion of the SdhD<sup>flox</sup> allele in the TH+ neurons of these regions. To this end, we generated a TH-SDHD/R26R mouse line (see Materials and Methods) in which we demonstrated co-localization in every TH+ neuron of X-gal staining (indicative of CRE recombinase-dependent DNA deletion at the floxed R26R locus) with TH immunoreactivity in brain sections including LC and A13 nuclei (Figure 6A). In addition, in situ hybridization analyses directly indicated that SdhD mRNA expression was abolished in LC neurons of TH-SDHD mice to a similar extent as in dopaminergic SNpc or VTA cells, whereas SdhD mRNA expression remained unaltered in non-catecholaminergic cells (Figure 6B). LC neurons seem to have an extraordinary resistance to mitochondrial complex II dysfunction as both CRE mediated R26R expression and SdhD ablation (as determined by in situ hybridization analysis) were tested to occur in LC neurons of juvenile TH-SDHD animals (of a month of life or younger). In contrast, LC TH+ neuron number remained unaltered in one-year old mutants (see Fig. 5C).
Altogether, these data unequivocally demonstrate that SNpc neurons have special sensitivity to genetic mitochondrial SdhD inhibition in comparison with cells in other catecholaminergic nuclei.

Previous studies with the Th-IRES-Cre mouse (29) have suggested that transient TH activation may occur during development in areas that are not TH+ in the adult. In our TH-SDHD/R26R animals we observed a very low level of R26R expression in striatum, cortex and hippocampus as compared with SNpc and other TH+ structures. As expected, all the non-catecholaminergic brain areas studied (particularly cerebral cortex and hippocampus) were found normal in the TH-SDHD mouse (data not shown). In addition, we also checked that the number of DARP32+ medium spiny neurons, the most numerous in the striatum, was normal in the TH-SDHD mouse (Fig. 7A). Moreover, the number of parvalbumin+ GABAergic interneurons, which provide most of the neurotrophic support for dopaminergic nigrostriatal neurons (23) was also unaffected by SdhD ablation (Fig. 7B). Finally, a quantitative analysis of the level of SdhD mRNA expression in striatum showed that CRE recombinase-dependent SdhD deletion was not aberrantly activated in non catecholaminergic striatal cells of TH-SDHD animals (Fig. 7C). Together, these data make unlikely the possibility that CRE expression during development in regions other than the TH+ catecholaminergic areas have any major contribution to the histological and functional phenotype of TH-SDHD animals

Progressive parkinsonian phenotype in TH-SDHD mouse
As a consequence of the loss of dopaminergic mesencephalic neurons projecting to the striatum, TH-SDHD animals exhibited intense striatal dopaminergic denervation, which seemed to be almost complete at 2-3 months of age (Fig. 8A). Measurement of striatal dopamine and its degradation metabolites indicated a profound neurochemical defect in SdhD-deficient animals. In normal mice, postnatal increase in the number of TH+ neurons of SNpc resulted in maturation of the nigrostriatal pathway with a marked rise in the caudate-putamen content of dopamine as well as in 3,4-dihydroxyphenylacetic (DOPAC) and homovanillic acid (HVA) levels. The postnatal increases of dopamine and its metabolites were almost abolished in striata from 2.5 months-old TH-SDHD animals (Fig. 8B). Striatal denervation in mutant animals seemed to be complete in 2.5 months-old animals even though a significant number of SNpc and VTA neurons still remained alive (see Figure 4A-C). This suggests that in the TH-SDHD mouse the nigrostriatal pathway does not reach full mature development and that the loss of dopaminergic axon terminals could precede death of SdhD−/− neurons. As indicated above, striatal parvalbumin+ cells, which provide most of the GDNF needed for trophic maintenance of nigrostriatal neurons (23, 38) were unaltered in the TH-SDHD mouse (see Fig. 7B), thus discarding possible non-cell autonomous effects of SdhD ablation on dopaminergic nigrostriatal neurons.

We also studied whether the anatomical and neurochemical nigrostriatal defects in TH-SDHD animals correlate with a behavioral phenotype. With this purpose we performed open-field tests at different ages. TH-SDHD mice showed a progressive bradykinetic syndrome characterized by marked decrease in the...
distance traveled in the open field and increase in time spent at rest (Fig. 8C and D).
In this paper we describe a new conditional mouse model of SdhD deficiency, which was designed to study tumorigenesis in paraganglionic cells as well as to compare the vulnerability of peripheral and central catecholaminergic neurons to primary mitochondrial ETC dysfunction. In humans, SDHD is considered a tumor suppressor gene because loss-of-function mutations are the cause of some hereditary paraganglioma mainly affecting the carotid body (3, 6). Individuals carrying the mutation are heterozygous and loss of the normal allele in the affected cells (LOH) is required for tumor transformation (5, 6, 22). SdhD+/− mice are non-viable and heterozygous SdhD+/− animals fail to develop tumors, thus suggesting that spontaneous LOH of this locus does not take place in mice paraganglia (4, 40). In our TH-SDHD mice we achieved complete deletion of both SdhD alleles in TH+ cells, a condition postulated to be the initiating event for tumor transformation. However, far from developing tumors, these animals underwent loss of catecholaminergic cells in the peripheral nervous system. Although TH-SDHD animals had a shortened lifespan, likely caused by the extensive central and peripheral catecholaminergic cell loss, examination of animals that survived even for one year never resulted in the detection of tumors.

Genetic and molecular studies have demonstrated that the inherited pattern of paraganglioma involves a parent-of-origin effect (6, 34), which indicates some form of genomic imprinting. Furthermore, it has been suggested that loss of a second imprinted locus in the same chromosome of SdhD is necessary for paraganglioma formation (22). In this regard, a double heterozygous mutant in
SdhD and H19, another imprinted locus proposed to be involved in hereditary paraganglioma, also failed to show any propensity to tumorigenesis (4). It could also be possible that complete maturation of the organs, AM and CB, is a prerequisite for tumor appearance. However, heterozygous SdhD\(^{\text{flo}\text{x/-}}\) animals in which we induced loss of the maternally inherited SdhD allele in adulthood (SDHD-ESR mice), also failed to show any tumoral or hypertrophic phenotype. The fact that both, the TH-SDHD and SDHD-ESR mice, despite being subjected to loss of the two copies of the gene in catecholaminergic tissues, do not display tumor features further strengthens the idea that a “second hit” is required for paraganglioma pathogenesis. Differences in chromosomal arrangements between man and mouse, or unknown distinct biochemical features between the two species, could account for the resistance to develop paraganglioma/pheochromocytoma in mouse.

As indicated above, SdhD deficient mice have also been used as a model of mitochondriopathy to investigate the effect of primary mitochondria ETC inhibition on postnatal maturation of SNpc dopaminergic neurons as well as their maintenance in adult life in comparison with other catecholaminergic cells. Although a vast literature exists suggesting that mitochondria play a central role in PD pathogenesis (14, 39, 47, 48), whether dopaminergic SNpc neurons, the cell group whose destruction is responsible for most of the motor symptoms in PD, are specially susceptible to mitochondrial dysfunction is not definitely established. Surprisingly, it is also little explored whether mitochondrial deficiencies alter the ontogenic maturation of ventral mesencephalic neurons, an elaborated sequence
of events occurring during the early postnatal period that involves programmed cell
death and acquisition of the dopaminergic phenotype thus determining the number
of TH+ SNpc neurons reaching adulthood (9, 26).

Systemic application of toxins, like MPTP or rotenone, which inhibit
mitochondrial complex I have been reported to induce a rather selective
dopaminergic SNpc cell loss (7, 28); see for review (14, 39). These actions have
mostly been observed in adult animals, although previous studies have described
alterations of adult mesencephalic dopaminergic neurons after application of low-
dose of MPTP and other mitochondrial inhibitors during the neonatal period (17). A
differential protective response of microglia to MPTP treatment between neonatal
and adult mice has also been reported (45). However, the effects of systemic
toxins largely depend on their distribution and uptake by the different cellular types
in the brain and therefore do not unequivocally reflect an inherent sensitivity of the
affected neurons to mitochondrial poisoning. Within the context of this discussion, it
may be also worth to consider that rotenone, a lipophylic substance, can block K+
currents in several cell types (1, 30, 50) and alter global oxygen homeostasis by
interfering with carotid body oxygen sensing (36). On the other hand, several
genes mutated in familial forms of PD have been shown to encode proteins located
in, or capable to interact with, mitochondria (49, 55). Deletion of these genes does
not induce SNpc cell death in mice (27), thus casting doubts on whether
mitochondrial bioenergetics alterations per se can lead to neurodegeneration. It
has previously been reported that genetic ablation of mitochondrial DNA-encoded
genes causing alteration in the function of complexes I, III, IV, and V results in
extensive death of ventral mesencephalic dopaminergic neurons. Nonetheless, the selective effect of this mitochondrial damage on SNpc cells, in comparison with other neuronal types, has not been studied in detail (16). In this report we show that mitochondrial dysfunction in mouse restricted to catecholaminergic brain areas causes a major inhibition of postnatal mesencephalic dopaminergic neuron phenotypic specification, thus causing a progressive PD-like syndrome due to cell loss affecting predominantly SNpc. Other catecholaminergic neuronal groups, such as the dopaminergic A13 and noradrenergic LC, already matured at birth were practically unaltered. Therefore, these data indicate that SNpc neurons are highly vulnerable to mitochondria dysfunction. Death of dopaminergic nigrostriatal neurons in TH-SDHD mice appeared to be a cell-autonomous phenomenon, since these mutant animals did not show abnormalities in other areas of the brain or in the striatal neurons innervated by the dopaminergic terminals.

We have shown in adrenal chromaffin cells that SdhD ablation leads to a decrease in ATP content. Therefore, the cell loss, particularly in the dopaminergic SNpc cell group, observed in the TH-SDHD mice could be a consequence of bioenergetic deficiency possibly aggravated by mitochondria ROS overproduction due to inefficient transference of electrons from Sdh to ubiquinone, similarly as it occurs in cells lacking SdhB or SdhC proteins (19, 25). The involvement of mitochondrial ROS in SNpc cell damage has been proposed long ago (46), and a causative link between ROS production and selective SNpc cell loss has recently been established. Adult dopaminergic SNpc neurons are autonomous Ca\(^{2+}\)-dependent pacemakers that require a proper mitochondrial ETC function for
cytosolic Ca\(^{2+}\)-buffering homeostasis. Therefore, these cells are normally exposed
to an oxidant stress, derived from the particularly active ETC, which not only
compromises their long-term survival but also makes them particularly vulnerable
to mitochondrial alterations (12, 18). In accord with this idea, systemic antioxidant
treatment of TH-SDHD mice with tempol appeared to have a protective role on
SNpc neurons and favored catecholaminergic phenotypic specification. However,
this observation must be taken as preliminary since, although we have been able
to measure higher level of lipid peroxidation in AM samples from TH-SDHD mice
compared to controls, other signs of oxidative stress, as for instance oxidative DNA
damage or increased protein oxidation, were undetectable. In addition, due to
technical limitations we do not have yet any direct measurement of ROS levels in
SNpc neurons of the mutant animals. Nevertheless, it is intriguing that other
catecholaminergic cells, as for example those in the LC that, as SNpc neurons,
posses large terminal axonal fields and exhibit Ca\(^{2+}\)-dependent spontaneous action
potential firing (11), are resistant to mitochondria dysfunction. Although Ca\(^{2+}\)-
dependent pacemaking is surely a major risk for neurodegeneration, our findings
suggest that additional metabolic and/or developmental differences between SNpc
and other catecholaminergic neurons must exist to explain their distinct sensitivity
to mitochondrial ETC inhibition. It is known that adult SNpc and LC neurons are
both highly sensitive to glial cell-line derived neurotrophic factor (GDNF) deficiency
(38), and this trophic factor protects LC neurons against oxidants (2). Therefore, it
could be that in TH-SDHD animals LC neurons are unaffected because they have
stronger trophic support and antioxidant defense than SNpc cells. Indeed, a
peculiar property of SNpc neurons with respect to LC and A13 cells is that the former reach full phenotype specification in postnatal life, which includes TH expression as well as final establishment of synapses at the distant striatum (9). In addition, we have recently shown that GDNF expression by neostriatal interneurons, necessary to support trophically SNpc cells, is not yet fully developed at birth but matures during the 2 to 4 postnatal weeks (23). Neurons of LC or A13, which mature during embryonic life, may have reached some of their targets at birth and therefore are provided with the trophic support that endows them resistance to Sdh deficiency. In contrast, the postnatal development of the nigrostriatal pathway and striatal GDNF-producing interneurons, leaves SNpc cells deprived of target-derived trophic support for a time lapse during which they are more susceptible to mitochondrial dysfunction. It is likely that in TH-SDHD animals the nigrostriatal pathway never reaches full maturation. This would explain why they have complete striatal denervation even at early juvenile stages despite numerous SNpc TH+ cells are still conserved. Lack of trophic support during postnatal maturation and strict dependence on mitochondrial ETC function are features that together could explain the exceptional vulnerability of SNpc to stressful conditions.

The data shown here provide providing unequivocal support for a major selective role of mitochondria in SNpc neuronal loss in PD, however it must be noted the TH-SDHD mouse does not exactly reproduce the neuronal loss seen in PD. For example, TH-SDHD animals had a clear loss of dopaminergic neurons in the VTA, whereas these neurons are spared or little affected in PD patients as well
as in the MPTP mouse model of PD. Furthermore, LC neurons, unaltered in TH-SDHD animals, are also affected in PD. The observations in this report reinforce a relatively little explored perspective in PD pathogenesis associated with early postnatal SNpc cell phenotypic specification, which could be also strictly dependent on a proper mitochondria function. In this regard it is worth to stress that TH-SDHD mice show death in peripheral sympathetic neurons and paraneurons, as it is also observed in early stages of PD (33, 54). Our results supports a long-standing notion (see 17) according to which subtle developmental alterations or exposure to environmental pollutants, occurring during the critical perinatal period, could diminish the final number of physiologically mature SNpc neurons. In accord with the proposed “multiple-hit” hypotheses for PD (10, 53), the affected individuals would surely be more susceptible to other hits damaging SNpc neurons later in life thus predisposing them to suffer the disease in adulthood.

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References


Figure Legends

FIG. 1. Generation of the TH-SDHD mutant mouse. (A) Scheme of the 16 kb targeting construct engineered to contain two loxP sites (grey triangles) that flank exons 2 to 4 (black boxes) of the SdhD gene, and a neomycin-resistance (NEO) cassette for clone selection. This construct was targeted to the wild-type SdhD genomic locus by homologous recombination. (B) CRE recombinase mediated excision of the SdhD<sup>flox</sup> allele. H, HindIII; E, EcoRV. Distances between relevant sites are indicated above dotted arrows. (C) Southern of HindIII-digested DNA from neomycin-resistant ES clones, hybridized with a probe against a 5' region (5'-p). (D) Southern of EcoRV-digested DNA from clones expressing the CRE recombinase, hybridized with a probe against the NEO gene. Band sizes are indicated with numbers aside the blots. The Δ symbol represents the SdhD excised allele. (E) Kaplan Meier survival curves of control and TH-SDHD mice.

FIG. 2. Histological analyses of peripheral catecholaminergic organs of control and TH-SDHD mice. (A) Representative photographs of adrenal glands from P75 mice. (B) Postnatal increase of volume of the adrenal medulla. (C) ATP levels in adrenal medulla of P75 to P105 mice. (D) Lipid peroxidation in AM of P75 mice for both separate controls (flox/+ and flox/- without CRE) and mutant groups. Similar analysis performed in liver tissue showed no difference between control and TH-SDHD mice (data not shown). (E, F) Representative photographs and number of TH<sup>+</sup> cells in carotid bodies (CB) from P75 mice. (G, H) Representative photographs
and density of TH⁺ cells in superior cervical ganglion (SCG) from P75 mice. All these tissues were immunostained for tyrosine hydroxylase (TH, red). Nuclei were stained with DAPI dye. AG: adrenal gland; AM: adrenal medulla; ca: carotid artery. Bar sizes: 100 µm in E; 200 µm in A and G. Number of individuals is 4 to 8 per group and age. Statistical significance: *: \(P \leq 0.05\); **: \(P \leq 0.01\); ***: \(P \leq 0.001\), between control and TH-SDHD groups.

FIG. 3. Lack of carotid body hypertrophy in the SDHD-ESR mouse. (A) Levels of \(SdhD\) mRNA in kidney and adrenal gland of both separate controls (flox/+ and flox/- without CRE) groups and mutant individuals five days after tamoxifen injection (at P50) demonstrating general loss of \(SdhD\). r. u.: relative units. Number of individuals is 3 to 7 per group. (B) Succinate ubiquinone oxi-reductase activity (MCII) in mitochondria isolated from kidney cells three weeks after tamoxifen injection. Number of individuals is 3 to 6 per group. (C) Carotid body (CB) immunostained against tyrosine hydroxylase (TH) three weeks after tamoxifen injection. Size bar: 100 µm. (D) Total and TH⁺ fraction volumes of carotid body from control and mutant, SDHD-ESR, animals three weeks after tamoxifen injection (at P50). Number of individuals is 3 and 4 respectively. *: \(P < 0.05\); ***: \(P \leq 0.001\).

FIG. 4. Loss of ventral mesencephalic dopaminergic neurons in TH-SDHD mice. (A) TH immunostaining of neurons in SNpc and VTA of control and TH-SDHD mice at postnatal days (P) 0, 30 and 180. Bar size: 200 µm. The insets show neurons at
higher magnification. Number of TH⁺ neurons in SNpc (B) and VTA (C) at different animal ages. (D) NeuN⁺ cell number in SNpc and VTA of P105 mice. Number of individuals is 3-8 per point. *: P<0.05; **: P<0.01; ***: P≤0.001, between control and TH-SDHD groups. #: P<0.05; ##: P≤0.001, between P30 and subsequent ages.

FIG. 5. TH immunostaining of noradrenergic neurons in locus coeruleus (A) and dopaminergic nucleus A13 of the zona incerta (B) of control and TH-SDHD mice at postnatal day 180. The insets show neurons at higher magnification. Axonal bundle of the nigrostriatal pathway (NSP) is shown. Bar size: 200 µm. Number of TH⁺ neurons in LC (C) and A13 (D) at different ages. Number of individuals is 3-8 per point.

FIG. 6. (A) Confocal microscope photographs of locus coeruleus and dopaminergic nucleus A13 showing co-localization of X-gal (blue dots) and TH⁺ (red) neurons in a TH-SDHD mouse carrying the R26R reporter construct. (B) In situ hybridization of SdhD mRNA in hippocampus, SNpc-VTA, and LC. Note the complete disappearance of mRNA in the catecholaminergic areas (encircled by dotted lines) of the TH-SDHD mouse. Note also that the level of SdhD mRNA expression in hippocampus of control animals (homozygous for SdhD) was higher than in heterozygous TH-SDHD mice (see Piruat et al., 2004). Bar sizes: Hippocampus 500 µm, SNpc/VTA 200 µm, LC 100 µm.
FIG. 7. Lack of striatal affectation in the TH-SDHD mouse. Number of DARP32 (A) and parvalbumin (B) positive neurons in striatum of control and TH-SDHD mice at P75. Immunostaining of striatal neurons was done as indicated in reference 23. Cell counts were done in 20 μm thick slices. Number of individuals is 3 per group. (C) SdhD mRNA levels in striatum of control and TH-SDHD mice at P30 as determined by RT-qPCR of total RNA. r. u.: relative units. Number of individuals is 3 to 4 per group. **: P<0.01; between control and TH-SDHD groups.

FIG. 8. Parkinsonian phenotype of TH-SDHD mouse. (A) TH immunostaining of striatum of control and TH-SDHD mice at P75. Bar: size 500 μm. CPu: Caudate putamen. Acb: Accumbens nucleus. (B) Dopamine (DA), 3,4-dihydroxyphenylacetic (DOPAC), and homovanillic acid (HVA) levels in striatum at P75 determined by HPLC. Number of individuals is 3 to 8 per point. (C) Walking traces of control and TH-SDHD mice in open-field test for 1 hour at the indicated ages. (D) Quantification of travelled distance and resting time (RT). Number of individuals is 3 to 6 per point. **: P<0.01; ***: P≤0.001, between control and TH-SDHD groups; ##: P≤0.001, between P40 and later ages.
Figure 1
Figure 2
Figure 4
Figure 5
Figure 7
Figure 8