Selective Deletion of PTEN in Dopamine Neurons Leads to Trophic Effects and Adaptation of Striatal Medium Spiny Projecting Neurons

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Abstract

The widespread distribution of the tumor suppressor PTEN in the nervous system suggests a role in a broad range of brain functions. PTEN negatively regulates the signaling pathways initiated by protein kinase B (Akt) thereby regulating signals for growth, proliferation and cell survival. PTen deletion in the mouse brain has revealed its role in controlling cell size and number. In this study, we used Cre-loxP technology to specifically inactivate Pten in dopamine (DA) neurons (Pten KO mice). The resulting mutant mice showed neuronal hypertrophy, and an increased number of dopaminergic neurons and fibers in the ventral mesencephalon. Interestingly, quantitative microdialysis studies in Pten KO mice revealed no alterations in basal DA extracellular levels or evoked DA release in the dorsal striatum, despite a significant increase in total DA tissue levels. Striatal dopamine receptor D1 (DRD1) and prodynorphin (PDyn) mRNA levels were significantly elevated in KO animals, suggesting an enhancement in neuronal activity associated with the striatonigral projection pathway, while dopamine receptor D2 (DRD2) and preproenkephalin (PPE) mRNA levels remained unchanged. In addition, Pten inactivation protected DA neurons and significantly enhanced DA-dependent behavioral functions in KO mice after a progressive 6OHDA lesion. These results provide further evidence about the role of PTEN in the brain and suggest that manipulation of the PTEN/Akt signaling pathway during development may alter the basal state of dopaminergic neurotransmission and could provide a therapeutic strategy for the treatment of Parkinson’s disease, and other neurodegenerative disorders.

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Introduction

Pten (for ‘phosphatase and tensin homologue deleted on chromosome ten’) is a tumor suppressor gene mutated in many human cancers, including glioblastomas, a highly malignant glial tumor in the CNS [1]. Individuals with germine Pten mutations are prone to tumors and may display brain disorders, including macrocephaly, seizures and mental retardation [2].

The tumor suppressive property of PTEN is dependent on its lipid phosphatase activity, which restrains the activation of the Akt (also called protein kinase B) signaling pathway. Upon activation, Akt phosphorylates a diverse spectrum of substrates known to regulate cellular functions related to cell cycle progression, cell growth and proliferation, cell death/survival and cell differentiation [3].

PTEN is widely distributed in the brain and is preferentially expressed in neurons, where it localizes to both the nucleus and cytoplasm [4–7]. The role of PTEN in the brain has been largely focused on the pathogenesis of glioblastoma; however, progress has recently been made in understanding the broader role of PTEN in neural circuits. A number of in vitro studies indicates a role for PTEN signaling in neuronal size, dendritic and axonal branching and neuronal polarization [8,9]. To analyze the role of PTEN in vivo and in specific neuronal populations mutant mice have been generated to induce the loss of the phosphatase in a tissue-specific manner by using the Cre-loxP technology. Conditional loss of Pten in the brain can have differing consequences depending on the cell type or state of differentiation. For example, the use of a Nestin promoter to induce Pten deletion in neural stem cells and glial progenitors resulted in neonatal death, as mice showed enlarged and histoarchitecturally abnormal brains [10]. Pten deletion in discrete mature neuronal populations in the cerebral cortex and hippocampus resulted in macrocephaly due to neuronal hypertrophy. In addition abnormalities in axonal growth and synapse number resulted in abnormal social behavior and inappropriate responses to sensory stimuli [11].

Recent studies have emphasized the association of PTEN with different parameters of the dopaminergic system in the ventral midbrain. For example, the continuous activation of the downstream PTEN pathway, Akt, in mesostriatal adult dopamine neurons by unilateral adеноiral injections in the substantia nigra compacta confers almost complete protection against apoptotic cell death in a dopamine toxin specific model [12]. In addition, a recent
study has shown Akt signaling in the mesolimbic dopamine system may also regulate functions intrinsic to dopamine neurons, such as cellular and behavioral responses to stressful stimuli [13]. Besides the inhibitory control on the activation of the Akt pathway, PTEN interacts with molecular substrates directly involved in neurotransmission such as glutamate and serotonin receptors [13,14].

Normally the activity of mesolimbic dopamine cells is under the tonic inhibitory control of the phosphorylated serotonin receptor 2c (5-HT2c). PTEN deletion in DA cells favors the phosphorylated state of the 5-HT2c receptor, inhibits dopaminergic transmission and abolishes the rewarding effects of nicotine and tetrahydrocannabinol [13]. These studies suggest PTEN function in dopamine neurons is not limited to the classical deregulation of the Akt pathway, and PTEN may also have an effect on distinct functions related to dopamine-mediated cognition. To further examine the correlation between PTEN and the dopaminergic system, we developed a mouse transgenic model to inactivate PTEN signaling specifically in DA neurons. We now report that PTEN ablation during development results in profound morphological, molecular and neurochemical changes in DA neuron maturation that translate into permanent adaptations of the dopaminergic system, including significant changes in postsynaptic brain areas. Additionally, and in agreement with previous studies [12], we show that PTEN deletion in dopamine cells provides significant protection against neurotoxic insults. Such adaptations will be documented in detail in this report. Future studies will be aimed at correlating the molecular adaptations mediated by PTEN deletion with behavioral aspects of dopamine neurotransmission.

Methods

Mice

Pten+/- mice were obtained from Jackson laboratories [14]. Mice were obtained in a background comprised of a mix of c57bl/6, 129S4/SvJae. All animals used in this study were approved by the Animal Care Committee at the National Institutes of Health. For the conditional inactivation of Pten in dopaminergic neurons of the ventral midbrain, we developed a dopamine transporter (DAT) promoter-driven Cre transgenic mouse line Slc6a3-Cre [15]. Cre recombinase binds to loxP target sequences and either deletes or inverts the intervening DNA depending on the individual orientation of the loxP sites [16]. In our model, Cre recombinase expression is driven by the DAT promoter and mediates deletion of exon 5 of the pten gene approximately at embryonic day 15 [15]. DAT is a molecular substrate specific to dopamine neurons and is highly expressed in dopamine neurons of the ventral midbrain, while lesser expression levels are present in the olfactory bulb and hypothalamic areas. To minimize interference with DAT function by preservation of both DAT alleles, Cre recombinase expression was driven from the 3′-untranslated region (3′UTR) of the endogenous DAT gene by means of an internal ribosomal entry sequence (IRES). The Pten+/- and Slc6a3-Cre lines were mixed to obtain regional knockout (Pten+/-loxP/Cre+/-) and control mice (Pten+/-loxP/Cre+/-). Animals were genotyped using Pten and Slc6a3-Cre primers described elsewhere [14,15].

To confirm if Pten deletion was specific to DAT expressing regions, primers specific to the recombination (delta5) were developed. Genomic DNAs were prepared from the olfactory bulb, motor cortex, striatum and SN/VTA. For ventral midbrain dissections, coronal cuts were made at the anterior and posterior boundaries of the mammillary nucleus. Dissection of the ventral midbrain region containing the SN/VTA was facilitated by removing the mammillary nucleus on the ventral surface and overlying cortex on the dorsal surface. A block containing the SN/VTA was then dissected. To further ensure specificity of Cre mediated recombination lung, heart, kidney and liver tissue was dissected and DNA extracted for PCR analysis.

Immunohistochemistry

Adult mice were deeply anesthetized with an intraperitoneal (i.p.) injection of chloral hydrate (30 mg/kg) and then perfused transcardially with saline solution followed by 4% paraformaldehyde (PFA). Brains were quickly removed from skull and post-fixed in 4% PFA for 4 hrs. After fixation, brains were rinsed in 0.1 M phosphate buffer (PB) and cryoprotected in 10% sucrose solution, overnight at 4°C.

Striatum and midbrain regions were cut in coronal sections at 40 μm in four series using a Leica CM3050S cryostat (Leica Microsystems, Bannockburn, IL). Sections were processed for Tyrosine Hydroxylase (TH) immunohistochemistry or for Pten or phospho-AKT immunofluorescence. For TH immunohistochemical studies, sections were rinsed with PB 3×10 min and then permeabilized and blocked with 0.25% Triton-X-100 and 4% bovine serum albumin (BSA) in 0.1 M PB. Sections were incubated overnight at 4°C with a rabbit polyclonal antibody against TH (1:1000, Chemicon, Temecula, CA). Sections were then rinsed 3×10 min in PB, and incubated for 1 hr with a biotinylated anti-rabbit antibody (1:200, Vector Labs, Burlingame, CA). Sections were rinsed with PB 3×10 min and incubated with avidin-biotinylated horseradish peroxidase for 2 h. Sections were rinsed and the peroxidase reaction was developed with 0.05% 3,3-diaminobenzidine-4 HCl (DAB) and 0.003% hydrogen peroxide (H2O2). Sections were mounted on coated slides, coverslipped and dried before analysis.

For immunofluorescence histochemistry, sections were rinsed in PB 3×10 min, and then incubated with 0.25% Triton-X-100 and 4% BSA in 0.1 M PB. Sections were incubated with a mouse monoclonal anti PTEN (Cell Signaling, Danvers, MA) or with anti-p-AKT rabbit monoclonal antibody (1:100, Cell Signaling, Danvers, MA) overnight at 4°C. Sections were rinsed with PB 3×10 min and incubated for 2 hrs at room temperature with one of the following secondary fluorescent antibodies: anti mouse or anti rabbit (Invitrogen, Carlsbad, CA). Sections were rinsed with PB 3×10 min and incubated with avidin-biotinylated horseradish peroxidase for 2 h. Sections were rinsed and the peroxidase reaction was developed with 0.05% 3,3-diaminobenzidine-4 HCl (DAB) and 0.003% hydrogen peroxide (H2O2). Sections were mounted on coated slides, coverslipped and dried before analysis.

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Stereologic Analyses

Unbiased stereological counts of TH-positive (TH+) neurons within the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) as well as fiber length in the substantia nigra pars reticulata (SNr) were performed using stereological principles [17] and analyzed with StereoInvestigator software (Microbrightfield, Williston, VT). The optical fractionator probe [18] was used to generate an estimate of neuronal TH+ number and area, nucleator probe [19] to estimate the size of TH+ neurons in SNc and VTA and space balls probe [20] to obtain an estimate of the total length of fibers in the SNr. SNc, VTA and SNr were outlined under a low magnification objective (5x) following landmarks from the Franklin and Paxinos mouse atlas [21] and all the stereologic analysis were performed under the 40x objective of a Leica DM5000B microscope (Leica Microsystems, Bannockburn, IL).

The total number of TH+ neurons, neuronal size and fiber density, was estimated for adult naive control and Pten KO mice. For each tissue section analyzed, section thickness was assessed in each sampling site and guard zones of 2.5 μm were used at the top
and bottom of each section. Systematic random sampling design was performed and generated with the following stereologic parameters: grid size: 131 μm, counting frame: 123 μm and dissector height of 25 μm. For space balls a 20 μm radius was used to intersect fibers that crossed the circle. The criterion for a TH+ fiber was a stained and in focus fiber at the crossing point in the circle. Our criterion for counting and measuring the area of an individual TH+ neuron was the presence of its nucleus either within the counting frame, or touching the right or top frame lines (green), but not touching the left or bottom lines (red). The area of TH+ neurons in the SNc and VTA was estimated using the dissector probe. Every time a neuron was counted with the optical fractionator probe, a set of four rays are extended from the middle of the cell and radiate with a random orientation in four orthogonal directions towards the edge of the neuron. The point at which each ray intersected the boundary of the neuron are used to define the area. Coefficients of error were calculated and values <0.10 were accepted.

Stereologic estimations were also performed with the same parameters in the SNC of control and Pten KO mice thirty days after receiving a unilateral 6OHDA lesion in the striatum. Results were presented as a percentage of surviving neurons on the contralateral side.

**Optical Density**

To determine fiber density in the striatum, the mean optical density (O.D.) was measured in TH positive stained sections from naive and 6OHDA-lesioned mice (thirty days after 6-OHDA unilateral lesion) in control and Pten KO mice. O.D. is a sensitive and reliable tool to measure levels of fiber innervation and to detect changes by experimental manipulations [22]. Images were captured with a Hitachi CCD HV-C20A camera and transformed to grey scale images of 8 bits. The O.D. quantification was perform using ImageJ software [23]. The O.D. measures were determined in each mouse at three coronal levels corresponding to the frontal striatum +1.5 mm, medial striatum +0.14 mm, and caudal striatum −0.50 mm, relative to bregma [21]. Nonspecific background was determined by readings made from the anterior commissure or corpus callosum. For naive animals, the O.D. value corresponds to the mean of both striatae minus the respective background level. For 6OHDA-treated animals, O.D. values were measured on the denervated and non denervated striatum in control and KO mice and the O.D. minus the background was expressed as a percentage relative to the contralateral side. In addition, striatal total area was determined with the same software and results expressed as square pixels.

**Behavioral Test: Locomotor activity induced by exposure to a novel environment**

All mice used were adult and kept on a 12:12 light – dark cycle (lights on 06:00 am) with food and water ad libitum. Behavioral tests were performed between 10:00–14:00 hrs. Locomotor activity assessed in the open field, is considered a well organized behavior in rodents determined by several factors, including anxiety, arousal, risk assessment, and exploration, and is an established tool for behavioral phenotyping in mice [24]. Control and Pten KO mice were brought to the testing room 30 minutes before the procedure began for acclimation. After this period, each mouse was gently placed inside a clean Plexiglas chamber (42x42x30 cm; Accuscan Instruments, Columbus, OH) for 30 minutes to assess locomotor activity in a new environment. Locomotor activity was measured in each cage by 16 horizontal and 16 vertical sensors (infrared beams) spaced 2.5 cm apart. Vertical sensors were located 7 cm above the floor chamber. Data was stored every 5 min and presented as cumulative values after 30 minutes.

**Real time PCR**

Striatal tissue was dissected and immediately processed for RNA isolation and DNAse I treatment using the RNaqueous-Micro A mbion kit (Applied Biosystems- Ambion, Austin, TX), following the manufacturers instructions. For cDNA synthesis total RNA was mixed following the manufacturers instructions for the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA). Real-time polymerase chain reaction was performed as duplicate determinations with specific Taqman probes from the mouse Probe Library (Exiqon A/S, Vedbaek, Denmark) designed for primers to mouse DRD1 (F: 5’- gag cgt aag ctc cca gat gc-3’ and B: 5’- agg tea aca tca gtc act tt tta ca-3’), DRD2 (F: 5’- ctc ttt gga ctc aac aac caa gc-3’ and B: 5’- aag ggc acg tag aac gac ac-3’), preproenkephalin (F: 5’- aag ggc acg tag aac gag ac-3’), proenkephalin (F: 5’- aag ggc acg tag aac gag ac-3’ and B: 5’- cgg cat tct gac tca ctt gtt gc-3’), and BDNF (F: 5’- gca tct gtt ggg gag caa gc-3’ and B: 5’- tgg tca tca ctc ttc tca cct g-3’). Hydroxymethylbilan synthase (Hmb, F: 5’- tcc cag aat gat ggt cct ac-3’ and B: 5’- aag gtt ttg ctc cct g-3’) and aminolevulinate synthase (ALAS, F: 5’- caa att cca aca gag tgc-3’ and B: 5’- tgg acc aag acg agc ttc a-3’) were used as housekeeping genes to normalize quantification data. The reproducibility of results was determined by inspection of duplicate samples. After an initial incubation step for 10 min at 95 °C, qRT-PCR was carried out using 40 cycles (95 °C for 15 seconds, 60 °C for 60 seconds). The standard curve method was used to compare mRNA expression levels between KO and control animals. Normalization to both endogenous control genes led to similar results.

**In Vivo Microdialysis**

Pen deficent mice and controls were anesthetized and implanted unilaterally with a microdialysis guide cannula (CMA/11, CMA microdialysis) in the dorsal striatum (AP: +0.4, L: -2.1, V: -2.2 mm from bregma) using standard stereotaxic techniques, and were allowed to recover for 5 days before the microdialysis experiments [25]. Fifteen hours before the start of experiments, a microdialysis probe (CMA/11, 2 mm membrane length, CMA microdialysis, North Chelmsford, MA) was connected to the dialysis system and flushed with artificial cerebrospinal fluid (aCSF: 145 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 0.25 mM ascorbic acid, and 5.4 mM D-glucose, pH 7.2 adjusted with NaOH 0.5 M). The mouse was gently restrained and the probe was slowly inserted into the guide cannula. The dialysis system consisted of FEP tubing (CMA microdialysis) that connected the probe to a 1 ml gastight syringe (Hamilton Co., Reno, NV) mounted on a microdialysis pump (CMA/102) through a quartz-lined, low-resistance swivel (375/D/ 22QM, Instech, Plymouth Meeting, PA). After probe insertion, the mouse was placed in the dialysis chamber with food and water freely available, and the probe perfused overnight with aCSF at a flow rate of 0.3 μl/min. The next morning, the perfusion syringes were loaded with fresh aCSF and probes were allowed to equilibrate for an additional 1 h at a flow rate of 0.6 μl/min before the start of experiments.

For no net flux experiments, five different concentrations of DA (0, 5, 10, 20, and 40 nM) in aCSF were perfused in random order through the dialysis probe. Each DA concentration was perfused for 30 min, and then 2 x 10 min samples were collected. Following completion of the no net flux experiments, normal aCSF was again perfused through the probe for 30 min, allowing for a period of equilibration. Consecutive 15 min samples were
The limit of detection was 0.25 nM. Under these conditions, retention time for DA was 3 min, and All the reagents used for the mobile phase were analytical grade.

standard curve calibration, using peak heights for quantification. vs. Ag/AgCl. Dialysate DA levels were quantified by external + carbon working electrode at an oxidation potential of methanol (vol/vol) was filtered through a 0.22-
sodium phosphate, 2.24 mM sodium octanesulfonic acid, ford, MA), a BAS PM-80 pump (BAS, West Lafayette, IN) and a detection. The chromatographic system consisted of a CMA/200 system at a flow rate of 0.6 ml/min. DA was separated in a BAS C18 column (100 mm 60 mM KCl, the average of the three baseline samples was calculated, and all the DA concentrations were expressed as % of that baseline. Differences between control and Pten KO animals in the appropriate variables were assessed by comparing both groups using a Student’s t-test.

DA was determined by HPLC coupled to electrochemical detection. The chromatographic system consisted of a CMA/200 refrigerated microinjector (CMA microdialysis, North Chelmsford, MA), a BAS PM-80 pump (BAS, West Lafayette, IN) and a BAS LC-4C amperometric detector. The mobile phase (0.15 M sodium phosphate, 2.24 mM sodium octanesulfonic acid, 0.94 mM EDTA, adjusted to pH 5.0 and containing 11% methanol (vol/vol) was filtered through a 0.22-µm nylon filter, degassed by a BAS online degasser, and pumped through the system at a flow rate of 0.6 ml/min. DA was separated in a BAS C18 column (100 mm x2.0 mm x3 µm) and detected on a glassy carbon working electrode at an oxidation potential of +700 mV vs. Ag/AgCl. Dialysate DA levels were quantified by external standard curve calibration, using peak heights for quantification. All the reagents used for the mobile phase were analytical grade. Under these conditions, retention time for DA was 3 min, and the limit of detection was 0.25 nM.

Tissue Levels of DA and metabolites

The midbrain and the left dorsal striatum were dissected after the microdialysis experiments and stored at −80°C for the determination of monoamine content. Tissue samples were homogenized by ultrasonication in 20 volumes of ice cold 0.05N HClO4 containing 0.5 µM of DHBA as the internal standard. Homogenates were centrifuged at 22000 g for 10 min at 4C. Aliquots from the supernatant were analyzed for monoamine content by HPLC coupled to electrochemical detection. The chromatographic system was similar to the one described for microdialysis experiments except for the column (100 x1 mm C18,5 um particle size, BAS) and the mobile phase (35.2 mM sodium phosphate, 26.4 mM citric acid, 1.66 mM sodium octanesulfonic acid and 0.1 mM EDTA adjusted to pH 4.2; containing 6% methanol and 0.4% tetrahydrofuran, vol/vol). The concentrations of monoamines were calculated against an external standard curve, normalized by the internal standard and corrected by the protein concentration in the homogenate.

6-Hydroxydopamine (6OHDA) Lesions

Mice were anesthetized with Avertin, and placed in a stereotaxic frame. 6OHDA (5.0 µg/µl in 0.9% NaCl/0.02% ascorbate) was injected using a microliter syringe at a rate of 0.5 µl/min for a total dose of 15.0 µg/3 µl. Injection was performed into the striatum at coordinates AP: +0.09 cm; ML: ±0.22 cm; DV: −0.25 cm relative to bregma. After 2 min, the needle was withdrawn slowly. For amphetamine-induced rotation, mice were injected with (+) methamphetamine HCl (2.5 mg/kg i.p. injection) and placed in a rotometer (Accuscan, Columbus, OH) for 120 min. Baseline amphetamine rotation tests were performed 7 days before 6OHDA lesions and the results used to assign the side of the subsequent 6OHDA lesion. Mice exhibiting net clockwise turns were lesioned in the left striatum, while mice exhibiting net counter-clockwise turns were lesioned in the right striatum. Following the 6OHDA lesion, amphetamine-induced rotational behavior was tested 14 and 28 days after lesion. The number of clockwise and counter-clockwise turns was counted and expressed as the number of net rotations per 120 minutes to the lesioned (ipsilateral) hemisphere.

Statistical analyses

Unless otherwise stated, statistical analyses between control and Pten KO animals were performed by using a Student’s t test with values of *p<0.05 considered significant.

Results

Pten deletion is specific for cells expressing the dopamine transporter (DAT)

To establish a mouse model carrying Pten deletion in cells expressing DAT, we crossed Pten loxP mice with a Slc6a3Cre transgenic line. Of the offspring generated, we used Pten loxP/loxP/Ctc/wt as knockouts (KO) and Pten wt/wt/Ctc/wt as experimental controls. Pten KO mice were born at the expected Mendelian ratio and were visibly indistinguishable from control mice.

To confirm Cre-mediated recombination in Pten loxP/loxP/Ctc/wt mice we used PCR and immunological strategies. As shown in figure 1A, Pten exon 5 deletion was specific to the SNc, VTA and olfactory bulb in adult animals (delta5 band), with no detectable leakage in other brain areas [15]. In addition, the extent of Pten inactivation in KO mice was examined by immunocytochemistry using antibodies against TH, PTEN and phospho-AKT. In KO animals the loss of PTEN immunoreactivity in the ventral mesencephalon was associated with increased TH and phospho-AKT staining (Figure 1B).

As Pten is a tumor suppressor gene and deregulation of this pathway has been associated with the formation of multiple tumor types, the animals used for morphological evaluations in this study were examined for the existence of brain tumors. Sections through the entire brain did not show evidence of neoplastic transformation in any of the brains analyzed (data not shown).

Pten deletion leads to neuronal hypertrophy and increased number of TH positive neurons and dendrites in the ventral midbrain

DA midbrain neurons are first generated near the midbrain-hindbrain junction and migrate radially to their final position in
the ventral midbrain [27,28]. Tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthetic pathway of catecholamines, can be first detected in the mouse at embryonic day (E) 11.5, suggesting initiation of DA differentiation [27]. In contrast, DAT gene transcripts are not detected in the mouse ventral mesencephalon until DA axons reach the target at about E15, suggesting that during ontogeny, DA synthesis, and high-affinity uptake develop asynchronously and in a non-correlated fashion [27]. In DAT-Cre conditional transgenic mice, Cre recombinase activity is induced shortly after DAT induction. In a previous report we first detected DAT-Cre mediated beta galactosidase expression in a conditional Rosa26LacZ reporter mice at embryonic day 17 [15], suggesting excision of Pten exon5 at around this developmental point. Stereological cell counts of TH positive profiles in the substantia nigra and VTA were significantly elevated already at postnatal day two (data not presented), indicating an early expression of anatomical changes mediated by Pten ablation.

A common phenotype associated with the loss of PTEN in neuronal populations is an enlargement of neuronal soma size, increased cell proliferation, inhibition of apoptosis, and the expression of thickened axonal and dendritic processes. We performed detailed morphological analyses of brain structures affected by the mutation in adult animals. PTEN ablation in DA neurons (n = 4) induced a significant increase in SNc and VTA total volume (Fig. 2A and B; Fig. 3A) as compared to controls (n = 4). At a cellular level, this increased regional volume was mainly attributed to a significant increase in the number of neurons (Fig. 3B), as well as an increase in neuronal soma size (Fig. 2A and 2B insets and 3C). Fiber density measurements also revealed a significant increase in the number of dendritic processes in the substantia nigra pars reticulata (SNr, Fig. 3D). In many cases, dendritic processes were so close together and thickened in the SNr that at low magnification may appear as cell bodies. Interestingly, changes in neuronal size and number were not accompanied by significant differences in striatal TH staining intensity (Fig. 4A and 4C). Area measurements suggest a modest but significant enlargement of the caudal striatum in KO animals as compared to controls (Fig. 4B).

Figure 1. Cre recombinase deletes Pten exon 5 in neurons expressing the dopamine transporter. (A) PCR screen for Cre-mediated Pten exon5 deletion. Genomic DNAs were prepared from the indicated brain tissues from a Pten loxP/loxP Cre/wt adult mouse. PCR analysis show Pten deletion (delta5) is specific to the olfactory bulb and SN/VTA. Pten deletion could not be detected in other brain areas analyzed. (B) Immunofluorescent staining shows TH and Pten staining in the ventral mesencephalon of control and Pten KO animals. In the mutant animals, loss of Pten activity is associated with increased TH and membrane phospho-AKT staining. Sections correspond to Bregma -2.95 mm with reference to Franklin and Paxinos (1997). Scale bar, 250 μm.

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Pten deletion alters the expression of postsynaptic markers in the striatum

Over 90% of neurons of the striatum are medium spiny projecting neurons. Distinct subpopulations of medium spiny neurons can be recognized based on their projections and expression of various markers. Direct and indirect striatal projection neurons selectively express the DRD1 and DRD2 dopamine receptor subtypes respectively [29]. Messenger RNAs encoding DRD1 are selectively localized in striatal neurons projecting to the substantia nigra (direct pathway) and co-localize with dynorphin. Conversely, mRNAs encoding DRD2 are selectively localized in neurons projecting to the lateral globus pallidus (indirect pathway) and colocalize with enkephalin. Real time quantitative PCR analyses were performed to define if Pten ablation altered the markers of the direct and indirect striatal pathways. Striatal mRNA levels for PPE, PDyn, and DRD1 and DRD2 receptors revealed a...
significant change in the expression of markers present in medium spiny neurons projecting to the substantia nigra (direct pathway). DRD1 and PDyn mRNA levels were significantly upregulated in KO animals as compared to controls (Fig. 5A and B). In contrast striatal mRNA expression levels for DRD2 and PPE did not differ between control and mutant animals (Fig. 5A and B). As striatal dynorphin positive neurons form connections with nigral DA neurons, we investigated the effects of the mutation on nigral BDNF messenger levels. BDNF messenger levels were significantly increased in *Pten* KO animals (Fig. 5C).

Extracellular DA Levels in Dorsal Striatum and correlation to locomotor activity

As previous studies have suggested levels of enkephalin and dynorphin are oppositely modulated by DA transmission, we next investigated if DA levels in the striatum were altered by *Pten* ablation in DA cells. We used the technique of in vivo microdialysis to investigate the consequences of genetic deletion of *Pten* in DA cells on basal extracellular DA dynamics as well as on potassium-evoked DA release. Quantitative no net flux microdialysis revealed no significant differences in basal dialysate levels (y-intercept) or in the estimated extracellular concentration (x-intercept) of DA in the dorsal striatum (Fig. 6A). Moreover, the DA extraction fraction, calculated as the slope of the no net flux regression line was unchanged in KO mice, suggesting that deletion of *Pten* in DA neurons did not alter the clearance of extracellular DA by the DA transporter [26]. Conventional microdialysis revealed a marked increase in basal dialysate DA levels in response to K⁺ evoked depolarization (Fig. 6C). No significant difference between control and KO mice in KCl-evoked DA levels was observed in the dorsal striatum (fig. 6C).

Mice lacking PTEN expression in DA cells showed an increase in catecholamine content in both the midbrain containing the DA cell bodies (fig. 6D) and in the terminal region in the dorsal striatum (fig. 6B). This increase was specific for DA and its metabolites since 5HT and 5HIAA levels were not altered (data not presented). Consistent with the dialysis data, no changes were observed in 3MT, a DA metabolite of primarily extracellular origin. In accordance with striatal DA release levels, no differences in locomotor activity were observed between control (*n* = 11) and *Pten* KO (*n* = 15) animals during exposure to a novel environment (Fig. 7).

**Pten influences neuroprotection after a neurotoxic insult**

A unilateral injection of 6OHDA into the striatum results in progressive loss of DA neurons in the ventral mesencephalon, and
Figure 4. Pten KO animals do not show differences in striatal TH fiber density measurements. (A, C) Optical density values did not reveal significant differences in striatal TH staining intensity between control (n = 4) and Pten KO animals (n = 4). (B) There were no significant differences in area measurements corresponding to rostral and medial striatum. A slight but significant increase in area was observed in the caudal striatal region of Pten KO animals as compared to controls. Scale bar, 500 μm. All data are mean±SEM. *p<0.05, Student’s t-test.

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Figure 5. Pten ablation in DA cells affect the direct striatal output system by significantly increasing mRNA expression levels for DRD1 and pDyn in striatal medium spiny neurons. BDNF mRNA levels were significantly elevated in the ventral mesencephalon (A) Dopamine receptor D1 (DRD1) and D2 (DRD2) mRNA expression levels in the striatum of control (n = 9) and Pten KO animals (n = 13). DRD1 mRNA expression levels were significantly increased in KO animals as compared to controls. DRD2 mRNA levels were unchanged between the groups. (B) Changes in DRD1 expression levels were accompanied by an elevation in prodynorphin (pDyn) mRNA in Pten KO animals. Preproenkephalin (PPE) mRNA expression levels were unchanged. (C) BDNF mRNA levels were significantly increased in the ventral mesencephalon in KO animals. All data are mean±SEM. *p<0.05, Student’s t-test.

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We found that Pten deletion protects DA neurons during this partial 6OHDA lesion and prevents fiber loss in the lesioned striatum when compared to control animals. Four weeks after the lesion, control 6OHDA-treated mice showed an extensive loss of TH positive SNc neurons in the ipsilateral or lesioned nigral region (Fig. 8A and B). Stereologic cell counts in control animals showed a 63% survival rate of TH positive neurons when compared to the contralateral side. In contrast, Pten KO mice showed an 89% survival rate on the lesioned side as compared to the contralateral nigra (Fig. 8C). Pten deletion also provided significant protection against axonal loss in the lesioned striatum. Four weeks after the lesion, quantification of TH fiber density in the lesioned striatum showed a significant decrease in optical density levels in control animals when compared to Pten KO animals. Significant differences in optical density were observed in rostral, medial and caudal sections (Fig. 8D).

The morphological preservation of the dopaminergic system observed in Pten KO animals was paralleled by a significant decrease in behavioral impairment developed after this partial 6OHDA striatal lesion. Unilateral 6OHDA striatal lesions result in an imbalance in the levels of DA between the two striatae [30]. We found that Pten deletion protects DA neurons during this partial 6OHDA lesion and prevents fiber loss in the lesioned striatum when compared to control animals. Four weeks after the lesion, control 6OHDA-treated mice showed an extensive loss of TH positive SNc neurons in the ipsilateral or lesioned nigral region (Fig. 8A and B). Stereologic cell counts in control animals showed a 63% survival rate of TH positive neurons when compared to the contralateral side. In contrast, Pten KO mice showed an 89% survival rate on the lesioned side as compared to the contralateral nigra (Fig. 8C). Pten deletion also provided significant protection against axonal loss in the lesioned striatum. Four weeks after the lesion, quantification of TH fiber density in the lesioned striatum showed a significant decrease in optical density levels in control animals when compared to Pten KO animals. Significant differences in optical density were observed in rostral, medial and caudal sections (Fig. 8D).

The morphological preservation of the dopaminergic system observed in Pten KO animals was paralleled by a significant decrease in behavioral impairment developed after this partial 6OHDA striatal lesion. Unilateral 6OHDA striatal lesions result in an imbalance in the levels of DA between the two striatae. As DA stores are depleted due to the loss of DA in axon terminals, the injection of drugs that act to release DA, such as amphetamine, will induce rotational behavior towards the denervated striatum. We examined the effects of Pten deletion on amphetamine-induced rotational asymmetry at different time points after 6OHDA lesioning. Amphetamine-induced rotational activity was recorded for 60 min following an injection of 2.5 mg/kg (+) methamphetamine HCl. The number of clockwise and counterclockwise turns were counted and expressed as the number of net rotations to the lesioned (ipsilateral) hemisphere (net rotations to ipsilateral hemisphere = ipsilateral rotations – contralateral rotations). KO animals exhibited a significant reduction in ipsilateral rotational behavior when compared to control animals at 2 and 4 weeks after the lesion (Fig. 9).

Discussion

Mice with targeted ablation of Pten in DA neurons provide a unique opportunity to study the correlation between deregulated PTEN signaling and specific changes in DA neurotransmission.
While the most described function of PTEN is mediated by the inhibition of cell-survival promoting PI3K/Akt-dependent signals, recent studies have shown PTEN regulates diverse functions in DA neurons, and its relationship to Parkinson's disease and drug addiction has been highlighted. In this study, we have further characterized PTEN function specific to postmitotic mouse mesencephalic DA neurons. We generated a knockin mouse expressing Cre recombinase under the transcriptional control of the Pten locus.

**Figure 8. Pten ablation shows neuroprotective effects in the intrastriatal 6OHDA mouse model.** Animals were treated with a unilateral 6OHDA injection in the striatum, and perfused 4 weeks after the injection to analyze the effect of Pten ablation on the magnitude of the lesion. (A) The SNc from control animals (n = 4) was markedly affected by the lesion as reflected by the significant loss of TH positive profiles in the ipsilateral side to the lesion as compared to the contralateral side. The dopaminergic neurons in Pten KO (n = 6) animals were largely spared after the lesion when compared to control animals. (B) The neuroprotective effects related to Pten ablation were observed at the level of the striatal axonal projections as well. Depletion of TH positive terminals in the striatum was reduced across the striatum in control animals, while axon terminal degeneration was much lower in Pten KO animals. (C) Survival rates for TH-positive neurons were quantified by stereologic counts. The number of surviving neurons (presented as percent surviving neurons when compared to the side contralateral to the lesion) was significantly increased in Pten KO animals (n = 6) when compared to controls (n = 4). (D) Optical density measurement in the injected striatum revealed a nearly complete preservation of TH terminals in Pten KO animals. TH staining density was significantly lower in control 6OHDA-treated animals with the biggest difference observed in the medial portion of the striatum, in accordance with stereotaxic coordinates used for the lesion. (A) Scale bar, 250 μm and (B) 500 μm. All data are mean ± SEM. *p < 0.05, Student's t-test.

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Figure 9. The morphological preservation of the Pten deficient nigrostriatal system after 6OHD treatment correlates with functional recovery after the lesion. To determine the functionality of the nigrostriatal system after the lesion, we examined methamphetamine-induced rotations. Methamphetamine treatment increases the extracellular availability of endogenous DA in the striatum, and it causes animals with a partial DA depletion to rotate against the lesion side (ipsilateral), due to the imbalance in DA release between the striatae. Strong ipsilateral rotational behavior was observed in control animals (n = 8) after treatment with (S)-methamphetamine HCl (2.5 mg/kg) at 14 and 28 days after the lesion. Ipsilateral rotational behavior was significantly reduced in Pten KO animals (n = 10). All data are mean±SEM. *p<0.05, Student’s t-test.

Ablation of PTEN in DA neurons did not only cause robust anatomical changes in the ventral mesencephalon, but also affected functional connections between DA neurons and target areas. Interestingly, PTEN ablation did not generate changes in TH optical density in the striatum or nucleus accumbens, suggesting an equal distribution of dopaminergic terminals between Pten KO and control animals. In vivo microdialysis studies showed no differences in basal extracellular DA levels or in the dynamics of neurotransmitter clearance in the dorsal striatum. In addition potassium evoked depolarizations did not show an increase in DA release in Pten KO animals when compared to controls.

While Pten deletion does not alter DA release, it induces permanent changes in striatal postsynaptic markers

Ablation of PTEN in DA neurons did not only cause robust anatomical changes in the ventral mesencephalon, but also affected functional connections between DA neurons and target areas. Interestingly, PTEN ablation did not generate changes in TH optical density in the striatum or nucleus accumbens, suggesting an equal distribution of dopaminergic terminals between Pten KO and control animals. In vivo microdialysis studies showed no differences in basal extracellular DA levels or in the dynamics of neurotransmitter clearance in the dorsal striatum. In addition potassium evoked depolarizations did not show an increase in DA release in Pten KO animals when compared to controls.

While no changes in DA release or uptake could be detected by microdialysis, KO animals showed a significant increase in total DA tissue content in dorsal striatum, an effect accompanied by a significant increase in striatal prodynorphin and DRD1 mRNA expression levels. As previous studies have shown enhancement of DA neurotransmission often results in elevated dynorphin and DRD1 mRNA levels in direct striatal projecting neurons, the unbalance between the direct and indirect striatal projection pathways observed in Pten KO animals could be explained by subtle but long-term changes in DA dynamics. In addition, we cannot rule out the possibility that the molecular changes specifically observed in dynorphin positive medium spiny neurons may be due to the direct synaptic interaction with DA neurons in the ventral mesencephalon. Medium spiny neurons may depend on trophic support and the availability of synaptic connections with their target, the SN, to define their molecular profile or even to survive during development. As Pten deletion creates a very robust change in substantia nigra, it is possible that the nigra may be in a better position to support afferent populations, such as the medium spiny neurons. Increased BDNF mRNA levels in the ventral mesencephalon in Pten KO animals, suggests that striatal afferents may have increased availability to trophic support in KO animals.

As locomotor activity and exposure to a novel environment are closely associated with mesocortical and mesolimbic activity [39,40], we compared the locomotor responses of control and Pten KO animals when exposed to a novel environment. Interestingly, and despite higher DA contents in the striatum,
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_Pten_ KO animals did not behave differently than controls when exposed to a novel environment. Increased DRD1 and dynorphin levels in medium spiny neurons may enhance the inhibition of DA neurons in the SNc, thereby normalizing overall DA release in the striatum.

Suppressing PTEN expression protects DA neurons against exposure to a neurotoxin

The relationship between PTEN and Parkinson’s disease has been emphasized in several studies often in reference to mitochondrial function. PTEN downregulation in cultured hippocampal neurons inhibits the release of cytochrome c during toxic insults [41]. PTEN directly regulates the activity of PTEN-induced putative kinase 1 (PINK1), a protein kinase that localizes to mitochondria where it inhibits cytochrome c release, and known to be mutated in certain forms of familial Parkinson’s disease [42]. In addition, as an upstream modulator of Akt, the phosphatase PTEN has been shown to play a role in the regulation of survival signaling of dopaminergic neuronal injury. A previous study overexpressed a constitutively active form of the oncoprotein Akt/PKB in neurons of the ventral midbrain of adult mice using adeno-associated virus transduction [12]. The authors reported a significant increase in cell size and number of TH positive profiles in the SNc on the injected side. In addition, TH immunostaining showed higher density in the striatum ipsilateral to the adeno viral injection. There was also near complete protection of DA neuron cell bodies after intrastralial 6OHDA lesion [12]. Our study provides further evidence that ablating PTEN activity leads to decreased dopaminergic neuronal death after a neurotoxic lesion. In agreement with the above mention study, we showed that _Pten_ deletion specific to DA neurons confers significant protection against 6OHDA mediated toxicity [43,44]. PTEN less DA neurons displayed a significant increase in survival rate as compared to control DA neurons. In addition, striatal TH density levels were significantly elevated in _Pten_ KO animals as compared to controls. This data correlates with behavioral parameters, as methamphetamine induced rotations were significantly decreased in KO animals, suggesting increased maintenance of DA release in the lesioned striatum.

The demonstration that PTEN/Akt mRNA expression is modulated in response to different apoptotic inducers [7] strongly suggests the association of this pathway with neuronal survival and death. However, in recent years investigations have shown cellular functions attributed to PTEN in various neuronal circuits go beyond interactions with the Akt pathway, and have strengthened the current view of this molecule as a potential therapeutic target. For example, it has been recently shown that PTEN can physically interact with serotonin receptors in dopamine neurons to modulate transmission [45,46]. Such interactions were able to affect the response of DA neurons to drugs of abuse, and therefore manipulation of the PTEN pathway could potentially affect the manner in which humans respond to reward and motivation. In addition, PTEN activity has been related to mitochondrial function in different neuronal systems, suggesting PTEN deregulation may provide a dual neuroprotective function in dopamine cells by the activation of survival pathways and maintenance of mitochondrial function. This molecule may thus have activity not only on cell death/survival, but also on other brain activities. Further studies defining the function of PTEN in dopamine cells may provide the molecular basis for the development of novel therapeutic strategies to treat CNS diseases.

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Author Contributions

Conceived and designed the experiments: ODR AZ LS YZ ACT CMB. Performed the experiments: ODR AZ LS YZ CMB. Analyzed the data: ODR AZ LS YZ CMB. Contributed reagents/materials/analysis tools: ODR AZ LS YZ ACT NM fdlc CMB. Wrote the paper: ODR CMB.

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