

Midbrain dopaminergic neurogenesis and behavioural recovery in a salamander lesion-induced regeneration model

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Death and lack of functional regeneration of midbrain dopaminergic (DA) neurons, decreased DA input in the target striatum and movement anomalies characterise Parkinson's disease (PD). There is currently no cure for PD. One way to promote recovery would be to induce or enhance DA neurogenesis. Whether DA neurogenesis occurs in the adult midbrain is a matter of debate. Here, we describe the creation of a salamander 6-hydroxydopamine model of PD to examine midbrain DA regeneration. We demonstrate a robust and complete regeneration of the mesencephalic and diencephalic DA system after elimination of DA neurons. Regeneration is contributed by DA neurogenesis, leads to histological restoration, and to full recovery of motor behaviour. Molecular analyses of the temporal expression pattern of DA determinants indicate that the regenerating DA neurons mature along a similar developmental program as their mammalian counterparts during embryogenesis. We also find that the adult salamander midbrain can reactivate radial glia-like ependymoglia cells that proliferate. The salamander model provides insights into the mechanisms of DA regeneration/neurogenesis and may contribute to the development of novel regenerative strategies for the mammalian brain.

KEY WORDS: Neurogenesis, Newt, Regeneration, 6-hydroxydopamine, Behaviour, Dopamine, Parkinson's disease

INTRODUCTION

One candidate treatment for Parkinson's disease is cell replacement therapy. Embryonic stem cells have been found to differentiate into dopaminergic (DA) neurons capable of integrating into the adult brain *in vivo*, and incorporating into functional circuits in animal models (Barberi et al., 2003; Bjorklund et al., 2002; Kim et al., 2002). Human foetal mesencephalic tissue has been tested in transplantation trials (Winkler et al., 2005), but methodological difficulties and ethical considerations have prevented the clinical development of these techniques (Bjorklund et al., 2003; Freed et al., 2003). Alternative approaches could involve adult somatic stem cell transplantation, or stimulation of endogenous neurogenesis *in situ* (Lindvall et al., 2004).

To address whether regeneration of DA neurons is feasible in the adult vertebrate brain, we studied an aquatic salamander, the newt, in which the mechanisms of cellular plasticity and functional tissue repair could be analysed. Following injury or tissue removal, adult newts regenerate large body structures, such as entire limbs and tails (Tanaka, 2003). Regeneration in salamanders could involve, depending on the site of the injury, both the activation of stem cells and/or reprogramming of differentiated cells (Brookes and Kumar, 2005; Grogg et al., 2005; Morrison et al., 2006). Tail regeneration, for example, involves lineage-shifting events in which radial glia-like cells (so called ependymoglia cells) give rise not only to neurons and glia, but also integrate into newly formed muscle and cartilage (Echeverri and Tanaka, 2002).

Little is known about how newts respond to brain injury, although available reports indicate increased proliferation and possibly neurogenesis after mechanical lesions (Minelli et al., 1990; Minelli

et al., 1987). A toxin model of DA cell loss in amphibia has been presented previously, but the issue of neurogenesis or behavioural recovery was not addressed (Barbeau et al., 1985).

We endeavoured to examine the regenerative/neurogenic capacity of the salamander brain following cell type-specific chemical lesioning with 6-hydroxydopamine (6-OHDA), a neurotoxin selective for catecholamine neurons and which is widely used to create parkinsonian animal models (Deumens et al., 2002).

MATERIALS AND METHODS

Animals

Adult red spotted newts, *Notophthalmus viridescens* (Charles Sullivan Co., Nashville, TN), were kept at 25°C and fed weekly. Animals were treated according to European Community and local ethics committee guidelines.

Immunohistochemistry

Animals were anaesthetised by immersion in 0.1% MS-222 (Sigma) and perfused with 4% paraformaldehyde in PBS. Brains were removed, post-fixed for 1 hour and left at 4°C overnight in 20% sucrose in PBS. Lesion size was assessed as the total number of tyrosine hydroxylase (TH)-positive neurons in a lesioned animal relative to sham-lesioned newts. A 1:5 series was cut on the coronal plane through the brain with a section thickness of 18 µm. The diencephalic-mesencephalic DA population extended through ~10–12 sections per brain (~1000 µm in length on a rostrocaudal axis). This DA population commenced immediately caudal to the preoptic area and ended at the rostral tegmental area of the midbrain (TM), and comprised 780 TH⁺ neurons in total. For an illustration of the DA midbrain region of the newt brain, as previously mapped by others (Gonzalez and Smeets, 1994; Marin et al., 1997), see Fig. S1 in the supplementary material. These cells project to the striatum, which was confirmed in our studies by the loss of striatal fibres corresponding to loss of DA cell bodies (see Fig. S1 in the supplementary material). The TM contained 1700 cells in total, of which 1200 were NeuN⁺ and 300 TH⁺. The TM extended through three sections per brain. Counts were made by overlaying a grid on the TM, aligning the midline and ventral pial surface of the brain in each section. The same grid area was counted for each animal. The grid overlay corresponded with the TH⁺ population within the TM. Repeatability and low standard deviation of counts within groups confirmed the consistency of the counting method.

Total engrailed 1 and Nurr1 counts were made in adjacent series of sections. All engrailed 1-labelled and Nurr1-labelled cells were counted within the sections of the TM for each animal.

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For immunohistochemistry, sections were incubated with one of the following primary antibodies: rabbit anti-TH (1:250, Chemicon); mouse anti-TH (1:250, Chemicon); mouse anti-NeuN (1:100, Ab Cam); mouse anti-GFAP (1:500, Sigma); rat anti-BrdU (1:250, Accurate Chemical and Scientific Corporation); rabbit anti-Nurr1 (1:250, Santa Cruz); mouse anti-engrailed 1 [1:10, Developmental Studies Hybridoma Bank (DSHB)]; mouse anti-Msx1/2 (1:3, DSHB); Sox2 (1:100, a kind gift from T. Edlund, Umeå University, Sweden); Foxa2 (1:10, DSHB). The following day, sections were incubated for 2 hours with the appropriate secondary antibodies (Alexa 546 and Alexa 448 IgG, 1:1000 dilution; Molecular Probes). TUNEL-staining was performed in accordance to the manufacturer's protocol for cryosections (Fluorescein-FragEL, Oncogene Research Products, MA). Cells were observed using a Zeiss upright microscope, and pictures were captured by a colour CCD camera. A confocal laser-scanning microscope (Zeiss) was used to detect and quantify BrdU⁺ TH⁺ and BrdU⁺ GFAP⁺ cells. Cell counts are presented as the number of cells per animal unless indicated otherwise in the figure legend.

6-OHDA lesions

Newts were anaesthetised by immersion in 0.1% MS-222 and the head secured in a neonatal stereotaxic head frame. A 6 µg/µl solution of 6-OHDA was prepared with ascorbic acid (0.2 mg/ml) and kept on ice until the time of injection. A glass micropipette was coupled with tubing to a 2 µl Hamilton syringe (with a 26-gauge needle) and mounted in the stereotaxic frame. The micropipette containing the 6-OHDA was inserted into the brain through a small (0.5 mm) hole drilled at the junction of the parietal and frontal bones in the cranial midline. The micropipette was inserted to a level 0.8 mm below the surface of the brain and the toxin (200 nl) was injected into the third ventricle, at the level of diencephalic/mesencephalic DA neurons. On completion of the injection, the needle was left in situ for 1 minute and then slowly withdrawn. Sham-lesioned animals were injected with 200 nl of 0.9% saline. Following surgery, the bone hole was sealed with dental cement and the animals left to recover overnight in 0.5% sulfamerazine (Sigma). Accuracy of injection was previously confirmed using injections of indian ink. TH counts revealed that we were able to obtain consistent, dose-dependent bilateral lesions of the newt DA cell groups with lesion sizes ranging from 5-97% of total TH⁺ cells.

Behavioural assessment

Behaviour was assessed between 13.00 and 16.00 hours CET with temperature and lighting similar to the newt's normal housing conditions. The observation plates were clear plastic 15 cm-diameter dishes with a 1.5 cm-high wall and a 3 cm-square grid on the base. The plates were filled with 50 ml tap water. On the day of testing, newts were injected intraperitoneally with amphetamine (25 mg/kg) and placed into the observation plate. Animal behaviour was captured on a digital video camera (Sony). Each animal was observed for 1 minute every 10 minutes, commencing 5 minutes after injection, for a 50-minute period. For regeneration/neurogenesis studies, only animals with lesions greater than 70%, based upon their behaviour, were used. Sham and control animals showed similar results at each of the analysed time points and data for sham groups were set as 100% for each data point.

BrdU pulse-labelling

BrdU (Sigma, 300 mg/kg) was injected intraperitoneally at day 1, 3, 5, 7, 10 and 19. Twenty-four hours after the single BrdU pulse, animals were anaesthetised, perfused and the brains processed for immunohistochemistry. For BrdU TH double-labelling experiments, newts were pulsed twice daily with BrdU (20 mg/kg) for 10 days and then daily from day 11-30. After 30 days, newts were sacrificed. In additional experiments, lesioned newts were pulsed daily on days 0-3 (during the period of DA cell death) or on days 4-23 (after cell death and during the neurogenic period) and analysed at 23 days for TH⁺ BrdU⁺ cells. Brains were sectioned and immunohistochemistry, using antibodies against TH and BrdU, was performed on all sections. Using confocal microscopy, the total number of TH⁺ BrdU⁺ cells was counted.

AraC treatment

Animals were lesioned and behaviour tests were performed at 3 days. AraC (500 mg/kg, Sigma) was administered by intraperitoneal injection three times daily (day 4-8) or twice daily (days 4-14) into lesion and control animals. Animals received two pulses of BrdU (150 mg/kg) either on day 7 or 13 and behavioural analysis was performed at day 8 or 14. All brains were sectioned and stained for BrdU and TH.

RESULTS

To identify DA neurons, we used immunohistochemical analyses with antibodies raised against tyrosine hydroxylase (TH, the rate-limiting enzyme in dopamine biosynthesis), Nurr1 and engrailed 1 (Arenas, 2005). We focused our study on 780 ventral diencephalic/mesencephalic TH⁺ cells (see Fig. S1 in the supplementary material), which were consistently affected by toxin administration into the third ventricle (see below). Whereas DA neurons in fish have only been observed in the diencephalon, the medial part of the diencephalic posterior tuberculum (TPm) in newts extends and forms a continuum with the mesencephalic DA cells of the rostral tegmental area (TM) (see Fig. S1G,H in the supplementary material). The catecholaminergic system in newts has been extensively studied (Gonzalez and Smeets, 1994; Marin et al., 1997), and these studies suggested that the continuous diencephalic and mesencephalic DA population is functionally homologous to the mammalian ventral tegmentum and substantia nigra.

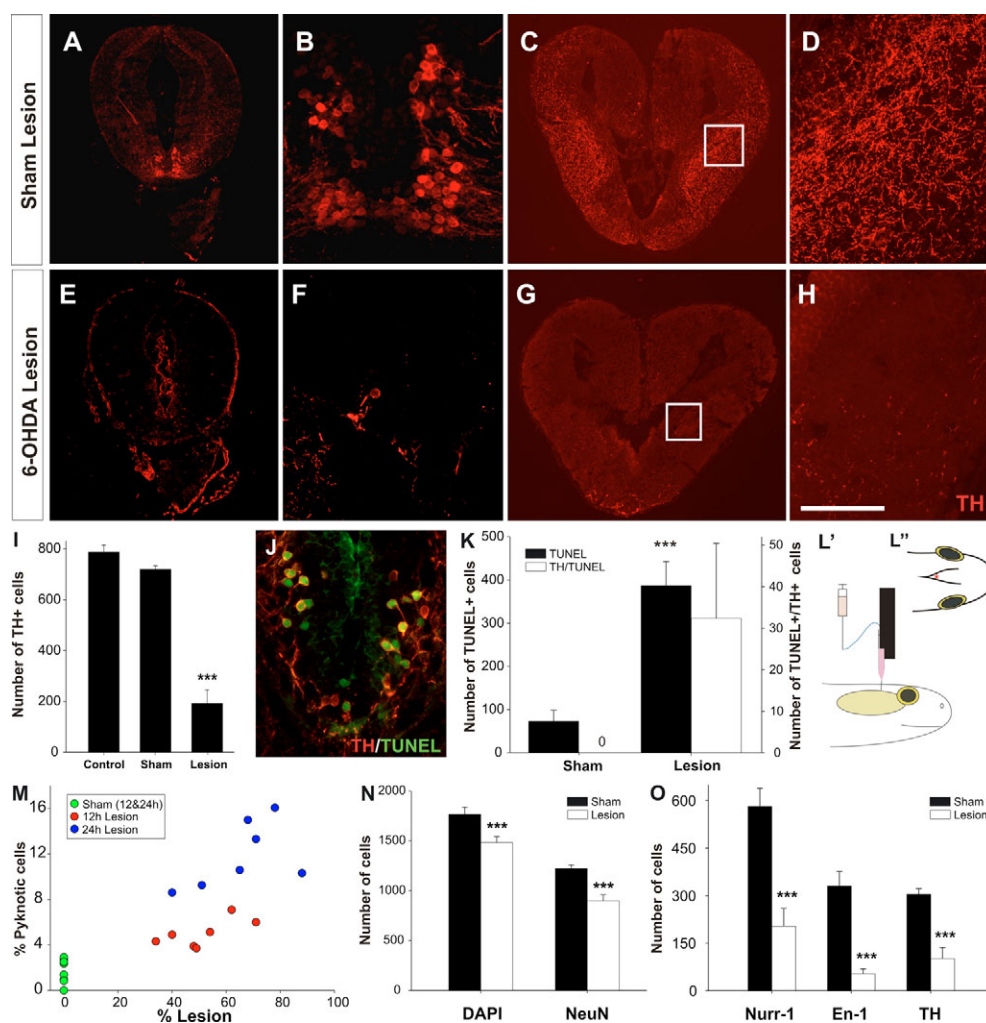
To lesion the DA system, 6-OHDA was stereotactically injected into the third ventricle, in the vicinity of diencephalic/mesencephalic DA neurons (Fig. 1L). Consistent bilateral lesions were seen in the DA groups shown in the supplementary material (see Fig S1E'-H' in the supplementary material). Three days after 6-OHDA injection, we observed the loss of TH⁺ cells and fibres in the diencephalic/mesencephalic regions, as compared with the uninjected controls (Fig. 1A-I). To confirm cell death, and not just a loss of phenotype, as a result of the neurotoxin, we performed double-immunostaining for fragmented DNA (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling, TUNEL) and TH. The presence of abundant TUNEL⁺ TH⁺ cells indicated that DA neurons died as a result of the 6-OHDA injection (Fig. 1J,K). Moreover, we found a significant increase in the number of pyknotic cells at 12 hours after lesioning, which further increased at 24 hours (Fig. 1M). Cell death was also evident from the loss of nuclei in the neuronal layers in the TM of the mesencephalon (Fig. 1N). In total, 1700 cells compose the entire TM as revealed by DAPI staining. Of these 1700 cells, 1200 are NeuN⁺ and 300 express TH. In addition to the loss of TH⁺ cells, we detected a significant reduction in the number of cells expressing the DA markers Nurr1 and engrailed 1, as compared with the sham injections (Fig. 1O). The number of TH-expressing cells was unchanged in control and sham-injected animals (Fig. 1I). Similarly sham injection did not alter the number of Nurr1- or engrailed 1-expressing cells (data not shown). These data indicate that 6-OHDA injection led to the death of DA neurons, not just to a loss of phenotype.

To assess the functional significance of the loss of DA cells, we devised a quantifiable, pharmacological locomotion assay. Animals received a single intraperitoneal injection of amphetamine, which stimulates DA release (Ungerstedt, 1971), and were placed in separate grid chambers. We quantified movement by counting the number of occasions a newt's tail crossed a line in a grid while swimming, as the typical movement of a newt can be described as a sine wave that starts at the head region and propagates to the tail (Davis et al., 1990). In addition, tail movement should not be

Fig. 1. Stereotaxic 6-OHDA injection leads to loss of DA cells in the diencephalon and the mesencephalon. (A,B) Low- and high-power photomicrographs of TH⁺ cells in the tegmental area of the newt midbrain. **(C)** TH⁺ fibres in the striatum from a sham-lesioned animal. **(D)** High-power photomicrograph of the boxed area in C. **(E,F)** TH⁺ cells in the TM and **(G)** TH⁺ fibres in the striatum from a 97% lesioned animal 3 days after 6-OHDA injection. **(H)** High-power photomicrograph of the boxed area in G. **(I)** A significant loss of TH cells was seen within the ventral diencephalon-mesencephalon 3 days post-lesioning ($n=16$ lesioned, $n=14$ sham, $n=7$ control; mean \pm s.d.; Student's t -test; ***, $P<0.001$).

(J) Colocalisation of TUNEL⁺ and TH⁺ cells in the TM 3 days after lesioning. **(K)** Significantly more TUNEL⁺ cells and numerous TUNEL⁺ TH⁺ cells were observed in lesioned as compared with sham-lesioned animals. No double-positive cells were seen in sham-lesioned animals (mean \pm s.d.; Student's t -test; ***, $P<0.001$). **(L')** Schematic representation of the lesioning apparatus. **(L'')** The location of the burr hole and injection site (*), at the junction of the frontal and parietal bones. **(M)** A significantly higher percentage of pyknotic cells (ANOVA with Tukey post-hoc test; $P<0.01$) was seen 12 hours (5 ± 1.2 ; $n=7$) and 24 hours (11.9 ± 2.9 ; $n=7$) after lesioning as compared with shams (12 and 24 hour sham-lesioned animals pooled; 1.7 ± 1.6 ; $n=10$). Note the increase in the percentage of pyknotic cells with increasing lesion size, and the increase in the percentage of pyknotic cells in the lesioned animals between 12 and 24 hours.

(N,O) 6-OHDA injection leads to a significant loss of cells (DAPI), including neurons (NeuN), within the TM, and more specifically to the loss of DA markers [Nurr1, engrailed 1 (En1), TH]. $n=3-7$; mean \pm s.e.m.; Student's t -test; ***, $P<0.001$. Scale bars: 1 mm in A,C,E,G; 150 μ m in B,F,J; 250 μ m in D,H.



affected by our regimen of 6-OHDA administration because the cell groups we lesion do not project to the spinal cord in salamanders (Sanchez-Camacho et al., 2001). Movies 1 and 2 (see Movies 1 and 2 in the supplementary material) show sham- and 6-OHDA-injected newts with or without an amphetamine challenge. Amphetamine induced a significant increase in locomotion only in uninjected and sham-injected animals, in which the DA system was intact, but not in 6-OHDA-injected animals (Fig. 2A). It was also evident that 6-OHDA-injected animals moved less than the shams, indicating that the damage was so extensive that it reduced baseline movements. Importantly, behavioural responses were in strong correlation with the number of TH⁺ cells (Fig. 2B); thus, in subsequent experiments, the degree of lesion could be estimated by this behavioural test.

We quantified the amphetamine response and the number of TH⁺ cells over time in those animals that, 3 days after 6-OHDA injection, showed a greater than 70% reduction in amphetamine-induced locomotion, as compared with shams. As shown in Fig. 2C, we observed substantial improvements in behavioural responses, with animals reaching normal motor scores by 30 days. The kinetics of behavioural improvement followed the timing of the reappearance

of TH⁺ cells (Fig. 2D). After 30 days, full recovery of the number and distribution of TH⁺ cell bodies along the entire anterior-posterior axis of the diencephalon and mesencephalon was detected (Fig. 2E-G, and see Fig. S2 in the supplementary material). We did not observe any difference in the recovery of the different TH⁺ subpopulations within the diencephalon/mesencephalon (as depicted in Fig. S1 in the supplementary material). Thus, newts were able to regenerate the DA system as assessed by cellular and functional analyses.

Several mechanisms could contribute to the restoration of the DA system after 6-OHDA injection. To test the hypothesis that neurogenesis was involved, we pulsed animals with 5-bromo-2'-deoxyuridine (BrdU), which incorporates into the replicating DNA. Single pulses of BrdU at various time points indicated that after 5 days the proliferative response was increased both in 6-OHDA-injected and sham-lesioned animals, compared with uninjected controls. At 7 and 10 days, the number of BrdU⁺ cells was significantly higher in ventral regions in lesioned animals than in shams, indicating a specific proliferative response evoked by the 6-OHDA injection (Fig. 3A).

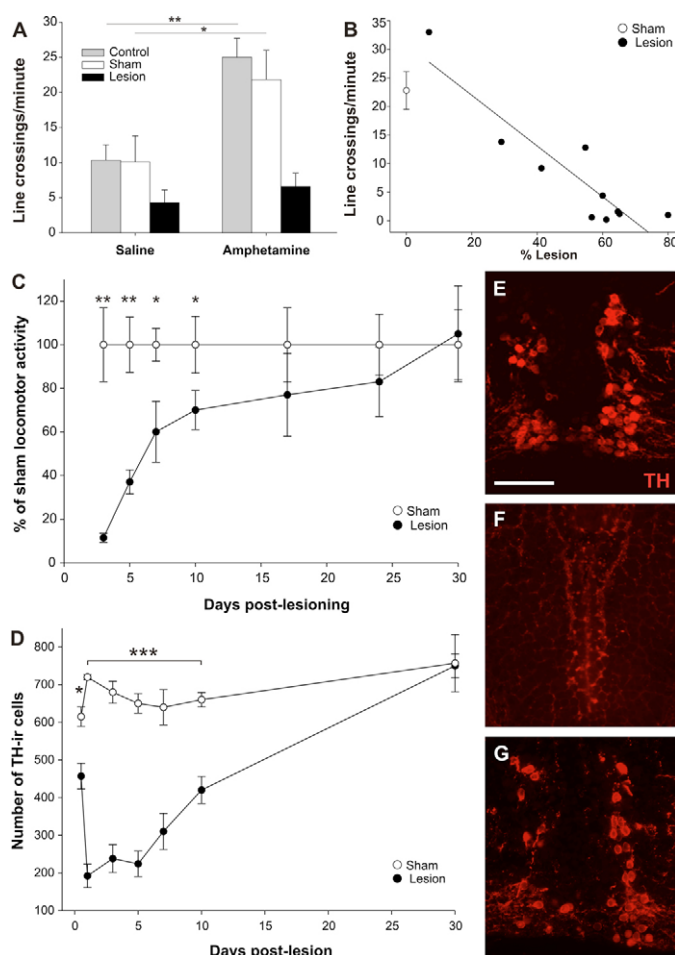


Fig. 2. Cellular and functional recovery after 6-OHDA injection.

(A) Behavioural response following amphetamine challenge in non-operated controls ($n=4$), and in animals 3 days after sham ($n=9$) or 6-OHDA injection ($n=21$). Data represent the number of times per minute that the newt's tail crosses a line in a grid (mean \pm s.d.; Student's t -test; *, $P<0.05$; **, $P<0.01$). (B) Correlation of motor behaviour with lesion size 3 days after lesioning. The loss of TH⁺ cells strongly correlates ($r^2=0.83$) with a decrease in locomotor activity in response to amphetamine challenge. Each data point represents 1 animal (for sham-lesioned animals, data represent mean \pm s.d.). (C) Time course of behavioural recovery as assessed by acute amphetamine challenge (lesion, $n=19$; sham, $n=9$). (D) Time course of TH⁺ cell regeneration following 6-OHDA injection. Sham, $n=4-14$ animals per group per time point (d3, $n=4$; d5, $n=6$; d7, $n=5$; d10, $n=6$; d17, $n=5$; d24, $n=4$; d30, $n=14$). Lesioned, $n=5-18$ animals per group per time point (d3, $n=5$; d5, $n=6$; d7, $n=5$; d10, $n=6$; d17, $n=5$; d24, $n=6$; d30, $n=18$). Mean \pm s.e.m.; Student's t -test; *, $P<0.05$; **, $P<0.01$.

(E-G) Photomicrographs showing TH⁺ neurons in TM following (E) sham operation, (F) 3 days post-lesion and (G) 30 days after 6-OHDA injection. Scale bar: 150 μ m.

During tail regeneration, ependymoglia cells of the spinal cord can act as neural stem cells (Echeverri and Tanaka, 2002). Similar to the central canal of the spinal cord, we found that glial fibrillary acidic protein (GFAP)-positive, radial glia-like ependymoglia cells line the third ventricle of the brain in the vicinity of neurons (Fig. 3B'). Cells lining the ventricle also expressed Sox2 (Fig. 3B''), which has been shown to be expressed in neural stem and progenitor cells and to repress proneural genes (Bylund et al.,

2003). Animals received a single pulse of BrdU immediately upon completion of the lesion. Six hours after the single BrdU pulse, BrdU⁺ cells were seen most prominently in the GFAP⁺ cell layer (Fig. 3C', C''). Thirteen days after a single BrdU pulse in lesioned animals, significantly more BrdU⁺ cells were observed in deeper cell layers (Fig. 3C', C'''), where NeuN⁺ cells are found (Fig. 3B').

In order to examine whether newborn cells gave rise to TH⁺ neurons, we performed sequential BrdU pulses, and examined the incorporation of BrdU. Animals were pulsed twice daily for 10 days and then daily from days 11-30. After 30 days, BrdU⁺ TH⁺ cells were detected only in the lesioned animals and not in the sham-operated animals (Fig. 3D, and see Movie 3 in the supplementary material), suggesting that DA neurogenesis was a specific response to the 6-OHDA-induced injury. It should however be noted that our BrdU⁺ TH⁺ counts most probably underestimate the number of newborn cells, both in sham and in 6-OHDA lesioned animals, because it is impossible to label all cells during S phase of the cell cycle with discontinuous BrdU pulses (see Materials and methods). The data however establish that there is a substantial increase in the number of TH⁺ BrdU⁺ cells after 6-OHDA administration. In order to confirm that BrdU only incorporated into new cells, and that BrdU incorporation was not due to toxin-mediated DNA damage or apoptotic DNA cleavage during the first 3 days, we administered BrdU every 12 hours to animals either during the period of cell death (days 0-3) or after the period of cell death (days 4-23). Results showed no BrdU⁺ TH⁺ cells at 23 days in animals that were pulsed during days 0-3, arguing against the possibility that BrdU was incorporated into damaged cells. Furthermore, animals that were pulsed during days 4-23 showed comparable numbers of TH⁺ BrdU⁺ cells to those previously observed in animals pulsed during days 0-30 (Fig. 3D').

To further examine whether neurogenesis was involved in the regenerative process, we examined the total number of cells and neurons in the TM after lesion. Whereas at 3 days the total cell number and the number of neurons were reduced, at 30 days both populations were restored, showing that cells were indeed eliminated and new cells appeared (Fig. 3E).

To determine the extent to which cellular proliferation was responsible for behavioural and cellular recovery, newts were treated with cytosine β -D-arabinofuroside (AraC), an antimetabolic compound used to block neurogenesis in rodent models (Doetsch et al., 1999). We did not detect any proliferating cells after AraC treatment (Fig. 3F''). AraC treatment had no effect on the behaviour of control animals (Fig. 3F'), indicating the lack of toxicity in our assay. However, AraC treatment from day 4-8 or day 4-14 blocked the behavioural recovery and significantly reduced cellular recovery as reflected in the number of TH⁺ neurons (Fig. 3F''). Thus, our results indicate that the behavioural recovery in the newt is indeed contributed by adult DA neurogenesis.

As neurogenesis was a response to the loss of TH⁺ cells evoked by 6-OHDA lesion, we examined whether the mechanisms of tissue repair in the parkinsonian salamander were similar, to some extent, to the process of epimorphic regeneration following limb or tail amputation. The transcriptional repressors Msx1/2 have been found to be regulated by and functionally linked to the process of regeneration in several models, including the limb of salamanders, the tail of frogs and the digit tip of mice (Carlson et al., 1998; Odelberg, 2004). Interestingly, the ablation of Msx1 in mice results in loss of Wnt1 (Bach et al., 2003), a factor that plays a crucial role in the generation of midbrain DA neurons (Prakash et al., 2006). Furthermore, Msx1 was recently found to be required for the development of DA neurons in the ventral mesencephalon

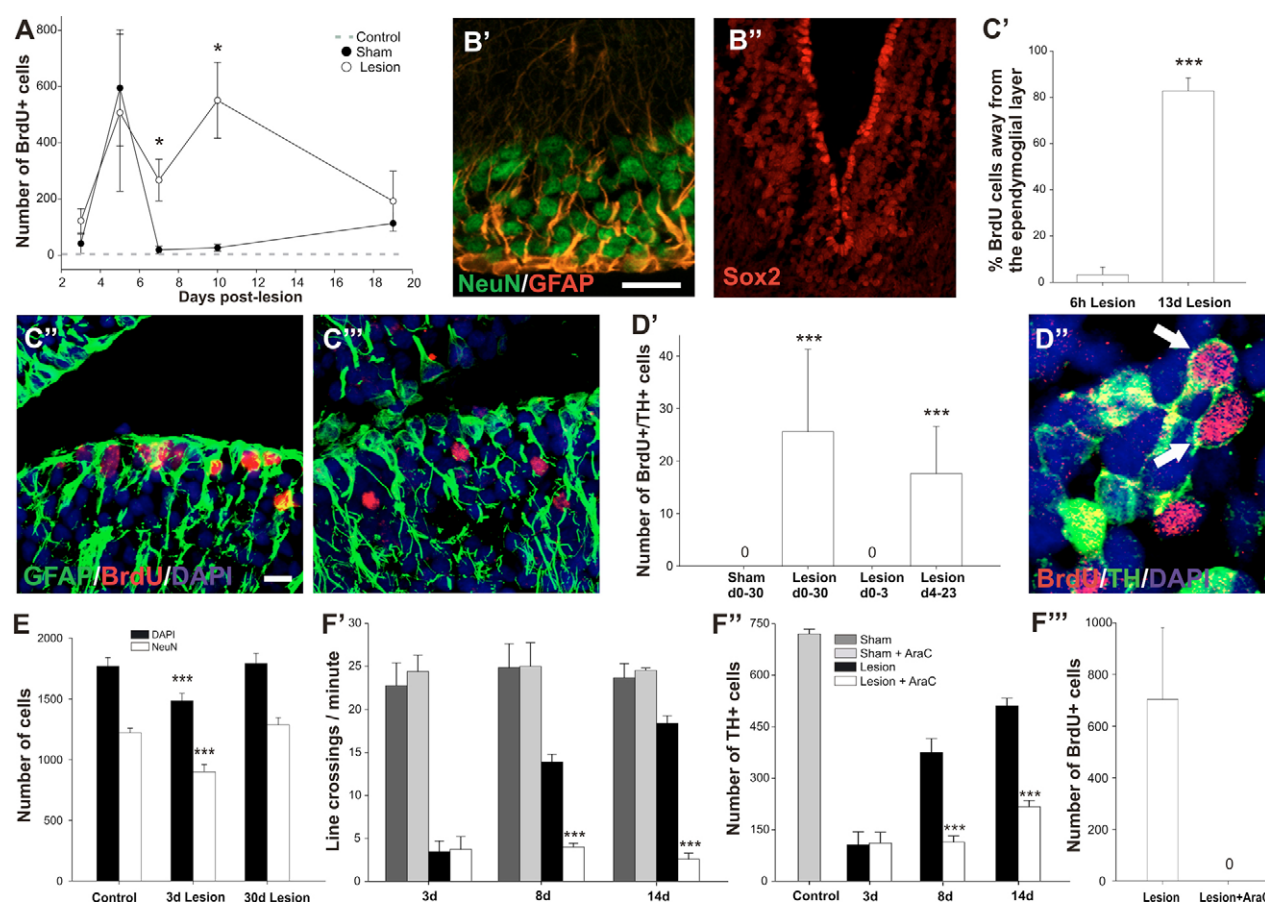


Fig. 3. Neurogenesis of TH⁺ cells after 6-OHDA injection. (A) Time course of cellular proliferation within the ventral diencephalon and mesencephalon following sham- or 6-OHDA-lesioning. At days 7 and 10, a significant increase was seen in the number of BrdU⁺ cells in 6-OHDA-lesioned as compared with sham-lesioned animals. Control (grey line) represents the number (3.1 ± 1.5) of BrdU⁺ cells in animals receiving no surgery/injection. Data represent mean \pm s.e.m.; $n=3-11$; Student's *t*-test; *, $P<0.05$. (B') Localisation of NeuN⁺ (neurons) and GFAP⁺ (ependymoglia) cells along the third ventricle. (B'') The cells lining the ventricle also express Sox2. (C') Significantly more BrdU⁺ cells were seen away from the ependymoglia layer at 13 days as compared with 6 hours after a single BrdU pulse ($n=3$ lesion; $n=3$ sham; mean \pm s.e.m.; Student's *t*-test; ***, $P<0.001$). (C'') Merged confocal image showing several BrdU⁺ GFAP⁺ cells lining the ventricle 6 hours after BrdU pulse. (C''') After a 13-day chase period, BrdU⁺ cells appear in neuronal layers. (D') Total number of BrdU⁺ TH⁺ cells following lesioning. Sham-lesioned animals pulsed days 0-30, $n=11$; lesioned animals pulsed days 0-30, $n=8$. Lesioned animals pulsed days 0-3, $n=8$. Lesioned animals pulsed days 4-23, $n=8$. Mean \pm s.e.m.; ANOVA with Tukey post-hoc test; ***, $P<0.001$. (D'') Confocal image of two BrdU⁺ TH⁺ neurons (arrows). (E) Total number of cells (DAPI) and neurons (NeuN) in the TM at 3 and 30 days after lesioning confirming that the number of cells returned to control levels by 30 days. (F) Behavioural response following amphetamine challenge at day 3, 8 and 14 after AraC treatment. AraC has no effect on the behaviour of control animals. However, it blocks all the behavioural recovery at 8 and 14 days normally seen in lesioned animals. (F') AraC blocks all TH recovery at 8 days and significantly reduces recovery at 14 days in lesioned animals as compared with lesioned animals not treated with AraC (mean \pm s.e.m.; Student's *t*-test; ***, $P<0.001$). (F'') No new cells (BrdU⁺) were seen at 8 days in AraC-treated lesioned newts confirming the action of AraC ($n=4-8$ newts per group).

(Andersson et al., 2006). We noted that *Msx1/2* expression was not restricted to the neuroepithelium, as in the developing mammalian midbrain (Andersson et al., 2006), but was also expressed in neuronal layers. We observed that the number of *Msx1/2*-expressing cells transiently decreased in the lesioned area at 24 hours (Fig. 4A,B'') and, subsequently, significantly increased above control levels at 7 days (Fig. 4A,B'''). No change was observed after sham injections (Fig. 4A,B'') and no increase in the number of *Msx1/2*-expressing cells was detected dorsally (see Fig. S4 in the supplementary material), arguing for the specificity of the regulation. We next investigated whether the expression of proteins found in mature DA neurons and DA precursors, *Nurr1* and *engrailed 1* (Arenas, 2005), changed during DA regeneration. We found that the number of *Nurr1*⁺ cells gradually increased in the ventral midbrain after the loss of DA neurons, and that *Nurr1*

expression preceded the expression of TH (Fig. 4C), implying that *Nurr1* is also a marker for DA precursors in newts. A similar temporal change was observed for *engrailed 1* (see Fig. S3 in the supplementary material). Together, these results indicate that adult DA regeneration in the newt recapitulates some of the key molecular events that take place during embryonic DA neurogenesis in mammals.

DISCUSSION

In this study, we demonstrated that a Parkinson-like model with cellular and functional readouts could be created in the salamander. By injecting 6-OHDA into the third ventricle, we revealed a previously unknown biological process and found that adult salamanders are able to regenerate DA neurons in the diencephalon and mesencephalon by mechanisms that involve neurogenesis.

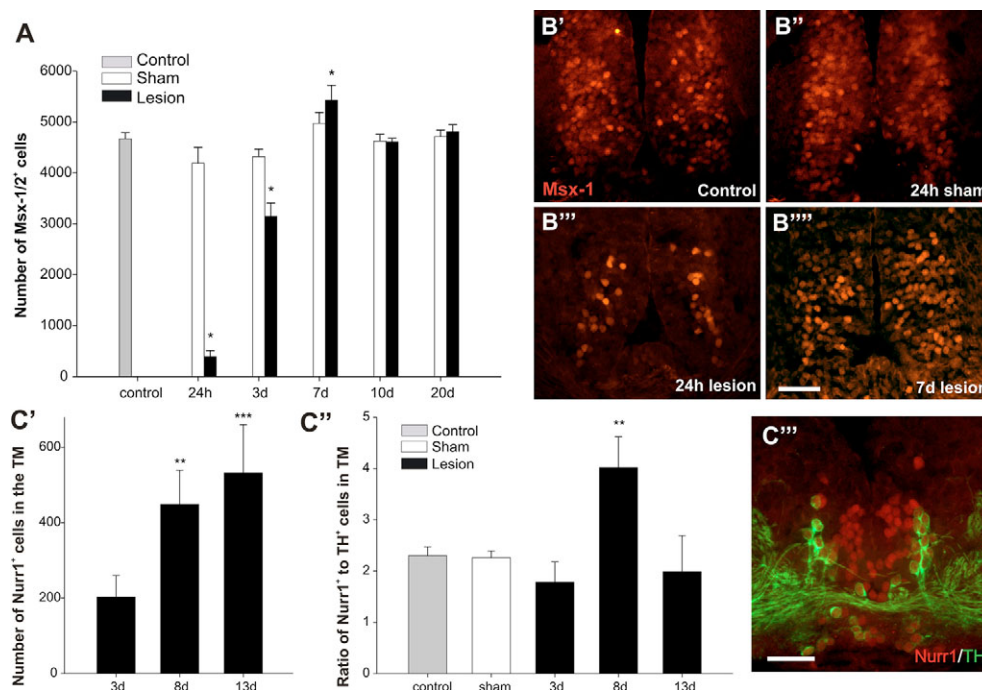


Fig. 4. De novo expression of DA determinants during DA regeneration in the newt. (A) Time course of Msx1/2 expression in the ventral diencephalon and mesencephalon following lesioning. Note the significant reduction in Msx1/2-expressing cells at 24 hours and the significant increase at 7 days. Mean \pm s.e.m.; ANOVA with Tukey post-hoc test; *, $P < 0.05$. (B') Msx1/2 expression in control animals and (B'') 24 hours after sham lesion and after (B''') 24 hours and (B''') 7 days in 6-OHDA lesioned animals. (C') Number of Nurr1⁺ cells in the TM 3, 8 and 13 days after lesioning. (C'') Proportion of Nurr1⁺ to TH⁺ cells in the TM. Note that at 8 days there are significantly more Nurr1⁺ than TH⁺ cells compared with controls, indicating that maturation of DA neurons occurs along the normal developmental pathway. Mean \pm s.e.m.; ANOVA with Tukey post-hoc test; **, $P < 0.005$; ***, $P < 0.001$. (C''') An example of TH and Nurr1 double-positive cells in the TM of a control animal, illustrating the expression of Nurr1 in all TH⁺ neurons. Scale bars: 200 μ m in A; 150 μ m in B.

Several sets of data suggest that neurogenesis is an important contributing mechanism that underlies DA regeneration. First, by actual cell counting in the TM, we show that neurons are lost 3 days after 6-OHDA injection and that the number of neurons returns to normal levels after 30 days (Fig. 3E). This experiment, together with the data showing the death of TH⁺ neurons after 6-OHDA administration (Fig. 1J,K,M), establishes that neurons are lost after injury and new neurons are born during regeneration. Second, a time course of BrdU incorporation revealed a specific increase in the number of BrdU⁺ cells after 6-OHDA lesioning (Fig. 3A). Furthermore, the end-point analysis shows the appearance of BrdU⁺ TH⁺ neurons as a response to 6-OHDA injury (Fig. 3D). These results are compatible with the involvement of neurogenesis, which starts by the proliferation of neural progenitor/stem cells, and these progenitors incorporate BrdU. Third, we see that blocking cellular proliferation with AraC significantly reduces or abolishes the histological and behavioural recovery (Fig. 3F), confirming the contribution of adult DA proliferating progenitors to neurogenesis. Fourth, BrdU pulse-chase experiments indicated that the ventricular GFAP⁺ cells were the first to start proliferating, and after 13 days of the chase period, the majority of these cells or their progeny appeared in deep layers (Fig. 3C). These data indicate that DA regeneration may be fuelled by the reactivation of ependymoglia cells, which line the ventricle and which mature into DA neurons. These ventricular cells express GFAP and Sox2 but are negative for Nurr1 and engrailed 1. Clearly, rigorous fate mapping studies will be needed in the future to examine this hypothesis. Fifth, we see that the temporal expression pattern of Nurr1, engrailed 1 and TH

recapitulates the sequential maturation process of DA neurons during mammalian embryonic DA development (Fig. 4C, and see Fig. S3 in the supplementary material). Finally, the transient increase in Msx1/2 (Fig. 4A,B, and see Fig. S4 in the supplementary material) also shows that adult DA regeneration shares some of the key players involved in mammalian DA neurogenesis.

Newt regeneration of some organs and body parts, such as the lens and limb, involves cellular dedifferentiation. In the future it will be interesting to address whether dedifferentiation plays a role in DA regeneration as well. In addition to its role in neurogenesis (Andersson et al., 2006), Msx1 has been implicated in skeletal muscle dedifferentiation (Kumar et al., 2004). At present we cannot exclude the possibility that dedifferentiation also plays a role in DA regeneration in the salamander brain. Dedifferentiation could in principle generate a proliferating DA progenitor that subsequently differentiates into DA neurons, as illustrated here. Future studies will aim at addressing this possibility. However, the observation that ependymoglia cells are first to incorporate BrdU, and that the label appears in deeper (neural) layers after a chase experiment, suggest that activation of GFAP/Sox2-expressing ependymoglia is one important component of the process. Interestingly, two recent protocols have reported that radial glia-like cells with neurogenic capacity can be derived from mammalian embryonic stem cells (Bibel et al., 2004; Conti et al., 2005). It remains to be determined whether endogenous or stem cell-derived mammalian cells can be differentiated into ependymoglia-like cells capable of undergoing DA neurogenesis and whether these cells might contribute to functional recovery.

We show that injury evokes increased cellular proliferation and midbrain DA neurogenesis. The existence of adult mammalian neurogenesis has been described and characterised previously in the subventricular zone-olfactory bulb pathway and in the dentate gyrus (Alvarez-Buylla and Lim, 2004; Kempermann et al., 2004). It has also been suggested that neurons could be generated in other parts of the brain, such as DA neurons in the adult midbrain (Frielingsdorf et al., 2004; Lie et al., 2002; Zhao et al., 2003). However, this is a controversial issue because adult DA neurogenesis in mammals is either undetectable or a rare and inefficient process that is difficult to study. By contrast, the salamander 6-OHDA model revealed robust DA regeneration/neurogenesis. A comparable process has not been observed in other adult vertebrate models of Parkinson's disease, making the salamander model a useful complement to other currently available models. We see that the adult newt brain can reactivate quiescent cells upon injury, and contains sufficient extracellular cues to direct activated neural progenitors towards a specific neural subtype within an existing brain structure. Hence, the 6-OHDA-induced regeneration model in salamander provides a basis for identifying and understanding the cues required for adult DA neurogenesis/regeneration. In the future, it will be possible to test whether activation or manipulation of these cellular and molecular programs could contribute to regeneration in mammalian models.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/15/2881/DC1>

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