Efficient regeneration by activation of neurogenesis in homeostatically quiescent regions of the adult vertebrate brain

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SUMMARY

In contrast to mammals, salamanders and teleost fishes can efficiently repair the adult brain. It has been hypothesised that constitutively active neurogenic niches are a prerequisite for extensive neuronal regeneration capacity. Here, we show that the highly regenerative salamander, the red spotted newt, displays an unexpectedly similar distribution of active germinal niches with mammals under normal physiological conditions. Proliferation zones in the adult newt brain are restricted to the forebrain, whereas all other regions are essentially quiescent. However, ablation of midbrain dopamine neurons in newts induced ependymoglia cells in the normally quiescent midbrain to proliferate and to undertake full dopamine neuron regeneration. Using oligonucleotide microarrays, we have catalogued a set of differentially expressed genes in these activated ependymoglia cells. This strategy identified hedgehog signalling as a key component of adult dopamine neuron regeneration. These data show that brain regeneration can occur by activation of neurogenesis in quiescent brain regions.

KEY WORDS: 6-OHDA, Adult neurogenesis, Dopamine, Midbrain, Neuronal stem cell, Salamander

INTRODUCTION

Abundant production of new neurons in the adult mammalian brain is limited to the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles in the forebrain (Alvarez-Buylla and Lim, 2004; Thored et al., 2007; Frielingdorf et al., 2004; Hermann et al., 2009; Zhao et al., 2003). Neurogenesis may be evoked in quiescent regions, but the number of persisting new neurons that are generated remains low and consequently the functional recovery of the animals limited (Lindvall et al., 2004). By contrast, the adult brain in non-mammalian vertebrates of certain fish and salamander species repairs damage via processes fuelled by neurogenesis (Zupanc, 2009). Previous studies have revealed several proliferation hotspots in the adult zebrafish brain from which neurons are continuously derived (Adolf et al., 2006; Chapouton et al., 2007; Grandel et al., 2006). From these and other observations it was hypothesised that the broad distribution of homeostatic neurogenesis in the brain is an underlying component of the extensive regenerative ability in these animals (Kaslin et al., 2008; Zupanc, 2009). This link, however, needs further testing, as it would have important implications for the possibility of engaging non-germinal zones for functional neuronal replacement in species where it naturally does not occur.

Cells that give rise to new neurons in mammals are of glial character in terms of morphology and gene expression pattern (Doetsch, 2003; Gotz and Barde, 2005; Kempermann et al., 2004).

Data indicated that glial cells are neural stem cells also in non-mammalian vertebrates (Benraiss et al., 1999; Pellegrini et al., 2007) but the glial origin of brain neurons has however not been directly demonstrated. A recent report suggested that stem cells in the adult non-mammalian brain have neuroepithelial rather than glial features (Kaslin et al., 2009).

Here, we have addressed whether the presence of constitutively active neurogenic niches is a prerequisite for extensive neuronal regeneration, and revisited the identity of cells that produce new neurons. We studied an aquatic salamander, the red spotted newt, which has the widest regenerative repertoire among vertebrates. Adult newts regenerate among other structures limbs, cardiac muscle, ocular tissues and tails. Central nervous system (CNS) regeneration in newts has mostly been studied after spinal cord transection, tail amputation, or by removing a piece of brain tissue (Chernoff et al., 2003; Okamoto et al., 2007).

Recently, we developed a chemical ablation model in newts by intraventricular injection of 6-hydroxydopamine (6-OHDA) (Parish et al., 2007). 6-OHDA acts as a selective neurotoxin and is used to model aspects of Parkinson’s disease in many species by the elimination of midbrain dopamine (DA) neurons. Similar to other species, midbrain DA neurons in newts express the evolutionarily conserved markers, tyrosine hydroxylase (TH) and Nurr1 (Marin et al., 1997; Parish et al., 2007; Wallen and Perlmann, 2003). Newts respond uniquely to the loss of midbrain DA neurons by full regeneration within 4 weeks. Regeneration of DA neurons depends on cellular proliferation and is characterised by the gradual birth of new neurons leading to complete histological and locomotor performance recovery (see Fig. S4 in the supplementary material) (Parish et al., 2007).

In the present study we show, unexpectedly, that the adult newt midbrain is essentially quiescent. Proliferation zones are normally restricted to the telencephalon and the most rostral areas of the diencephalon in the newt brain. We see no sign of caudal migration from the constitutively active germinal zones towards...
quiescent regions. Elimination of midbrain DA neurons by 6-OHDA injection on the other hand leads to cell cycle re-entry by midbrain ependymoglia cells that line the ventricular lumen. Using cell-type specific labelling, we observed that many of these cells exited from the ventricular layer and underwent neurogenesis to replace the lost TH-expressing neurons. By contrast, intraventricular sham injection induced a mitotic response but the majority of these cells remained locally in their ventricular niche. We further gained molecular insight into the processes that accompany ependymoglia activation during DA neurogenesis using a cross-species oligonucleotide based microarray strategy. This approach identified a large set of candidate genes and also led to the demonstration that hedgehog signalling is required for adult DA regeneration.

MATERIALS AND METHODS

Animals

Adult red spotted newts Notophthalmus viridescens (Charles Sullivan, Nashville, TN, USA) were maintained in a humidified room at 15-20°C. All experiments were performed according to European Community and local ethics committee guidelines.

Immunohistochemistry

Animals were anaesthetised by immersion in an aqueous solution of 0.1% MS-222 (Sigma) and perfused with 4% formaldehyde in PBS. Animals were dissected and the brains were rapidly placed in 4% formaldehyde. After 1 hour of post fixation, brains were cryoprotected in 20% sucrose in PBS for 12 hours and then embedded in OCT compound. Coronal sections (20 μm) were collected alternating on five slides. Sections were then incubated with one of the following antibodies: mouse anti-PCNA (1:500, Chemicon), rat anti-BrdU (1:500, Accurate Chemical and Scientific Corporation), mouse anti-GFAP (1:500, Chemicon), rabbit anti-GFAP (1:500, Chemicon), mouse anti-NeuN (1:500, AbCam), mouse anti-GFAP (1:200, Millipore), rabbit anti-TH (1:500, Chemicon) and rabbit anti-MCM2 antibody (1:200, Abcam). The following day, sections were incubated with appropriate secondary antibody: Alexa 594 or Alexa 448 IgG (1:1000; Molecular Probes). Cells were observed using a Zeiss upright microscope, and pictures were captured by a colour CCD camera. For confocal microscopy, an LSM 510 Meta laser microscope with LSM 5 Image Browser software (both Carl Zeiss MicroImaging) was used. All images were manipulated using Adobe Photoshop according to the guidelines in Development.

BrdU pulse labelling

BrdU (Sigma, 20 mg/kg) was injected intraperitoneally five times with 12 hour intervals. Animals were sacrificed 3 and 15 days after the first BrdU injection and brains processed for immunohistochemistry.

6-OHDA and sham ablations

Newts were anaesthetised by placing them in an aqueous solution of 0.1% MS-222 (Sigma) for 20 minutes. Animals were placed in a neonatal stereotaxic head frame. 6-OHDA (200 nl of 6 M) was injected into the third ventricle with a glass micropipette through a small hole drilled in the skull. Sham ablated animals were injected with 200 nl 0.9% saline. Subsequently, the surgery cavity in the skull was sealed with dental cement and animals were left to recover overnight in a shallow container of water before being placed back into a 25°C water environment. For BrdU-tracing experiments, BrdU was injected intraperitoneally five times with 12 hours interval starting 48 hours after lesion. Locomotion performance was assessed as previously described (Parish et al., 2007).

Electroporation

pCMV H2BYP (a kind gift from Claire Acquaviva, Welcome Trust/CR UK Gordon Institute, UK) was modified as described previously (Loof et al., 2007; Morrison et al., 2007). pGFAP Gal4-VP16-Gal4UAS GFP (Echovirus and Manataka, 2002) was purified using high purity maxiprep system (Marligen) and was resuspended in 10 mM Tris HCl (pH 8.5) at 5 μg/μl. Plasmid solution (400-500 nl) was injected into the third ventricle of the newt, as previously described for the injection of 6-OHDA above. Electroporation was carried out using round (0.5 cm diameter; TR Tech) electrodes and an electroporator CUY21 EDIT (TR Tech). Five 50 msecond pulses at 150 V/cm with 950 ms intervals and current of 0.1-0.15 A were used.

Array analyses

Ambystoma mexicanum and Ambystoma tigrinum sequences available in EST databases were assembled and annotated using the TIGR assembler and by blasting against the Refseq database. Oligonucleotides (60 mer) derived from these sequences and from available Notophthalmus viridescens cDNA sequences were designed by e-Array (Agilent). To optimise oligonucleotide selection for Notophthalmus viridescens, total RNA was prepared from Notophthalmus viridescens whole brains or laser microdissected ventral midbrain. RNA quality was assessed using Bioanalyzer (Agilent), before amplified RNA (aRNA) was synthesised using two rounds of linear amplification using Amino allyl message amp II aRNA amplification kit (Ambion) according to the manufacturer’s recommendations. Hybridisations were carried out on duplicate arrays. Oligonucleotides that had a signal higher than background after normalisation (Gry et al., 2009; Smyth, 2004) were selected and from this set two oligonucleotides for each EST were printed on 44 K arrays. RNA from laser microdissected ventral ventricular zone of the midbrain from four control and four 6-OHDA-injected brains were individually analysed. Principal component analysis (Ozvar et al., 2008) was used to group individual samples, which led to the exclusion of the data from one control and one 6-OHDA injected sample (see Fig S6 in the supplementary material).

Cloning, qRTPCR and in situ hybridisation

cDNA fragments were generated from whole brain RNA extracts using the following primers.

nrAD: Forw, GGC TTG TCT TCT TTG CTR TC; Rev, TWY GAC ATW TGG GAR CAG GA
Annexini: Forw, T AR TCT CCT TGG GTK TCA TCA A; Rev, TAY GAA GCW GGA GAA ARG AGA
ODC1: Forw, TGT AGT GTG CMA GYA AGA CTG; Rev, RGC CCC CAR ATC AAA GAC AM
Jarid2: Forw, GAT TTC CTY ACG CTT CTV TGY C; Rev, TTC CTT AAG WCC TGA GCC GA
HNRNPK: Forw, AAT GCC AGT GTT TCA GTC CC; Rev, CGA TCA GTC GAA TGA GGR CAR
Shh: Forw, GAG CGC TTC AAG GAG CTA AC; Rev, ACC AGT GGA CTC CCT CTG AC
FGF2: Forw, AAG MGGCTS TAC TGC AAR AA; Rev, GTT CKY TTY AGH GCC ACA TAC CA
Sox1: Forw, TTY TTV AGC AGS GTC TTG GT; Rev, CCY ATG AAC GCC TTY ATG GT

qRT-PCRs were performed on 7500 fast real-time PCR system (Applied Biosystems), using experimental design template for comparative Cₚ experiment where the reference sample was uninjured tissue and the endogenous control was 40S ribosomal protein S21. Out of the 14 primer sets used, seven gave products that could be accurately analysed based on Cₚ value and fragment size, and these are marked with an asterisk. The following primers were used.

40S_S21: Forw, AAG TAA CCA TGC AGA AGC ATG; Rev, GCC TAA CCG AAG GAT AGA GTC
*nrAD: Forw, AGG AGG ATG ATT GGA ATG T; Rev, TAT GGG AGC AGG ATG AGA C
*Collagenase: Forw, CTT GGC ACT TAA TGG GTA CG; Rev, ATG GTG CGG GTA CTT TCA T
*Cytokeratin 8: Forw, GGA GGC AGC ACT GAA TAA GG; Rev, TCC AGC AGC TCT CTG TAG GT
*Annexini: Forw, CGC TTG TAA TGT GCC TTG A; Rev, CCT GAC CGC TAT TGT GAA A
*JARI2D: Forw, TGG GTA CAG CAA ATC ACC AA; Rev, TAT GTG GAG CAG GAT TG
RESULTS

Constitutive mitotic activity is restricted to the forebrain

To reveal actively dividing cells in the adult newt brain, we first identified cells that express the proliferating cell nuclear antigen (PCNA). In general, we saw that PCNA+ cells were in contact with the ventricles, had radial glia-like morphology and expressed the glial fibrillary acidic protein (GFAP). These cells are generally referred to as ependymoglia cells in newts and provide the only cell type expressing GFAP given the lack of astrocytes (Benraiss et al., 1996; Lazzari et al., 1997). A detailed analysis of proliferation zones revealed distinct patterns of clusters of actively dividing cells, which were restricted to regions located rostral to the ventral thalamic region. The telencephalon harbours several mitotic clusters located caudal to the rostral part of the olfactory bulb (OB) parenchyma. The rostral OB is devoid of mitotic cells (Fig. 1B). Along the rostrocaudal axis, starting from the accessory olfactory bulb, proliferating cells are present in the lateral walls of the lateral ventricles (Fig. 1C). Proliferating cells are also found in the dorsolateral wall of the lateral ventricle throughout the telencephalon, situated adjacent to the border between the dorsal pallium and the lateral pallium (Fig. 1D-E). A ventrally located accumulation of PCNA+ cells is visible in the region of the bed nucleus of stria terminalis. This area was dense in PCNA+ cells, stretching ~300 μm along the rostral-caudal axis of the brain (Fig. 1F,G, lower arrows). An accumulation of proliferating cells is also apparent in the lateral wall of the lateral ventricles adjacent to the lateral and medial amygdala (Fig. 1F,G, upper arrows). The walls of the third ventricle were found to contain two proliferation zones.
situated in the rostral diencephalon. The most ventral of these two zones starts at the optic recess and extends caudally through to the ventricular cells medial to the suprachiasmatic nucleus (Fig. 1H, lower arrow). The more dorsally located proliferation zone is found in the ventricular layer bordering to the ventral thalamus (Fig. 1H, upper arrow). We did not find any other accumulation of proliferating cells in the adult newt brain located caudal to this region. With the exception of a maximum 3±2 scattered PCNA+ cells/brain, the caudal diencephalon, the entire mesencephalon, hindbrain and cerebellum were found to be essentially quiescent (Fig. 1I,J). To confirm the conclusions based on the distribution of the PCNA+ cells, we looked for cells positive for minichromosome maintenance protein, MCM2, which is involved in DNA replication (Maslov et al., 2007). As shown in Fig. S2 in the supplementary material, although the midbrain is devoid of MCM2+ cells, the forebrain harbours numerous MCM2+ cells. In addition most cells that are MCM2+ also express PCNA (data not shown). These results together show that during normal homeostatic conditions, dividing cells are restricted to the telencephalon and rostral diencephalon.

**Constitutive neurogenesis in the adult newt forebrain**

In order to trace the progeny of the proliferating cells, we first performed a pulse-chase experiment using the nucleotide analogue 5-bromo-2-deoxyuridine (BrdU), which incorporates into the replicating DNA. Animals received five pulses of BrdU for 3 days and were analysed either 1 day later or after a 13 days chase period (Fig. 2A). One day after the BrdU pulses 96±0.7% of the BrdU+ cells were also PCNA+ (see Fig. S2 in the supplementary material), and the majority (74.5±4.5%) of the BrdU+ cells were immuno reactive for the GFAP and had a radial glia-like morphology (Fig. 2B). At this time point, 62±1.5% of the PVCA+ cells were still positive for BrdU (Fig. 2D) and these cells were either lining or were found in close proximity (within two cell layers) to the lateral ventricles. Fifteen days after the first BrdU pulse, the majority (81.3±1.9%) of the BrdU+ cells were found away from the lateral ventricles, in the parenchyma of the lateral pallium and dorsal pallium, suggesting that the progeny of the dividing cells had migrated laterally. 72±6.6% of the BrdU+ nuclei were positive for the pan-neuronal marker NeuN (Fig. 2C; see Fig. S3A in the supplementary material), showing that the progeny of the ventricular GFAP+ cells entered a neuronal differentiation program. At this time point, ~1.3% of the ventricular, PCNA+ cells were retaining BrdU-labelling, suggesting that they might represent a label retaining stem cell population (Fig. 2D). Several BrdU+ cells expressing NeuN were also observed in the rostral olfactory bulb (Fig. 2F; see Fig. S3B in the supplementary material), where no cycling cells could be detected at the earlier time point (Fig. 1B), suggesting a rostral migration and neuronal differentiation of cells originating from the walls of the lateral ventricles. By contrast, analysing four brains we could not detect any BrdU+ cells after the chase periods in the caudal diencephalon, midbrain, hindbrain or cerebellum showing the lack of caudal migration. These results show that constitutive proliferation and neurogenesis are essentially restricted to the forebrain under normal homeostatic conditions.

**Activation of ependymoglia cells and ablation-responsive exit from normally quiescent midbrain niches**

We previously showed that selective ablation of diencephalic and mesencephalic DA neurons by stereotaxic injection of 6-OHDA leads to complete regeneration in adult newts (Parish et al., 2007) (see Fig S4 in the supplementary material). Following the death of DA neurons, regeneration involves extensive neurogenesis and depends on cellular proliferation (Parish et al., 2007). In contrast to the non-ablated animals, ependymoglia cells in the midbrain exit quiescence and proliferate (Parish et al., 2007). Both sham and 6-OHDA injections evoked mitotic activity with higher proliferation index after 6-OHDA injection compared with the sham control (Parish et al., 2007). We compared the cellular dynamics of the proliferation response by BrdU pulse-chase experiments. We observed a marked difference between 6-OHDA- and sham-injected animals after a 3-day chase. 91±6% of the BrdU-labelled cells were found distant from the ventricles and were GFAP+ following 6-OHDA injection, and only 35±16% following sham injection (Fig. 3B; see Fig. S5 in the supplementary material). 6-OHDA injection significantly shifted the equal distribution of the BrdU label towards GFAP+ non-ventricular cells when comparing 1-day and 3-day chase periods. By contrast, we did not see any difference in the distribution of the BrdU label in sham injected midbrains (Fig. 3B). These results showed that neuron ablation stimulated the activation and exit of ependymoglia cells from quiescent niches.
Neurogenesis by ependymoglia progeny

The above results suggested but did not prove that activated ependymoglia cells give rise to neurons after neuron ablation. Hence, we genetically labelled quiescent ependymoglia cells prior to the elimination of DA neurons by 6-OHDA. We marked GFAP+ cells lining the ventricle by in vivo electroporation of a DNA construct encoding for a histone2b-yellow fluorescent fusion protein (H2B2-YFP) under the control of the ubiquitously expressed CMV promoter. H2B2-YFP was stereotaxically injected into the third ventricle and an electrical current was applied via external electrodes. Fifteen hours after electroporation, a clear unilateral expression of YFP was observed in ventricular cells throughout the dorsoventral axis of the third ventricle. YFP expression was nuclear, and 952 out of the 960 YFP+ cells (n=3) were found in the ventricular GFAP+ cell layer (Fig. 3D-D′). Four days after electroporation, we injected 6-OHDA into the third ventricle. After 14 days, 26±3% of the H2B2YFP-labelled cells were non-ventricular and GFAP+ compared with 7.3±3.5% in the controls (Fig. 3E-G). In accordance with this finding, we found 16 times more H2B2YFP/NeuN cells in 6-OHDA-injected animals compared with control animals (data deposited in http://www.ebi.ac.uk/arrayexpress/; Accession Number, E-MEXP-2752; see Table S1 in the supplementary material). Out of these 1063 oligonucleotides, 739 were upregulated and 324 were downregulated. Six-hundred and forty-five oligonucleotides could be annotated to 444 open reading frames (ORFs), out of which 121 ORFs had multiple probe hits, i.e. they were represented by several ESTs. Probes representing 113 out of the 121 ORFs with multiple hits showed

analysed animals after 2 weeks. Because of the gradual neurogenesis of TH+ cells during regeneration (Parish et al., 2007) it is likely that the time frame under which the progeny of individual ependymoglia cells mature into neurons is substantially shorter than 14 days. In contrast to sham-injected animals, which were lacking double-labelled cells (n=4), we found in average 5±1.6 (n=5) GFAP+/TH+ cells in the 6-OHDA injected animals, which corresponds to 5% of the new TH+ cells that are produced in the midbrain during 14 days (Fig. 4C-C′). However, given that only a fraction of ependymoglia cells are labelled by electroporation, these results are best interpreted in qualitative terms. These data show that the progeny of the activated GFAP+ ependymoglia have migrated away from the ventricle and differentiated into TH+ neurons.

Molecular characterisation of ependymoglia cells

Next we looked at molecular events occurring in ependymoglia cells after DA ablation by analysing changes in gene expression. We prepared RNA and subsequently cDNA from laser microdissected tissue corresponding 80-120 ventricular cells in the midbrain, and analysed amplified RNA (aRNA) on oligonucleotide microarrays, which were derived from salamander EST and cDNA databases. Two oligonucleotide features represented each EST. The analyses revealed 1063 oligonucleotides out of 20,839, which were differentially regulated (P<0.01) in 6-OHDA-injected compared with control animals (data deposited in http://www.ebi.ac.uk/arrayexpress/; Accession Number, E-MEXP-2752; see Table S1 in the supplementary material). Out of these 1063 oligonucleotides, 739 were upregulated and 324 were downregulated. Six-hundred and forty-five oligonucleotides could be annotated to 444 open reading frames (ORFs), out of which 121 ORFs had multiple probe hits, i.e. they were represented by several ESTs. Probes representing 113 out of the 121 ORFs with multiple hits showed...
either up- or downregulation but no mixed patterns of regulation. Furthermore, in no cases were individual ESTs represented by probes, where one probe was up- and the other downregulated. Fig. 5A shows that the differentially regulated ORFs represented a broad range of biological processes. To validate the array data, we set out to analyse the expression of 14 candidates using quantitative real-time polymerase chain reactions (qRTPCR). Out of those 14, we obtained products with analysable \( \Delta C_t \) values and of expected size in 7 cases, all of which confirm the array data (Fig. 5C; see Table S1 in the supplementary material). Notably the array results identified several genes implicated in remodelling, neuronal stem cell regulation and DA neurogenesis (Fig. 5B), reinforcing the validity of the data.

Sonic hedgehog (\( Shh \)) was one of the genes whose differential regulation we could not validate using qRTPCR and hence we used in situ hybridisation. \( Shh \) has been implicated in regulating the fate of neural stem cells in general, and in DA neurogenesis in particular (Arenas, 2008; Traiffort et al., 2010). In addition \( Shh \) signalling has been implicated controlling aspects of appendage and lens regeneration in salamanders (Schnapp et al., 2005; Tsonis et al., 2004). We first cloned a fragment of \( Notoptalus \) \( Shh \) encoding for a 174 amino acid long ORF (see Fig S7 in the supplementary material). Subsequently, we performed in situ hybridisations, which showed upregulation of \( Shh \) after 6-OHDA injection (Fig. 6A,B). In accordance with these observations, inhibiting \( Shh \) signalling with cyclopamine reduced DA regeneration, as measured by the regeneration of the number of TH+ cells (Fig. 6C-E). As a control, we saw that cyclopamine treatment did not reduce the number of TH+ cells in non-ablated brains (data not shown). Furthermore, interference with TGF\( \beta \) signalling using an inhibitor of activin receptor-like kinase did not inhibit DA regeneration (data not shown). This is consistent with the array result, which did not show differential regulation of TGF\( \beta \) (see deposited data http://www.ebi.ac.uk/arrayexpress; Accession Number, E-MEXP-2752). Treatment with cyclopamine did not reduce the proliferation of ependymoglia cells (data not shown), nevertheless our data show that hedgehog signalling is required for adult midbrain DA regeneration.

**DISCUSSION**

Regenerative therapies aiming to replace lost neurons could be achieved either by transplantation of exogenous cells or by stimulating self-repair. The identification of neural stem cells in the adult brain that continuously give rise to neurons has coaxed the...
hope for involving these cells in functional repair after injury or in various degenerative diseases (Lindvall et al., 2004). Germinoval zones are, however, restricted mainly to two areas in the mammalian brain. Although some neuroblasts derived from these regions are attracted to injury sites (Brill et al., 2009; Carlen et al., 2009), the extent of neuronal replacement is modest in regions that normally do not produce new neurons.

In contrast to mammals, some non-mammalian vertebrate species regenerate neurons more efficiently in the entire brain (Becker and Becker, 2008). Careful mapping of the zebrafish showed that this capacity correlated with the sustained and widespread production of neurons in the adult brain (Grandel et al., 2006), which suggested a correlation between homeostatic and injury-responsive cell replacement (Zupanc, 2009). In this report, we showed that the adult brain is capable of maintaining as well as awakening the efficient neurogenic and regenerative potential of quiescent niches. Neurogenesis is normally limited to the forebrain in newts, but neuronal loss leads to activation of neuronal stem cells in otherwise quiescent areas. Thus, the newt is akin to mammals in terms of the extent of normal homeostatic cell turn over, but distinct in terms of its injury response, which is manifested in complete regeneration.

The exact fate of the stem cells in the various constitutively active proliferation zones is unclear. However, three different fate-mapping methods identified that ependymoglia cells that have radial glia morphology and express GFAP give rise to neurons in the newt brain. Although ependymoglia cells and neuroepithelial cells are closely related, our results to some extent contrast a recent study in zebrafish, which suggested that the cells giving rise to neurons in the adult non-mammalian brain have neuroepithelial rather than glial characteristics (Kaslin et al., 2009). It is possible that species variations exist between newts and zebrafish, not only in terms of the cellular turn over during normal homeostatic conditions, but also when it comes to the identity of stem cells. The type of neuronal stem cells that are retained in adults may also reflect the degree of maturation of the brain during embryonic development and postnatally. The identity of neuronal stem cells may also be different in different regions of the CNS as spinal cord regeneration studies in zebrafish indicated radial-glial like cells as a source of new neurons (Reimer et al., 2008).

A key question is how the cell cycle block in ependymoglia cells is lifted upon injury and neuronal loss. Quiescence may be an intrinsic cellular property that is undermined by extrinsic factors generated upon injury, similar to newt limb regeneration (Tanaka et al., 1999). Alternatively, stem cells may constantly and actively be kept out of the cell cycle by extracellular signals that disappear after injury.

Irrespective of how stem cells are activated, we see a specific response in terms of the cellular dynamics when comparing 6-OHDA-ablated animals with sham-ablated animals. We see increased exit from the niche after DA ablation compared with sham ablation. Activated ependymoglia cells that do not produce neurons after sham ablation may participate in the complete restoration of the ependymoglia layer. We see that the ependymoglia layer recovers both after sham and DA ablation, and is able to support a second round of regeneration following repetitive DA ablation (data not shown). Ependymoglia cells that remain locally after sham ablation may also undergo higher rate of cell death than after DA ablation. Although the fate of the activated ependymoglia cells after sham ablation is not clear at present, the observations indicate the existence of attractants produced specifically after the DA ablation. Such signals may act on cells that are analogous to transit amplifying cells, immature neuroblasts or on fully differentiated neurons. Alternatively, neuronal stem cells in the midbrain may undergo one asymmetric division producing one stem cell and one post-mitotic progenitor that differentiates into a mature DA neuron as described during embryonic DA neurogenesis in the mammalian midbrain (Bonilla et al., 2008). In mammals, neuroblasts are attracted by factors derived from astrocytes (Mason et al., 2001). The newt brain lacks astrocytes (Benraiss et al., 1996; Lazzari et al., 1997); thus, such attractant signals are either different compared with mammals or are produced by other cell types.

The extent of the genomic information currently available for newt research is currently limited but rapidly increasing (Borchardt et al., 2010; Maki et al., 2010). Here, we used a cross-species approach to start deciphering adult DA regeneration at the molecular level. The data indicate that DA regeneration in an existing brain structure demands to some extent similar cues that are used during embryonic midbrain development. Further analyses are likely to reveal necessary cues for neuron replacement in the adult midbrain and thereby contribute to novel restorative strategies in neurodegenerative diseases, such as Parkinson’s disease.

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Competing interests statement
The authors declare no competing financial interests.

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