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The Neurogenic Factor *NeuroD1* Is Expressed in Post-Mitotic Cells during Juvenile and Adult Xenopus Neurogenesis and Not in Progenitor or Radial Glial Cells

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**Abstract**

In contrast to mammals that have limited proliferation and neurogenesis capacities, the *Xenopus* frog exhibit a great potential regarding proliferation and production of new cells in the adult brain. This ability makes *Xenopus* a useful model for understanding the molecular programs required for adult neurogenesis. Transcriptional factors that control adult neurogenesis in vertebrate species undergoing widespread neurogenesis are unknown. *NeuroD1* is a member of the family of proneural genes, which function during embryonic neurogenesis as a potent neuronal differentiation factor. Here, we study in detail the expression of *NeuroD1* gene in the juvenile and adult *Xenopus* brains by in situ hybridization combined with immunodetections for proliferation markers (PCNA, BrdU) or in situ hybridizations for cell type markers (*Vimentin*, *Sox2*).

We found *NeuroD1* gene activity in many brain regions, including olfactory bulbs, pallial regions of cerebral hemispheres, preoptic area, habenula, hypothalamus, cerebellum and medulla oblongata. We also demonstrated by double staining *NeuroD1*/BrdU experiments, after long post-BrdU administration survival times, that *NeuroD1* gene activity was turned on in new born neurons during post-metamorphic neurogenesis. Importantly, we provided evidence that *NeuroD1*-expressing cells at this brain developmental stage were post-mitotic (PCNA−) cells and not radial glial (*Vimentin*+) or progenitors (*Sox2+*) cells.

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**Competing Interests:** The authors have declared that no competing interests exist.

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**Introduction**

Adult neurogenesis is a fascinating biological trait, which has captivated researchers since many years. In mammals and under normal conditions, adult neurogenesis has been identified in two anatomical regions: the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (reviewed by [1]). Interestingly, adult neurogenesis seems to be more abundant in birds, reptiles, amphibians and fish than in mammals (reviewed by [2–5]). Recently, the detailed neuroanatomical mappings of proliferative activity in the adult brain were provided in two non-mammalian amphibians and fish than in mammals (reviewed by [6–8] and the competive Proceedings of the National Academy of Sciences of the United States of America.

In *Drosophila melanogaster* embryos, *NeuroD1* can convert non-neuronal cells into neurons, and consequently, adult neurogenesis in vertebrate species undergoing widespread neurogenesis are unknown. *NeuroD1* is a member of the family of proneural genes, which function during embryonic neurogenesis as a potent neuronal differentiation factor. Here, we study in detail the expression of *NeuroD1* gene in the juvenile and adult *Xenopus* brains by in situ hybridization combined with immunodetections for proliferation markers (PCNA, BrdU) or in situ hybridizations for cell type markers (*Vimentin*, *Sox2*).

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particularly for the generation of granule cells in the hippocampus and cerebellum [19,20].

To gain further insight into the transcriptional program that controls adult neurogenesis in non-mammalian vertebrates, this study examined in details the NeuroD1 gene expression, together with various cellular markers (Vimentin, Sox2, PCNA and BrdU), in post-metamorphic (juvenile and adult) Xenopus brains. The data show that a high number of NeuroD1-expressing cells can be detected in various brain areas and that NeuroD1 gene is up-regulated during post-metamorphic neurogenesis. Moreover, we provide evidence that NeuroD1 is expressed in post-mitotic (PCNA+) neuronal cells and not in radial glial (Vimentin+) or neural progenitors (Sox2+) cells.

**Results**

**Strong and spatially restricted expression of the NeuroD1 gene in juvenile and adult Xenopus brains**

In order to identify the brain sub-divisions that express the NeuroD1 gene, in situ hybridization experiments, using high stringent conditions, were performed on coronal sections throughout the whole juvenile and adult brains. The detailed NeuroD1 expression pattern is illustrated in figures 1 and 2 and described below. There were no significant differences between males and females in any brain regions.

In the more rostral portions of the telencephalon, NeuroD1 expression was strongly detected in the glomerular and mitral cell layers of the olfactory bulbs (Figs. 1, A1–A3 and B1–B3). No expression of NeuroD1 was detected in the inner granule cell layer. Moving caudally towards mid-telencephalic levels, NeuroD1-positive cells were predominantly found in the dorsal, lateral and medial (arrows) territories of the pallia (Figs. 1, B1–B3 and C1–C3). Scattered cells could be also observed in the medial septum (arrowheads in Fig. 1, C2 and C3). No NeuroD1 labeling was observed in ventricular cells directly at the ventricle but rather in the migrated cells in the mantle zone. In more caudal portions of the telencephalon, NeuroD1-expressing cells were still detected in the dorsal, lateral and medial pallium (Figs. 1, D1–D3). In addition, NeuroD1 was expressed in part of the medial amygdala and bed nucleus striae medullaris (arrow in Figs. 1, D2). Ventrally, NeuroD1-expressing cells were also present in the medial portion of the anterior preoptic telencephalic area, such labeling being manifest in the adult brain section (arrow in Figs. 1, D3). In both pallial and preoptic areas, numerous NeuroD1-expressing cells were localized close to the ventricles, mainly in the subventricular layers from where the newborn post-mitotic neuronal cells migrate to their more peripheral final destination.

In the diencephalon, the level of NeuroD1 expression was moderate compared to telencephalon. The most densely labeled cell group was located in the epithalamus, namely, in the dorsal and ventral nuclei of the habenula (Figs. 1, E1 and E2). This expression pattern was maintained at adult stage (Figs. 1, E3). More caudally, a low number of moderately labeled cells was also found in posterior and ventromedialthalamic areas (Fig. 2, F2), which was no longer detected at adult stage (Fig. 2, F3). In addition, a discrete cell population located dorsally in the hypothalamic region, and very close to the infundibular recess, obviously expressed NeuroD1 in both juvenile and adult brains (arrows in Figs. 2, G1–G3). At mesencephalic level, only very few and dispersed NeuroD1-expressing cells were present in the tectum and tegmentum at juvenile stage (Figs. 2, H2). This weak labeling was not found in corresponding adult mesencephalic sections (Fig. 2, H3).

Within the metencephalon, very high levels of NeuroD1 labeling were found in the region of the cerebellum, such expression being restricted to the granular layer (Figs. 2, I1–I3). Interestingly, this strong NeuroD1 expression was maintained in adult cerebellum (Figs. 2, I4). Few NeuroD1-expressing cells were also found in areas corresponding to the nuclei of the trigeminal nerves (Figs. 2, J1 and J5). Posterior to the cerebellum, in the medulla oblongata (Figs. 2, J1–J3), NeuroD1 expression was still detected in nuclei of trigeminal nerves and, more dorsally, close to the lateral vestibular area (arrows in Figs. 2, J2 and J3). Importantly, throughout the juvenile and adult brains, we never observed NeuroD1-expressing cells in the ependymal layer.

**NeuroD1-expressing cells are not radial glial or progenitors cells**

As previously described, NeuroD1-positive cells could be observed both in the parenchyma and in the subventricular layers, but never in the ventricular zone adjacent to the ventricle.

To more precisely define the cellular expression of NeuroD1 within the brain, serial in situ hybridizations were performed on adjacent sections using the NeuroD1 probe, but also Vimentin and Sox2 probes, as markers of radial glial cells and neural progenitor cells, respectively [21,22]. Radial glial cells, known to behave as neural stem cell (reviewed in Kriegstein and Alvarez-Buylla, 2009), were previously identified in the ventricular layers of post-metamorphic Xenopus brain [9]. We focused our analyses on the pallial and cerebellar regions of juvenile brains because these two regions displayed heavy NeuroD1 expressions (see Figs. 1 and 2). As confirmed in figure 3, strong NeuroD1 expression domains were detected in the dorso-lateral pallium (Fig. 3, A) and in the cerebellum (Fig. 3, H). Using adjacent sections, in situ hybridizations with Vimentin (Fig. 3, B and I) and Sox2 (Fig. 3, C and J) probes showed strong expressions of both genes in ventricular cells of the pallium and sub-pallium. Sox2 labeling was also detected in few cells localized in the parenchyma (Fig. 3, C and J). Most importantly, higher magnifications of the dorso-lateral pallium (Figs. 3, D–F) and the granular layer of the cerebellum (Figs. 3, K–M) provided evidence that NeuroD1-positive cells were detected in large amount outside the ventricular layer cells that expressed Vimentin and Sox2 markers (compared Figs. 3 D to E and F; Figs. 3 K to L and M). These observations were reinforced by performing double labelings NeuroD1/DAPI on the same sections (Figs. 3, G and N). Taken together, these in situ hybridization experiments demonstrated that expression domains of NeuroD1 were excluded from the ventricular layers as these domains did not overlap with radial glia or neural progenitor markers.

**NeuroD1-expressing cells are post-mitotic cells**

Our detailed analysis of NeuroD1 gene activity have revealed that, throughout juvenile and adult brains, NeuroD1 transcripts were never detected in the ventricular layers, where both neural stem cells and mitotic neuronal precursor cells were located (Figure 3). This consistent NeuroD1 expression pattern, together with our previous published data that identified proliferating cells specifically in the ventricular layers of juvenile and adult brains [9], strongly suggested that NeuroD1-expressing cells were post-mitotic cells. To further investigate this hypothesis, we designed double labeling experiments combining NeuroD1 RNA in situ hybridization and immunocytochemistry for the PCNA proliferation marker on coronal sections of pallial and cerebellar regions (Figure 4). As shown at high magnifications of the cerebellum (Fig. 4, A–H), the PCNA positive cells were clearly found restricted to the ventricular layer (Fig. 4, B–D and F–H) while NeuroD1-expressing cells were observed both in sub-ventricular layers and
Figure 1. Expression pattern of NeuroD1 in the juvenile (A2–E2) and adult (A3–E3) X. laevis brains. (A1–E1) Schematic coronal illustrations of the corresponding transverse sections of a juvenile X. laevis brain (NF stage 66). The drawing at the top of the figure shows a dorsal view of the X. laevis brain. The letters correspond to the rostro-caudal location of sections as depicted in the whole brain drawing. Arrows and arrowheads in C2, C3, D2, and D3 highlight less conspicuous areas of labeling. Abbreviations are defined in Table 1. The anatomical drawings are from [55], with modifications of basal ganglia subdivisions according to [56]. For all images, dorsal is to the top. Scale bar = 400 μm in A2–E2, and 100 μm in A3–E3. doi:10.1371/journal.pone.0066487.g001

Discussion

To gain further insight into the activation of NeuroD1 gene in non mammalian vertebrates, we examined the precise expression pattern of NeuroD1 by in situ hybridization on coronal sections throughout the whole juvenile and adult Xenopus laevis brains. Expression data analysis revealed NeuroD1 gene activity in various brain regions, including olfactory bulbs, pallial regions of cerebral hemispheres, preoptic area, habenula, hypothalamus, cerebellum and medulla oblongata. In previous reports, NeuroD1 gene expression has only been studied in embryonic and larval Xenopus stages (up to stage 48) in the context of primary neurogenesis and secondary neurogenesis, respectively [17,23–26]. At larval stage, NeuroD1 gene activity was identified in pallia, dorsal thalamus (habenula), pretectum, posterior tuberculum, nucleus of the medial longitudinal fascicle, mesencephalic optic tectum, torus semicircularis, tegmentum and medulla oblongata [23]. Thus, our study demonstrates that some of the previously identified larva brain regions maintained NeuroD1 gene activity until juvenile and adult stages. This is obvious for the pallial, habenula and cerebellar regions in which we detected the heaviest NeuroD1 expressions. Importantly, our study also revealed that two additional post-metamorphic brain regions, compared to larva stage, expressed NeuroD1, namely the preoptic area and the hypothalamus. Previously, our laboratory performed studies to identify proliferation in the brain of both juvenile and adult Xenopus [9]. Interestingly, the patterns of NeuroD1 expression were consistent with the majority of the proliferation zones that we mapped. In mammals, where most of NeuroD1 studies were performed, it was shown that structures such as the olfactory bulbs, cerebellum and hippocampus, maintained significant levels of NeuroD1 mRNA expression at post-natal stages and also throughout adulthood in humans and mice [14,27–30]. Recent studies in adult zebrafish telencephalon have also identified NeuroD1 gene activity in the pallial region [31]. Wether or not the Xenopus NeuroD1 protein is regionally expressed at a similar level than the NeuroD1 transcripts remains to be demonstrated. Unfortunately, due to the lack of available valid NeuroD1 antibodies, this could not be investigated.

In juvenile and adult Xenopus brains, we identified a large amount of NeuroD1 expressing cells in the cerebellum, in particular in the granular layer. Interestingly, this strong NeuroD1 gene activity is both conserved during Xenopus cerebellar development and across species. Indeed, in Xenopus and zebrafish larvae, a very strong NeuroD1 expression in the developing cerebellum was previously detected [23,32]. In mouse, at post-natal stages, NeuroD1 was also clearly detected in both external and internal granular layers of the cerebellum, and the internal granular layers expression was shown to stably persist until adulthood [19,27,28]. In addition, systemic or conditional NeuroD1 null mice experiments have shown that the absence of NeuroD1 leads to a lack of foliation and the complete loss of granular cells in the posterior half of the cerebellum, whereas a substantial number of granular cells survive and differentiate in the anterior lobules [19,33,34]. It will be interesting to investigate, in nonmammalian vertebrates, if there is a similar anterior-posterior differential requirement of NeuroD1 for granular cell maintenance.

The present study also shows that the medial pallium was another domain of abundant gene activity in juvenile and adult
In perfect agreement, we previously identified migrating cells and new born mature neurons in the medial pallium of brains. In perfect agreement, we previously identified migrating cells and new born mature neurons in the medial pallium
and adult brains, we could never find NeuroD1 expressing cells in the ventricular wall, in particular no co-localization with the radial glia or neural progenitor cells markers could be identified; 2) NeuroD1 in situ hybridization combined with PCNA immunodetection did not allow identifying any co-localization of both factors; 3) Positive NeuroD1/BrdU double stainings were only found after long (14 days) post-BrdU administration survival time but never with short survival time (2 days). Therefore, we conclude that NeuroD1 expressing cells in frog post-metamorphic brains are postmitotic. Interestingly, this feature seems to be conserved during Xenopus central nervous development. In Xenopus embryo, during primary neurogenesis, it was clear established that NeuroD1 is expressed transiently in a subset of neurons in the central and peripheral nervous systems at the time of their terminal differentiation into mature neurons [17]. During Xenopus secondary neurogenesis, i.e. at early larval stages, NeuroD1 gene expression was also excluded to the most ventriculally located cells in proliferation zones, in particular mitotic cells expressing Ngn-1 and Delta1 genes [23]. Interestingly, in post-embryonic and adult zebrafish brains, NeuroD1-expressing cells were also identified from one to several cell rows away from the ventricular surface [31,32,49]. Taken together, NeuroD1 expression studies in non-mammalian vertebrates have indicated that NeuroD1-expressing cells were post-mitotic at embryonic, larval and adult brain stages. Surprisingly, in murine NeuroD1 expression was detected not only in post-mitotic but also in mitotic cells, as was evident in its expression in external granular layer of cerebellum and granule cells of the dentate gyrus during post-natal development. This observation suggested that NeuroD1 protein may have a unique role in proliferation and/or differentiation of granule cells of the cerebellum and dentate gyrus [19,20,27,29,30]. As NeuroD1 expression was restricted to post-mitotic cells in the sub-ventricular zone and parenchyma, the present study suggests that NeuroD1 may play an important role in neuronal cell differentiation in the late stages of neurogenesis rather than proliferation stages in post-metamorphic Xenopus brain.

(D’Amico et al., 2011). Interestingly, the amphibian medial pallium is regarded as the homologue of the mammalian hippocampus [26,35]. In other vertebrates, the hippocampus is also known to be one of the very few brain regions in which adult neurogenesis continues into adult stages of development, as demonstrated in reptiles, birds, fish and mammals [10,36–43]. In mammals, after birth, NeuroD1 is prominently expressed in the hippocampus, particularly in the granule cells of the dentate gyrus and pyramidal cells in CA1 and CA3 [27,44–46]. As expected from the high expression level in the granule cell of the dentate gyrus, mice lacking the NeuroD1 gene activity in the hippocampus of post-metamorphic brains, several arguments strongly support that NeuroD1 gene activity. The observation suggested that NeuroD1 protein may have a unique role in proliferation and/or differentiation of granule cells of the cerebellum and dentate gyrus [19,20,27,29,30]. As NeuroD1 expression was restricted to post-mitotic cells in the sub-ventricular zone and parenchyma, the present study suggests that NeuroD1 may play an important role in neuronal cell differentiation in the late stages of neurogenesis rather than proliferation stages in post-metamorphic Xenopus brain.
In an effort to understand the molecular cascade involved in frog adult neurogenesis, further investigations with other proneural and/or neurogenic factors have to be conducted in the future. During primary neurogenesis, and retinal neurogenesis, expression of *NeuroD1* is known to follow the expression of the bHLH gene *neurogenin-related-1 (X-ngnr-1)*, a vertebrate neuronal determination gene, also known as *Neurogenin 2 (Ngn2)* in mammalians [17,50,51]. During primary neurogenesis, overexpression of *X-ngnr-1* induces formation of ectopic neurons in nonneuronal ectoderm and induces ectopic expression of *NeuroD1*

**Figure 3. Expression patterns of NeuroD1, Sox2 and Vimentin in the juvenile *X. laevis* brain.** In situ hybridizations on coronal sections of cerebral hemispheres (A–G) and cerebellum (H–N). In cerebral illustrations, D, E and F are high magnifications of A, B and C, respectively. In cerebellum illustrations, K, L and M are high magnifications of H, I and J, respectively. To allow merge with the DAPI staining, colors of high magnification illustrations D and K were negatively inverted in photos G and N, respectively. For all images, dorsal is to the top. Scale bar = 220 μm in A–C and H–J; 95 μm in D–G and K–N.

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These data demonstrate that X-ngnr-1 and NeuroD 1 function to regulate successive stages of neuronal differentiation in the developing neural plate. Preliminary studies in our laboratory suggest that X-Ngnr-1 might not be a key factor during adult neurogenesis as we were not able to detect X-Ngnr-1 expression, using stringent hybridization conditions, in any juvenile or adult brain areas, including regions with high proliferative and neurogenic capacities (data not shown). Whether or not other members of the Neurogenin family, such as Xenopus Neurogenin 1 or Neurogenin 3 [52], are able to compensate X-Ngnr-1/Ngn2 expression in the adult neurogenic network remains an open question.

Materials and Methods

Animals

For the present study, juvenile and adult Xenopus laevis of both sexes were used. Juveniles (NF stage 66) stage were classified according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). All procedures involving animals were conducted in accordance with the guidelines of Ethical Committee at our institutions (University of Rennes 1, CNRS and INSERM) and in accordance with European Union regulations concerning the protection of experimental animals (Directive 86/609/EEC) and in accordance with Ethical Committee CEEA (Comité Rennais d’Ethique en matière d’Expérimentation Animale) and performed under the supervision of authorized investigators (Permit number: 75-390). All steps have been taken to reduce suffering of animals. Animals were deeply anesthetized with 0.05% tricaine methane sulphonate (MS-222; Sigma) and killed by decapitation. The whole heads of juvenile frogs were fixed 2 hours in freshly prepared 0.5M, pH 7.4 phosphate–buffered saline (PBS) containing 4% paraformaldehyde (three specimens per stage). After two washes in PBS, brains were then carefully removed from the skull, post-fixed in fresh fixative overnight at 4°C and then stored, for no more than one week, in PBS until sectioning.

BrdU incorporation

To follow the fate of proliferating cells, juvenile X. laevis were anesthetized (as described above) and injected intraperitoneally with approximately 50 μL/g body weight of labeling reagent (Amersham Cell Proliferation kit; RPN20). After survival periods ranging between 2 days and 14 days post-injections, frogs were deeply anesthetized and killed as described above.

In situ hybridization and immunohistochemistry

Brains were embedded in paraffin and sectionned coronally. Consecutive/adjacent thin sections of 8 μM thickness were placed on different slide sets allowing each individual brain to undergo in situ hybridization with different probes and/or antibodies (as described in the following). Sections were subjected to stringent in situ hybridization (ISH) as described [53]. The following ISH probes were used: NeuroD1 [17], Vimentin [21] and Sox2 [54]. For double ISH/immunodetections, the brains were first processed for ISH, then for immunocytochemistry. PCNA and BrdU immunodetections were performed as previously described [9]. All sections were photographed and analyzed under a Olympus PROVIS AX70 microscope with a digital camera (Olympus SP71), and a Nikon multizoom AZ100 macroscope with a DS-Ri1 color

Figure 4. NeuroD1/PCNA and Vimentin/PCNA double stainings in the cerebellar and pallial regions of juvenile X. laevis brain. Coronal sections at the level of cerebellum (A–H) or pallium (I–K). In situ hybridization using a NeuroD1 (A–D and I–K) or a Vimentin (E–H) probe combined with PCNA immunohistochemistry. For all images, dorsal is to the top. Scale bar = 30 μm.
Camera. Cell counting was done manually under the microscope by two of the authors and by an observer unfamiliar with experiments. Red blood cells were clearly identified using 20× and 40× microscope objectives and were not counted.

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**Author Contributions**

Conceived and designed the experiments: PC DB. Performed the experiments: PC LAD. Analyzed the data: PC LAD. Contributed reagents/materials/analysis tools: PC. Wrote the paper: PC DB.

Figure 5. **NeuroD1/BrdU double stainings on telencephalic and cerebellar sections of a juvenile* X. laevis* brain.** (A–J) Telencephalon high magnifications of NeuroD1 in situ hybridizations (A, C, F and H) combined with BrdU immunodetections (B, C, F and H) after 14-days BrdU post-administration time. Arrows indicate double stained cells. DAPI stainings are indicated to certify the presence of the nucleus. (K–T) Low magnifications of the above NeuroD1/BrdU/DAPI triple labelling experiments showing larger view of telencephalon (K–O) and cerebellum (P–T). For all images, dorsal is to the top. Scale bar = 15 μm in A–J; 45 μm in K–T.
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