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Reprogramming of neonatal SVZ progenitors by Islet-1 and Neurogenin-2

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ABSTRACT

The subventricular zone (SVZ) lining the lateral walls of the lateral ventricles is one of the major neurogenic areas in the postnatal brain. Precursor cells in the SVZ migrate via the rostral migratory stream to the olfactory bulb where they differentiate into neurons. Cell replacement strategies utilizing the recruitment of these endogenous progenitors and their progeny to different areas of the brain hold great promise for the future, but much research is needed in order to understand the sequence of molecular signals necessary to induce proliferation, migration and site-specific differentiation of these cells.

In this study we show that the SVZ cells can be redirected from their normal migration route and directed towards other brain regions when they are infected with retroviruses encoding the developmentally important transcription factors Islet-1 and Neurogenin-2. After co-transduction with these transcription factors, transduced cells could be detected in several areas of the brain. When located in the striatum, the reprogrammed cells displayed neuroblast-like morphology. Once removed from the striatal parenchyma and allowed to further differentiation *in vitro* they developed into β-III-tubulin positive neurons.

Introduction

Neurogenesis occurs during the embryonic period and is completed around birth in most brain regions. Some neurogenesis, however, persist in the postnatal and adult brain, but it is restricted to two regions: the subventricular zone of the lateral ventricles (SVZ) and the hippocampus (Alvarez-Buylla and Lim, 2004). The progenitor cells within these regions generate migrating neuroblasts. Neuroblasts originating from the SVZ migrate over long distance; via the rostral migratory stream (RMS) to olfactory bulb (OB), where they differentiate into interneurons (Lois and Alvarez-Buylla, 1994). This migration pattern can be visualized by retroviral labeling of dividing cells within the SVZ (Rogelius et al., 2005; Yamada et al., 2004). Under normal conditions the neuroblasts migrate specifically to the OB and no significant migration to other areas such as cortex, striatum, or external capsule (CE) occurs. However, neurogenesis and migration from the postnatal and adult SVZ can be influenced under experimental conditions by changes in the brain environment such as by brain lesions (Arvidsson et al., 2002; Parent et al., 2002; Yamashita et al., 2006), addition of trophic factors (Benraiss et al., 2001; Chmielnicki et al., 2004; Pencea et al., 2001), or expression of transcription factors important during embryogenesis (Hack et al., 2005; Rogelius et al., 2006). Taken together, these studies illustrate the plasticity and capacity of the SVZ cells to be recruited to alternative fates which make cell replacement therapies utilizing these endogenous progenitors a possible alternative to transplantation of *in vitro* expanded neural stem cells in the future.

Our group has previously studied what effect ectopic expression of the LIM homeodomain transcription factor Islet-1 (Isl1) in the SVZ cells has on their migration and final differentiation (Rogelius et al., 2006). In the ventral forebrain, Isl1 expression is restricted to the embryonic period where it is expressed in the SVZ from embryonic day 12 to birth in cells that differentiate into striatal projection neurons (Olsson et al., 1995; Toresson et al., 2000; Wang and Liu, 2001). Viral-driven ectopic expression of Isl1 in the neonatal SVZ cells resulted in a new migration pattern as the Isl1 expressing cells, in addition to the OB, also migrated specifically to the striatum. The newly generated cells in the striatum however, did not differentiate into striatal neurons, instead they adopted a Ng2 positive glial phenotype.

In the present study we hypothesized that the proneural basic helix-loop-helix transcription factor Neurogenin-2 (Ngn2), which during the embryonic development contribute to neurogenesis, may be sufficient to direct the progeny from ectopic Isl1 expressing SVZ progenitors in the striatum and further instruct them to adopt a neuronal fate. Ngn2 is an activator of neuronal gene expression (Morrison, 2001), participates in neuronal specification (Fode et al., 2000), and is known to promote neuronal differentiation of embryonic and adult neural stem cells in culture (Berninger et al., 2007; Falk et al., 2002; Jensen and Parmar, 2006). To test our hypothesis we injected two retroviruses into the

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Table 1
Presence of transduced cells in the olfactory bulb

	Score
Rv.GFP, *2w*	++
Rv.ISL, *2w*	++
Rv.Ngn2, *2w*	–
Rv.Isl/Rv.Ngn2, *2w*	+

– = 0 cells, + = 1–50 cells, ++ = 51–100 cellsand +++ = more than 100 cells.

lateral ventricle of neonatal rats, one encoding Isl1and the other Ngn2, thus infecting the proliferating cells of the SVZ with both Isl1 and Ngn2 at the same time.

The results presented in this study show that ectopic expression of Ngn2 in combination with Isl1 in the proliferating SVZ cells results in the appearance of cell populations primarily in the striatum, CE, and septum in addition to the OB. When the newly formed cells in the striatum were isolated and further differentiated *in vitro* they displayed neuronal morphology and expressed the neuronal marker β-III-tubulin. Thus, our results suggest that Ngn2 prevents the cells from adopting a glial fate and further that Ngn2 and Isl1 act synergistically and their combined expression affects which migratory cues and differentiation signals that the cells responds to.

Results

Retroviral injections to the lateral ventricles of neonatal rats target the proliferating cells of the SVZ (Rogelius et al., 2005). In this study we injected four combinations of retrovirus; the control virus containing the marker gene GFP only (Rv.GFP), the Rv.Ngn2 virus which contain the coding sequence for Ngn2 and IRES-GFP (Falk et al., 2002), the Rv.Isl1 virus (Rogelius et al., 2006) which also contain IRES-GFP and finally co-injections of Rv.Ngn2 and Rv.Isl1 (Rv.Ngn2/Rv.Isl1).

Ectopic expression of Ngn2 in combination with Isl1 results in recruitment of cells to the striatum, external capsule, and septum

The normal fate of the neuroblasts in the SVZ is to migrate to the OB and form interneurons (Lois and Alvarez-Buylla, 1994; Luskin, 1993). As we have previously reported (Rogelius et al., 2006), and also shown in this study, this same migration and differentiation pattern were seen in the control (Rv.GFP) animals 2 weeks after injection. Also in agreement with previous observations, the animals in the control group did not have transduced GFP positive cells in the striatum, CE, or septum (not shown). In the co-injected group that received both Rv. Ngn2 and Rv.Isl1 (Rv.Ngn2/Rv.Isl1) the animals had the normal migration of cells in the RMS to the OB, as detected by their expression of GFP. However, the number of cells in the OB was reduced compared to control-injected animals (Table 1). In addition to this, and in contrasts to the control animals, an extensive amount of GFP-expressing cells could be detected in both the striatum and in the CE 2 weeks after transduction in the co-injected group. Small populations of GFP positive cells could also be detected in the septum, contralateral striatum and contralateral CE 6 weeks after injection (Table 2). Within the striatum, the newly formed cells derived from the co-transduced SVZ were evenly distributed in all regions of the striatum, whereas in the CE the cells were clustered in tight association with each other (Fig. 1A). 6 weeks after injection, the cells in the striatum had a tendency to be concentrated within the striatal fiber tracts. In agreement with this, co-staining for GFP and the neuronal marker MAP2 show that the majority of the GFP-expressing cells reside in the low-MAP2 expressing fiber bundles (Figs. 2A and 4A).

We also studied the distribution of cells in animals that received injections with only Rv.Ngn2 or only Rv.Isl1. When Ngn2 is expressed alone, GFP-expressing cells were mainly found in the striatum close to the SVZ and in the CE (Table 1) but not in the OB. In the Rv.Isl1 injected group the migration of cells to the OB was undisturbed (Table 1). In addition, a large population of SVZ-derived cells had migrated to the striatum. In some animals (3/12) a few cells could also be detected in CE (Table 2). The distribution of cells in the striatum was markedly different, though, between animals injected with Isl1 only and Isl1/Ngn2 in combination. In the co-injected group the cells were found evenly distributed in all areas of the striatum, whereas the cells in the Isl1-only group were primarily located in the ventrolateral striatum (Figs. 1A and B).

Taken together, ectopic expression of Isl1 and Ngn2 affects the migration of the cells, and expression of both Ngn2 and Isl1 in combination contributes to recruitment of cells from the SVZ mainly to the to the striatum, CE and septum.

Cells recruited to striatum after co-expression of Isl1 and Ngn2 have a neuroblast-like morphology

In the control-injected group transduced cells in the RMS and OB were indistinguishable from non-transduced cells based on morphology, localization and immunostainings for DCX and Er81. In the RMS the cells had elongated cell bodies and a leading process where as the cells in the OB displayed differentiated neuronal morphologies (not shown and Rogelius et al., 2006). In the co-injected group, the transduced cells in the RMS and OB had the same morphology as the cells in the control (Fig. 2C). Interestingly, the transduced cells recruited to the striatum and CE also had an elongated cell body and a long process, thus resembling a migrating neuroblast (Figs. 2A and B). In the Rv.Ngn2 group, the striatal and CE cell populations had the same neuroblast-like morphology (Figs. 2D and E), as the cells that received injections of both Rv.Ngn2 and Rv.Isl1. In the RV.Isl1 only group, however, the striatal cells did not have a neuroblast-like morphology but had a typical glial morphology (Fig. 2F) and expressed Ng2 as previously shown (Rogelius et al., 2006). No such cells with this glia morphology and Ng2 expression could be detected in any of the co-injected animals.

In this study, GFP is the marker gene for the transduced cells in both the Rv.Ngn2 and Rv.Isl1 vectors. Thus we cannot easily determine if a cell is in fact expressing from both viruses or just from one in the co-injected group. However, after immunohistochemistry analysis followed by confocal analysis of transduced cells in striatum, CE and RMS in the Rv.Ngn2/Rv.Isl1 group, the Isl1 protein was co-detected with GFP in virtually all cells indicating that the great majority of GFP-expressing cells have received Rv.Isl1 viruses (Fig. 3). Further, if the transduced cells in the Rv.Ngn2/Rv.Isl1 group only had received the Rv.Isl1 vector and not the Rv.Ngn2 one would expect the presence of some GFP positive, Ng2 expressing glia-like cells in the striatum as this is the only cell type present in the animals that received only Isl1 injections. As this cell phenotype was never detected in the co-transduced group, we can therefore conclude that a vast majority of the GFP-expressing cells in the Rv.Ngn2/Rv.Isl1 injected group have received both viruses.

Table 2
Relative distribution of cells in different brain regions after genetic manipulation of SVZ cells

	Striatum	CE	cl CE	cl striatum	Septum	Average total number of cells per animal
Rv.ISL, *2w*	96±5%	4±3%	0	0	0	873
Rv.Ngn2, *2w*	73±30%	27±26%	0	0	≤1%	317
Rv.Isl/Rv.Ngn2, *2w*	87±5%	13±5%	0	0	≤1%	1005
Rv.ISL, *6w*	100±0%	0	0	0	0	1334
Rv.Ngn2, *6w*	na	na	na	na	na	na
Rv.Isl/Rv.Ngn2, *6w*	46±14%	34±22%	3±2%	11±12%	7±7%	1073

CE = Capsula Externa, cl = side contralateral to injection, na = not analyzed.

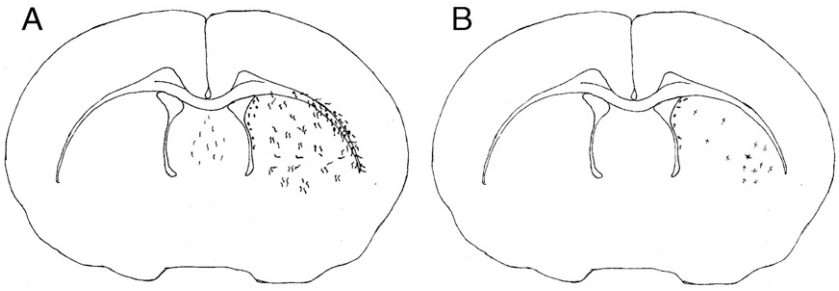

Fig. 1. Differential distribution of cells on striatum after Ngn2/Isl and Isl1 only injections. Camera Lucida drawings of representative brain sections showing the difference in distribution of cells after Ngn2/Isl co-injections (A) and Isl1 only injections (B). Cells in the co-injected group were found evenly distributed in the striatum whereas cells in the Isl1 only group were clustered in the ventrolateral striatum.

Transduced cells located in striatum and CE lack neuronal and glial markers in the RV.Ngn2/Rv.Isl1 injected group

To determine the phenotype of the transduced cells in the striatum and CE of the Rv.Ngn2/Rv.Isl1 group, we stained with cell-type specific antibodies for immature glia (Ng2), mature oligodendrocytes (APC), immature migrating neurons (DCX), and the neuron maker MAP2. We found that even though the transduced cells after Rv.Ngn2/Rv.Isl1 injection displayed morphology of migrating neuroblasts with a typical elongated cell body and leading process they did not express DCX (Fig. 4C) or MAP2 (Fig. 4A). They were also negative for the expression of Ng2 (Fig. 4C) and APC (Fig. 4D) leaving the molecular phenotype of the cells undetermined.

Striatal cells from the co-injected group differentiate into neurons in vitro

Since no mature neurons could be detected in the striatum after Rv. Isl1 and Rv.Ngn2 co-injection of the proliferative SVZ cells, we hypothesized that the gliogenic environment in the striatum is not permissive for terminal neuronal differentiation of the SVZ-derived cells. To test this hypothesis, we isolated the GFP-expressing cells migrating in the striatum 2 weeks after viral targeting of the SVZ, and differentiated them *in vitro* in order to study their neuronal differentiation potential once removed from the gliogenic environment of the neonatal striatum.

Retroviruses were injected into the lateral ventricle of neonatal rats in the same four injection groups as described above, namely; control, RV.Ngn2/Rv.Isl1, RV.Ngn2, and RV.Isl1. Two weeks after injection, the animals were sacrificed and the part of the striatum that contained GFP positive cells was isolated under a dissection microscope. Single cell suspensions were made from the tissue pieces and the cells were plated in culture dishes for further differentiation *in vitro*. The cells were differentiated for 7 days under conditions normally used for primary neuronal cultures. At the time of isolation, the cells were negative for DCX. However, upon inspection using standard light microscope during the culture period, the cells underwent the same morphological changes normally seen in maturing neurons in mixed primary cultures. After 7 days, the cells were fixed and analyzed for their expression of GFP in combination with neuronal markers and morphology (Fig. 5A).

Interestingly, once removed from the striatum, cells transduced *in vivo* with Rv.Ngn2/Rv.Isl1 showed a high capacity for neuronal differentiation (Fig. 5B). 44±17.5% of the isolated GFP positive cells expressed the neuronal marker β-III-tubulin in the co-injected group (n=420), whereas only a few transduced cells, 2±0.85%, expressed β-III-tubulin in the Rv.Ngn2 group (n=163) (Fig. 5C). Animals that received control virus were processed in parallel but since the transduced cells in this group do not migrate to the striatum no GFP positive cells could be obtained for further analysis. In the group that received Rv.Isl1 only, we obtained fewer surviving GFP positive cells than in the Isl1/Ngn2 or Ngn2 only group after 7 days in culture. None of the GFP positive cells that were present in this group expressed β-III-tubulin or showed a neuronal morphology. Thus, only cells originating from the SVZ transduced with both Rv.Ngn2 and Rv.Isl1 are able to form a substantial number of neurons when isolated from the postnatal

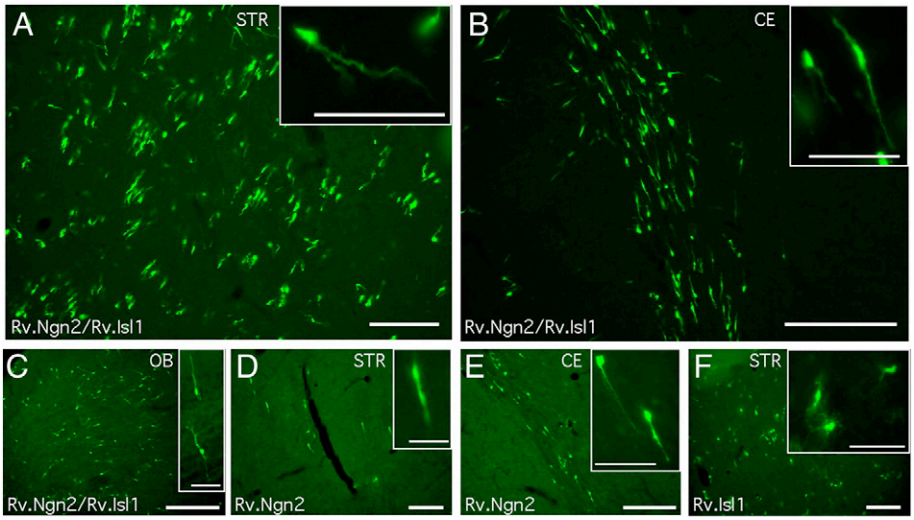

Fig. 2. Neuroblast-like morphology of the transduced cells in striatum and CE. The cells found in striatum (A) and CE (B) in the Rv.Ngn2/Rv.Isl1 injected group displayed neuroblast-like morphologies. The cells found in OB in this group had the normal morphology of newly derived OB cell (C). In the Rv.Ngn2 injected group (D and E) the cells found in striatum (D) and CE (E) also had a neuroblast-like morphology. The morphology of the transduced cells in striatum after Rv.Isl1 (F) is glial-like. Scale bar 100 μm.

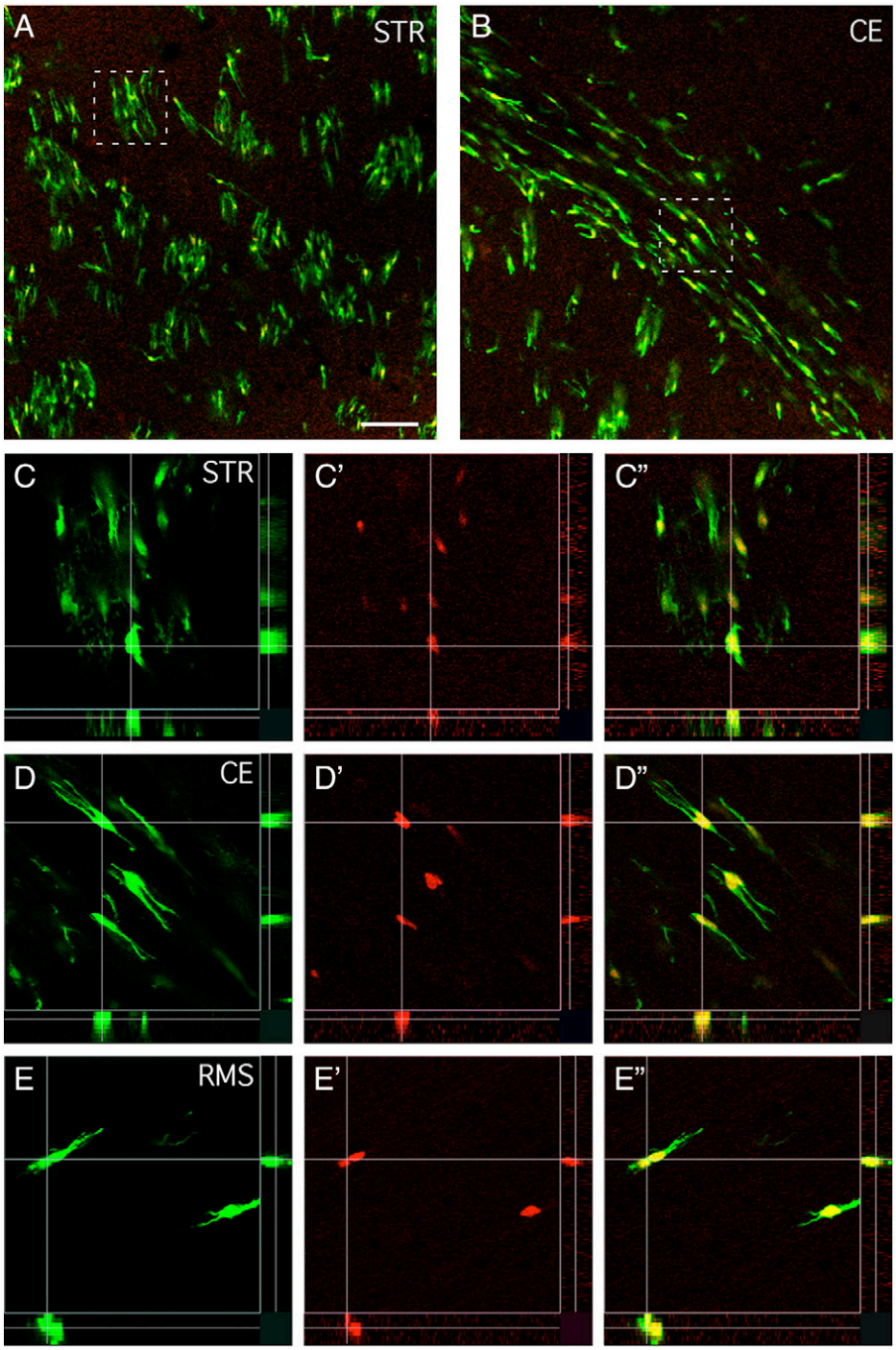

Fig. 3. Transduced GFP-expressing cells in the striatum, CE, and OB express Isl1 in the Rv.Ngn2/Rv.Isl1 injected group. Immunohistochemically staining for GFP (green) and ISL1 (red) followed by confocal analysis showed that in the co-injected group (Rv.Ngn2/Rv.Isl1) virtually all transduced GFP-expressing cells co-labeled with Isl1 in the striatum (A and C–C"), CE (B, D–D") and OB (E–E"). Dotted boxes shows area in striatum and CE that is used for high magnification picture in C–C" and D–D". Scale bar 100 μm.

striatum and allowed to differentiate *in vitro* under conditions that are permissive for neuronal differentiation.

Discussion

Neural replacement therapies based on recruitment of endogenous precursors *in situ* have several advantages over transplantation-based approaches (Emsley et al., 2004), but much remains to be elucidated about the potential limitations in differentiation fate of endogenous progenitors as well as their migration and integration capacity. Another big challenge is to understand which developmental events an adult progenitor cell undergo in order to form a specific neuronal subtype and then to learn how to provide the precise combination of molecular signals and environmental cues necessary to induce the formation of such neurons in response to injury (Emsley et al., 2004; Lie et al., 2004). Knowledge of how progenitor cells and their progeny can be redirected into a certain fate could be important for future development of therapies for neurodegenerative disorders.

In the present study we demonstrate that the progenitor cells in the SVZ are plastic and can adopt a new fate. By expression of Ngn2 in combination with Isl1, the progeny from SVZ precursor can populate the striatum, CE and septum. Once removed from the non-permissive striatal environment, the cells can differentiate to neurons *in vitro*. The knowledge of how plastic the progenitor cells of the SVZ are and what

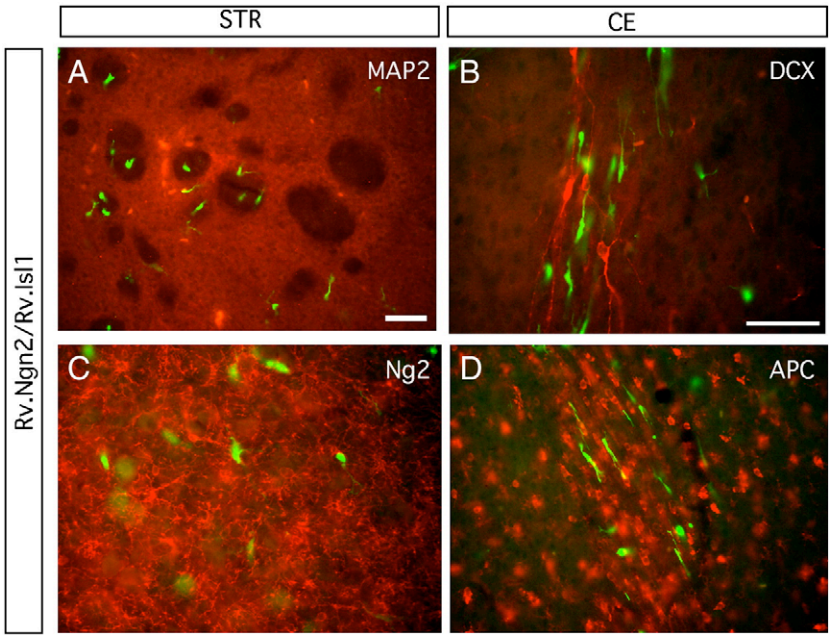

Fig. 4. No expression of neuronal or glia markers of the transduced cells in the striatum and CE in the RV.Ngn2/Rv.Isl1 injected group. Endogenous cells expressing MAP2 (A), DCX (B), Ng2 (C), or APC (D) could be detected. In the Rv.Ngn2/Rv.Isl1 injected group the transduced cells found in the striatum and CE did never overlap with any of these neuronal or glia markers. The transduced cells could, however be detected within the fiber bundles as seen in the MAP2 staining where the staining is not intense in this area (A).

factors they respond to is important for devising future strategies for redirecting them to a desired fate with the aim to achieve brain repair from these resident precursors.

The question addressed in this study was if ectopic expression of Ngn2 in combination with Isl1 in progenitor cells of the neonatal SVZ could contribute to new neuronal cells in the striatum. The progeny of Rv.Ngn2/Rv.Isl1 co-transduced cells populated the striatum and had morphology compatible with migrating neuroblasts. However, despite their neuroblast-like morphology, expression of the neuronal markers DCX or MAP2 could not be detected while the cells were residing within the striatum. After birth, no neurogenesis occurs in the striatum under normal conditions and the postnatal striatum is normally seen as gliogenic environment that is not permissive for neuronal differentiation. We therefore removed the newly formed cells from the striatum and differentiated them *in vitro*. We found that once removed from the non-permissive striatal environment, approximately half of the SVZ-derived striatal cells differentiated into neurons. These results show that ectopic expression of Ngn2 in combination with Isl1 in SVZ-derived cells mobilizes a population of cells that migrate to the striatum and that harbors a neuronal differentiation potential once the cells are removed from the non-permissive environment of the intact postnatal striatum. This is in agreement with previous studies that show that non-neurogenic environments can override the neurogenic effect of Ngn2 (Hofstetter et al., 2005). Also in this study we see that the ectopic expression of Ngn2 and Isl1 is not sufficient to generate striatal neurons *in vivo*, where extrinsic factors in the gliogenic environment of the neonatal striatum have a strong effect of the final differentiation fate of the newly derived cells, likely overriding the neurogenic effect of Ngn2.

Other groups have shown that Ngn2 actively cooperate with Isl1 to specify motor neuron identity in the spinal cord (Lee and Pfaff, 2003). The results from our study show that Ngn2 and Isl1 have a synergistic effect on the neuronal production from SVZ-derived postnatal progenitor cells *in vivo* and *in vitro*. Co-expression of Ngn2 and Isl1 seems to be important for generation of neurons, as only transduced cells from the co-injected group differentiated with a high percentage into neurons. If the neuronal differentiation potential of the striatal cells was the effect of Ngn2 alone one would expect an equally high amount of β-III-tubulin positive neurons in the Rv.Ngn2 group as in the Rv.Ngn2/Rv.Isl1 group which was not the case.

The *in vivo* results also suggest that the ectopically expressed intrinsic factors interact, not necessary physically but maybe through downstream signaling, thereby affecting cell migration and differentiation. Transduction with only Isl1 did not contribute to any cells in the CE or septum, and transduction with only Ngn2 gave no GFP-expressing cells in OB. In contrast, many cells could be detected in both striatum, CE and OB when both viruses are co-transduced. The limited migration of cells and the complete lack of transduced cells in OB when Ngn2 is expressed in SVZ cells are interesting. As Ngn2 is a proneural gene that upon high expression drives the cells out of cell cycle and promotes neuronal differentiation (Bylund et al., 2003; Sun et al., 2001), the lack of cells in the OB and in the distal parts of striatum in Ngn2-only transduced animals could be an effect of premature cell cycle exit that is not compatible with migration in the RMS or striatal parenchyma. When Isl1 is expressed in combination with Ngn2 this migration defect seems to be at least partly overcome as GFP-expressing cells in these animals are found in the OB, albeit a lower numbers than in the Isl1 only or control groups. The morphology of the cells was also markedly different between the groups. Expression of Ngn2 (alone or in combination with Isl1) prevents the expression of the glia precursor marker Ng2 and also shifts the morphology of the glia-like cells seen in the striatum after Isl1 transduction to a more neuronal-like morphology. Thus, Ngn2 seem to promote cell cycle exit and block glial differentiation and at the same time priming the cells towards a neuronal fate, which is only fully revealed once the transduced cells are removed from the non-neurogenic environment of the striatum and cultured *in vitro*.

Cell fate is governed by both intrinsic and extrinsic factors. In postnatal striatal neurogenesis the extrinsic environment seems to play a large role. Change in striatal environment with lesions like stroke (Arvidsson et al., 2002) or addition of BDNF and/or bFGF (Benraiss et al., 2001; Chmielnicki et al., 2004; Pencea et al., 2001) contribute to new neurons in the adult striatum. Recruitment of new neurons to the striatum apparently also needs a dramatic insult to occur since a small excitotoxic quinolinic acid lesions and glioma fail to contribute to this ectopic neurogenesis (own unpublished results and

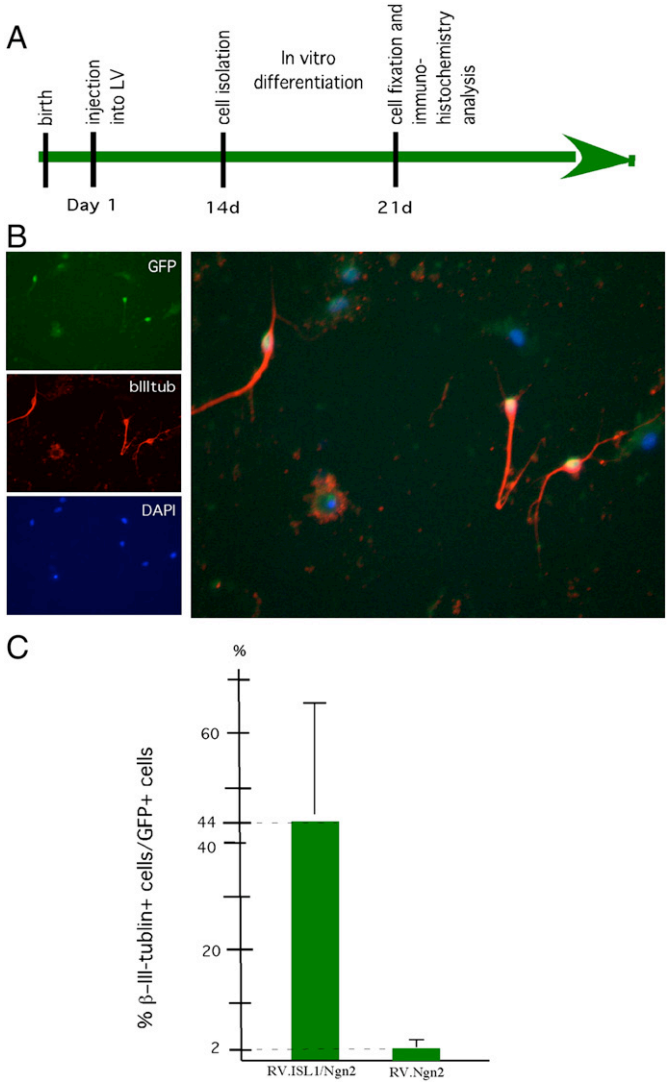

Fig. 5. *In vivo* Rv.Ngn2/Rv.Isl1 co-transduced cells express β-III-tubulin after *in vitro* differentiation. One day after birth the neonatal rats were injected with Rv.GFP, Rv.Ngn2/Rv.Isl1, Rv.Ngn2, or Rv.Isl1 to the lateral ventricle (A). Two weeks later the transduced GFP-expressing cells located in striatum were dissected out and cultured for further differentiation for 1 week. Immunohistochemical analysis showed co-expression of GFP and β-III-tubulin in cells in the Rv.Ngn2/Rv.Isl1 injected group (B). In this group 44 ± 17.5% of all isolated GFP-expressing cells (n = 420) expressed β-III-tubulin (C). In the Rv.Ngn2 group, only 2 ± 0.85% of the GFP-expressing cells (n = 163) were β-III-tubulin positive. Values are expressed as mean ± SEM.

Staflin et al., 2007). It would therefore be of great value if cells in the SVZ could become more susceptible to change their fate, thus permitting striatal neurogenesis even after no or minor insults. In this study we show that the intrinsic properties of the SVZ cells can be altered by ectopic expression of Ngn2 and Isl1 in combination, however, not strongly enough to completely override the non-neurogenic environment of the intact postnatal striatum. It would be interesting to study the behavior and differentiation potential of the Ngn2 and Isl1 transduced cells in a lesion model or after BDNF administration. Such combination of intrinsic and extrinsic factors may lead to replacement of a sufficient number of striatal neurons from endogenous progenitors *in situ*.

Experimental methods

Retroviral production

The constructs used here, CMMP-GFP (Rogelius et al., 2005), CMMP-Ngn2-IRES-GFP (Falk et al., 2002), and CMMP-Isl1-IRES-GFP (Rogelius et al., 2006) have previously been described. All retroviruses used in this study (Rv.Ngn2, Rv.Isl1, and Rv.GFP) were produced as previously described (Ory et al., 1996). Briefly, the constructs were transfected using lipofectamin (Life Technologies) to the packaging cell line 239GPG (a gift from Dr RC Mulligan). Virus containing media were collected every day for 7 days after transfection and the harvested supernatants were concentrated by ultracentrifugation (Beckman L-70), spun at 16500 rpm for 1.5 h at 4 °C. Viral titers were determined based on green fluorescence protein (GFP) expression, and less than 30% GFP positive cells were used to calculate transduction units (tu)/μl. The viral titers were between $1.4–4.5 \times 10^8$ 10^8 tu/μl, 5×10^8 10^8 tu/μl, and 4.5×10^8 10^8 tu/μl for Rv.Isl1, Rv.Ngn2, and control (Rv.GFP), respectively.

Retroviral injections and tissue processing

Sprague–Dawley rat pups (P1) received unilateral 1 μl injections of the following viral vectors: control (Rv.GFP), Rv.Isl1, Rv.Ngn2 or Rv.Ngn2 and Rv.Isl1 in combination into the lateral ventricle. Using a stereotactic frame, the rats were injected at the following coordinates measured from the dura, ML = −1.5 mm DV = −1.5 mm (Rogelius et al., 2005). The injections have been done on four separate occasions. A total of 10, 14, 20, and 23 animals were injected in the control, Rv.Isl1, Rv.Ngn2, and Rv.Ngn2/Rv.Isl1groups, respectively The animals were divided into groups with different survival times: 2, 6, and 12 weeks. In all experiments, control animals were injected with vector containing only GFP, and control-injected animals were always processed in parallel with the other injection groups.

The animals were perfused through the ascending aorta with isotonic saline, followed by ice-cold 4% paraformaldehyde (PFA). The brains were then postfixed for 6 h in 4% PFA and placed in 25% sucrose in 0.1 phosphatase buffer. The brains were cut in 40 μm-thick sections on a freezing microtome (1400 Leitz, Germany). The cerebral hemispheres were cut as coronal sections and the OB (including the rostral part of RMS) into sagittal sections.

Culturing of striatal cells, derived from in vivo transduced SVZ cells

Cultures were generated from striatal cells of 2 week old rats, that have received unilateral injections with either Rv.GFP, Rv.Ngn2/Rv.Isl1, Rv.Ngn2, or Rv.Isl1 into the lateral ventricle at P1. Animals were anesthetized with pentobarbital and sacrificed by cervical dislocation. The brain tissues were placed in ice-cold L-15 dissection medium. To detect the area of GFP-expressing cells a fluorescence dissection microscope were used to dissect out the striatum. The tissue piece were incubated at 37 °C for 20 min in 0.05% DNase and 0.1% trypsin and subsequently mechanically dissociated into a single cell suspension. The cells were plated in Poly-L-Lysine (PLL)-coated plastic wells at the concentration 200 000 cells/well in differentiating medium containing 1% FBS and no growth factors. After 5–7 days of differentiation the cells were fixed in 4% PFA for 15 min at room temperature.

Immunochemistry

After rinsing in PBS, cultures or sections were preincubated in 5% normal serum (Chemicon International, UK and Göteborgs Termometerfabrik, Sweden) and 0.25% triton-X100 (Sigma-Aldrich, USA) for 1 h at room temperature. Primary antibodies used were; chicken anti-GFP (1:5000 Chemicon International, UK), mouse anti-ISL1 (1:100, Hybrydoma Bank), mouse anti-β-III-tubulin (1:1000, Promega), rabbit anit-Ng2 (1:500, Chemicon International, UK), goat anti-DCX (1:3000 Santa Cruz, USA), mouse anti-APC (1:200, Calbiochemi), and mouse anti-MAP2 (1:200, Chemicon). Incubations in primary antisera were carried out in preincubation solution overnight at room temperature. The FITC and Cy3 conjugated secondary antibodies (1:400, Jacksson ImmunoResearch, USA) were diluted in the preincubation solution and incubated for 2 h at room temperature. Antigen-retrieval by boiling in 10 mM citrate buffer (pH 6) was performed on sections prior to incubation with mouse anti-ISL1.

Quantification

In vivo

4–6 animals per group were selected for quantification. Sections from each group were subdivided into the following zones: striatum, CE, septum, cl striatum, cl CE and OB. Cell number in each zone (except OB) was counted in 3 consecutive sections, covering virtually all transduced cells as rostrocaudal migration was limited. There was a significant variation of the total number of cells per animal (288–1107 for Rv.Isl1 2 weeks, 302–1919 for Rv.Isl1/RV.Ngn2 2 weeks and 162–423 for Rv.Ngn2 2 weeks; 137–2990 for Rv.Isl1 6 weeks and 624–1523 for Rv.Isl1/Rv.Ngn2). Because of this large variation in total cell number, the proportion of cells in each structure was calculated as a percentage of the total cell number and is reported as the average proportion of cells in each region ± SEM per group.

For the OB, the number of cells in the central section was estimated using a semi-quantitative scoring system where + was assigned to sections containing 1–50 cells, ++ was assigned to sections with 51–100 cells and +++ was assigned to sections with more than 100 cells.

In vitro

All GFP-expressing cells were quantified in 3–4 wells in each group. Then all cells co-expressing β-III-tubulin and GFP were quantified in these same 3–4 wells. A mean value

was calculated and the numbers presented as percentage β-III-tubulin of GFP-expressing cells ± SEM.

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