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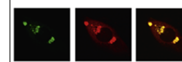

Research Report

Nop2 is expressed during proliferation of neural stem cells and in adult mouse and human brain

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ABSTRACT

The nucleolar protein 2 gene encodes a protein specific for the nucleolus. It is assumed that it plays a role in the synthesis of ribosomes and regulation of the cell cycle. Due to its link to cell proliferation, higher expression of Nop2 indicates a worse tumor prognosis. In this work we used *Nop2*^{gt1gaj} gene trap mouse strain. While lethality of homozygous animals suggested a vital role of this gene, heterozygous animals allowed the detection of expression of Nop2 in various tissues, including mouse brain. Histochemistry, immunohistochemistry and immunoelectron microscopy techniques, applied to a mature mouse brain, human brain and on mouse neural stem cells revealed expression of Nop2 in differentiating cells, including astrocytes, as well as in mature neurons. Nop2 was detected in various regions of mouse and human brain, mostly in large pyramidal neurons. In the human, Nop2 was strongly expressed in supragranular and infragranular layers of the somatosensory cortex and in layer III of the cingulate cortex. Also, Nop2 was detected in CA1 and the subiculum of the hippocampus. Subcellular analyses revealed predominant location of Nop2 within the dense fibrillar component of the nucleolus. To test if Nop2

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expression correlates to cell proliferation occurring during tissue regeneration, we induced strokes in mice by middle cerebral artery occlusion. Two weeks after stroke, the number of Nop2/nestin double positive cells in the region affected by ischemia and the periventricular zone substantially increased. Our findings suggest a newly discovered role of Nop2 in both mature neurons and in cells possibly involved in the regeneration of nervous tissue.
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1. Introduction

The nucleolar protein 2 (Nop2, Nol1, p120) gene encodes a protein specific for the nucleolus with moderately strong ribosomal RNA methyl transferase activity (Freeman et al., 1988). In addition to the presumed role in the synthesis of ribosomes, expression of Nop2 depends on the phase of the cell cycle. The highest Nop2 activity is observed in late G_1 and S phase, which corresponds with the synthesis of DNA and cell division (Fonagy et al., 1993). The fact that the gene is expressed in malignant tumors is used in the histopathologic diagnosis of neoplasms (Saijo et al., 2001). Stronger expression of Nop2 indicates a more rapidly proliferating tumor, which is a sign of poor prognosis (Bantis et al., 2004). Nop2 has so far been used as a marker in a number of tumors, such as breast and lung carcinomas (Liu et al., 2009; Talvinen et al., 2010). Despite these findings and its prognostic application, the exact role of Nop2 is still unknown.

In the brain, glial cell populations are the only cell types which can multiply. Mature neurons are in a permanent G_0 phase and as such cannot divide. However, in some limited areas of the mammalian brain, neurogenesis continues throughout life: the subependymal zone of the lateral ventricle in humans and the supragranular zone of the hippocampal dentate gyrus and olfactory region in some animals exhibit constant mitosis of neuronal precursors (Altman and Das, 1965; Bernardino et al., 2012; Christie and Turnley, 2013).

An attractive approach to elucidate brain regenerative potential is to analyse neurogenesis as a response to brain diseases (e.g. stroke, neurodegeneration, injury). It is known that some cells can exit from their remnant state and contribute to cell regeneration (Kirby et al., 2013). Thus discovery and elucidation of genes involved in cellular responses to diseases of or damage to the brain represent an attractive goal for furthering understanding of cellular processes taking place in normal nervous tissue as well as nervous tissue affected by disease.

In this work we used a unique *Nop2^{gt1gaj}* gene trap mouse strain previously designed and constructed by our group (Mitrečić et al., 2008). While homozygous embryos die during early embryo development, suggesting a significant importance of this gene, heterozygous animals appear normal and allow the detection of Nop2 expression through lacZ activity (Mitrečić et al., 2008). In this work we confirmed our hypothesis that Nop2 should be expressed during the proliferation of neural stem cells in vitro. Furthermore, we clearly showed that after the onset of stroke, the number of Nop2 expressing cells in the mouse brain markedly increased. In addition and unexpectedly, we found that this protein is expressed in mature neurons of the somatosensory and cingulate cortex and in the hippocampus, in both mouse and human brain. This suggests a new, so far unknown role of this gene in postmitotic cells.

2. Results

2.1. *β-Galactosidase staining revealed the presence of Nop2 expression in adult mouse brain*

Adult mice heterozygous for wild type Nop2 were used in order to examine whether there is expression of Nop2 in the adult mouse brain. 60 μm thick sections of *Nop2^{gt1gaj}* heterozygotes showed a clearly visible green signal in some cells in the brain of the adult mouse. The greatest density of these cells was found in the cortex (Fig. 1), while a less pronounced signal was found in the hippocampus and the cerebellum.

Higher magnification of these sections revealed that the signal was mainly located in and around the nucleus. In many sections we found cells with the morphology, shape and size of neurons (Fig. 1B). The same method applied in other tissues, such as heart muscle (Fig. 1C) and the control WT (C57Bl/6) mice (Fig. 1D) did not reveal the presence of this protein.

2.2. *Nop2 expression in mature mouse brain was confirmed by immunohistochemical staining for β-galactosidase and Nop2*

Immunohistochemical staining on β-galactosidase confirmed the findings obtained by histochemical staining on β-galactosidase (Supporting information, Fig. 1A and B). Although the antibody showed the presence of small amounts of a constant background staining, the number and the shape of cells that were positive for the presence of the Nop2/β-galactosidase construct were comparable to the results obtained using the histochemical reaction.

Nop2 immunohistochemistry in the brains of adult mice confirmed the findings found by histochemical staining and immunohistochemical confirmation on the β-galactosidase. We found a large number of positive cells in the cerebral cortex of adult mice, most of which morphologically resemble neurons (Supporting information, Fig. 1C and D).

As an additional control of our results, we performed immunohistochemistry with Nop2 antibody using control C57Bl/6 mice. The number, shape and arrangement of positive cells was the same as in Nop2 heterozygotes (not shown).

Control immunohistochemistry on liver, kidney and lungs did not show any Nop2 positive cells (not shown).

2.3. Nop2 expression in mature human brain

Immunohistochemical staining of post mortem sections of different areas of human brain from healthy adults demonstrated expression of Nop2 protein in different populations of neurons. Overall staining of Nop2 in the cortical neurons was not uniform, showing different levels of protein expression between pyramidal neurons of same population, with the most pronounced expression being in the largest pyramids. Strong expression was found in the somatosensory cortex, in both supragranular and infragranular pyramidal neurons and in the pyramids of precingulate layers III and Vb. The posterior

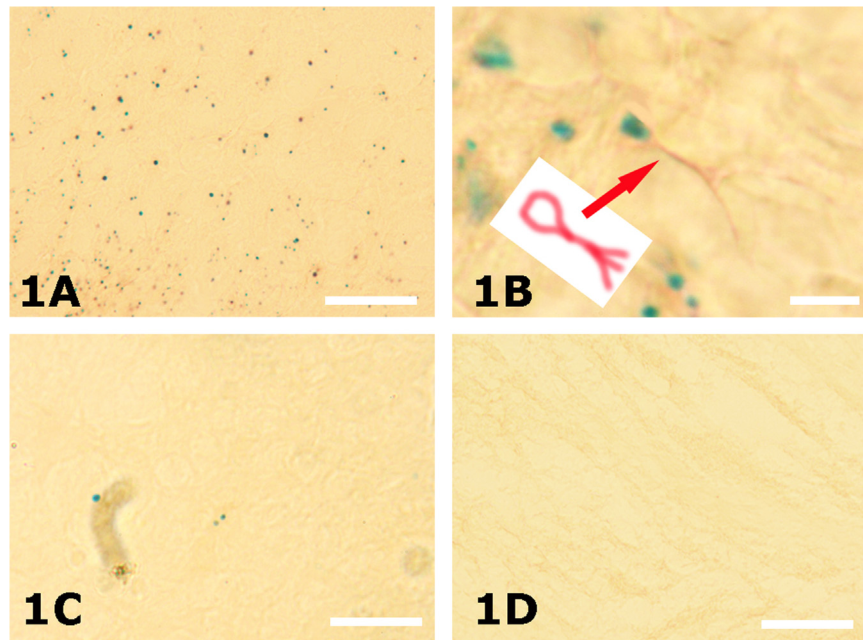

Fig. 1 – Beta galactosidase positive staining was detected in the adult brain of Nop2 heterozygous mouse. Histochemical reaction to beta galactosidase in the brain of Nop2 heterozygous (A and B), heart muscle Nop2 heterozygous (C) and brain control C57Bl/6 mouse (D). In (A) a large number of cells is shown in the cortex containing Nop2 protein and at higher magnification, a neuron with a labelled nucleus is visible (B). (C) shows the absence of positive cells in the heart muscle of Nop2 heterozygotes, while the D negative control was performed using a control C57Bl/6 mouse. Magnification bar: (A, C and D): 1 mm, (B): 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

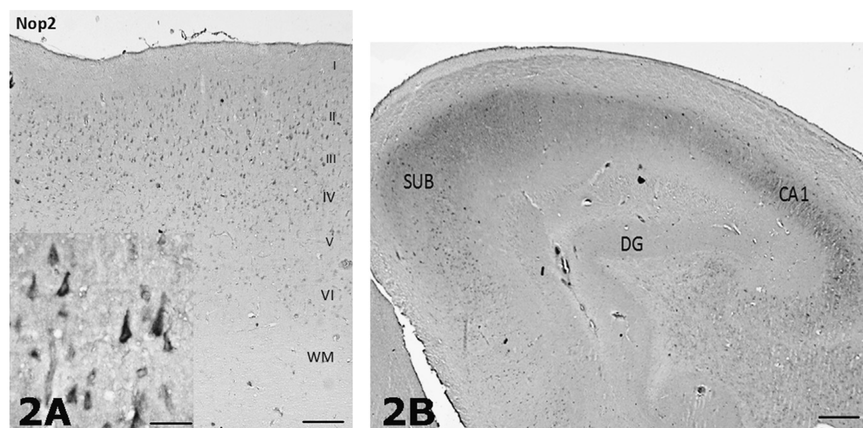

Fig. 2 – Expression of Nop2 was found in neurons of the adult human brain cortex and hippocampus. In (A) immunohistochemical staining of the expression of Nop2 protein in the neurons of the posterior cingulate cortex of the human brain is shown. There is a strong expression in some cells visible in the layers II–IV. (B) Shows expression of Nop2 protein in the pyramidal layer of the human brain, hippocampal formation. While the subiculum and CA1 exhibit a strong expression of Nop2, the dentate gyrus does not express this gene. WM—white matter, I–VI layers of cortex. CA1-Cornu Ammon's 1, DG-dentate gyrus. Magnification bar 250 μm. Inlet Y demonstrates cellular localization of the Nop2 protein in pyramids. Magnification bar 75 μm.

cingulate expressed reactivity in layer III (Fig. 2A, inlet) and weak sporadic expression in layer V pyramids (Fig. 2A, low magnification). Labelled cells displayed pyramidal morphology. We did not detect expression of Nop2 in the motor cortex.

Cornu Ammon's gyrus (CA1) and subiculum (SUB) exhibited moderate to weak expression of Nop2 in the pyramidal layer (Fig. 2B). Expression of Nop2 protein in proliferating cells in the subependymal zone or dentate gyrus (DG) was not detected.

2.4. *Nop2/Map2 double immunohistochemical staining revealed that the majority of Nop2 positive cells were neurons*

To determine which cells in the brain of adult mouse expressed Nop2, double immunohistochemistry was performed with antibodies against Nop2 and Map2. Approximately 50% of cells that were positive for Nop2 exhibited the presence of Map2 (Fig. 3, white arrows) demonstrating their neuronal identity. At the same time, 35% of Map2 positive cells were not Nop2 positive (Fig. 3, blue arrows). Analyses at higher magnification revealed that the presence of Nop2 signal was limited to the nucleus and initial segment of the axon (Fig. 3, inlet).

2.5. *Double immunohistochemical staining using Nop2/NeuN confirmed that most of the Nop2 positive cells were neurons*

In order to confirm that many neurons in the brain of adult mice express Nop2 gene, an additional comparison was made using an antibody to NeuN. Unlike Map2, which is a protein of neuronal cytoskeleton, NeuN protein is specific for the nucleus, and sometimes perikarya of neurons.

Since the number of cells positive for NeuN matched the one found with Map2, our finding that a large number of neurons in the cerebral cortex of adult mice express Nop2 was confirmed (Fig. 4, white arrows). We also observed NeuN positive neurons that did not contain protein Nop2 (Fig. 4, blue arrows).

2.6. *Nop2 is present in Purkinje cells of the cerebellum, but not in its granular layer*

Immunohistochemical analysis of the cerebellum with antibodies against NeuN and Nop2 revealed that only Purkinje nerve cells contained Nop2 protein, while the neurons of granular layer of the cerebellum were negative for Nop2 (Supporting information, Fig. 2). Also, this finding indicates that Nop2 is expressed in one type of neurons, while not in others, as we have already described in the cortex of adult mouse. Our finding that Purkinje cells were negative for NeuN was already reported in the literature (Mullen et al., 1992).

2.7. *Some of the cells that express Nop2 in the adult mouse brain are astrocytes*

In order to examine if some of the cells that express Nop2 in the adult mouse brain were astrocytes, we performed Nop2 and GFAP immunohistochemistry. By using careful examination of z-stacks we found Nop2 in the bodies of some astrocytes (Supporting information, Fig. 3).

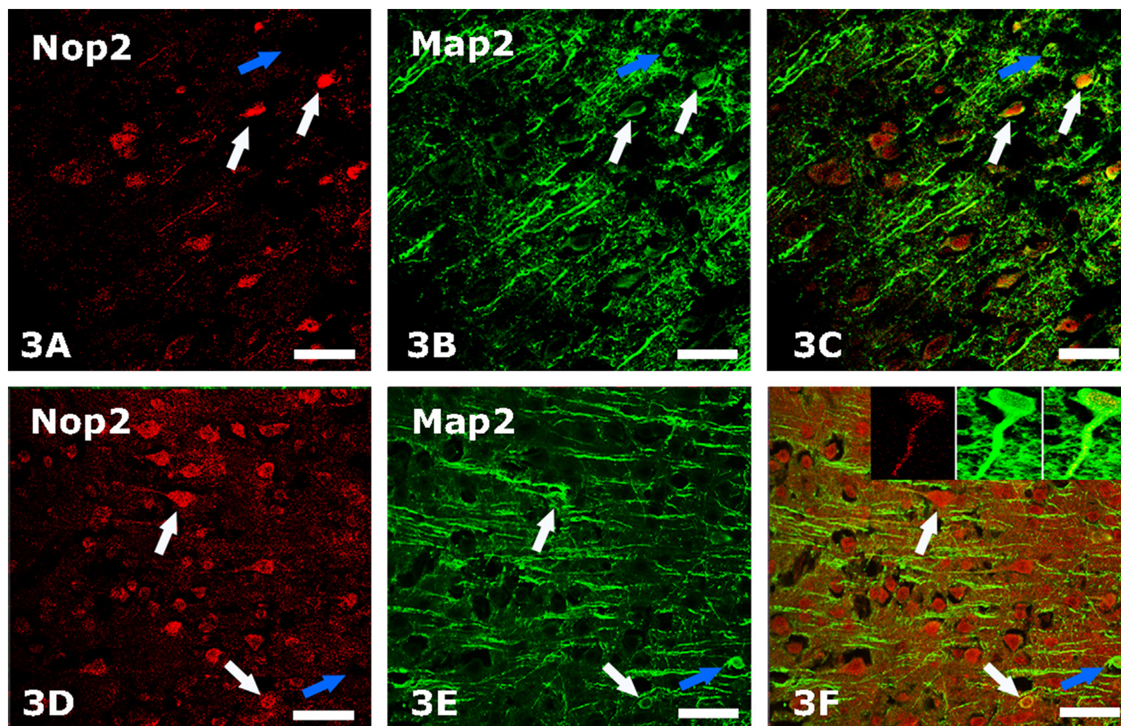

Fig. 3 – Co-localization of Nop2 and Map2 revealed that the majority of Nop2 positive cells were neurons. Double immunohistochemical reaction with Nop2 (A and D) and Map2 (B and E) revealed that many Nop2 positive cells in mouse cortex are also Map2 positive (white arrows). However, we found Map2 positive neurons (blue arrow) that were not Nop2 positive. Magnification bar: 200 μm. Inlet Y within (F) demonstrates a presence of Nop2 in the neuronal body and in the initial segment of the axon. Magnification bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

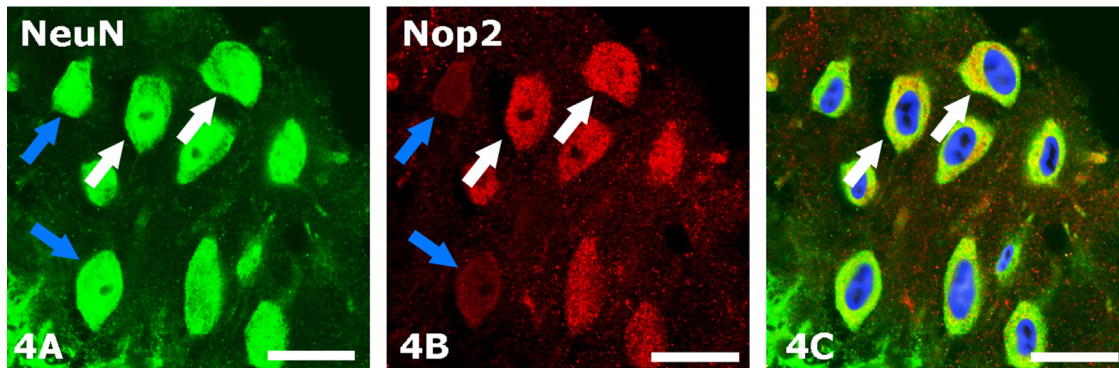

Fig. 4 – Presence of Nop2 protein in cortical neurons was confirmed by double Nop2/NeuN immunohistochemical staining. Double immunohistochemical reaction NeuN (A) and Nop2 (B) revealed that many Nop2 positive cells in the mouse somatosensory cortex were as well NeuN positive (white arrows). However, we found NeuN positive neurons (blue arrow) that were Nop2 negative. NeuN immunohistochemistry showed that in addition to the nucleus (reactive to NeuN antibody, blue) Nop2 was located in the wider area of the perikaryon (C). Magnification bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.8. *Nop2 is expressed in neural stem cells and continues to be present in some neurons during cell differentiation*

To gain further insight into the appearance and presence of Nop2 in cells of nervous system, we used neural stem cell cultures. Within 24 h after isolation, many, but not all neural stem cells from the telencephalic wall of 14.5 days old mouse embryos positive for nestin also expressed Nop2 (Fig. 5A–C). The signal was almost exclusively present in the perinuclear region of the cells.

After 3 days of differentiation, correlating to the onset of Map2 expression, Nop2 signal was present in a majority of cells (Fig. 5D–F). Nop2 in immature neurons was present mostly in perinuclear region, the same localization observed in neural stem cells.

After 9 days of differentiation, the overall strength of the Nop2 signal decreased. In cells with the morphology of mature neurons, Nop2 was not only present in perinuclear, but in the nuclear region as well (Fig. 5G–I).

2.9. *Brain regeneration after stroke correlates to the appearance of Nop2/nestin positive cells*

Because we hypothesized that brain regeneration might be linked to increased expression of Nop2 we induced strokes in 2 month old C57Bl/6 control mice by occlusion of the middle cerebral artery (MCAO). Analyses of the brain region affected by stroke (the corticostriatal border) revealed numerous nestin and Nop2 double positive cells (Fig. 6A–F). In comparison to the unaffected half of the brain, and with the brains of control mice not subjected to MCAO (Fig. 6G–I), the number of nestin positive cells was much higher, especially in the region affected by stroke. Moreover, the morphology of nestin positive cells was different. While in healthy brains nestin positive cells exhibited an astrocytic shape, this morphology was not present in the region affected by stroke. In ischemia-affected regions cells were rounded, with morphology resembling differentiating neuroblasts. Analyses of stroke—affected regions revealed that every nestin positive cell was also Nop2 positive, which suggests a role for Nop2 in proliferation of neural stem cells. In the same region, not every Nop2 positive cell was nestin positive. These Nop2+/nestin-cells probably represented the Nop2 population described on healthy brain tissue. The observation that the majority of Nop2 positive cells coexpress nestin after stroke suggests that Nop2 positive cells in the brain exhibit the potential to express nestin after the onset of brain damage.

2.10. *In vitro cultured neural stem cells and Nop2 positive cells occurring after stroke revealed high BrdU positivity*

Since we hypothesized that Nop2 marks proliferating cells linked to neural regeneration, we analyzed the presence of BrdU positive cells in both in vitro culture and in the brain after stroke. In vitro cultures of neural stem cells revealed that majority of neural stem cells show positive signal for BrdU, suggesting their proliferative capacity. Indeed, only a small portion of cells were BrdU negative and they all exhibited elongated cell processes, typical for more differentiated cells (Fig. 7A–C).

Analyses of BrdU in the mouse brain 10 days after stroke revealed that majority of Nop2 positive cells were also BrdU positive. This confirmed our hypothesis that Nop2 marks cell populations with the proliferative capacity needed for brain regeneration (Fig. 7D–F).

In this region, in a normal brain, the number of Brdu/Nop2 double positive cells was around 16 per 0.5 mm^2; after stroke, this number increased to 289. This suggests that the majority of newly born Nop2 cells are BrdU positive and that their number is highly linked to brain damage (Fig. 8).

2.11. *Immunoelectron microscopy revealed that Nop2 is primarily located in the nucleolar dense fibrillar component of neural stem cells and neurons*

Based on the finding of Nop2 protein in some neurons, astrocytes and neural stem cells it was necessary to precisely define the subcellular localization of this protein.

For this purpose, we performed immunoelectron microscopy on neural stem cell cultures and on primary cultures of 8 days

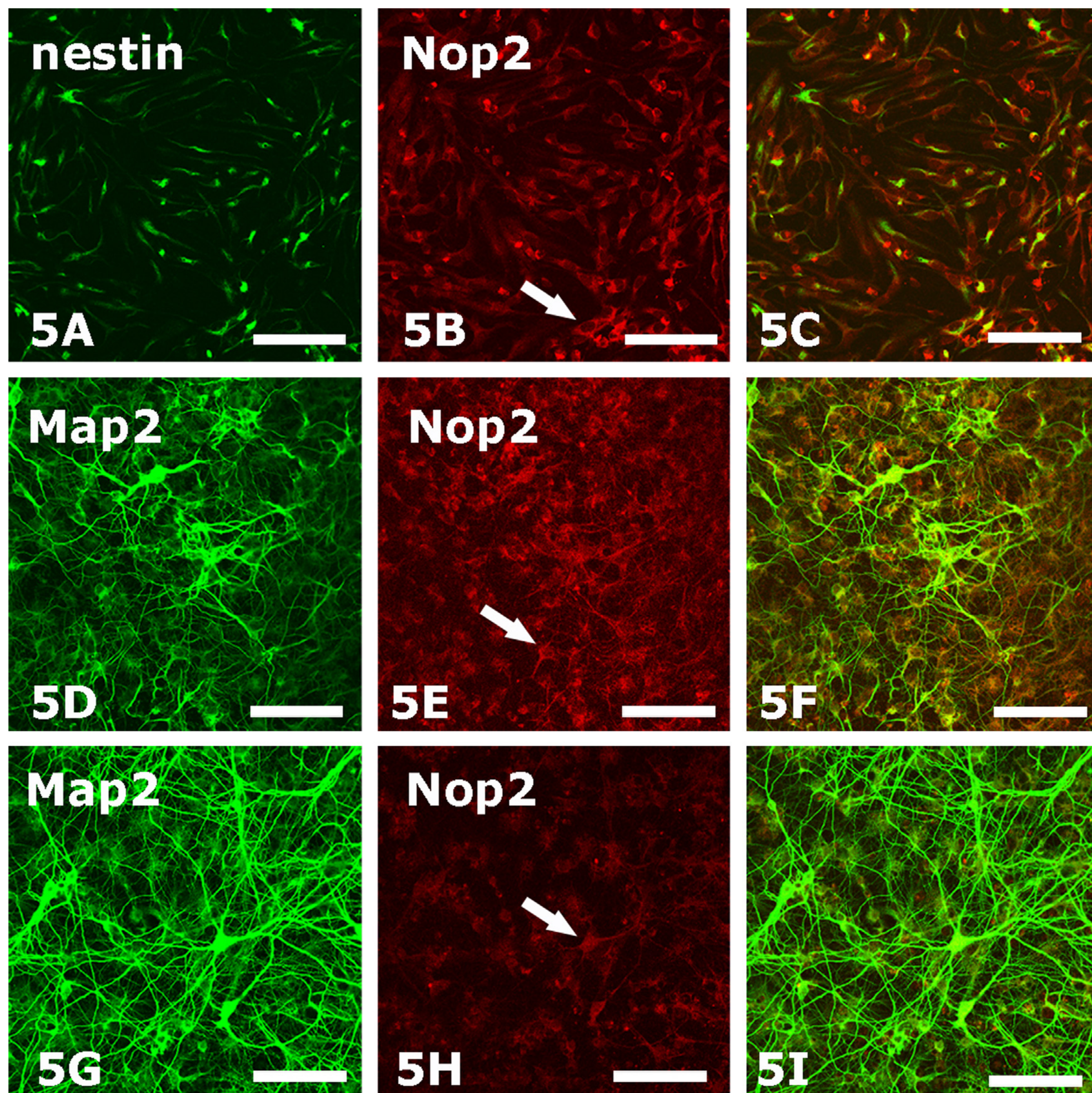

Fig. 5 – Nop2 protein was detected in cultured neural stem cells as well as differentiating neurons. Double immunohistochemical reaction against nestin (A) and Nop2 (B) on mouse neural stem cell cultures (day 0: A–C, day 3: D–F, day 9: G–I) revealed that some neural stem cells express Nop2 (white arrows). Double immunohistochemical reaction against Map2 (D and G) and Nop2 (E and H) revealed that during differentiation of neural stem cells Nop2 expression was initially visible in the perinuclear region (3 days of differentiation, (D–F)) and then in nuclear region of neurons (9 days of differentiation, (G–I)). Magnification bar: 100 μm.

old neurons. By isolation of the cortex of newborn mice and their cultivation in a medium highly selective for nerve cells, we obtained a nearly 100% homogeneous culture of neurons that served as a test for the presence of Nop2 in these cells.

Immunogold electron microscopy on neural stem cells revealed Nop2 protein in the dense fibrillar component of the nucleolus, suggesting a potential role of Nop2 in the synthesis of ribosomes (Fig. 9A). Immunoelectron microscopy of primary neuronal cultures confirmed this finding (Fig. 9B).

3. Discussion

Based on its expression linked to the cell cycle, we suspected that Nop2 might serve as a marker of neural stem cell proliferation, both in vitro and possibly, in the brain, after ischemic injury. In this work, we confirmed our hypothesis. Neural stem cells express Nop2 highly and the expression decreases during cell differentiation. Moreover, after onset of ischemic brain damage, many reactive nestin-positive cells co-express Nop2. These cells are as well BrdU positive. However, unlike in the majority of adult tissue, such as liver, lung or kidney, where Nop2 is not expressed, we found that this gene is active in many post-mitotic cells of the healthy mature mouse and human brain: these cells were mostly present in the supragranular and infragranular layers of the somatosensory cortex, in the cingulum and in the CA1 region of the hippocampus.

The observation that the majority of Nop2 positive cells are mature differentiated neurons fits with known role of Nop2 in the synthesis of ribosomes. Indeed, Nop2 binds the 28S subunit of the ribosome through its arginine rich domain (Woolford and Warner, 1991). Our immunoelectron microscopic data

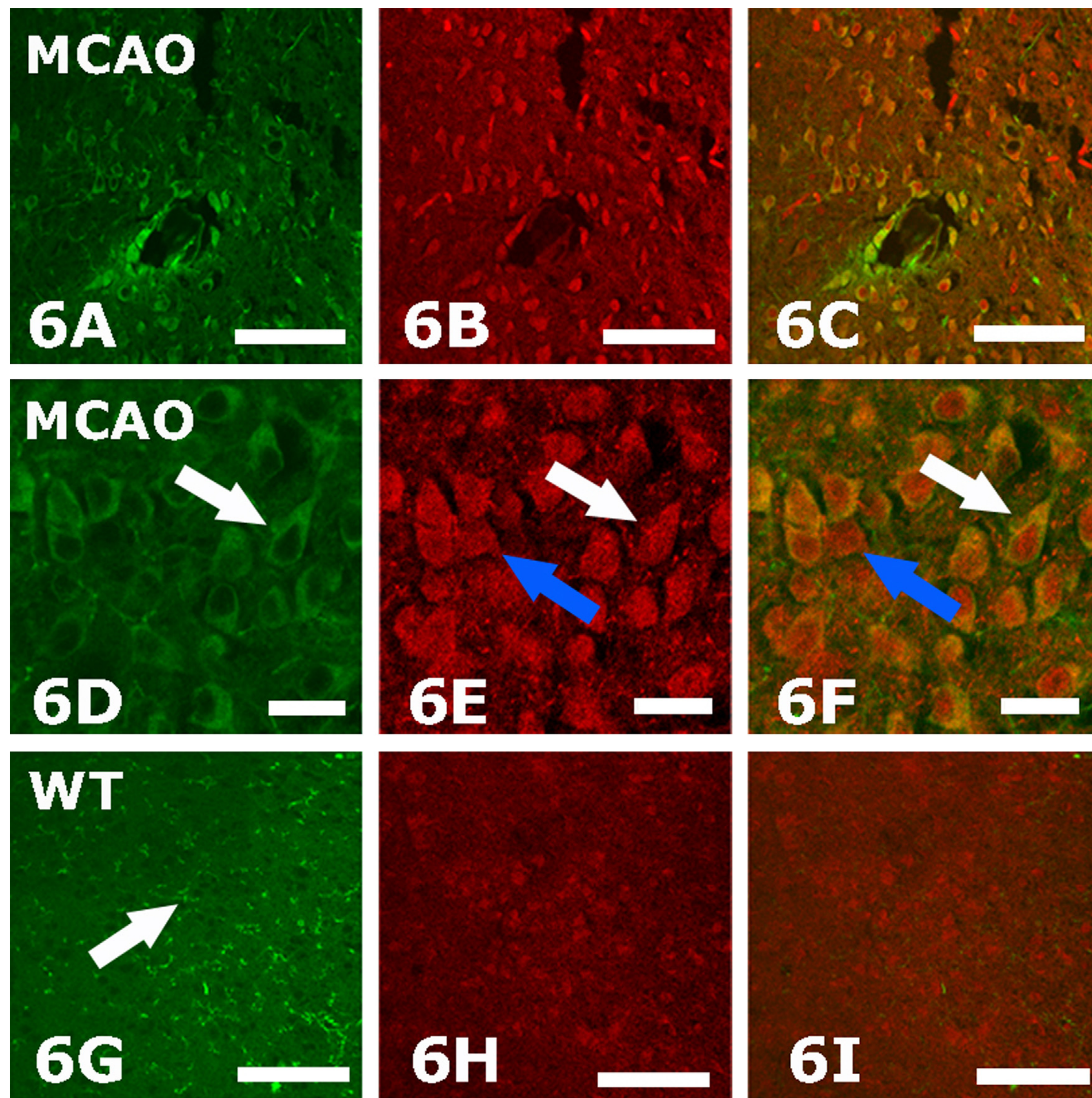

Fig. 6 – The number of Nop2/nestin double positive cells increased in the adult mouse brain affected by stroke. Double immunohistochemical reaction against nestin (A and D) and Nop2 (B and E) in the region affected by stroke in the mouse brain revealed numerous nestin/Nop2 double positive round shaped cells (white arrow). Some Nop2 positive cells were not nestin positive (blue arrow). In the healthy mouse brain nestin positive cells were spindle shaped (6 G, arrow), resembling astrocyes (G–I). The majority of these nestin positive cells were not Nop2 positive. Magnification bar: (A–C and G–I) 100 μm; (D–F) 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

further validates this. However, it remains unclear why many neurons in the same or other areas of the brain are Nop2 negative, and why some cells with intensive synthesis of ribosomes (e.g. hepatocytes) do not express Nop2.

Our finding that after stroke, many cells co-express Nop2 and nestin, a marker of neural stem cells, and are simultaneously BrdU positive, turned our attention towards brain regeneration and its correlation to the cell cycle. Indeed, Nop2 is linked to the cell cycle regulation (Fonagy et al., 1992, 1993; Freeman and Busch, 1991), and intensity of its expression is a reliable indicator of the rate of proliferation of both normal proliferative and malignant cells, thus indicating a worse prognosis of tumors. This has shown for tumors of the prostate, breast, lung and oral cavity (Bantis et al., 2004; Fonagy et al., 1994; Uchiyama et al., 1997; Ventura et al., 1999). Accordingly, it was proposed to block Nop2 with a goal to slow down tumor growth (Busch et al., 1991). Our finding that a complete lack of Nop2 is lethal is possibly linked to an inability of cells to multiply in its absence.

Nop2 expression in mature neurons, given that other postmitotic cells are apparently devoid of the protein, may indicate a new, so far unknown role for Nop2, exclusively linked to the central nervous system. The emergence of new cells (neurogenesis) in the brain of mammals is still a topic under investigation. So far, neurogenesis in most mammals has been found within the subventricular zone, the olfactory bulb and the hippocampus, with the phenomenon generally less robust in humans. In the human brain, there is reliable proof of neurogenesis only in a very limited region next to the ventricles (Curtis et al., 2012). On the other hand, very little is

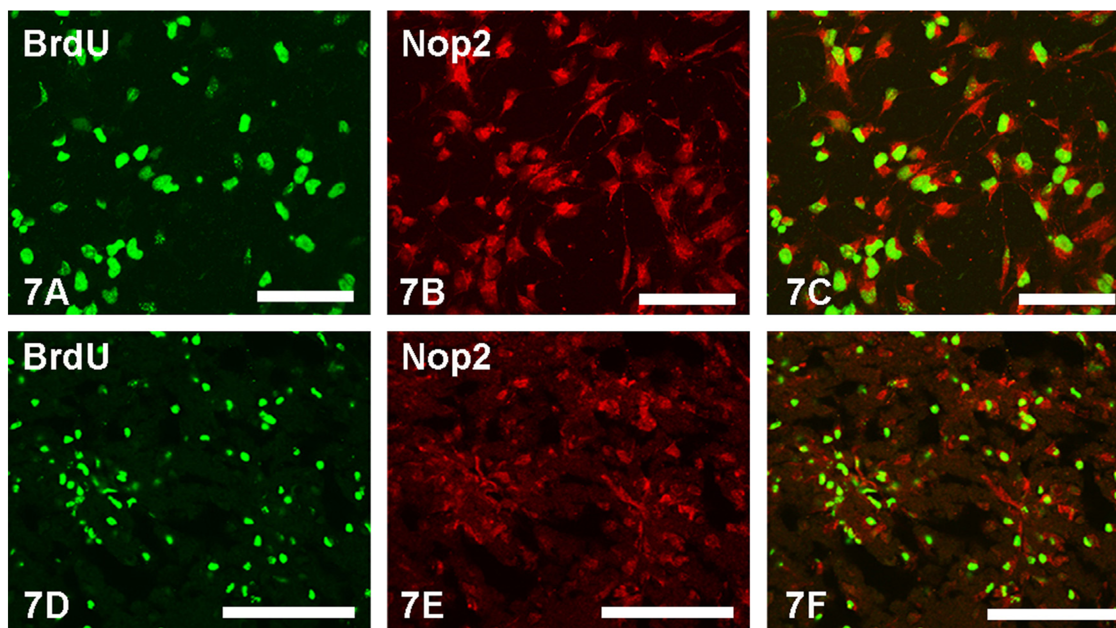

Fig. 7 – The majority of Nop2 positive cells in both in vitro cultures and in the mouse brain after stroke were BrdU positive. Double immunohistochemical reaction against BrdU (A and D) and Nop2 (B and E) in in vitro cultures (A–C) and in the mouse brain region affected by stroke (D–F) revealed that more than 90% Nop2 positive cells were, simultaneously, BrdU positive. The only cells which were Nop2+/BrDU—resembled neurons at a higher stage of differentiation with elongated processes. Magnification bar: 100 μm.

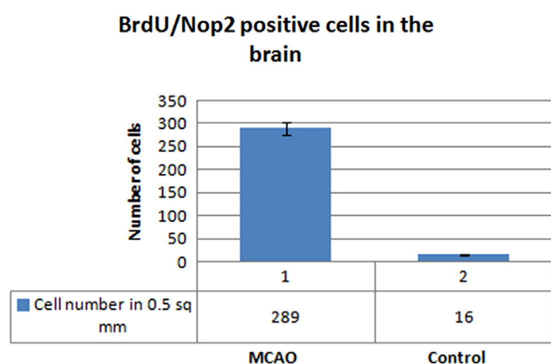

Fig. 8 – Number of Nop2/BrdU positive cells in the brain affected by stroke increased by a factor of 18 as compared to the healthy mouse brain.

known about processes occurring during disease or brain injury: there are suggestions that a pathological condition might bring reactivation of expression of certain genes that are normally specific for neural stem cells during embryonic development (Garcia et al., 2004).

Taking into account the role of Nop2 in the cell cycle, our finding that only some neurons and some astrocytes express Nop2 may indicate either different levels of expression between individual cells or the presence of specific subpopulations of cells dedicated to tissue repair in the case of tissue injury. This is strongly supported by our finding that the majority of Nop2 positive cells after stroke express nestin and are BrdU positive. Similar findings on the expression of nestin after onset of stroke was recently published (Shin et al., 2013).

In conclusion, histochemical and immunohistochemical analysis, double checked by several markers for neurons and astrocytes in the mature mouse and human brain has convincingly demonstrated that Nop2 is expressed and its protein is present in some neurons and astrocytes of the cerebral cortex, thalamus and hippocampus and in Purkinje cells of the cerebellum. We did not detect any oligodendrocytes or microglia positive for Nop2. Light and electron microscopy revealed that Nop2 in the adult mouse brain is located mainly in the nucleus and nucleoli, and to a smaller extent in the perikarya of neurons and in the initial segment of the axon. Furthermore, after the onset of brain damage, the number of Nop2 cells strikingly increased. The majority of those Nop2 positive cells in the region affected by stroke also expressed nestin, which suggested a possible link between Nop2 expression and the regenerative potential of the brain tissue. Based on the proven role of Nop2 in the transition from the inactive to the active phase of the cell cycle and the synthesis of ribosomes, and based on our own discovery of expression of Nop2 during the proliferation and differentiation of neural stem cells, both in normal and pathological conditions, we suggest that Nop2 should be further explored as a marker of proliferative and regenerative potential of the cells within nervous tissue.

4. Experimental procedures

4.1. Nop2 transgenic mouse strain (*Nop2*^{gt1gaj})

A transgenic mouse strain with a modification in the Nop2 gene (*Nop2*^{gt1gaj}) was obtained by gene trap. The method involves the insertion of a non-homologous DNA vector which

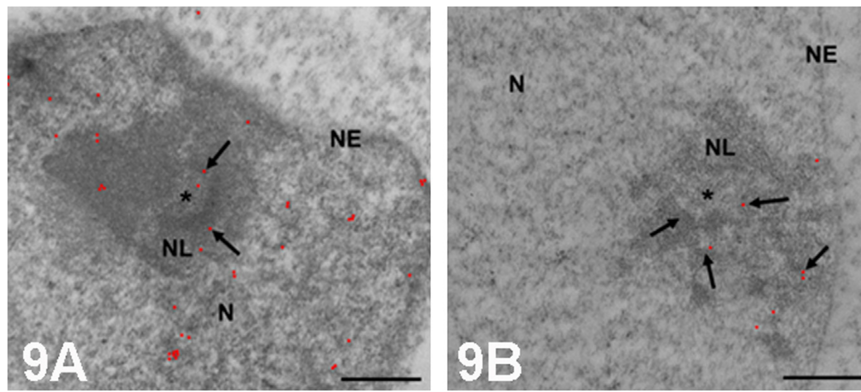

Fig. 9 – Immunogold labelling revealed the presence of Nop2 in the nucleolus and nucleoplasm of cultured neural stem cells. Immunoelectron microscopy image of a cultured neural stem cell (0 day) labelled with anti-Nop2 antibody shows the location of Nop2 protein (red dots) both in the dense fibrillar component (arrows) of the nucleolus (NL) and in the nucleoplasm (A). Nop2 protein is present in cultured 8 day old neurons mainly in the dense fibrillar component of the nucleolus (B). N—nucleus, NE—nuclear envelope; the asterisk points out the fibrillar center. Magnification bar: 500 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

carries a selector (NeoR) and marker (lacZ) in embryonic stem cells. The altered Nop2 gene is marked by the lacZ gene and its expression can be monitored via lacZ activity (Mitrečić et al., 2008).

All experiments on both transgenic and wild type animals described in this work received approval of the Internal Review Board of the Ethical Committee of the School of Medicine, University of Zagreb. All experiments were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

4.2. *Genotyping Nop2 strain*

In order to genotype the mice, two pairs of primers were used (common primer for both Wt and mutant sequence: 5′TCT GCC TGC CTT GTT TCT T3′, transgene sequence primer: 5′CGC CAT ACA GTC CTC TTC AC3′ and wt primer: 5′GGA CAG GAC CCT CCT TAG A3′). The length of the amplified DNA segment of the first pair, specific for transgenic construct is 370 bp. The length of the amplified DNA segment of the second pair, specific for the wild type construct, is 400 bp.

4.3. *Animal sacrifice, perfusion, fixation and isolation of the brain*

Ten male transgenic mice (*Nop2^{gt1gaj}*) and two wild type males (C57Bl/6, Charles River) were anesthetised via intraperitoneal injection of 2.5% tribromethanol (Avertin). Systemic circulation was first washed with 50 ml phosphate buffered saline (PBS) followed by fixation. Mice designated for β-galactosidase histochemical staining (five mice of the Nop2 strain and two mice of the C57Bl/6 strain) were fixed with 30 ml of fixative consisting of 2% formaldehyde and 0.2% glutaraldehyde. Mice designated for immunohistochemical staining (five mice of the Nop2 strain and two mice of the C57Bl/6 strain) were fixed with 20 ml of 4% paraformaldehyde. Brain, heart, kidney and liver were isolated. After isolation of organs, tissue was further left in a fixative overnight. In order to isolate neural stem cells, 14.5 day pregnant females were used. These animals were sacrificed by cervical dislocation.

4.4. *Middle cerebral artery occlusion (MCAO)*

Ischemic brain injury was induced by transient left middle cerebral artery occlusion (MCAO) in adult C57Bl/6 mice, age 2 months and weight 20 to 25 g. Animals were anesthetized with 2% isoflurane in 100% O_2. During surgery, animal body temperature was maintained with a heating pad. Following a ventral midline incision, the common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed and dissected. An intraluminal monofilament (Doccol Corporation) was inserted through the ECA into the ICA and advanced to the origin of middle cerebral artery where it was left in place for 60 min. After 60 min the intraluminal monofilament was withdrawn and perfusion was restored. After surgery mice were kept in heated cages for 24 h.

4.5. *X-gal staining*

After 2% formaldehyde and 0.2% glutaraldehyde fixation, the brain and other tissues (liver, kidney, lungs) designated for X-gal staining were quickly frozen at −80 °C and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek). Sections were cut at 60 μm with a Leica cryostat and mounted on Superfrost slides (Fischer Scientific). Mounted sections were washed in PBS and placed at 37 °C overnight in a hybridization oven in a solution containing X-gal as a substrate for β-galactosidase (0.5 mg/ml X-gal, 2 mm $MgCl_2$, 10 mm $K_4Fe(CN)_6$, 10 mm $K_3Fe(CN)_6$ in PBS).

4.6. *BrdU administration*

Ten adult C57Bl/6 mice (five MCAO mice and five control mice) were used for the BrdU experiment. BrdU solution (Sigma-Aldrich, 5-Bromo-2′-deoxyuridine (BrdU) ≥99% (HPLC), Cat. no. B5002-1G) was prepared in sterile PBS, concentration 2 mg/ml. Twenty four hours after MCAO all animals were intraperitoneally

injected with 50 μg/g BrdU solution. Animals were injected every 12 h throughout 10 days. Twenty four hours after the last injection animals were sacrificed and brain sections were prepared for immunohistochemical staining. Before immunohistochemical staining brain sections were incubated with 10 mM sodium citrate, pH 6.0 at 97 °C/15 min. For immunohistochemical staining we used the primary antibody BrdU (Bu20a) Mouse mAb (Cell Signaling, CST-5292S-100 μl, Cat. no. 00011534).

For BrdU labelling in vitro the cells were incubated with 10 μM BrdU solution for 8 h, after which the medium was aspirated, replaced with fresh medium and cells were placed on special substrates containing poly-D-lysine and laminin. After fixation cells were prepared for immunohistochemical staining.

4.7. *Immunohistochemical staining of mouse tissue*

Tissue fixed in 4% paraformaldehyde was first passed through graded sucrose solution 10% and 30%. Afterwards it was embedded in Tissue-Tek OCT Compound (Sakura Finetek), cut at 20 μm with a Leica cryostat and mounted on Superfrost slides (Fischer Scientific). Mounted sections were then rinsed in PBS, permeabilized in 0.2% Triton and blocked with a blocking solution (5% goat serum, 0.2% Triton, PBS). After blocking, sections were incubated with primary antibody solution (2% goat serum, 0.2% Triton, primary antibody, PBS) overnight at 4 °C. Next day the sections were first washed in PBS and then incubated in secondary antibody solution for 2.5 h at room temperature. Primary antibodies used were following: chicken anti-*βgal* (Abcam, ab9361), mouse anti-NeuN (Millipore, MAB377), chicken anti-GFAP (Abcam, ab4674), chicken anti-Map2 (Abcam, ab5392), mouse anti-nestin (Millipore, MAB353), rabbit anti-Nop2 (Santa Cruz Biotechnology, sc-292098) and mouse anti-BrdU (Cell Signaling, CST-5292S-100 μl, Cat. no. 00011534). For secondary antibodies, either Alexa Fluor® 488 or 546 antibodies were used, depending on the primary antibody. Sections were analyzed and recorded with confocal microscope Zeiss LSM 510 META.

4.8. *Immunohistochemical staining of the human tissue*

After dewaxing in alcohol, and rehydrating in PBS, the sections were pre-treated for 20 min in 0.3% hydrogen peroxide in a 3:1 mixture of methanol and re-distilled water, washed for 10 min in PBS, and immersed for 2 h in the blocking solution (PBS containing 3% bovine serum albumine, BSA and 0.5% Triton X-100, all from Sigma, St. Louis, MO) at room temperature to prevent non-specific background staining. Sections were then incubated with primary antibody for 18 h at 4 °C, washed again, and further incubated with secondary biotinylated anti-mouse or anti-rabbit antibodies, respectively, diluted in blocking solution (1:200) for 1 h at room temperature (Vectastain ABC kit, Vector Laboratories, Burlingame, USA). Afterward, sections were incubated in Vectastain ABC reagent (streptavidin-peroxidase complex) for 1 h at room temperature, rinsed in PBS for 10 min, and finally, the peroxidase activity was visualized with Ni-3,3-diaminobenzidine (Sigma, St. Louis, MO). Sections were dehydrated in a graded series of alcohol, cleared in xylene, and cover-slipped with Histamount (National Diagnostic). Negative controls were included in all immunohistochemical experiments: (i) by replacing the primary antibody with blocking solution, or preimmune goat or horse serum; or (ii) by omitting the secondary antibody or replacing it with an inappropriate secondary antibody. No immunolabeling was detected in the control sections. Qualitative analysis of stained sections was performed using an upright microscope Olympus Provis AX70, and images were captured with a Nikon DXM1200 digital camera.

4.9. *Immunoelectronic microscopy*

The procedure for immunoelectronic microscopy involved fixation in 3% PFA and 0.1% GA in Sörensen's phosphate buffer (SB), pH 7.2–7.4) cell medium. After washing in phosphate buffer SB, dehydration was performed in ascending series of ethanol alcohol with a gradient (30, 50, 70, 90, 96% ethanol). The cells were embedded in LR White with a gradual increase in gradient. Finally, the cells were left in 100% LR White overnight. Polymerization was performed in UV light for 48 h. After cutting the 70–80 nm thick thin sections, immunogold labeling was performed by the standard procedure (Sobol et al., 2011). Briefly, rabbit polyclonal anti-NOP2 antibody was used as a primary antibody followed with goat anti-rabbit IgG (H+L) antibody coupled with 12 nm colloidal gold particles (Jackson ImmunoResearch Laboratories Inc). The sections were examined using a FEI Morgagni 268 transmission electron microscope operated at 80 kV, and the images were captured with Mega View III CCD camera. Multiple sections of at least three independent immunogold labeling experiments were analyzed. To facilitate the visualization of the immunolabeling in images, Adobe Photoshop CS3 was used to identify the geometrical centres of 12 nm gold particles and cover them with red dots.

4.10. *Culture of stem cells and neurons*

In order to verify and compare the expression of Nop2 in neural stem cells and mature neurons we used both neural stem cell and primary neuronal cultures. For neural stem cell isolation pregnant wild-type C57Bl/6 females were sacrificed. On the 14.5th day of pregnancy, embryos were isolated and parts of the telencephalic wall of 14.5 days old embryos were dissociated using Accutase (Gibco). After a series of centrifugations and filter purification, large numbers of homogeneous and equally large round stem cells were obtained, which were then placed in a specific proliferation supporting medium comprising of: DMEM/F12, GlutaMAX (Gibco), the growth factors EGF and FGF2 (Gibco), factors B27 and N2 (Gibco), and antibiotics (Penicillin and Streptomycin, PAA). After cultivation for a few days, neurospheres formed. Dissociated neurospheres were placed on special substrates containing poly-D-lysine and laminin (Sigma) to enable differentiation of neurons. At the same time primary neuronal culture was obtained from newborn mice cortices. Isolated cells were cultured in a neuron-specific media: Neurobasal (Gibco), factors B27 and N2 (Gibco), glutamine (PAA) and antibiotics (Penicillin and Streptomycin, PAA), which enabled the growth of a culture with a high percentage of neurons. In parallel, the

isolation of the cortex of newborn mice was performed and a primary culture established in a neuron-specific media, which enabled the growth of a culture of pure neurons.

4.11. *Human brain tissue sources and preparation*

Human brain samples were obtained during obligatory autopsy procedures, approved by the Internal Review Board of the Ethical Committee of the School of Medicine, University of Zagreb, in accordance with the Declaration of Helsinki 2000, after written consent of legal representatives of the human subjects. Human subjects were aged 27 years, 36 years and 39 years, had no neurological, psychiatric or psychological medical records, and had died suddenly in accidents that did not included brain trauma. The brain tissue was fixed for 24 h in 4% phosphate-buffered paraformaldehyde (0.1 M phosphate buffer, pH 7.4), and small tissue blocks were embedded in paraffin and cut in 12 μm-thick serial sections in the coronal plane. To delineate cytoarchitectural boundaries and cellular compartments of the telencephalic regions, every tenth section of the series was stained by the Nissl method (not shown).

4.12. *Cell counting*

For the purpose of cell quantification, we used an approach in which positive cells were marked using the Photoshop CS5 application in which cells were enumerated and subsequently their number was declared as an absolute count in 0.5 mm^2. For this purpose we analyzed 8 brains, surveying a volume of approx. 5 mm^3 for each brain.

Conflict of interest

Authors have no conflict of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2014.11.040.

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