Pannexin 2 Is Expressed by Postnatal Hippocampal Neural Progenitors and Modulates Neuronal Commitment

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The pannexins (Panx1, -2, and -3) are a mammalian family of putative single membrane channels discovered through homology to invertebrate gap junction-forming proteins, the innexins. Because connexin gap junction proteins are known regulators of neural stem and progenitor cell proliferation, migration, and specification, we asked whether pannexins, specifically Panx2, play a similar role in the postnatal hippocampus. We show that Panx2 protein is differentially expressed by multipotential progenitor cells and mature neurons. Both in vivo and in vitro, Type I and IIa stem-like neural progenitor cells express an S-palmitoylated Panx2 species localizing to Golgi and endoplasmic reticulum membranes. Protein expression is down-regulated during neurogenesis in neuronally committed Type IIb and III progenitor cells and immature neurons. Panx2 is re-expressed by neurons following maturation. Protein expressed by mature neurons is not palmitoylated and localizes to the plasma membrane. To assess the impact of Panx2 on neuronal differentiation, we used short hairpin RNA to suppress Panx2 expression in Neuro2a cells. Knockdown significantly accelerated the rate of neuronal differentiation. Neuritic extension and the expression of antigenic markers of mature neurons occurred earlier in stable lines expressing Panx2 short hairpin RNA than in controls. Together, these findings describe an endogenous post-translational regulation of Panx2, specific to early neural progenitor cells, and demonstrate that this expression plays a role in modulating the timing of their commitment to a neuronal lineage.

Neural stem and progenitor cells (NPCs)3 in the hippocampal formation generate new granule neurons throughout adult life. Multipotential Type I and Type IIa NPCs located in the subgranular zone of the dentate gyrus give rise to intermediate progenitor populations (Type IIb and Type III) before terminal neuronal differentiation (reviewed in Ref. 1; see also the schematic in Fig. 4B). The physiological production (and deletion) of these new neurons is implicated in the consolidation of spatial learning and memory (reviewed in Ref. 2), whereas aberrant or impaired neurogenesis is associated with multiple neurological and cognitive disorders, including epilepsy, mood disorders, neurodegenerative diseases, and ischemia (reviewed in Refs. 3 and 4).

The signaling systems that maintain multipotential NPC populations in their “stem-like” state within the adult hippocampal network have only begun to be elucidated (2). One mechanism, the expression of ion-permeable single membrane (ion channels and gap junction hemichannels) as well as double-membrane (gap junction intercellular) channels, is implicated in the control of embryonic and postnatal NPC migration, proliferation, and specification (5–11). Channel proteins, in part, regulate metabolic coupling, adhesion, and calcium wave propagation between NPCs and adjacent terminally differentiated cells (5–11). Pannexins are a family of membrane channel proteins discovered through their homology to the invertebrate family of gap junction proteins, the innexins. Panx1 is expressed in multiple tissues. Panx2 is enriched in the central nervous system. Panx3 is found in skin and cartilage (12–14). Given their sequence homology with innexins and their structural homology with connexins, pannexins were originally proposed to form intercellular channels; however, recent studies suggest that Panx1 and Panx3 more likely form single membrane channels, with Panx1 channels permeable to ions, ATP, and arachidonic acid-related lipids (15–23) (reviewed in Ref. 24).

Little is known about the biology of central nervous system expressed Panx2. Intriguingly, Panx2 mRNA is detected only in postnatal tissue (25). In the hippocampus, Panx2 mRNA levels increase during the peak period of granule cell neurogenesis (25). Although Panx2 is primarily detected in terminally differentiated neurons, transient expression is observed in glial fibrillary acidic protein (GFAP)-positive cells following brain injury (25–28). No physiological function has yet been ascribed to Panx2 beyond reports that, upon heterologous expression in Xenopus oocytes, Panx2 physically interacts with Panx1 to reduce current amplitude (12, 29). Upon ectopic expression in short hairpin RNA; siRNA, small interfering RNA; RIPA buffer, radioimmunoprecipitation assay buffer; Endo H, endo-β-N-acetylglucosaminidase H.
HEK293 cells, only a small increase in dye uptake is observed in comparison with Panx1 and Panx3 transfection (30).

In this study, we asked whether Panx2 is expressed by postnatal NPCs and whether its expression plays a role in regulating neurogenesis. Using both primary NPC cultures and Neuro2a (N2a) cells to provide functional insight, we show that Panx2 protein expression and post-translational modification modulates the timing of NPC commitment to a neuronal lineage.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary NPC cultures were isolated from postnatal day 3 (P3) C57BL/6 mouse hippocampus and expanded as neurospheres for 14 days in vitro (DIV) as described (10). Hippocampal neurons were cultured as previously described (31) for 7 DIV. N2a cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells (1.8 × 10⁶ cells/60-mm diameter dish) were induced to differentiate to a neuronal phenotype in low serum medium (2% fetal bovine serum) supplemented with 10 μM retinoic acid (32). To label cell membranes, cells were treated with 5 μg/ml Alexa-488-conjugated wheat germ agglutinin (Invitrogen/Molecular Probes) for 5 min at 37°C in Hanks’ buffered salt solution (137 mM NaCl, 5.4 mM KCl, 0.25 mM NaH₂PO₄, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃), washed in Hanks’ buffered salt solution, and fixed and processed for immunostaining as described below. In loss-of-function experiments, a Panx2-specific (accession number NM_001002005.2) siRNA target sequence (AAGAGCAACTTCATCCTTGAGACCATCCGCCAC-3’) and 5’-GGTGAGCAGCATGGAATGA-3’ defining a 269-bp PANX2 amplicon (accession number NM_001002005.2) and 5’-TTGTGCTGAGTGTTTGCAGTAGTGATGG-3’ defining a 292-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplicon (accession number NM_008084.2).

**Protein Analyses**—Murine hippocampus and cortex were isolated from P0–P90 C57BL/6 mice, P3 NPCs cultured as neurospheres for 14 DIV, N2a cells cultured in complete medium, or N2a cells differentiated for 48 h to a neuronal phenotype. Western analysis was performed as described (10). All procedures were carried out in agreement with the guidelines of the Canadian Council for Animal Care and the University of Ottawa Animal Care Committee. Samples were homogenized in RIPA buffer (10 mM PBS, 150 mM NaCl, 9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 30 μl/ml aprotinin, 10 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonl fluoride, and 1 μM NaF) for 30 min and centrifuged for 20 min at 12,000 rpm to remove debris. To assess N-linked glycosylation, lysates were further incubated in 0.5% SDS, 40 mM dithiothreitol and denatured by boiling for 10 min. This protocol also partially depalmitoylates target proteins without significant impact on other post-translational modifications (33, 34). Aliquots of these same lysates were incubated with or without 40 units of N-glycosidase F (New England Biolabs, Pickering, Canada) in a buffer containing 1% Nonidet P-40 and 50 mM sodium phosphate, pH 7.5, for 1 h at 37°C. A similar protocol was used for Endo H treatment. For dephosphorylation experiments, cells were lysed in a Tris-based RIPA buffer (50 mM Tris-HCl (pH 9.0 at 37°C) and 10 mM MgCl₂ supplemented with 1% Nonidet-P40, 0.1% SDS, 30 μl/ml aprotinin, 100 μg/ml phenylmethylsulfonl fluoride, and 1 μM NaF) and treated without or with 20 units of shrimp alkaline phosphatase (Promega, Madison, WI) for 1 h at 37°C. For Western blots, SDS-PAGE was performed under reducing conditions (dithiothreitol and β-mercaptoethanol) without heating. Palmitoylation was directly assessed by treatment of RIPA lysates with buffer (10 mM PBS) or 1 M hydroxylamine, pH 7.4, a chemical depalmitoylating agent that cleaves thioester linkages, for 20 min at room temperature, followed by Western blot analysis as described (15). Palmitoylation was further quantified by metabolic labeling, immunoprecipitation, and depalmitoylation essentially as described (36) with the following modifications. Neurospheres were labeled with 10 μCi BODIPY FL hexadecanoic acid (37) (Invitrogen/Molecular Probes) for 1 h at 37°C in 95% O₂, 5% CO₂ followed by lysis in RIPA buffer and immunoprecipitation with polyclonal anti-Panx2 C-term (Zymed Laboratories Inc./Invitrogen). Immunoprecipitation was performed as described (38). After washing with RIPA buffer followed by PBS or 1 M hydroxylamine, pH 7.4, lipids were extracted using a modified Bligh and Dyer protocol (39).
Chloroform extracts were evaporated under a constant stream of N₂ gas, lipids were resuspended in ethanol, and the concentration of BODIPY FL-C16 released from immunoprecipitates was measured using a BioTek fluorescence microplate reader referenced to a standard curve (485-nm excitation/528-nm emission; BioTek, Winooski, VT). Data were expressed as pM of BODIPY FL-C16/immunoprecipitate.

Primary antibodies used for Western blots were polyclonal anti-Panx2 (1:800; Aviva Systems Biology, San Diego, CA) previously characterized (26) (supplemental Fig. S1), monoclonal anti-GAPDH (1:2000; Imgenex, San Diego, CA), mouse monoclonal anti-Tuj1 (1:10000; Fitzgerald Industries, Concord, MA), mouse monoclonal anti-Sumo-1 (1:1000; Zymed Laboratories Inc./Invitrogen), mouse monoclonal anti-NeuN (1:500; Millipore/Chemicon, Temecula, CA), polyclonal anti-phospho-GSK3β (glycogen synthase kinase 3β, phospho-Ser9) (1:1500; Cell Signaling Technology, Danvers, MA), and polyclonal anti-transferrin (1:2000; Assay Designs/Stressgen, Ann Arbor, MI). Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit and mouse IgGs) diluted 1:2000 were incubated in secondary antibodies: Cy3-conjugated anti-rabbit IgG (1:600), fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:100), Cy3-conjugated anti-guinea pig IgG (1:800), fluorescein isothiocyanate-conjugated anti-mouse IgG (1:100), Cy5-conjugated anti-mouse IgG (1:400; Jackson ImmunoResearch). Hoechst 33258 (1 µg/ml) was used as a nuclear counterstain. Confocal immunofluorescence imaging was performed on a Zeiss 510 META laser-scanning confocal microscope, and images were captured with Zeiss LSM 5 software version 4.0 SP2 using EC plan-neofluor ×40/1.30 oil DIC M27 or plan-apochromat ×63/1.4 oil DIC M27 objectives with a pinhole size of 1 Airy unit (or equal optical slices for multiple fluorophores) through z-stacks in 0.5-µm optical sections (Zeiss Canada, Toronto, Canada). Three-dimensional digital reconstruction and AVI movies were rendered using Zeiss LSM 5 software version 4.0 SP2 and Axiovision software version 4.7.

Flow Cytometry—NPCs in neurosphere cultures were disaggregated into single cells in versene (Invitrogen) before fixation in 2% formaldehyde. Cell suspensions were permeabilized in 2% fetal bovine serum (PBS/fetal bovine serum) supplemented with 0.18% saponin. Primary antibodies were added directly to aliquots of 10⁵ cells at the following concentrations: Panx2, 1:50 (Aviva Systems Biology); nestin, 1:20; GFAP, 1:20. Following several washes, cells were incubated in secondary antibodies: fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:25), Cy5-conjugated anti-mouse IgG (1:25), and R-phycocerythrin-conjugated anti-rat IgG (1:25; AbD Serotec, Raleigh, NC). Analysis was performed on a Beckman Coulter FC500 flow cytometer with CXP software (Beckman Coulter Canada, Mississauga, Canada).
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**RESULTS**

Panx2 Protein Is Expressed by Type I NPCs in the Neonatal Hippocampus—Hippocampal Panx2 protein levels were assessed by Western blotting between postnatal day 0 (P0) and P90 (Fig. 1). In P0–P30 hippocampus, multiple Panx2 species were observed ranging from 60 to 85 kDa (Fig. 1A, black and white arrowheads). An increase in protein expression was evident at P7. In adult brain (P90), the predominant Panx2 species migrated as a single ~60 kDa band (Fig. 1, lower black arrowhead).

Pyramidal neurons of CA fields of the hippocampus are born embryonically, whereas the majority of granule neurons in the dentate gyrus are born between P3 and P10 (41, 42). Neuronal maturation and synaptogenesis is complete by P30 (43–46). To identify Panx2-expressing cells during the peak period of neurogenesis, double immunolabeling was performed on P7 hippocampal tissue for Panx2 and antigenic markers expressed by stem-like multipotent Type I NPCs as well as mature neuronal markers. In addition to the expected expression in neurons, we found discrete Panx2+ punctae localized to nestin+/GFAP+ Type I NPCs in the developing dentate gyrus (Fig. 1B).

*Postnatal NPCs Intrinsically Express Panx2 in Vitro*—To assess whether Type I hippocampal NPCs intrinsically express Panx2 protein or whether expression depends upon the extrinsic cues provided by the postnatal hippocampal network (47), Type I NPCs were removed from P3 hippocampi and cultured for 14 DIV in suspension (Fig. 2A). Both Panx2 mRNA (Fig. 2B) and protein (Fig. 2C) were readily detected in neurosphere cultures on DIV 14. Panx2 migrated as a ~60-kDa species in both NPC cultures and adult tissue (Fig. 2B, black arrowhead). Protein expression was elevated in adult tissue compared with NPC cultures (Fig. 2B). As observed in early postnatal hippocampal tissue lysates (Fig. 1A), a higher molecular mass Panx2 protein species (~85 kDa) was also observed in NPC cultures that was not present in adult tissue (Fig. 2C, white arrowhead). Alternative splicing was ruled out as a potential source of the NPC-specific Panx2 species by Northern blotting (data not shown).

Confocal immunofluorescence revealed a similar punctate expression profile in cultured NPCs (Fig. 2D) as observed in vivo (Fig. 1B), confirmed using two Panx2 antibodies directed against two different epitopes, in the first extracellular loop and in the C-terminal intracellular tail, respectively (Fig. 2D and supplemental Fig. S1A). Panx2 invariably appeared as prominent intracellular punctae (Fig. 2D and supplemental Fig. S1B). At higher magnification, these punctae were found to form perinuclear oblong structures ~1.5 μm in length with a flattened rod-like morphology (supplemental Movies M1 and M2).

Panx2 Is S-Palmitoylated in NPCs—To characterize Panx2 protein expressed in NPCs, we performed biochemical tests for multiple putative post-translational modifications. Online prediction tools identified potential Panx2 phosphorylation, sumoylation, N-glycosylation, and palmitoylation sites that...
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FIGURE 3. NPCs express an S-palmitoylated Panx2 species. Protein lysates were prepared from P3 neurosphere cultures on DIV 14. A, the mobilities of the ∼85-kDa (white arrowhead) and ∼60-kDa species (black arrowhead) were not markedly altered by treatment with alkaline phosphatase (AP). Blots were stripped and reprobed with anti-phospho-GSK3β (Ser9) (pGSK-3β) polyclonal antibody to confirm efficient dephosphorylation (bottom panel). B, the ∼85-kDa species is not N-glycosylated. i, for deglycosylation experiments, lysates were either incubated at 4 °C (first lane) or boiled in the presence of SDS and dithiothreitol (DTT) and incubated at 37 °C with control buffer (second lane) or N-glycosidase F (third lane). Conditions that promote partial depalmitoylation (second and third lanes) induced a shift in the mobility of the ∼85-kDa Panx2 species (white arrowhead) to the ∼60-kDa species (black arrowhead, compare first and second lanes). A small shift to below ∼60 kDa was detected after treatment with N-glycosidase F (asterisk). Blots were stripped and reprobed with anti-transferrin antibody to verify effective deglycosylation (bottom panel). ii, Endo H treatment did not alter Panx2 migration. C, the NPC-specific ∼85-kDa species was markedly reduced following hydroxylamine (HA) treatment. ii, quantitation of this reduction in three independent experiments. Data represent densitometric analysis of the upper band (∗) normalized to GAPDH and expressed as a percentage of the buffer-treated samples after these incubations were extracted and quantified. Data represent mean ± S.D. from three independent experiments. Differences were analyzed by the Student’s t test. *, p < 0.05, compared with the buffer-treated samples. Data represent pM BODIPY FL hexadecanoic acid extracted from each sample. Could account for the delayed migration of the NPC-specific species (available on the ExPASy web site).

Lysates, prepared from postnatal neurosphere cultures, were dephosphorylated with alkaline phosphatase (AP) (Fig. 3A). Dephosphorylation had no visible effect on the mobility of the NPC-specific species (Fig. 3A, upper panel, white arrowhead). To confirm the efficiency of alkaline phosphatase treatment, blots were stripped and reprobed for phospho-GSK3β (Ser9) (Fig. 3A, lower panel).

To assess sumoylation, Panx2 was immunoprecipitated and blotted with antibodies raised against Sumo1. Although sumoylation was readily detected in input lysates, no evidence of specific sumoylation was observed between the Panx2 and control IgG immunoprecipitates (supplemental Fig. 3).

Previous studies have shown that ectopically expressed Panx1, -2, and -3 in human embryonic kidney cells are glycosylated (16, 30). A minor, potentially N-glycosylated component, of the Panx2 NPC profile was detected following treatment with N-glycosidase F (Fig. 3B, panel i, asterisk). A faint band, migrating just below the mature ∼60-kDa Panx2 species, was consistently observed (Fig. 3B, asterisk, panel i, lane 3), which may indicate endogenous N-glycosylation of the mature species, consistent with previous reports (30). However, deglycosylation did not markedly alter the migration of the NPC-specific upper doublet species (Fig. 3B, upper panel i, white arrowhead, compare lanes 2 and 3). Immunoblots were stripped and reprobed for transferrin to confirm effective deglycosylation (Fig. 3B, lower panel i). Likewise, treatment with Endo H had no effect on Panx2 mobility (Fig. 3C, panel ii).

During the preparation of samples for both N-glycosidase and Endo H treatment, we consistently found that the process of boiling, under reducing conditions, reduced the intensity of the upper Panx2 species and increased the intensity of lower molecular weight species in NPC lysates (Fig. 3B, compare lanes 1 and 2, panel i). This procedure is one means of depalmitoylating proteins (33, 34). To test this hypothesis, we examined the Panx2 sequence for potential palmitoylation sites. Although there is no single palmitoylation consensus sequence, probably due to the large size of the newly discovered DHHC (Asp-His-His-Cys) palmitoylating protein family, conserved patterns can still be predicted (48).

Our in silico analysis (CCS-Palm version 2.04) (49) identified at
least one potential palmitoylation consensus site (SYLCTYY) at amino acid 246, and several additional sites were detected when analyses were performed at a lower stringency. To directly assess \( S \)-palmitoylation, lysates prepared from NPC cultures were treated with 1 M hydroxylamine. Hydroxylamine releases palmitic acid from palmitoylated proteins by chemically cleaving the thioester linkages of fatty acyl groups to cystine (Fig. 3C, panels i–iii). Panx2 lysates were clearly sensitive to hydroxylamine treatment as demonstrated by the reduction in the intensity of the upper Panx2 species to 37.9/11006 3.6% of control (i.e., reactions carried out in the absence of hydroxylamine) quantified in triplicate experiments (Fig. 3C, panels i and ii).

These data were further confirmed by in vivo metabolic labeling of neurosphere cultures using fluorescently labeled palmitate (BODIPY FL hexadecanoic acid; Fig. 3C, panel iii). Protein lysates prepared from labeled cultures were immunoprecipitated with either control IgG or anti-Panx2 C-term antibody. The lipids bound to each sample were extracted (50), and the amount of fluorescent palmitic acid was quantified. We found that 109/11006 34 pM of BODIPY FL palmitate was bound to protein in the Panx2 immunoprecipitates as compared with 15/11006 4 pM associated with the IgG control samples. Furthermore, hydroxylamine completely liberated the \( S \)-linked palmitic acid from the Panx2 immunoprecipitates, reducing the Panx2-linked BODIPY FL palmitate concentrations to that of control immunoprecipitates (21/11006 3 pM) (Fig. 3C, panel iii).

Panx2 Protein Expression Is Restricted to Type I and IIa Early Stem-like Hippocampal Neural Progenitors and Terminally Differentiated Mature Granule Neurons—Although each neurosphere is derived from a single Type I or, more rarely, a single Type IIa NPC, daughter cells spontaneously adopt distinct lineages over several DIV (supplemental Fig. S2), recapitulating the multistep neurogenic
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FIGURE 5. Panx2 localizes to different subcellular compartments in Type I and IIa NPCs and mature neurons. Subcellular localization was assessed by confocal microscopy using antigenic markers of or carbohydrate-binding proteins labeling plasma membrane (syntaxin, wheat germ agglutinin), Golgi apparatus (giantin, and endoplasmic reticulum (calnexin). A–C, in NPCs, Panx2 does not localize to the plasma membrane (A) but is found at the Golgi apparatus (B) and associated with ER membranes (C). D–F, in mature granule neurons, Panx2 is found at the plasma membrane (D) but not at Golgi (E) or ER (F) membranes. G–K, this shift in localization is associated with neuronal differentiation. Panx2 mRNA (G, reverse transcription-PCR) and protein (H, Western analysis; I–K, confocal immunofluorescence) are expressed by undifferentiated and differentiated N2a cells (U, undifferentiated; D, differentiated). Both the 85-kDa palmitoylated (white arrowhead) and 60-kDa unpalmitoylated (upper black arrowhead) species are present in undifferentiated N2a cells with intensity of the 85-kDa form reduced following differentiation. A novel Panx2 species is also detected (lower black arrowhead) (H). Abbreviations and controls are as described in the legend to Fig. 2. J and K, further subcellular localization comparisons were made using fluorescently labeled wheat germ agglutinin as a plasma membrane marker. In undifferentiated N2a cells, Panx2 is not detected at the plasma membrane (J), following 48 h of differentiation with 10 μM retinoic acid, Panx2 colocalizes with wheat germ agglutinin at the plasma membrane in neuritic extensions (K, arrows). This shift in localization is associated with a reduction in Panx2 protein expression, in particular the ~85-kDa species (white arrowhead, H). In all micrographs, Hoechst 33258 was used as a nuclear counterstain (blue). Scale bars, 10 μm. Polyclonal anti-Panx2 (Aviva Systems Biology) was used.
Panx2 Localizes to Different Subcellular Compartments in Multipotential NPCs and Terminally Differentiated Neurons—Palmitoylation of neuronal transmembrane proteins, including G-protein-coupled receptors, neuronal cell adhesion molecules, and glutamate receptors, regulates their shuttling between intracellular compartments (reviewed in Ref. 48). To test whether the change in palmitoylation is associated with a shift subcellular localization in Type I NPCs and neurons, we localized Panx2⁺ rod-like structures to discrete organelles by confocal analysis in combination with immunolabeling for plasma, Golgi apparatus, and ER membranes (Fig. 5). In hippocampal NPCs, Panx2 was not detected at the plasma membrane (Fig. 5A). Instead, Panx2⁺ rod-like structures colocalized with the Golgi apparatus marker, giantin (Fig. 5B), and also within ER membranes labeled with calnexin (Fig. 5C). In mature hippocampal granule neurons, Panx2 primarily formed rod-like structures in plasma membrane (Fig. 5D) and did not co-localize with giantin (Fig. 5E) or calnexin (Fig. 5F).

To provide further evidence of a shift in subcellular localization following neuronal differentiation, we employed the mouse neuroblastoma N2a cell model. N2a cells differentiated with retinoic acid in low serum medium express the mature neuronal marker NeuN yet can be easily transfected in their undifferentiated state (32). We found that N2a cells endogenously expressed Panx2 mRNA by reverse transcription-PCR (Fig. 5G) and protein by immunofluorescence and Western analysis (Fig. 5, H and J). In undifferentiated N2a cells, Panx2 formed intracellular rod-like punctae similar to those observed in hippocampal NPCs (Fig. 5, I and J, boxed z-stacks and arrows) but also displayed a dispersed intracellular punctate vesicular pattern not detected in NPC cultures (Fig. 5I, arrowheads). Following a 48-h differentiation to a neuronal phenotype (retinoic acid/low serum), Panx2 relocalized to the plasma membrane of both cell soma and neurites (Fig. 5K). This subcellular redistribution was associated with a reduction in the intensity of the upper species and shift in protein mobility consistency with depalmitoylation (Fig. 5H, upper white arrowhead) as well as the appearance of a de novo ~60-kDa lower molecular mass species (Fig. 5I, black arrowheads) that may reflect deglycosylation of the mature Panx2 or alterations in other putative post-translational modifications (30). An overall reduction in protein expression was also observed in differentiated N2a cultures. To confirm these data, Panx2 expression was examined in cultured hippocampal neurons by confocal microscopy (Fig. 6, A and B). We found Panx2 immunostaining restricted to NeuN⁺ neurons at 7 DIV and not the glial cells in our cultures, and it was observed at the plasma membrane of the cell soma as well as in varicosities on neuritic processes.

Panx2 Knockdown Accelerates Neuronal Differentiation—Stable expression of Panx2-targeted shRNA in N2a cells resulted in a ~50% reduction in protein expression of both the palmitoylated and unpalmitoylated Panx2 species in undifferentiated cultures (Fig. 7, A and C). Remarkably, knockdown accelerated morphological differentiation to a neuronal phenotype following exposure to neurogenic stimuli (Fig. 7B). Eight hours after exposure to retinoic acid, significantly more Panx2 shRNA-expressing cells exhibited a neuronal morphology than empty vector control transfectants (empty vector N2a transfectants, 17.4 ± 3.4%; shRNA-mediated Panx2 knockdown N2a transfectants, 40.4 ± 5.2%; Fig. 7B). Although Panx2 knockdown did not further enhance morphological differentiation at later time points (48 h), stable Panx2 shRNA transfectants expressed higher levels of the mature neuronal marker, NeuN, and lower levels of the immature neuronal marker, Tuj1, than empty vector controls (Fig. 7C). Together, these data demonstrate that the loss of Panx2 from N2a cells accelerates their progression to a neuronal phenotype.

DISCUSSION
In this study, we show that Panx2 is dynamically expressed over the course of postnatal hippocampal neurogenesis in vivo and in vitro. At least two Panx2 species are present in stem-like Type I and IIa NPCs that are not detected in their neuronally committed Type IIb and III progeny or in immature neurons. These represent S-palmitoylated and unpalmitoylated species, with S-palmitoylated Panx2 uniquely detected in multipotential NPCs. Only the unpalmitoylated Panx2 species is re-expressed in adult hippocampus following granule neuron maturation. In keeping with evidence that S-palmitoylation regulates intracellular sorting and protein-membrane interactions (48), we found that Panx2 forms rod-like structures in the ER and Golgi, whereas, in adult hippocampal granule cells, Panx2 is expressed at the plasma membrane. This is the first evidence of a cell type-specific post-translational modification of a pannexin associated with a shift in subcellular localization.

In addition to changes in post-translational modification and subcellular localization, we found that shRNA-mediated reduction of Panx2 expression accelerated both morphological and biochemical indices of differentiation in N2a cells. This, along with the absence of Panx2 expression in DCX⁺ neuronally committed hippocampal NPCs and Tuj-1⁺ immature neurons, provides evidence that intracellular, palmitoylated Panx2 itself probably plays a modulatory role in neurogenesis, participating either in maintaining “stemness” of multipotential NPCs or, alternatively, in the regulation the timing of their transition to differentiation once exposed to neurogenic stimuli. This potential function is novel for...
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Recent work by Yeh et al. (51) has demonstrated that the Caenorhabditis elegans-expressed innexin UNC-7 (and UNC-9) may act as a “corral” sequestering multiple protein components required for presynaptic active zone differentiation to discrete plasma membrane (and perisynaptic) regions during synaptogenesis. It is tempting to speculate that an analogous function for S-palmitoylated Panx2 may be to sequester proteins that promote synaptogenesis at the ER and Golgi in multipotent NPCs, with the loss of Panx2 enabling their redistribution in intermediate progeny, and the re-expression of unpalmitoylated Panx2 at the plasma membrane of mature neurons facilitating the localization of the required presynaptic neuronal machinery to developing active zones over the course of synaptogenesis. Clearly, this hypothesis will require a comprehensive proteomic analysis of Panx2-interacting proteins and their subcellular localization in Type I NPCs and mature neurons.

The formation of rod-like structures at the ER and Golgi apparatus is unusual for a gap junction-associated protein and highlights an important spatial and temporal regulation of protein expression associated with neurogenesis. A growing body of evidence indicates that purine nucleotides act as short range autocrine factors enhancing NPC proliferation via purinergic receptor-mediated mobilization of intracellular calcium stores (52–54). In this context, it is also possible that the regulation of pannexin-mediated hemichannel activity underlies the modulatory role of Panx2 on NPC fate. Not only has Panx1 been shown to form functional hemichannels in GFAP+ glia that release ATP in response to purinergic receptor stimulation (55, 56); ectopically expressed protein has also been shown to form functional calcium-permeable channels in the ER (40). A potential interaction between S-palmitoylated Panx2 and Panx1 in NPCs could target Panx1 to the ER to act as a Ca2+ leak channel (29, 40). Alternatively, expression of unpalmitoylated Panx2 at the plasma membrane could facilitate Panx2-mediated reduction of Panx1 permeability and prime NPCs for neuronal specification (29). It is interesting to note that pharmacological inhibition or siRNA-mediated knockdown of P2X7 ionotropic purinergic receptors also accelerates morphological neuronal differentiation of N2a cells (32), similar to what we observed in the present study. These putative physical and functional interactions may represent redundant mechanisms of regulating Panx1 channel-forming activity and thus modulate the timing of NPC specification. In summary, these data provide the first evidence that Panx2 protein expression plays a role in regulating the timing of neuronal differentiation.

Acknowledgments—We thank L. Mitchell for assistance in Northern blotting, A. Strom and S. Imbeault for advice in confocal microscopy, R. DesRoches for assistance with lineage assessment, J. Bennett for editorial assistance, Dr. C. Thompson and the Centre for Stroke Recovery for confocal access, Dr. L. Filion for advice in quantitative flow cytometry analysis, and Dr. J. Woulfe for helpful discussion.

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