Neonatal Stroke in Mice Causes Long-Term Changes in Neuronal Notch-2 Expression That May Contribute to Prolonged Injury

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Background and Purpose—Notch receptors (1–4) are membrane proteins that, on ligand stimulation, release their cytoplasmic domains to serve as transcription factors. Notch-2 promotes proliferation both during development and cancer, but its role in response to ischemic injury is less well understood. The purpose of this study was to understand whether Notch-2 is induced after neonatal stroke and to investigate its functional relevance.

Methods—P12 CD1 mice were subjected to permanent unilateral (right-sided) double ligation of the common carotid artery.

Results—Neonatal ischemia induces a progressive brain injury with prolonged apoptosis and Notch-2 up-regulation. Notch-2 expression was induced shortly after injury in hippocampal areas with elevated c-fos activation and increased cell death. Long-term induction of Notch-2 also occurred in CA1 and CA3 in and around areas of cell death, and had a distinct pattern of expression as compared to Notch-1. In vitro oxygen glucose deprivation treatment showed a similar increase in Notch-2 in apoptotic cells. In vitro gain of function experiments, using an active form of Notch-2, show that Notch-2 induction is neurotoxic to a comparable extent as oxygen glucose deprivation treatment.

Conclusions—These results suggest that Notch-2 up-regulation after neonatal ischemia is detrimental to neuronal survival. (Stroke. 2010;41[suppl 1]:S64-S71.)

Key Words: neonatal stroke ■ hippocampus ■ apoptosis ■ c-fos ■ Notch-2
mouse line is on a CD1 background and is phenotypically comparable to wild-type CD1 mice. Litters of P12 pups were bred at the JHU animal facility and housed in polycarbonate cages on a 12 hour light dark cycle; food was provided ad libitum. On P12, mice of both sexes received permanent unilateral (right-sided) double ligation of the common carotid artery under isoflurane anesthesia or sham surgery as previously described.13,14 Seizure activity was scored during the 4 hours after injury as previously described in this model.13

**Histological Preparation**

The brains were perfused with 4% PFA and cryoprotected by sequential immersion in 15% and 30% sucrose for 24 hours each. Coronal brain sections 20 μm thick were cut on a cryostat in serial order to create 10 series of sections that were mounted on super frost plus glass slides and stored at −20°C.

**In Vitro Transfection, DNA Constructs**

High efficiency Ca²⁺-phosphate transfection was carried out on 10 days in vitro neuronal cultures as previously described.15 Cotransfections of pCAG-GFP and pCLEN2 (Notch-2 intracellular domain, NICD2, CDS: 5350 to 6684 cloned into pCLE), or pCLE16 alone as control, were carried out. Transfection efficiency was evaluated by GFP expression, and alkaline phosphatase immunostaining was performed on randomized samples within each experiment (a ratio ≥3:1, pCLE or pCLEN2 to pCAG-GFP, gave a 100% colabeling).

**OGD and Cell-Death Scoring**

OGD was performed on 12 days in vitro neuronal cultures as previously described.17 Neuronal death was assessed 6 hours after OGD treatment and determined by nucleus condensation/fragmentation after staining with 1 μg/mL of DAPI (Roche). Dishes were counted by an investigator blinded to the experimental condition. Percent cell-death per dish was calculated as follows: ([Number of dead GFP+cells/Total number of GFP+cells]×100). Average percent cell-death was then calculated for each condition (n=8 per condition).

**Antibodies**

Antibodies used to detect Notch-2 were rabbit anti-Notch-2 (intracellular portion, 1:500 Abcam, Cambridge, Mass; for immunohisto-

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**Figure 1.** Experimental layout of perinatal ischemia in P12 mice, time-course of injury, and correlation of the acute seizure score with the extent of injury after stroke. A, For the acute analysis we selected T30 minutes, T3 hours, T6 hours, and T24 hours to detect early molecular changes. T7, T14, and T21 days were used to monitor the long term effects of neonatal stroke. (For each time-point we used n=10 ligated mice. n=4 shams were used at the time points indicated with S). B and C, Mean ipsilateral percent hemispheric (B) and hippocampal (C) asymmetry, compared to shams for each time-point analyzed, shows a negative value at T24 hours, representing swelling, followed by a progressive brain atrophy after ischemia (see positive percent asymmetry at T7 to T21 days) (*P<0.05, **P<0.01, ***P<0.001). D, Plots representing the correlations between acute seizure scores and percent hemispheric atrophy for animals euthanized at T7 days (P19) (r²=0.58, P=0.01), T14 days (P26) (r²=0.78, P<0.01) and T21 days (P33) (r²=0.79, P<0.01) after ligation. E, Plots representing the correlations between acute seizure score and severity of hippocampal atrophy at T7 days (P19, no linear correlation), T14 days (P26; r²=0.72, P=0.01) and at T21 days (P33; r²=0.85, P<0.01) after stroke at P12.
chemistry on sections), goat anti-Notch-2 (1:500, Santa Cruz, Calif; for western blot and immunohistochemistry in cell culture), rabbit anti-Notch-1 (1:500 Abcam, Cambridge, Mass), rabbit anti-c-fos (1:20,000 Calbiochem, San Diego, Calif), mouse anti-GFAP (1:500, Chemicon, Temecula, Calif), rabbit anti-cleaved caspase-3 (1:1000, Cell Signaling, Danvers, Mass), sheep anti-PLAP (1:1000, American Research Product, Bemont, Mass), mouse anti-Arc/Arg 3.1 (1:1000, gift Worley P.), and mouse anti-β-actin (1:5000, Sigma, St. Louis, Mo).

Immunohistochemistry
20 µm thick coronal brain sections and neuronal cultures were fixed in 4% PFA, and postfixed in ice-cold acetone-methanol (1:1) at −20°C for 10 minutes. The immunostainings with anti-Notch and anti-c-fos antibodies on sections were performed according to the instructions included in the TSA fluorescence amplification kit (Perkin Elmer). For all other applications, primary antibodies were visualized with directly conjugated donkey secondary antibodies (Alexa 488, Alexa 555, Alexa 647, Invitrogen, Carlsbad, Calif). TUNEL labeling was carried out using DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer’s instructions. Nuclei were counterstained with 1 µg/mL DAPI (Roche). Images were taken using a Zeiss AxioScope microscope connected to an AxioCam, or Zeiss confocal LSM 510. All images were processed using Adobe Photoshop.

Western Blot Analysis
Neuronal cultures were washed in ice cold PBS and harvested using RIPA buffer, and protein concentrations were determined using the BCA method (BioRad). Protein samples were subjected to denaturing SDS-PAGE and then transferred from the gel to an Immuno-Blot PVDF membrane (BioRad). Membranes were probed with primary antibodies and HRP-conjugated secondary antibodies. A chemiluminescent substrate (ECL, GE Amersham) and film were used to visualize the HRP signal.

Computerized Brain Asymmetry Analysis
Fixed cresyl violet stained mouse brain slices, photographed after calibration using an AxioCamcolor camera and AxioVision 2.05 software, were measured using MCID 7.0 Elite (InterFocus Imaging Ltd). Brain asymmetry scores were measured as previously described.[14]

Fluorescent Image Analysis
TUNEL labeled cells were counted from images, acquired with a 20× objective from 3 consecutive sections per animal. C-fos and Notch-2 immunolabeling were quantified, using ImageJ-software (National Institutes of Health), as pixel counts (pixels were fitted to represent positive c-fos cells) and area fraction, respectively, on images (20× objective) from 3 consecutive sections per animal. Data
were normalized to control condition fluorescence. C-fos pixel counts and area fractions of Notch-2 measurements were sampled over the entire picture area of constant resolution.

Data Analysis
ANOVA was used to calculate variance among animals for a given time point. Student t test was then used for comparisons between the ipsilateral hemisphere from the ligation-injured group and from the sham group. Correlation analysis was performed between the acute seizure score and the extent of injury. A linear relationship was considered $r^2 > 0.5$. For the Student t test and correlation analysis, $P < 0.05$ was considered significant.

Results
Time-Course of Injury and Apoptosis After Neonatal Ischemia
Before the 24 hour time-point, neither edema nor evidence of injury was grossly or microscopically visible by cresyl violet (Nissl) staining. Twenty-four hours after unilateral carotid ligation, however, edema was detectable in the ipsilateral hemisphere from the ligation-injured group and from the sham group. Correlation analysis was performed between the acute seizure score and the extent of injury. A linear relationship was considered $r^2 > 0.5$. For the Student t test and correlation analysis, $P < 0.05$ was considered significant.

C-fos Expression in the Hippocampus Acutely and Chronically After Ischemia
For all the data points and regions analyzed, expression of c-fos protein in the contralateral hemisphere from injured animals was low and comparable to sham animals; for this reason only sham control data are shown (Figure 3, panels A and B). At T3 hours in ligated pups that seized (n = 4/4 mice), c-fos expression was significantly increased in the ipsilateral DG, CA3, and CA1 areas (Figure 3, panel B). At T24 hours c-fos expression was intense in scattered cells in the ipsilateral hippocampus and neocortex (n = 6/10; Figure 3, panel B). Analysis at later time-points revealed that at P19, 83% of the mice with acute poststroke seizures (n = 5/6 mice) had ectopic c-fos expression in ipsilateral CA1 regions adjacent to niches of cell-death (yellow arrows in Figure 3, panels A and B). Fourteen days after ligation, in 100% of the animals with...
acute poststroke seizures (n=4/6 mice), c-fos expression was still elevated in scattered pyramidal neurons of the injured hippocampus (Figure 3, panel B).

**Notch-2 Is Ectopically Up-regulated in the Hippocampus After Neonatal Stroke**

In control perinatal brains and contralateral hemispheres of ligated brains, at all time-points analyzed, Notch-2 and Notch-1 expression was restricted to the neurogenic zones of the subventricular zone and the SGZ (Figure 4, panels A-A” and D-D”, and not shown). Acutely at T6 and T24 hours after ischemia, in animals with acute poststroke seizures (n=4/6 mice per group), Notch-2 expression extended to the granule cell layer of the DG (Figure 4, panels B to B’ and panel F) and colocalized only in few GFAP+ astroglia (Figure 3 panels B’, putative glia are indicated with yellow arrows). Notch-1 expression, on the other hand, was elevated in the SGZ (Figure 4, panel B”). At T6 and T24 hours after injury, expression of Notch-2 was also significantly elevated in ipsilateral CA3 and CA1 areas (Figure 4, panel F). At T24 hours, Notch-1 expression appeared punctuate in the granule cell layer of the DG. E-E”, 7 days after injury, at P19, Notch-2 is strongly increased as compared to sham control (D-D”) in ectopic niches in the ipsilateral CA1 in and around areas with elevated c-fos expression (yellow arrows, and see Figure 3, panel A). Orthogonal views of stacked images reveal that Notch-2 is expressed in the granule cell layer in and around condensate nuclei. E”, Double labeling with Notch-2 and GFAP staining shows that Notch-2 and GFAP expression only modestly colocalize. E”, At T7 days Notch-1 is only moderately expressed in invading glia and in the granule cell layer. F, Table summarizing the time course of Notch-2 overexpression in hippocampal region CA1, CA3, and DG. Highlighted boxes indicate regions with elevated apoptosis and c-fos expression (all scale bars are 50 μm; *P<0.05, **P<0.01, ***P<0.001).
present in reactive glia (data not shown) and despite being reduced, continued to be present in the partially intact ipsilateral SGZ (Figure 4, panel F).

Physiological Notch-2 Expression Is Critical for Neuronal Survival

In order to address the effect of Notch-2 expression after hypoxic-ischemic injury, we used a gain-of-function approach using OGD in hippocampal primary neuronal cell culture, as an in vitro hypoxia-ischemia model.17 Six hours after OGD, Notch-2 processing was induced (NICD2) in the neuronal cultures (Figure 5, panel A, n=3 experiments), when neuronal activity was elevated (as indicated by the presence of the activity regulated gene Arc/Arg 3.1) and cell-death was ongoing, as shown by the presence of cleaved caspase-3 (Figure 5, Panel A). In addition, 6 hours after OGD, Notch-2 was up-regulated and colocalized with cleaved-caspase-3 in the majority of the cells (Figure 5, panel B). Transfection with pCLEN2 led to a 6-fold increase in Notch-2 expression (data not shown). When we overexpressed NICD2 for 72 hours we observed a significant increase in cell-death in untreated cultures (no OGD) as compared with the control (pCLE) transfected cells (38±12% versus 23±9%, P<0.05, n=6). Six hours after OGD treatment, neurons transfected with either pCLE or pCLEN2 had similar levels of cell-death (Figure 5, panel D; n=6). However, interestingly, overexpression of NICD2 in neurons subjected to OGD did not increase cell death compared to control-transfected neurons (Figure 5, panel D).

Discussion

As reported in other immature animal hypoxia-ischemia models, the evolution of the neonatal stroke injury is quite prolonged.18,19 In this model, we observed progressive atrophy in the ipsilateral hemisphere over the 3 weeks after neonatal stroke at P12. Acutely, the majority of cells died in an environment where edema developed; later, over the following weeks increased apoptosis was visible in the injured hippocampus and neocortex. These results suggest that neonatal stroke has long lasting effects on neuronal viability and supports the existence of a prolonged potential therapeutic-window for alleviating the progression of cell-death after such an injury.

In order to better understand how ischemia affects neuronal activity, we monitored the temporal profile of c-fos expres-
sion. In this context, c-fos expression can serve as a marker of several processes, including neuronal hyper-activity after seizure, excessive glutamate response after cerebral ischemia, and cell-death. A clear up-regulation in c-fos expression was observed 3 hours after stroke in all regions of the ipsilateral hippocampus. The CA3 and CA1 areas are the regions most susceptible to excitotoxic cell-death in the immature brain, whereas the DG remains partially preserved. At 1 week after ligation (P19), ectopic foci of c-fos expression in CA1 were noted in and around areas of cell-death. At T14 (P26) and T21 days (P33) after ligation, when hippocampal atrophy peaked, we observed c-fos expression restricted to scattered pyramidal cells of the ipsilateral hippocampus. This delayed and abnormal c-fos expression can be interpreted as resulting from hyper- or abnormal activity after neonatal ischemia that can contribute to the brain injury, as it has been proposed by others working with rat perinatal models of hypoxia ischemia.

Several recent articles have reported that Notch-1 activation occurs in response to cerebral ischemia in very different cell types: in the germinial zones of the subventricular zone and SGZ which have been shown to contribute to the maintenance of the progenitor pool, and in the neurons of the cortex where it is thought to contribute to neuronal damage. Neonatal mice with acute seizures after double unilateral carotid ligation showed a strong increase in Notch-2 receptor expression in the granule and pyramidal layers of the ipsilateral hippocampus, specifically in regions with ectopic c-fos expression, which subsequently became atrophic. Interestingly Hes5, a conical target of the Notch pathway, was also up-regulated in the hippocampus after ischemia (data not shown), indicating that up-regulation of Notch-2 led to pathway activation.

The widespread induction of Notch-2 in the granule cell layer acutely after neonatal stroke injury was distinct from Notch-1 which remained largely restricted to the SGZ. Interestingly, 1 week after injury, Notch-1 and Notch-2 still had very different cellular patterns of expression in the ipsilateral hippocampus; Notch-2 was aberrantly increased in injured hippocampal neurons, whereas Notch-1 was localized to reactive glia. This finding suggests a differential role for the 2 receptors in response to neonatal ischemia.

We have shown that OGD challenges in vitro induced Notch-2 activation in primary hippocampal neurons, similarly to what has been seen with Notch-1 in cortical cultures, and we have shown that most of the cells that had aberrant Notch-2 activation were also positive for the apoptotic marker cleaved-caspase-3. Utilizing a gain of function experiment, we demonstrated that overexpression of the transcriptionally active form of Notch-2 (NICD2) was neurotoxic under basal conditions to a comparable level as after OGD treatment alone. Furthermore, under OGD conditions the control transfected and NICD2 transfected cultures had similar levels of cell-death.

In conclusion, this work demonstrates that neonatal ischemia induced by unilateral carotid ligation in P12 mice is a clinically relevant model that produces long lasting anatomic and molecular changes in the hippocampus and cortex. The prolonged and ectopic c-fos expression in regions of ongoing cell death is of particular interest for the possible identification of sites with prolonged abnormal neuronal activity and/or cell demise. Future research using this model may link these sites to the process of postischemic epileptogenesis, or alternatively to the focus of new regenerative strategies. In addition, Notch-2 appears to be rapidly and persistently induced in postmitotic neurons by ischemic injury. The work reported here suggests that this aberrant induction of Notch-2 may be neurotoxic. We anticipate that identifying the downstream effectors of Notch-2 after ischemic brain injury could lead to the development of better therapeutic agents, which might help contain the neuronal damage resulting from Notch-2 overactivation.

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Disclosures

None.

References


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