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Wnt Expression in the Adult Rat Subventricular Zone After Stroke

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Abstract

Introduction: In the adult brain, neurogenesis occurs in the subventricular zone (SVZ) of the lateral ventricle. During development, the Wnt pathways contribute to stem cell maintenance and promote neurogenesis. We hypothesized that the Wnt family genes are expressed in neural progenitor cells of the non-ischemic and ischemic SVZ of the adult rodent brain after middle cerebral artery (MCA) occlusion.

Methods: Non-ischemic and ischemic cultured SVZ cells and a single population of non-ischemic and ischemic SVZ cells isolated by laser capture microdissection (LCM) were analyzed for Wnt pathway expression using real-time RT-PCR and immunostaining.

Results: The number of neurospheres increased significantly ($p < 0.05$) in SVZ cells derived from ischemic ($32 \pm 4.7/\text{rat}$) compared with the number in non-ischemic SVZ cells ($18 \pm 3/\text{rat}$). Wnt family gene mRNA levels were detected in SVZ cells isolated from both cultured and LCM SVZ cells, however there was no upregulation between non-ischemic and ischemic SVZ cells. Immunostaining on brain sections also demonstrated no upregulation of Wnt pathway protein between ischemic and non-ischemic SVZ cells.

Conclusions: Expression of the Wnt family genes in SVZ cells suggests that the Wnt pathway may be involved in neurogenesis in the adult brain. However, ischemia does not upregulate Wnt family gene expression.

Keywords

Wnt pathway; Stroke; SVZ; Laser Capture Microdissection

Introduction

In the adult brain, neurogenesis occurs in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus [5,10,11]. The SVZ has been identified as a “neurogenic niche” in which a quiescent stem cell population prospers. Adult stem cells, by definition, are able to self-renew and generate differentiated cell types specific for that particular organ. Neural progenitor cells (NPCs), a type of adult stem cell in the brain, are present in the SVZ and these NPCs differentiate into neuroblasts that travel the rostral migratory stream to the olfactory bulb where they differentiate into inter-neurons throughout

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rodent life. A fundamental observation which has sparked the interest and curiosity of both neuroscientists and stroke researchers is that after ischemic stroke, NPCs in these “niches” proliferate and without hesitation, migrate towards the ischemic boundary region and may contribute to recovery [2,13,18]. The cell proliferation pathways and growth factors responsible for this incredible self repair mechanism are unclear. One particular proliferation pathway that has been well described in the developmental literature is the Wntless-type (Wnt) pathway. The Wnt pathway regulates embryonic neural stem cell patterning, cell fate determination and cell proliferation [4]. The Wnt pathway has been demonstrated to play important roles in neuronal migration, axon pathfinding, dendritic morphogenesis and synaptic differentiation during neuronal development in both invertebrates and vertebrates [6]. In general, the canonical Wnt pathway is activated when Wnt glycoproteins bind “frizzled” receptors (fzd), activating “disheveled” (dvl) that in turn induces the disassembly of a protein complex which when assembled degrades β -catenin. Activation of Wnt, therefore, involves the preserving the transcription factor β -catenin. Wnt can also signal through the noncanonical pathway which releases intracellular calcium [4]. Wnt 3 signals through the canonical pathway and is expressed in the hippocampal niche [8]. Wnt 5a signals through the noncanonical pathway and has been shown to regulate the development of midbrain dopaminergic neurons [3]. Moreover, the canonical Wnt signaling pathway regulates stem cells in many adult tissues including the skin and blood. [1,15]. Studies in general demonstrate that the canonical Wnt pathway may play an important role in the maintenance of pluripotency of stem cells and inhibition of Wnt induces neuronal differentiation of embryonic stem cells [7].

Parallel to the developmental literature, Wnt signaling was observed to regulate adult rat hippocampal neurogenesis. Wnt family members were expressed by hippocampal astrocytes whereas hippocampal stem/progenitor cells expressed receptors and signaling components for Wnt proteins [8]. This finding was evidence that the Wnt pathway was involved in adult stem cell maintenance and neurogenesis. Based on these observations and employing a rat model of stroke, we measured genetic expression of the Wnt pathway after stroke in cells isolated from the SVZ as well as astrocytes in and around the SVZ. We hypothesized that the Wnt pathway may be involved in SVZ neurogenesis after stroke.

Material and Methods

All experimental procedures were approved by the Institutional Animals Care and Use Committee of Henry Ford Hospital.

Animal Model

The middle cerebral artery (MCA) of Male Wistar rats (320 to 380 g) was occluded by placement of an embolus at the origin of the MCA, as previously described [19]. Rats were sacrificed 7 days after MCA occlusion and brain coronal sections (8 μ m thick) were cut on a cryostat set at -20°C and kept at -80°C until processing.

SVZ Cell Culture

SVZ cells were dissociated from normal (n=24) and 7 day MCA occlusion (n=24) rats as previously described [18]. Three separate cultures each containing SVZ cells from eight rats were grown. The cells were plated at a density of 20,000/ml in medium containing 20 ng/ml epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). The generated neurospheres (primary spheres) were passed by mechanical dissociation and reseeded as single cells at a density of 20/ul in EGF-containing media. Total numbers of neurospheres in 1 ml media were counted 7 days after in culture.

Immunofluorescent Staining

Brain coronal sections localized to the territory supplied by the MCA were air-dried for 30 secs and fixed in 4°C cold acetone for 2 mins [9]. The coronal sections were incubated with prodium iodide (nuclei marker, 1:3000 dilution) for 5 mins. For detection of astrocytes, coronal sections were incubated with antibody against glial fibrillary acidic protein (GFAP) (an astrocyte marker, 1:50, santa cruz biotechnology) for 5 mins followed by CY3 (1:100) for 5 mins. The slides were subsequently dehydrated in graded ethanol solutions (75%, 95%, 100% each once for 30 secs) and cleared in xylene for 5 mins. All procedures were performed at room temperature and completed within 30 minutes.

Laser Capture Microdissection and RNA isolation

After air drying for 10 minutes, SVZ cells were located by visually identifying prodium iodide positive cells in the anterior horn of the lateral ventricle under fluorescence [9]. Figure 1A is an image (20X) under light microscopy before laser capture while figure 1B is an image (20X) after SVZ cells was harvested. In a similar fashion, GFAP positive cells (astrocytes) in and in close proximity to the SVZ were identified under fluorescence. Immunofluorescent reactive cells were captured onto a thermoplastic film mounted on optically transparent laser capture microdissection (LCM) caps using the PixCell II LCM System (Arcturus Engineering). The LCM device was set using a 7.5- μ m laser spot size, 70-mW power and 750- μ s duration. SVZ cells were located in a distinct area located in the anterior horn of the lateral ventricle. After collecting 2000-3000 SVZ cells per hemisphere (contralateral and ischemic), the thin transfer film on the LCM cap was peeled off the cap and placed in 350 μ l of RLT lysis buffer and vortexed for 30 sec. Total RNA from the captured cells was isolated using RNeasy Micro Kit (Qiagen, Inc). cDNA was prepared from total RNA using oligo (dT), dNTP mix, First-Strand Buffer, DTT, RNaseOut and Superscript III (Invitrogen).

Real-Time PCR

Real Time PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems). The RT-PCR reaction system contained TaqMan® Universal Master Mix with the Wnt pathway pre-made primers/probes purchased from Applied Biosystems (ABI): Wnt5a, catenin beta-1 (catnb), casein kappa (csnk), dishevelled homolog 1 (Dvl1), frizzled 3 (fzd3), glycogen kinase synthase beta-3 (gsk 3b) and protein phosphatase 2 catalytic subunit alpha isoform (PPP2ca). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Relative gene expression was determined using the 2^{- $\Delta\Delta$ CT} method. GAPDH and Wnt 3 were custom designed: GAPDH forward primer- CCAGCAAGGATACTGAGAGCAA, reverse primer-GATGGAATTGTGAGGGAGATGCT and FAM labeled probe- CAACTGAGGGCCTCTCT; Wnt 3 forward primer-GCCTCTGACAAGCCCGAAA, reverse primer-GCGACGCCCAATAGTT and FAM labeled probe-TTGAGGTTGGAAATGA.

Immunohistochemistry

Single immunostaining was performed on brain coronal sections with antibodies against Wnt pathway proteins. Briefly, after blocking in normal serum, adjacent coronal sections were treated with polyclonal antibodies against Wnt 3 (1:50), Wnt 5a (1:500), Dvl1 (1:500) (Santa Cruz Biotechnology) diluted in phosphate-buffered saline (PBS) for 60 minutes at room temperature. A monoclonal antibody against Catenin beta-1 (1:100) (Xymed) was incubated overnight at 4°C. Following sequential incubation with biotin-conjugated anti mouse or goat IgG (dilution 1:200, Vector laboratories, INC), the sections were treated with an ABC kit (Vector laboratories, INC). Diaminobenzidine (DAB) was then used as a sensitive chromogen for light microscopy.

Semi-Quantitative Image Analysis

For semi-quantitative measurements, immunoreactive cells in the non-stroke and stroke SVZ were digitized under a 40x objective (BX40; Olympus Optical) using a 3-CCD color video camera (DXC-970MD; Sony, Tokyo, Japan) interfaced with an MCID image analysis system [9,17]. After establishing a threshold parameter that covers positive regions on a control image, the same parameter was applied to all images that were obtained at equal objectives and light intensities on slides that were processed at the same time. The data are presented as a percentage of positive immunoreactivity area within the total SVZ area (pixel) [9,17].

Statistical Analysis

Comparison of eight gene variables (Wnt3, Wnt5a, Catnb, Csnk, Dvl1, Fzd3, Gsk3b, PPP2ca) was performed in rats with and without stroke. Ratio of stroke versus non-stroke was calculated for each gene variable. We hypothesized that gene measurement was altered after stroke. Repeated measure analysis of variance (ANOVA) was used to test the ratio difference from 1 (no alteration) on data collected from the SVZ neurospheres cultures. Student t-test was used to test differences in the LCM and immunostaining.

Results

Proliferation of SVZ cells

The number of neurospheres increased significantly ($p < 0.05$) in SVZ cells derived from stroke ($32 \pm 4.7/\text{rat}$) compared with the number in non-stroke SVZ cells ($18 \pm 3.0/\text{rat}$), suggesting that stroke increases NPC proliferation (Fig. 2).

Expression of Wnt pathway

To measure Wnt pathway gene profile changes in stroke-induced NPC cell proliferation, total RNA was isolated from non-ischemic and ischemic SVZ cells cultured in the growth medium. Real-time RT-PCR analysis showed that mRNA levels of Catnb, Csnk, Dvl1, Fzd3, Gsk 3b, PPP2ca, Wnt 3 and Wnt 5a mRNAs were detected in both non-ischemic and ischemic SVZ cells (Table 1). Levels of Wnt 3 mRNA were significantly decreased in ischemic SVZ cells compared with levels in non-ischemic SVZ cells, whereas other genes showed no differences between non-ischemic and ischemic SVZ cells (Table 1). To measure *in vivo* Wnt pathway gene profile changes in SVZ cells, single population of SVZ cells in brain coronal slices was isolated using LCM. Real-time RT-PCR analysis revealed that the isolated SVZ cells yielded strong GAPDH signals for each animal with Ct values in the 25-30 cycle range (Table 1), which is consistent with our previous findings that this protocol is sensitive for quantification of gene profiles [9]. In contrast to cultured SVZ cells, Wnt 3 and Wnt 5a mRNAs were not detected in non-ischemic and ischemic SVZ cells isolated from brain slices using LCM (Table 2). Although mRNA levels of Catnb, Csnk, Dvl1, Fzd3, Gsk 3b and PPP2ca were detected, there was no significant change of gene expression between non-ischemic and ischemic SVZ cells (Table 2).

Astrocytes stimulate Wnt/ β catenin signaling resulting in hippocampal neurogenesis [8]. Accordingly, a single population of astrocytes identified by GFAP positive cells was captured using LCM. Real-time RT-PCR analysis showed that mRNA levels of Catnb, Dvl1, Fzd3, Gsk3b but not Wnt3 and Wnt 5a were detected in astrocytes (Table 3). However there was no significant change in expression between non-ischemic and ischemic astrocytes (Table 3).

To measure Wnt pathway proteins, immunohistochemical staining was performed on brain slices. SVZ cells were Wnt 3, Wnt 5a, Catnb and Dvl-1 immunoreactive (Table 4). However, semi-quantitative analysis showed immunoreactivity of these proteins did not differ between

ischemic and non-ischemic hemispheres, suggesting that ischemic stroke does not alter Wnt pathway proteins examined in the present study.

Discussion

The adult brain is particularly venerable to injury because of its limited capacity to repair itself, unlike tissues such as the skin which has remarkable repair mechanisms. However, recent findings of the “neurogenic niche” have created excitement that manipulation of the adult stem cells in this “niche” may promote recovery in ischemic insults such as stroke. We hypothesized that the Wnt pathway would be upregulated in SVZ cells after stroke based on observations of embryonic stem cells in development and cancer research [8,14,15].

Our results demonstrated expression of Wnt family genes in cultured (in-vitro) and laser captured (in-vivo) SVZ cells after stroke, however, stroke does not induce an upregulated expression of the Wnt pathway genes as originally hypothesized. The in-vitro model demonstrated a decreased expression of the canonical Wnt3 of ischemic SVZ cells while the noncanonical Wnt 5a was unchanged from normal SVZ cells. Moreover, it appears that decreases of Wnt 3 expression (canonical) and expression of Wnt 5a (noncanonical) are coincident with increases of ischemic SVZ cell proliferation, indicating that the canonical pathway may be downregulated in NPC proliferation after ischemia. It is important to mention however, that decreases of Wnt3 in our in-vitro model may result from a number of factors inherent to cell culture systems. Manipulation of the SVZ cells outside of its natural environment may induce artifacts not observed in in-vivo models. In addition, cell culture conditions may alter expression of certain genes, especially since the SVZ cells are placed in a proliferative medium.

Our study suggests that ischemic brain does not upregulate the Wnt pathway genes for NPC proliferation as observed in developing tissue. For instance, the Wnt pathway has been well described in patterning of the developing nervous system, specifically the neural crest and midbrain dopaminergic neurons [3]. Based on these studies we hypothesized that the Wnt pathway would at least be upregulated in the “neurogenic niche.” Further study of different proliferation pathways or at least variations of other Wnt proteins (ie, Wnt 1, 8b) may be involved, however, we would expect an upregulation of β -catenin, frizzled and dishelved had these specific Wnts been involved in promoting neurogenesis in ischemic adult neural tissue. Furthermore, glycogen synthase kinase 3 is an important component of Wnt signaling which regulates neurogenesis during development [16], which in our study was not elevated in either the in-vivo or in-vitro model.

Based on Lie et al observations, we investigated if a paracrine effect of the Wnt pathway existed between astrocytes and NPCs after stroke. It was possible that the source of Wnt3 or Wnt 5a could have been GFAP positive cells which after ischemia could have secreted Wnt proteins to send guidance and proliferative signals to NPC. We did not detect Wnt3 (or Wnt 5a) as Lie et al observed in normal adult rat brain, however levels of other components of the Wnt pathway were detected (table 2). These results provided further evidence that components of the Wnt pathway is expressed in ischemia but not upregulated.

We detected no RNA expression of Wnt3 or Wnt 5a using LCM (in-vivo), however, we did detect protein expression of Wnt 3, Wnt 5a using immunohistochemical staining. This discrepancy may be due to the smaller amounts of RNA that is isolated in LCM versus neurospheres (larger amounts) isolated in cell culture. The lack of expression in Wnt3 or Wnt 5a in the in-vivo model further supports the view that the Wnt pathway is not upregulated.

We chose to measure the Wnt pathway 7 days after stroke because at 7 days maximal migration and proliferation is observed. We did not measure Wnt pathway expression in a temporal

fashion. It is conceivable that the Wnt pathway in SVZ cells may be expressed earlier in stroke, however expression of Wnt in the sub-acute phase of stroke is unlikely as recovery is generally not observed until day 7.

Persistence of stem cells in any tissue is critical for organisms to replace cells that are victims to injury or disease [12]. Furthermore, these stem cells must proliferate and navigate through a potentially hostile environment to reach their final destination. The brain's inability to upregulate the Wnt pathway after injury could contribute to the high morbidity and mortality of stroke in the general population. That is not to say that other proliferation pathways may be involved. Further investigation of the expression of the bone morphogenetic pathway (BMP), Notch and Sonic Hedge Hog (SHH) is warranted. Moreover, a speculative treatment option may be to pharmacologically upregulate the Wnt pathway to promote recovery after stroke.

In conclusion, our results show that the Wnt pathway is expressed in both normal and ischemic SVZ cells, however, ischemic SVZ cells do not demonstrate an upregulation of this developmentally important pathway. Furthermore, the canonical Wnt pathway may be downregulated in ischemic SVZ cell.

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Figure 1.
20X Magnification of anterior horn of lateral ventricle of ischemic hemisphere before laser capture microdissection (A). 20X Magnification of anterior horn of lateral ventricle of ischemic hemisphere after laser capture microdissection (B).

Figure 2.

20X Magnification of cultured neurospheres. The number of neurospheres increased significantly ($p < 0.05$) in SVZ cells derived from stroke compared with the number in non-stroke SVZ cells.

Table 1**Cultured Neurospheres**

Wnt pathway mRNA Expression (ischemic/non-ischemic)

	Mean	SD
Wnt 3	0.20 [*]	± 0.01
Wnt 5a	1.20	± 0.69
Catnb	0.30 [*]	± 0.09
Csnk	0.60	± 0.21
Dvl1	0.70	± 0.23
Fzd 3	0.60	± 0.27
Gsk 3b	0.80	± 0.21
PPP 2ca	0.70	± 0.14

*
P<0.05

Table 2**Laser Capture Microdissection**

Wnt pathway mRNA Expression (ischemic/non-ischemic)

	Mean	SD
Wnt 3	not detected	
Wnt 5a	not detected	
Catnb	1.01	± 0.54
Csnk	1.03	± 0.39
Dvl1	0.62	± 0.18
Fzd 3	0.78	± 0.24
Gsk 3b	0.81	± 0.17
PPP 2ca	1.16	± 0.28

P>0.05 for all values

Table 3
Laser Capture Microdissection (GFAP positive cells)
Wnt pathway mRNA Expression (ischemic/non-ischemic)

	Mean	SD
Wnt 3	not detected	
Wnt 5a	not detected	
Catnb	0.88	± 0.13
Dvl1	0.70	± 0.31
Fzd 3	1.05	± 0.16
Gsk 3b	0.84	± 0.11

P>0.05 for all values

Table 4

Wnt pathway protein expression in SVZ cells (%)

	Contralateral	Ischemic
Wnt 3	0.081%	0.075%
Wnt 5a	0.068	0.120
Catnb	0.142	0.140
Dvl1	0.085	0.138

P>0.05 for all values