Original Article

Expression of Wnt 3a, β-Catenin, Cyclin D1 and PCNA in Mouse Dentate Gyrus Subgranular Zone (SGZ): a Possible Role of Wnt Pathway in SGZ Neural Stem Cell Proliferation

(β-catenin / cyclin D1 / subgranular zone / neural stem cell)

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Abstract. In mammalian dentate gyrus subgranular zone, the addition of new neurons throughout adulthood is a remarkable form of structural plasticity. Yet, the molecular controls over subgranular zone neural stem cell proliferation, survival, and differentiation are poorly understood. In this study we analysed the expression of Wnt 3a, β-catenin, cyclin D1 and proliferating cell nuclear antigen in mouse subgranular zone to elucidate the involvement of Wnt pathway in subgranular zone neural stem cell proliferation. We performed immunohistochemistry and RT-PCR for the above molecules on adult and postnatal developing hippocampal tissues of mice, respectively. RT-PCR analysis showed a gradual increase in expression of mRNA of Wnt 3a, β-catenin, cyclin D1 and proliferating cell nuclear antigen as the postnatal hippocampus developed, and immunohistochemical analysis showed a highly positive immunoreactive expression for Wnt 3a, β-catenin, cyclin D1 and proliferating cell nuclear antigen in the subgranular zone cells. Together, our data suggested that the Wnt pathway is activated in subgranular zone and could play an important role in regulating subgranular zone neural stem cell proliferation in mouse hippocampus.

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Abbreviations: CDK – cyclin-dependent kinase, DG – dentate gyrus, GSK – glycogen synthase kinase, LRP – lipoprotein-receptor-related protein, PCNA – proliferating cell nuclear antigen, Rb – retinoblastoma, RT-PCR – reverse transcription polymerase chain reaction, SGZ – subgranular zone, TCF/LEF – T-cell factor/lymphoid enhancer factor.

Introduction

The subgranular zone (SGZ) of the hippocampus contains neural stem cells that divide, differentiate and migrate to produce functional neuron that becomes incorporated into the hippocampal circuitry (Jessberger and Kempermann, 2003; Schinder and Gage, 2004). Evidence suggests that each step of this process is highly regulated and many manipulations that alter hippocampal neurogenesis do so by influencing neural stem cell proliferation in the SGZ. Information about how SGZ neural stem cells divide would allow more detailed exploration of the regulation of adult neurogenesis.

Cyclin D1, originally identified as a molecule that links growth factor signalling and cell cycle machinery, is a critical molecule in the regulation of progression through the G1 phase of the cell cycle (Baldin et al., 1993; Matsushime et al., 1994). Cyclin D1 and its catalytic enzymes, cyclin-dependent kinase 4 (CDK4) and CDK6, promote G1-to-S phase progression by retinoblastoma (Rb) protein phosphorylation, a process that inactivates the ability of Rb protein to suppress the transcription factor E2F that regulates genes required for DNA replication and cell cycle progression (Weinberg, 1995; Sherr and Roberts, 1999). Earlier reports have suggested that cyclin D1 expression is significantly correlated to β-catenin expression (Meirmanov et al., 2003; Nakashima et al., 2004). β-Catenin is a key downstream effector of the Wnt signalling pathway that regulates cell proliferation (Nelson and Nusse, 2004). The Wnt pathway is initiated by the binding of the Wnt protein to a receptor complex, consisting of a member of the Frizzled family, and the low-density lipoprotein-receptor-related protein (LRP). Subsequently, the cytoplasmic adaptor protein disheveled is phosphorylated and inhibits glycogen synthase kinase (GSK)-3β activity through its association with axin. Unphosphorylated β-catenin accumulates in the cytoplasm and translocates into the nucleus, where it interacts with members of the family of T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors

and activate genes, such as cyclin D1 (Clevers, 2006; Willert and Jones, 2006).

Many reports have supported the important role of Wnt pathway in controlling stem cell proliferation. It has been shown to play a crucial role in regulating stem cell expansion in various tissues such as the intestine (Pinto et al., 2003), skin (Alonso and Fuchs, 2003), haematopoietic (Reya et al., 2003) and nervous system (Ciani and Salinas, 2005). Studies have indicated that blocking the Wnt signalling pathway perturbs progenitor cell proliferation and causes severe reduction in the hippocampus development (Lee et al., 2000). Conversely, over-expression of Wnt signalling has been shown to cause uncontrolled cell proliferation and tumour formation (Behrens and Lustig, 2004).

In the present study, we analysed the existence of Wnt 3a, β-catenin, cyclin D1 and PCNA in mouse SGZ to elucidate the involvement of the Wnt pathway in SGZ neural stem cell proliferation. In our results, we found prominent membranous expression of Wnt 3a and strong nuclear signals for β-catenin, cyclin D1 and PCNA in the SGZ cells. Also, we found a gradual increase in the mRNA expression level of Wnt 3a, β-catenin, cyclin D1 and PCNA as the postnatal hippocampus develops. Together, our data suggest that Wnt signalling is activated in SGZ and can play an important role in regulating SGZ neural stem cell proliferation in the mouse hippocampus.

Material and Methods

Material

The anti-Wnt 3a antibody was obtained from Chemicon International (Temecula, CA). Anti-β-catenin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-cyclin D1 antibody was a generous gift from Dr. Heather E. Kleiner (Louisiana State University Health Sciences Center, Shreveport, LA). Anti-PCNA antibody was a kind gift from Dr. Alan R. Lehmann (University of Sussex, Brighton, UK). The secondary antibodies conjugated to HRP, PCR buffer, reverse transcriptase enzyme were purchased from Genei (Bangalore, India). All the other materials and chemicals were of highest degree of purity obtained from Sisco Research Laboratories (Mumbai, India).

Animals

Male mice were used for the study. They were obtained from Kings Institute of Preventive Medicine, Chennai, India. The mice were housed in a specific pathogen-free environment under strictly controlled light cycle conditions, fed a standard rodent lab chow, and provided water *ad libitum*. All procedures were performed in accordance to the laws and conditions of the ethical committee board in India (Ethical clearance IAEC/No. 01/013/2010).

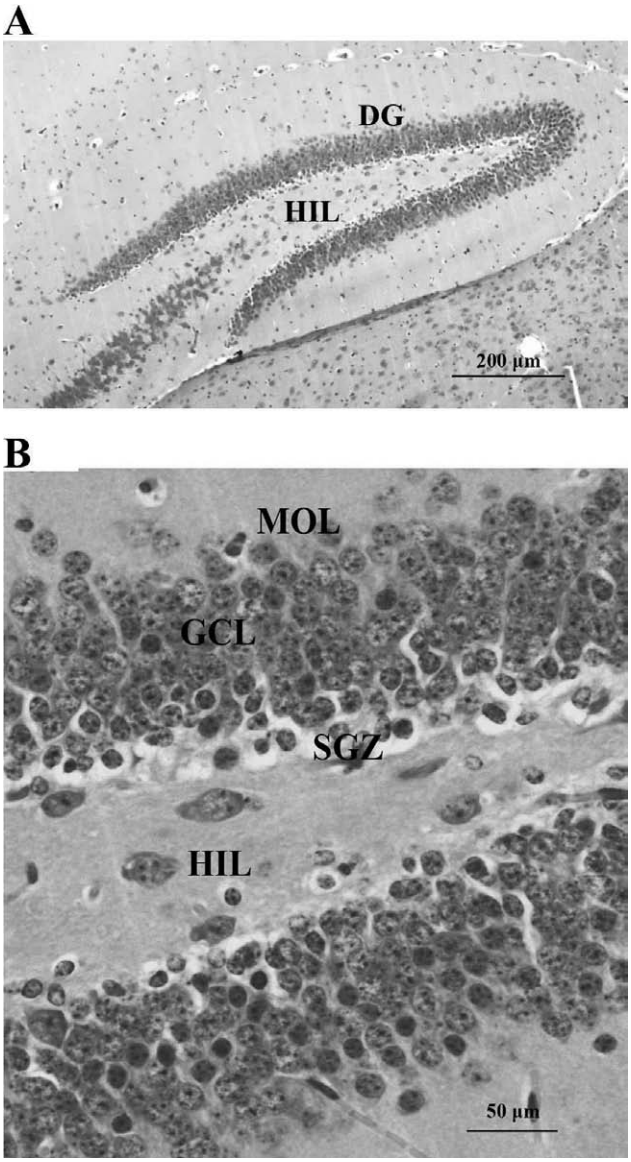

Fig. 1. Histological analysis of mouse hippocampus: coronal sections were stained with haematoxylin and eosin. Note the dentate gyrus (DG), hilus (HIL), subgranular zone (SGZ), granular cell layer (GCL) and molecular layer (MOL) **(A)** and **(B)**. Scale bars: A, 200 µm; B, 50 µm.

Histology

Two month old mice were anaesthetized with a combination of xylazine/ketamine (10 mg/kg and 75 mg/kg, respectively) in 0.9% NaCl and immediately perfused with 4% paraformaldehyde in PBS at 4 °C. The brains were stored in the same fixative for 24 h, serially dehydrated in alcohol and embedded in paraffin wax. Coronal paraffin sections of 5 µm thickness were made using an ultra microtome and stained with haematoxylin and eosin. The histological analysis of hippocampal dentate gyrus clearly showed two cellular layers (Fig. 1A, B).

Immunohistochemistry

The paraffin tissue sections containing the hippocampal formation were de-waxed in xylene, rehydrated in a

series of ethanol solutions and incubated in 10 mM sodium citrate buffer, pH 6.0, for 10 min at 100 °C for antigen retrieval. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide and non-specific binding was blocked with 3% bovine serum albumin and 0.3% Triton X-100 in PBS for 60 min. The sections were incubated overnight at 4 °C with specific primary antibodies for Wnt 3a (dilution 1 : 250), β-catenin (dilution 1 : 250), cyclin D1 (dilution 1 : 200) and PCNA (dilution 1 : 200). Slides were washed with PBS and incubated with corresponding secondary antibodies conjugated with HRP for 2 h at room temperature. They were developed with DAB solution containing 0.05% DAB, 10 µl H_2O_2 in PBS and counter-stained with haematoxylin. Finally, sections were dehydrated, mounted with DPX and visualized using a phase-contrast microscope (Carl Zeiss, Germany). Negative controls included substituting the primary antibody with a similar dilution of BSA and buffer, which resulted in negative staining.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from 3, 5, 7 and 9 week old mouse hippocampus using TRIzol reagent (Invitrogen, CA) according to the instructions from the manufacturer's protocol. The RNA was preheated for 5 min at 65 °C, immediately cooled on ice and reverse transcribed for 50 min at 42 °C in a 20 µl reaction mixture containing 10 mM dNTPs, 10 mM oligodeoxynucleotides, 25 units of reverse transcriptase and 2 µl of assay buffer. The RT reaction was terminated by heating for 5 min at 70 °C. The resulting cDNA templates were subjected to PCR amplification (Eppendorf, Germany). The primers used for PCR were the following: Wnt 3a – sense: 5′-CATCGCCAGTCACATGCACCT-3′, antisense: 5′-CGTCTATGCCATGCGAGCTCA-3′; β-catenin – sense: 5′-GATTTGATGGAGTTGGACATGG-3′, antisense: 5′-TGTTCTTGAGTGAAGGACTGAG -3′; cyclin D1 – sense: 5′-TGGAGCCCCTGAAGAAGAG-3′, antisense: 5′-AAGTGCGTTGTGCGGTAGC-3′; PCNA – sense: 5′-GACGCGGCGGCATTAAAC-3′, antisense: 5′-GTTCACGCCCATGGCCAG-3′; β-actin – sense: 5′-GGCATCGTGATGGACTCCG-3′, antisense: 5′-GCTGGAAGGTGGACAGCGA-3′. The amplification conditions were 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and final extension at 72 °C for 7 min. The PCR reaction products were electrophoresed on 2% agarose gels with ethidium bromide and visualized in UV light (Gel documentation, Amersham, Hong Kong).

Results

Wnt 3a protein localization in dentate gyrus tissue

Wnt 3a was expressed throughout most of the cells in dentate gyrus (DG), but immune signals were more prominent in the subgranular zone. The function of Wnt 3a in granular cell layer is unclear; Wnt 3a might have a role in survival or maintenance of the granular cells in granular cell layer, and this needs further investigation (Fig. 2E).

β-catenin, cyclin D1 and PCNA localization in subgranular zone cells

Immunohistochemical analysis of mouse hippocampal DG showed a highly positive immunoreactive expression for β-catenin, cyclin D1 and PCNA in subgranular zone (Fig. 2F, G and H). The immunoreactivity of β-catenin, cyclin D1 and PCNA was observed prominently in the nucleus of SGZ cells. A few cells in granular cell layer also showed immunoreactivity for β-catenin, cyclin D1 and PCNA, but their role in the granular cell layer is not clear.

Detection of expressional variation of Wnt 3a during postnatal hippocampal development

RT-PCR analysis of RNA extracted from 3, 5, 7 and 9 week old mouse hippocampus showed a progressive increase in Wnt 3a mRNA expression. In the 9 week old mouse hippocampus, Wnt 3a mRNA expression was high when compared with 3, 5 and 7 week old stages (Fig. 3A).

Differential expression of β-catenin and its effector cyclin D1 during postnatal hippocampus development

RT-PCR analysis showed upregulation in β-catenin and cyclin D1 expression as postnatal hippocampus developed. β-Catenin and cyclin D1 mRNA levels gradually increased from 3 week to 9 week old hippocampus (Fig. 3B and C).

Differential expression of cell proliferation marker PCNA during postnatal hippocampus development

PCNA mRNA expression was high in 9 week old mouse hippocampus when compared with 3, 5 and 7 week old stages (Fig. 3D).

Discussion

The Wnt/β-catenin signalling pathway is an evolutionarily conserved pathway (Logan and Nusse, 2004; Sancho et al., 2004) that plays a major role in various processes during development including cell proliferation and differentiation (Nelson and Nusse, 2004). During subsequent development, Wnt signalling is required in the entire central nervous system for expanding the progenitor cell population by simultaneously promoting cell proliferation and blocking apoptosis and differentiation (Zechner et al., 2003). Deregulation and

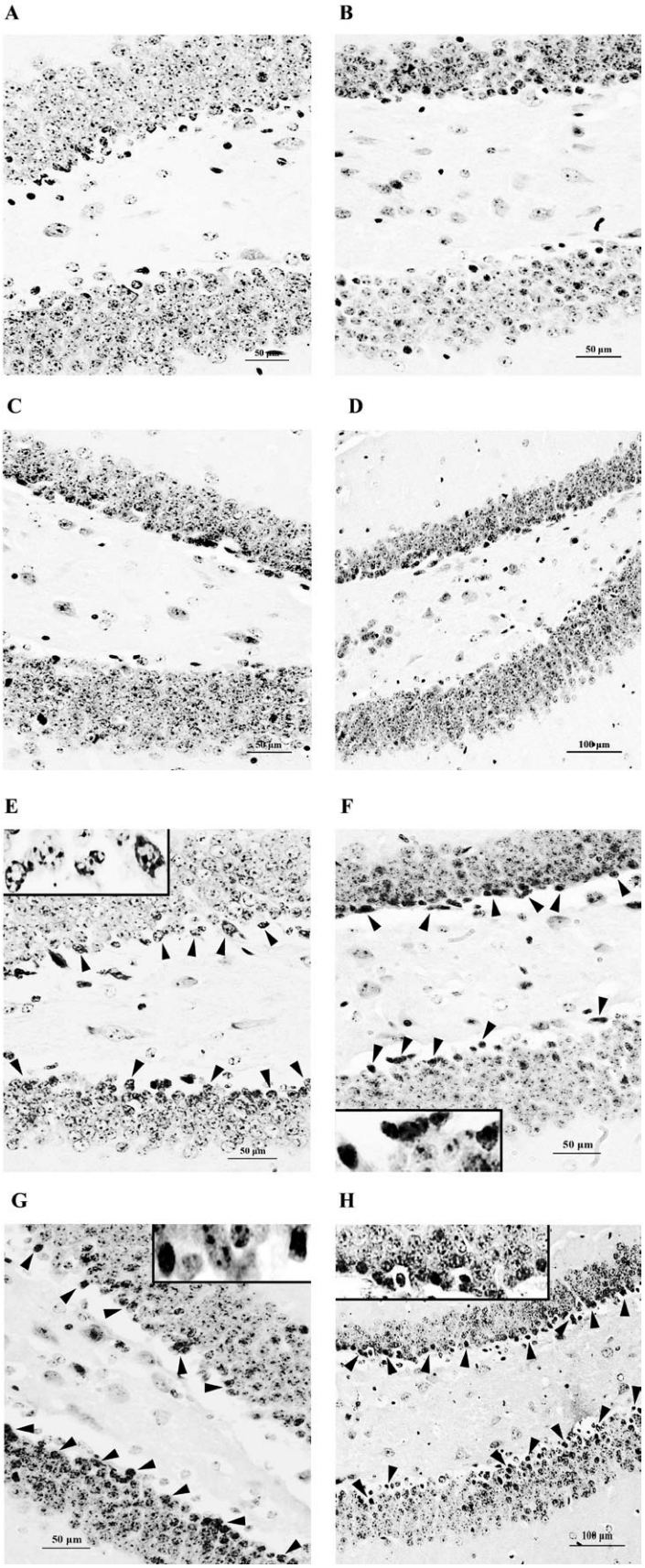

Fig. 2. High-magnification view of Wnt 3a **(E)**, β-catenin **(F)**, cyclin D1 **(G)** and PCNA **(H)** in mouse dentate gyrus. Note the strong membranous staining of Wnt 3a and prominent nuclear staining of β-catenin, cyclin D1, and PCNA in neural stem cell niche, SGZ (black arrows indicate protein expression). Sections with no primary antibody for Wnt 3a **(A),** β-catenin **(B),** cyclin D1 **(C)** and PCNA **(D)** served as negative controls. Scale bars: A, B, C, E, F and G, 50 µm; D and H, 100 µm.

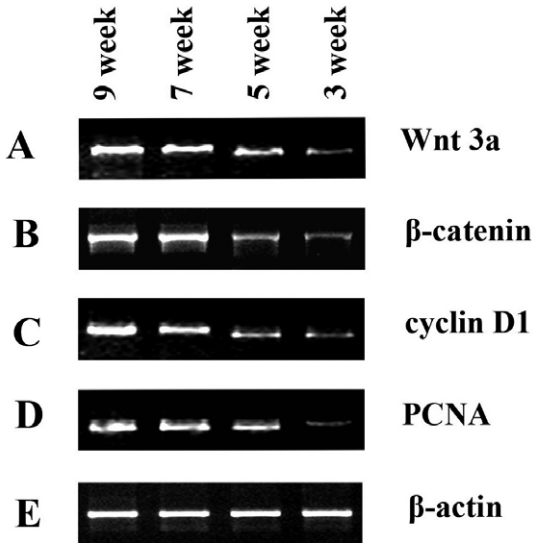

Fig. 3. Wnt signalling components at different stages of developing hippocampus: RT-PCR analysis of RNA from 3, 5, 7 and 9 week old mouse hippocampus show gene expression for Wnt 3a **(A),** β-catenin **(B),** cyclin D1 **(C)** and PCNA **(D)**. β-actin gene product was used as the positive control **(E)**.

inappropriate activation of the Wnt pathway are associated with several diseases including neurological disorders and cancer (Clevers, 2006). In this study, we analysed expression of the key Wnt signalling components, Wnt 3a, β-catenin and cyclin D1 in different stages of postnatal hippocampus and in adult SGZ, to elucidate the involvement of the Wnt pathway in SGZ neural stem cell proliferation.

Cyclin D1 is a major transcriptional target of β-catenin/Wnt signalling (Shtutman et al., 1999). Studies suggest that the expression of cyclin D1 is highly regulated throughout the cell cycle and that its expression level in each phase of the cell cycle helps determine the overall proliferative characteristics of the cell. Cyclin D1 expression must be high for passage through G1 phase and for initiation of DNA synthesis (Hitomi and Stacey, 1999; Guo et al., 2005). Its expression is vital for normal cell cycle progression and cell proliferation (Baldin et al., 1993). Our study revealed the expression of cyclin D1 in SGZ of mouse hippocampus. Studies have reported that cyclin D1 expression can promote cell proliferation (Sherr, 1996) in model systems and humans (Lin et al., 2006; Barbash et al., 2008). There are also reports suggesting that over-expression of cyclin D1 can induce re-entry into the cell cycle from the terminally differentiated state in various types of established cells, including neurons derived from P19 embryonal carcinoma cells (Latella et al., 2001). The results presented here indicate nuclear localization of cyclin D1 in SGZ. To assess the influence of nuclear cyclin D1 in SGZ neural stem cell proliferation, PCNA was utilized. PCNA is a well-characterized proliferation marker expressed by all cells undergoing cell cycle. The present findings demonstrate that cyclin D1 and PCNA expression was enhanced in neural stem cell niche SGZ when compared to the granular cell layer. Cyclin D1 localization correlated with the cell proliferating marker PCNA. Also, cyclin D1 gene up-regulation during the postnatal hippocampus development highly correlated with up-regulation of PCNA. Combined, these data suggest that cyclin D1 may influence neural stem cell proliferation in SGZ.

Wnt 3a is a secreted glycoprotein that interacts with cell membrane-associated proteins. Mice in which Wnt 3a had been deleted exhibited hippocampal progenitor cell pool depletion and severe reduction in hippocampal development (Lee et al., 2000). This finding probably indicates that Wnt 3a can mediate progenitor pool expansion. Such an action is also suggested by our observation that the Wnt 3a expression level in hippocampus increases as it develops, which raises the possibility that the Wnt 3a-mediated signalling can also help in neural stem cell expansion in SGZ.

β-Catenin is a key downstream effector of the Wnt pathway. Stabilization of β-catenin results in persistent activation of signalling, which can increase expression of genes that drive cell proliferation such as cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999). We found that SGZ cells express β-catenin. The nuclear localization of β-catenin suggests that it may transcribe cyclin D1 in SGZ cells; this data is consistent with the idea that nuclear β-catenin can activate cyclin D1 (Glass and Karsenty, 2006).

In the present study, the existence of Wnt 3a, β-catenin and cyclin D1 in SGZ suggests that the Wnt pathway is activated in SGZ, because all the data presented here are consistent with the canonical pathway in which cytoplasmic stabilization of β-catenin leads to translocation of β-catenin into the nucleus and subsequent activation of cyclin D1 transcription. With reference to mammalian hippocampus, evidence suggests that neural stem cells in SGZ expand as the hippocampus develops. In our results we found that Wnt signalling components and proliferating marker PCNA gradually increase as the postnatal hippocampus develops. Furthermore, we found that the increase in Wnt3a expression was followed by an increase in β-catenin, cyclin D1 and PCNA, showing that cyclin D1 is the target of Wnt3a and can possibly help in cell proliferation.

In conclusion, we suggest that the Wnt pathway can play an important role in regulating the SGZ neural stem cell proliferation in the murine hippocampus.

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