Neuroscience 213 (2012) 19–28

SUPPRESSION OF FRIZZLED-2-MEDIATED WNT/CA^{2+} SIGNALING SIGNIFICANTLY ATTENUATES INTRACELLULAR CALCIUM ACCUMULATION *IN VITRO* AND IN A RAT MODEL OF TRAUMATIC BRAIN INJURY

L.-J. NIU,[a,b,c] R.-X. XU,[a,b,c*] P. ZHANG,[a,b] M.-X. DU[a,b] AND X.-D. JIANG[a,b]

[a] *Institute of Neurology, Southern Medical University/Zhujiang Hospital, Guangzhou 510282, PR China*

[b] *Laboratory of Brain Function and Repair, Guangdong Province 510282, PR China*

[c] *Affiliated Brain Hospital, Military General Hospital of Beijing PLA, Beijing 100041, PR China*

Abstract—Traumatic brain injury (TBI) can dramatically increase levels of intracellular calcium (Ca^{2+}). The association between Wnt5a/Frizzled-2 (wingless-type mouse mammary tumor virus integration site family member 5a/Fzd2) signaling and Ca^{2+} cellular homeostasis in lower vertebrates has been well documented. However, little is known about Wnt5a/Fzd2 signaling in mammalian nerve cells, or whether Ca^{2+} accumulation after TBI is mediated through this pathway. We hypothesized that an activated Wnt5a/Fzd2 pathway following TBI may play a role in Ca^{2+} overloading. To elucidate the influence of Fzd2 and the Wnt5a signal transduction pathway on an increase in intracellular Ca^{2+}, we assessed the expression of Wnt5a/Fzd2 in rat hippocampal cells both *in vitro* and *in vivo.* We found that transfection of the rat *Fzd2* gene in rat neonatal hippocampal astrocytes significantly increased gene expressions of both *Wnt5a* and *Fzd2* by fourfold when compared to non-transfected cells ($P < 0.01$ in both cases). Expressions of the proteins Wnt5a and Fzd2 were significantly increased approximately two- and threefold, respectively, when compared to non-transfected control cells ($P < 0.01$ in both cases). Moreover, intracellular Ca^{2+}, as manifested by the fluorescent intensity of the intracellular Ca^{2+} indicator Fluo-3/AM, was significantly increased by 1.75-fold ($P < 0.01$). The blocking of Fzd2 signaling using Stealth RNAi markedly inhibited the elevated gene and protein expression of Wnt5a in the transfected cells by two- and fourfold, respectively ($P < 0.01$), and suppressed intracellular Ca^{2+} by 1.5-fold ($P < 0.01$). Furthermore, *in vivo*, we demonstrated that TBI-induced dramatic upregulation of gene and protein expression of Wnt5a/Fzd2 by two- and five-fold ($P < 0.01$) in injured hippocampi, and intracellular Ca^{2+} increased in isolated injured hippocampal cells. Whereas, the *in vivo* blocking of Fzd2 signaling by hippocampal delivery of Stealth RNAi and Invivofectamine significantly suppressed the increased gene and protein expression of Wnt5a and Fzd2 induced by TBI by 1- to 3.5-fold ($P < 0.01$) and also inhibited Ca^{2+}accumulation by 1.5-fold ($P < 0.01$). These findings demonstrated that the Wnt5a/Fzd2 signaling pathway contributed to increasing intracellular Ca^{2+} in nerve cells under physiological and pathological conditions. Furthermore, our findings provide evidence that specifically expressed components of this signal pathway, such as Wnt5a and Fzd2, are potential therapeutic targets following brain trauma.

Key words: Wnt5a, Frizzled-2, Wnt/Ca^{2+} pathway, calcium, astrocytes, TBI.

INTRODUCTION

Intracellular calcium (Ca^{2+}) is an important secondary messenger of the nervous system (Farber, 1981). It regulates diverse cell functions including cell signaling, gene expression, neurotransmitter release, and assembly of the cytoskeleton (Carafoli, 1987; Racay et al., 1996), and it is a key element in maintaining physiological functions of nerve cells (Verkhratsky et al., 1998). Under normal conditions, levels of Ca^{2+} are tightly regulated, with free intracellular concentrations maintained at 50–90 nM (Racay and Lehotsky, 1996).

After neuronal injury neurons and other brain cells are excited abnormally, following which Ca^{2+} channels open, Ca^{2+} traverses the membrane, and cytosolic Ca^{2+} is also released; [Ca^{2+}] accumulates until cell death occurs (Sullivan et al., 2004, 2005; Alberdi et al., 2005). This is the model of the well-known calcium hypothesis of cell injury. According to this model, pathological conditions such as traumatic brain injury (TBI) disrupt Ca^{2+} homeostasis dramatically and ultimately lead to cell death due to an increase in the concentration of Ca^{2+} (Schanne et al., 1979).

*Corresponding author. Address: Affiliated Brain Hospital, Military General Hospital of Beijing PLA, 5 South Gate, Beijing 100041, PR China. Tel: +86-10-66721024; fax: +86-10-64036310.

E-mail address: jz_xuruxiang@163.com (R.-X. Xu).

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; CaMKII, calcium–calmodulin-dependent protein kinase II; cDNA, complementary DNA; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FBS, fetal bovine serum; Fzd2, Frizzled-2; IP, intraperitoneal; LEF1, lymphoid enhancer-binding factor 1; LRP, low density lipoprotein receptor-related protein; p-CaMKII, phosphorylated CaMKII; PMSF, phenylmethylsulfonyl fluoride; Q-PCR, quantitative real-time PCR; SDS, sodium dodecyl sulfate; TBI, traumatic brain injury; TBST, Tris-buffered saline with Tween 20; TCF, transcription factor; Wnt5a, wingless-type mouse mammary tumor virus integration site family member 5a.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.03.057

Although the role of Ca^{2+} in secondary cell injury and death has been established, little is known of the specific underlying molecular mechanisms involved. Studies with *Xenopus* and Zebrafish embryos have demonstrated that the frizzled family receptor 2 (Fzd2) protein mediates a non-canonical wingless-type murine-mammary-tumor virus integration site Wnt/Ca^{2+} signal pathway (also known as Wnt family member 5a (Wnt5a)/Fzd2) (Ishitani et al., 2003; Sheldahl et al., 2003), which correlates with intracellular Ca^{2+} release (Sheldahl et al., 1999). Related studies with rats have demonstrated that as a specific receptor, Fzd2 protein transduces binding of the cellular secreting ligand Wnt5a to increase intracellular Ca^{2+} release (Slusarski et al., 1997a). Moreover, it has been found that Fzd2 in lower vertebrates stimulates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Kuhl et al., 2000a,b), a marker protein of the activation of the Wnt5a/Fzd2 pathway. The active form of CaMKII is phosphorylated (p-CaMKII) (Peifer and Polakis, 2000).

To date, mechanisms underlying the binding and downstream signaling pathways of Wnt5a/Fzd2 have been widely studied in the cells of lower animals like *Xenopus* or *Drosophila* and in some mammalian tissues (Miller et al., 1999). However, little is known about the roles of these pathways in mammalian nerve cells, or whether Wnt5a/Fzd2 signaling mediates Ca^{2+} accumulation in injured nerve cells. In this study, we examined whether TBI induced the expressions of Wnt5a and Fzd2 proteins, promoted their binding to each other, and/or activated the Wnt5a/Fzd2 signal pathway. We also sought to determine if the accumulation of intracellular Ca^{2+} after TBI was partially through activation of this signal pathway. Here we present evidence that the Wnt5a/Fzd2 signaling pathway is activated following TBI and is involved in accumulation of intracellular Ca^{2+} in injured nerve cells and tissues.

EXPERIMENTAL PROCEDURES

Synthesis of the rat Fzd2 gene

Full-length *Fzd2* (GenBank accession No. NM_172035.1) complementary DNAs (cDNAs) were synthesized by the Shenggong Company (Shanghai, PR China). The synthesized full-length *Fzd2* constructs were cloned into the pEGFP-C1 report vector (Prasher et al., 1992) and all constructs were confirmed by enzyme digestion analysis and sequencing. After linearization, the vectors were co-transformed into competent cells (DH5a; Invitrogen Life Technologies, Carlsbad, CA, USA). The plasmids conferred resistance to kanamycin (30 μg/mL). The recombinant *Fzd2* cDNA was extracted from competent cells using an Endofree Plasmid Maxi kit (Qiagen, Hilden, Germany).

Synthesis and selection of the small interfering RNA, Stealth RNAi, for blocking Fzd2 signaling

Desalting was performed using *In Vivo*-Purity Stealth RNAiTM duplexes designed with Block-ItTM RNAi Designer (Invitrogen Life Technologies, Carlsbad, CA, USA), specifically formulated for blocking the target gene *Fzd2*, *in vivo* or *in vitro*. Three different and non-overlapping RNAi duplexes are offered by Invitrogen, and each differed in the efficiency of suppression. The sense and anti-sense strands of the three Stealth RNAis were: Fzd2_311, beginning at nt 311: sense 5′-UGCAUCAAUUCUACCCGCUGGUGAA-3′ and antisense 5′-UUCACCGCGGGUAGAAUUGAUGCA-3′; Fzd2_954, beginning at nt 954: sense 5′-CACCAUGGUGUCAGUGGCCUACAUU-3′ and antisense5′-AAUGUAGGCCACUGACACCAUGGUG-3′; and Fzd2_1053, beginning at nt 1053: sense 5′-GGGCACUAAGAAAGAAGGCUGUACU-3′ and antisense5′-AGUACAGCCUUCUUUCUUAGUGCCC-3′. The suppression efficiency of the three sequences was evaluated using human embryonic kidney (H293) cells. The RNAi sequence Fzd2_954 had the highest efficiency of suppression, 79.83% at 50 nM (Fig. 1), and therefore it was chosen for the remaining experiments.

Isolation and culture of rat neonatal primary hippocampal astrocytes

The method of isolation of hippocampal astrocytes from one-day-old neonatal rats (n = 5) was the same as described previously (Liu et al., 2006). The rats were sacrificed by decapitation. The hippocampi were isolated and transferred into Dulbecco's modified Eagle's medium (DMEM; GIBCO, Invitrogen Life Technologies, Carlsbad, CA, USA), and minced with scissors. Ten hippocampi were transferred into 21.5 mL of Hank's balanced salt solution (Invitrogen Life Technologies), digested in 2.5 mL of trypsin–EDTA (2.5%, Invitrogen) and 1 mL of pancreatin (2.5%, ICN Biochemicals) for 30 min at 37 °C with occasional shaking. Trypsin was deactivated by adding 7.5 mL of DMEM with 10% fetal bovine serum (FBS; Invitrogen) and 1.5% penicillin/streptomycin (Invitrogen). Digested hippocampi were collected by centrifugation at 500×g for 5 min, resuspended in 20 mL of DMEM with 10% heat-inactivated FBS and 1% penicillin/streptomycin, and plated onto poly-D-lysine-coated 75 cm^2 tissue-culture flasks. The media was changed on day 2 and every third day thereafter. After 10–14 days, when astrocytes reached confluency, microglia and oligodendrocytes were removed by shaking the flasks at 240 rpm for 6 h. The adherent astrocyte cultures were rinsed with PBS, and passaged using trypsin–EDTA (1:3). Once astrocytes were confluent, they were used for experiments.

Transfection and target gene silencing with Stealth RNAi + Lipofectamine 2000

One day before transfection, 10^5 cells/well in 6-well plates, or 10^6 cells in 100 mm dishes, were seeded in the growth medium without antibiotics. At the time of transfection, the cells reached 50–60% confluency. Transfections were performed using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's protocol. Cells were incubated in Opti-MEM I Reduced Serum Medium (Invitrogen Life Technologies) with or without Stealth RNAi-Lipofectamine 2000 complexes (50 nM) or plasmid-Lipofectamine 2000 complexes (2 μg/mL) for 6 h, and then replaced with fresh DMEM. Cells that underwent transfection with empty vector-Lipofectamine 2000 complexes were set as the control group (referred to as ''non-transfection''). Cells were incubated for another 24 h before RNA isolation for quantitative real-time PCR (Q-PCR) analysis, or 48 h before collecting protein samples for Western blot.

Animals

A total of 96 male Sprague–Dawley rats (10–12 weeks old, Beijing Vital River Laboratory Animal Technical Company) weighing 300–350 g were used in the *in vivo* (TBI) experiments. They were randomly separated into three groups: the sham control group (n = 32), TBI group (n = 32), and TBI + Stealth RNAi injection group (n = 32). All animal procedures were performed under the guidelines set by the Southern Medical University, Guangdong