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Original Article

GSK3β expression and phosphorylation during neuronal maturation in the rat dorsal root ganglion

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Abstract: Glycogen synthase kinase 3β (GSK-3β) protein is a key regulator of neurogenesis, neuronal differentiation and polarisation during neurodevelopment. Sensory neurons in dorsal root ganglion (DRG) undergo a series of development stages during its maturation. In this study, we investigated the dynamic changes in GSK-3β expression and phosphorylation of its N-terminal serine-9 residue (p-GSK-3β (S9)) during DRG development. Sprague-Dawley (SD) rats were divided according to the following ages: Embryonic 13th (E13), E15, E19, Postnatal 1st (P1), P3, P7, P14, P21 and P60 days. GSK-3β was detected by immunohistochemistry and double immunofluorescence on DRGs. Western blotting was used to determine the quantity of GSK-3β and p-GSK-3β (S9) expression. It was found that GSK-3β immunopositive cells in the DRG appeared as early as E13 development phase, and gradually increased to a peak level at P3, at which almost all neurons were GSK-3β positive, and then stayed at a high level to the experiment day 60. GSK3β expression was cell-type-specific during DRG maturation and exhibited cytoplasmic staining in the neuronal cell body and the axon. Glial cells consistently remained negative in DRGs at all stages. Western blot analysis revealed that GSK3β expression stayed the same during DRG maturation. In contrast, p-GSK-3β (S9) expression was stage-specific and decreased from E13 to P60 ($P < 0.01$). Taken together, these results suggest that GSK-3β expression is stage-specific and cell-type-specific during DRG maturation.

Keywords: GSK-3β, p-GSK-3β (S9), development, dorsal root ganglion, neurogenesis

Introduction

GSK-3β and its upstream and downstream regulators have key roles in many fundamental processes during neurodevelopment including neurogenesis, neuronal migration, neuronal polarisation and axon growth guidance [1]. The status of GSK-3β activity has been associated with many psychiatric and neurodegenerative diseases, such as Alzheimer's disease, schizophrenia and autism spectrum disorders [1-3].

GSK-3β is abundant in nervous system and displays more than 90% sequence similarity within the kinase domain between flies and humans [4-7]. It acts as a serine/threonine kinase and was identified as a key regulatory kinase that phosphorylates glycogen synthase and participates in the regulation of protein synthesis, microtubule dynamics, cell proliferation, differentiation, motility, and apoptosis [1, 4, 6, 8]. Phosphorylation of the N-terminal serine 9 residue of GSK-3β results in inactivation of the kinase. Recently, extensive evidence suggested that GSK-3β is a major signalling node that regulates many fundamental processes during neurodevelopment [1, 7]. GSK-3β not only regulates the gene expression of transcription factors throughout neurodevelopment and the activity of several microtubule-associated proteins to reorganise the cytoskeleton, but also coordinates movement of the leading process and the soma during neuronal migration, and directs growth cone advancement during axon growth and guidance [1]. Regulating GSK-3β activity has been shown to profoundly influence the neuronal axon growth and interfere with the neuronal dendrite/axon specification [5]. Deleting GSK-3β in the developing nervous system markedly enhances neural progenitor prolifera-

tion and inhibits neurogenesis [9]. Although GSK-3β has been shown to be a key regulator of central nervous system development, its effect on peripheral nervous system development still remains to be determined. During dorsal root ganglion (DRG) maturation, sensory neurons undergo proliferation, polarisation and transformation from bipolar to pseudo-unipolar neurons that transmit sensory information from the periphery to the central nervous system [10].

Therefore, a better revealing the magnitude and time course of GSK-3β expression in neurodevelopment of DRG could provide insight into the physiology of GSK-3β and/or etiology of nerve disorders and possibly open up a new potential library of therapeutic targets.

Methods

Experimental procedure

The experimental protocols were performed as approved by the local Ethical Committee of Animal Experimentation of Tongji University. After being anaesthetised with 4% chloral hydrate (400 mg/kg, i.p.), the animals at P1, 3, 7, 14, 21, 60 days (n = 5, each stage/group) were transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4); the embryonic rats at 13, 15, 19 days (n = 5, each stage/group) were cut out from pregnant mater; the DRG tissues and fetus were collected and postfixed in the same fixative and were transferred to 30% sucrose 24 hours later. For western blotting, the DRG tissues were harvested immediately after saline perfusion and were frozen in liquid nitrogen and stored at -80°C.

Western blot

The DRG samples were homogenised in RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and a phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Thirty μg of total protein was loaded in each well and subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The specific GSK3β (27C10) and p-GSK-3β (S9) antibodies (Cell Signaling Technology, Danvers, MA, USA) at dilutions 1:1000 or β-Actin (Earthox, LLC, San Francisco, CA, USA) as an internal standard 1:10,000 were used to incubated the membrane. Immunoreactive signals were detected by a Chemiluminescence Reagent (Thermo Fisher, Rockford, IL, USA) and quantified by densitometry using the Image J software.

Immunohistochemistry

The DRGs were flash frozen, and horizontal sections (thickness 20 μm) were cut on a cryostat. Free floating immunohistochemistry was performed by the ABC (avidin-biotin complex) technique as described previously [11]. Briefly, DRG sections were incubated in 0.3% H_2O_2 in methanol for 30 minutes, then treated with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min in 0.01 M phosphate buffered saline (PBS), pH 7.2. After blocking with PBS containing 0.5% bovine serum albumin (BSA) for 1 h, the sections were incubated overnight at 4°C with specific primary antibodies against GSK3β, then immersed in a biotinylated goat anti-rabbit IgG (H+L) antibody (KPL, MD, USA), diluted 1:200 for 2 h. After that, streptavidin-HRP (Invitrogen, California, USA) diluted 1:200 was applied for 2 h. At last, the samples were incubated in a solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (50 mg/100 mL, Sigma) and 0.005% (v/v) H_2O_2. Negative controls by omitting the primary antibody were included in these experiments.

Double immunofluorescence

After blocking, the DRG sections were incubated overnight at 4°C with primary antibody against GSK3β, NeuN (mouse monoclonal, 1:100, GeneTex, San Antonio, TX, USA), Iba1 (goat polyclonal, 1:500, Abcam, Cambridge, MA, USA), or GFAP (mouse monoclonal, 1:1000, Abcam). The sections were washed and incubated for 2 h at RT with secondary antibodies, Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 594 donkey anti-mouse IgG, and Alexa Fluor 594 donkey anti-goat IgG (1:500, Invitrogen, Carlsbad, CA, USA) respectively. Following washing, the sections were mounted under glass coverslips using mounting media containing 4'6'-diamidino- 2-phenylondole dihydrochloride (DAPI) serving as a counterstain.

Statistical analysis

In immunohistochemistry study, 10 sections in each DRG were selected randomly, and the

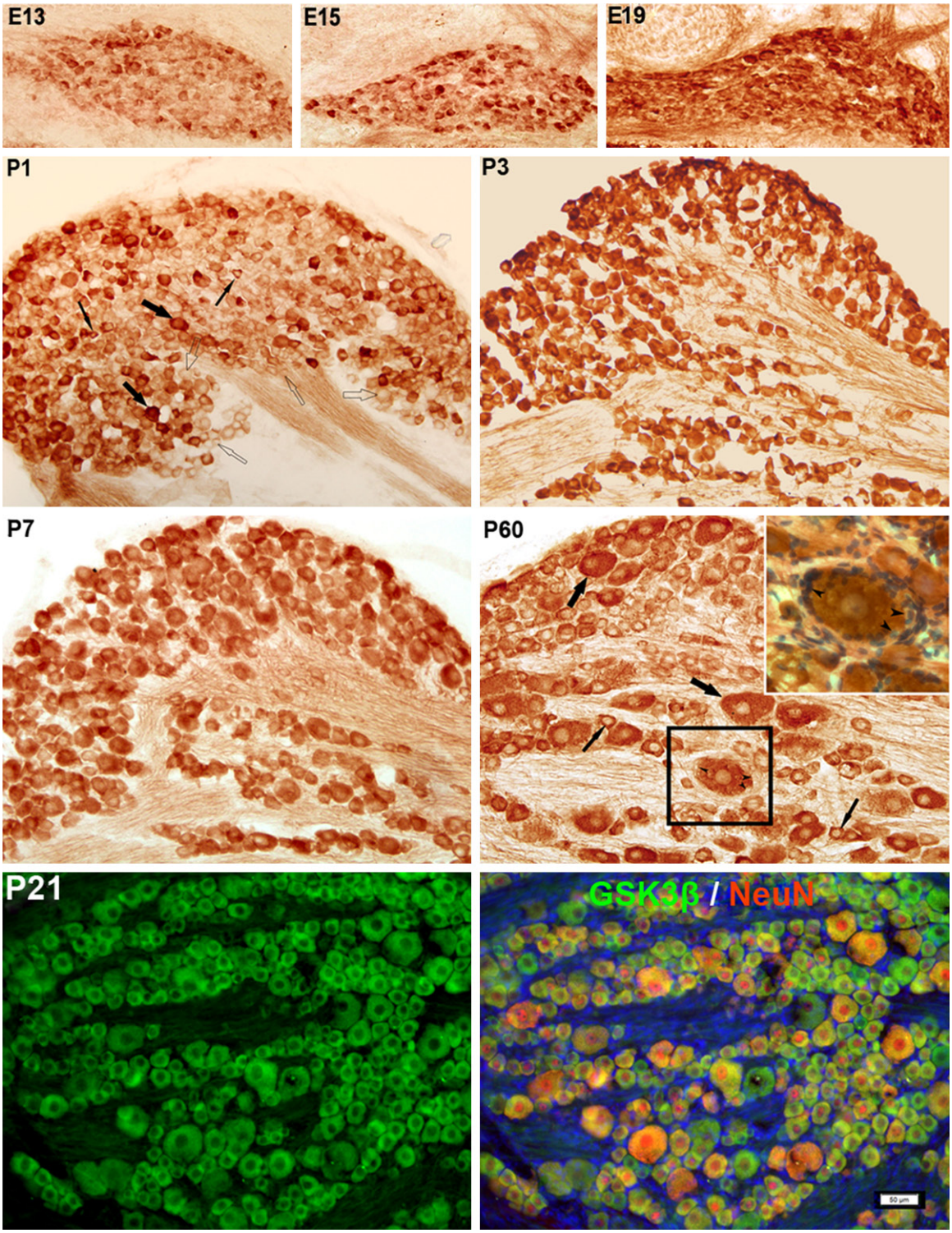

Figure 1. Immunohistochemical staining for GSK-3β in DRGs from different developmental stages. GSK-3β exhibited intense cytoplasmic immunolabeling in the large and small neuronal body. At E13, E15, E19, and P1, the GSK-3β immunopositive neurons were 15% , 47%, 69%, 86% respectively, from P3 and later on, almost all the neurons in the DRG were GSK-3β positive. Thick solid arrow, GSK-3β positive large sensory neuron; thin solid arrow, GSK-3β positive small sensory neuron; thick empty arrow, GSK-3β negative large sensory neuron; thin empty arrow, GSK-3β negative small sensory neuron. The boxed area shown at higher magnification in the inset at P60 showing the same section stained with H&E confirms that the SGCs lied beyond the brown GSK-3β positive sensory neurons with bright blue nucleus and obscure cell bodies. Satellite glial cells remained negative in DRGs from all stages. Arrow heads, pointing the margin of GSK-3β positive neurons. In all DRGs from different development stages, GSK-3β (green) colabelled with neuron specific marker NeuN (red).

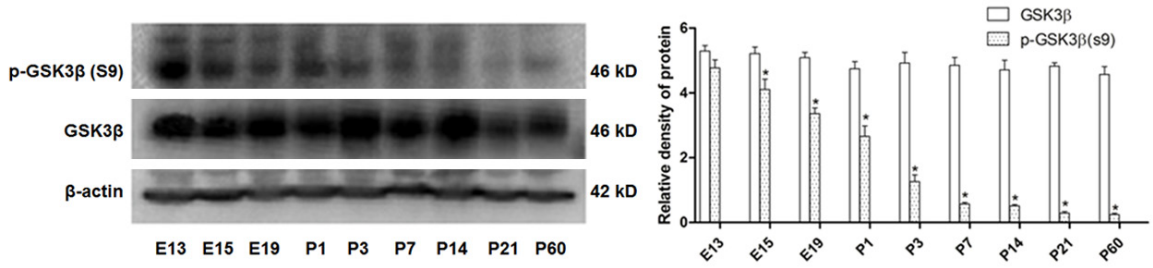

Figure 2. Western blotting analysis of GSK-3β and p-GSK-3β (S9) expression in DRGs from different development stages. From E13 to P60, GSK-3β expression remained unchanged. p-GSK-3β (S9) expression decreased with age and peaked at E13, after birth, p-GSK-3β (S9) levels decreased sharply, and from P3 to P60, p-GSK-3β (S9) remained at a basal level. Data are expressed as means ± s.e.m (n = 5 each group). *P < 0.01 vs. E13. E13, E15, E19 indicate sampling days of embryo, and P1, P3, P7, P14, P21, P60 postnatal days.

numbers of GSK-3β immunoreactive neurons were counted by two independent observers who were blinded to this experiment. The results of this study were expressed as the means ± s. e. m. Significant differences among groups were assessed using one-way analysis of variance (ANOVA). Differences between the groups were evaluated by Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

GSK-3β localised to DRG neurons

GSK-3β immunohistochemistry DAB staining revealed the DRGs from different developmental stages of rats. The GSK-3β immunopositive cells demonstrated shallow to deep brown, and the reaction precipitate was mainly localised to the cytoplasm. The positive cells were round or oval, either large or small, distributed scatteredly over the DRG, with pale nucleus and distinct cell border, which suggested that the GSK-3β immunopositive cells in DRG were large light and small dark sensory neurons. From P3 and afterwards, almost all the neurons in DRG were GSK-3β positive with consistent intensity, but during E13 to P1, only some neurons in DRG of each stage expressed GSK-3β. The rates of the GSK-3β immunopositive cells were E13 (15 ± 2.5%), E15 (47 ± 8.9%), E19 (69 ± 10.4%), and P1 (86 ± 7.1%) respectively. Axons displayed weak positive staining during DRG development. After counterstaining with H&E, the GSK-3β immunopositive cells in adult DRGs were still in brown under the microscope, and showed the clear cell boundary. The surrounding satellite glial cells (SGCs) lied on the outside of the brown neurons with bright blue nucleus, but pale cytoplasm and unsharp cell margin, indicating SGCs were GSK-3β immunonegative (**Figure 1**).

GSK-3β expression was neuron-specific during DRG maturation

GSK-3β localisation in DRGs neurons was further confirmed by immunofluorescent photomicrographs to avoid any possible artifactual staining from immunoperoxidase staining. In all DRGs from different developmental stage rats, GSK-3β colabelled with NeuN indicating that GSK-3β was expressed in neurons (**Figure 1**) and was strong in neuronal bodies. In contrast, Iba1-positive resident macrophages/microglia did not express GSK-3β. Satellite glial cells (SGCs) were identified by their immunofluorescent staining for GFAP. However, the basal level of GFAP expression is quite low in SGCs of normal animals [12]. In our study, there was also an absence of GFAP labeling of SGCs in DRGs from E13 to adult rats (Data not shown).

p-GSK-3β (S9) expression was decreased during DRG maturation

Western blot analysis (**Figure 2**) revealed that although GSK-3β expression remained invariable from E13 to P60, at E13, GSK-3β was significantly phosphorylated at the N-terminal serine 9, and from then on, the p-GSK-3β (S9) level decreased with age ($P < 0.01$). Moreover the p-GSK-3β (S9) expression level decreased sharply after birth, but from P3 to P60, it remained at a basal level.

Discussion

GSK-3β is involved in neural development and neuronal polarisation and has been considered to be a convergent point for regulatory path-

ways to control neurogenesis [1, 5, 7, 13]. In the present study, we found that GSK-3β was expressed in the DRG sensory neurons, and GSK-3β positive neurons increased gradually during embryonic and early postnatal days. While, p-GSK-3β (S9), the inactivated form of GSK-3β, showed a peak level in E13 and decreased afterwards during the DRG developmental process, suggesting a close relationship between GSK-3β and DRG development.

DRG contains both neurons and large number of glial cells including Iba1/OX-6 positive microglias [14, 15], Schwann cells and SGCs. The sensory neuron and its surrounding SGCs form a distinct morphological and probably functional unit [15]. DRG neurons, which can be identified and characterized into at least 25 subgroups, morphologically mainly divided into two types, large light and small dark neurones with physiological, biochemical and functional differences [16, 17]. Originally, in rat, DRG cells migrate ventrally from the neural crest on embryonic day 11, and discrete ganglia can usually be observed at day E13, when proliferation and division of neurons are remarkable, meanwhile the sensory fibers reach the spinal cord or grow into the ventral root to form mixed spinal nerves [18, 19]. In detail, the last cell division of most of the small DRG neurons is on day E13, all large neurons have ceased to divide by day E15, and later undergo pseudo-unipolarization [13, 20].

In our study, we detected that the GSK-3β positive neural cells were limited to neurones, either large light or small dark. The microglias and SGCs in DRG were GSK-3β negative. These results were similar to the previous findings in which the authors found that GSK-3β expression was primarily localized in neurons of rat cerebellum or in pyramidal cells of the hippocampus, and during development was distinctly abundant in young neurites [21].

GSK-3β plays important roles for neuron development, and suggested that GSK-3β activation promotes neuronal differentiation [22]. This present study indicated that from E13 to P1, the quantity of GSK-3β positive neurons was increased gradually, until P3 at which almost all the neurons were GSK-3β positive. From their early embryonic development, the neural crest stem cells in DRG are continuously differentiating, with remarkable morphological changes from the bipolar to the more mature pseudo-unipolar shape [10], and give rise to the large neurons express neurotrophic tyrosine receptor kinase (Trk) C, medium-sized TrkB and small-sized TrkA [23]. Our present results suggested that the GSK-3β positive neurons maybe the maturing or differentiated neurons, which needs further study.

Otherwise, GSK-3β inhibition promotes the proliferation of neural progenitor cells [1, 9, 10]. And GSK-3β has been shown to be involved in neuronal polarisation; inhibition of GSK-3β by small-molecule inhibitors or knock-down promoted microtubule assembly and axon branching [24], and localised inactivation of GSK-3β at the nascent axon is required for both the establishment and maintenance of neuronal polarity, however over-expression of p-GSK-3β (S9) prevents axon formation [25]. In this study, we observed that p-GSK-3β (S9) expression peaked at E13, a remarkable proliferation and polarity transformation period, and during DRG maturation phase, p-GSK-3β (S9) decreased steadily till P3 at which to a basal level. These lines of evidence suggest that GSK-3β activity plays a key role in neuronal development in DRGs and may be regulated by phosphorylation of serine-9. In the present study, the overall p-GSK-3β (S9) level decreased with increased neuronal polarity, in agreement with previous studies. Collectively, this evidence suggests that GSK-3β plays a key role in neurons maturation in DRG development through the regulation of serine-9 phosphorylation on GSK-3β.

According to recent study, extracellular factors can regulate GSK-3β activity to initiate neuronal development and polarisation [1]. However, to date, it is not known how GSK-3β activity is regulated in DRG development. Satellite glial cells wrap completely around and intertwine with the sensory neuronal cell body. During DRG maturation, satellite glial cells continue to divide rapidly, resemble those in neurons [18, 19], but glial cells are negative for GSK-3β expression. Activation of satellite glial cells might influence neighbouring neurons and control the morphology of neurons in the DRG [16]. It remains to be determined whether a relation exists between satellite glial cells and the regulation of GSK-3β in sensory neurons.

Conclusions

The expression of GSK-3β and its inactivated form p-GSK-3β (S9) is stage-specific and cell-

specific during DRG maturation. There is a close relationship between GSK-3β and sensory neuronal development during rat DRG maturation, which may be regulated by the phosphorylation of serine-9 on GSK-3β.

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Disclosure of conflict of interest

None.

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