Glycogen synthase kinase 3β functions as a positive effector in the WNK signaling pathway

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Abstract

The with no lysine (WNK) protein kinase family is conserved among many species. Some mutations in human WNK gene are associated with pseudohypoaldosteronism type II, a form of hypertension, and hereditary sensory and autonomic neuropathy type 2A. In kidney, WNK regulates the activity of STE20/SPS1-related, proline alanine-rich kinase and/or oxidative-stress responsive 1, which in turn regulate ion co-transporters. The misregulation of this pathway is involved in the pathogenesis of pseudohypoaldosteronism type II. In the neural system, WNK is involved in the specification of the cholinergic neuron, but the pathogenesis of hereditary sensory and autonomic neuropathy type 2A is still unknown. To better understand the WNK pathway, we isolated WNK-associated genes using Drosophila. We identified Glycogen synthase kinase 3β (GSK3β)/Shaggy (Sgg) as a candidate gene that was shown to interact with the WNK signaling pathway in both Drosophila and mammalian cells. Furthermore, GSK3β was involved in neural specification downstream of WNK. These results suggest that GSK3β/Sgg functions as a positive effector in the WNK signaling pathway.

Introduction

The with no lysine (WNK) protein kinases are atypical members of the serine/threonine kinase family, and are conserved among many species [1–3]. The mammalian WNK family has four members: WNK1–4. WNK1 and WNK4 have been identified as causative genes of pseudohypoaldosteronism type II (PHAII) [4], and WNK1 is also a causative gene of hereditary sensory and autonomic neuropathy type 2A (HSAN2A) [5]. Several groups including ours have attempted to identify the functions of the WNK family. In the kidney, WNK1 and WNK4 phosphorylate and activate STE20/SPS1-related, proline alanine-rich kinase (SPAK) and oxidative-stress responsive 1 (OSR1) kinases, which in turn regulate ion co-transporters [6–9]. Because knock-in mice of Wnk4 ΔS661A (the mutation found in PHAII patients) display similar phenotypes to PHAII, dysregulation of this WNK signaling pathway was thought to cause hypertension in PHAII patients [10]. In the neural system, a neural-specific alternatively spliced isoform of WNK1 is expressed, which includes the neural-specific exon HSN2. In
HSAN2A patients, mutations were found in this HSN2 exon [5, 11], but HSN2 knock-out mice have no discernable morphological phenotype [11, 12]. Furthermore, in other familial HSAN2A patients, mutations were found outside the HSN2 exon in WNK1 [13]. Thus, the pathogenesis of HSAN2A remains unclear.

WNK kinases are required for EGF-mediated ERK5 activation, and WNK family members are also involved in proliferation, migration, and differentiation [14–16]. Recently, we found that WNK1 and WNK4 induced Lhx8 expression and were important for neural specification [17]. Moreover, WNK was identified as a positive regulator of the Wnt signaling pathway; however, the detailed mechanisms of this are unknown [18]. Although WNK has a range of functions during many developmental processes, little is known about the components of the WNK signaling pathway, except for the main molecules WNK1/4–SPAK/OSR1. In the kidney, ASK3 inhibits WNK1 [19], and the PI3K/AKT signaling pathway activates the WNK–SPAK/OSR1–NCC pathway [20]. Other upstream or downstream component(s) are still unknown.

Glycogen synthase kinase 3β (GSK3β) is a ubiquitously expressed serine/threonine kinase that was originally identified as the regulatory kinase of glycogen synthase. Since then, GSK3β has been shown to be involved in many biological processes [21]. GSK3β plays important roles in several signaling pathways, especially PI3K/AKT and Wnt signaling pathways. In the PI3K/AKT signaling pathway, AKT phosphorylates Ser9 of GSK3β which inhibits its activity, thus phosphorylating cyclin D1 and regulating the cell cycle [22]. In the Wnt signaling pathway, GSK3β is a major component of the destruction complex that phosphorylates β-catenin, which in turn is degraded by proteasomes [22].

In this study, we attempted to identify a new component of the WNK signaling pathway using Drosophila, and identified the shaggy gene (sgg) as a possible candidate. Sgg is a Drosophila homolog of mammalian GSK3β. We found that GSK3β worked as a positive effector downstream of WNK in both mammalian and Drosophila cells.

Materials and methods

Ethics statement

All the animal experiments were performed under the ethical guidelines of Tokyo Medical and Dental University, and animal protocols were reviewed and approved by the animal welfare committee of the Tokyo Medical and Dental University.

Fly stocks and genetics

Fly strains used in this study were; Canton-S, y w, EY10165 (UAS-Wnk; Bloomington Stock Center #16970), UAS-fray [17], Wnk [18] FRT2A [17], Akt1 [19] (Bloomington Stock Center #11627), sgg [1] (Bloomington Stock Center #9095), sgg [2] (Bloomington Stock Center #31308), UAS-sgg (Bloomington Stock Center #5435), hh-Gal4 (Bloomington Stock Center #67046), arm-Gal4 (Bloomington Stock Center #1560), hsGFP hsCD2(y+8) M(3)i55 Tub-Gal80 FRT2A (provided by G. Struhl).

We made hh-Gal4 EY10165 recombinant flies for screening. We crossed these flies with the fray mutant and confirmed the suppression as described previously [17]. For initial screening, we crossed several mutants and isolated candidate suppressor genes (data not shown), including sgg.

Histology and staining

All wings were mounted in GMM [23]. Images were obtained using SteREO Discovery and Axioscope microscopes (Carl Zeiss), and were processed using Axiovision with extended focus (Carl Zeiss) and Photoshop (Adobe).
Cultured cell lines

Neuro2A cells [17] were grown in DMEM with 20% FBS. Polyethylenimine (Polysciences) was used to transfect plasmids, and the TransIT-X2 Dynamic Delivery System (Mirus Bio) was used to transfect small interfering (si)RNA and co-transfect siRNA and plasmids. The plasmids we used are: pRK5-Flag-hWNK1, pRK5-Flag-hWNK1<sup>D368A</sup>, pRK5-Flag-OSR1, pRK5-T7-OSR1, pRK5-Flag-OSR1<sup>K46M</sup>, pRK5-T7-OSR1<sup>K46M</sup>, pRK5-Flag-OSR1<sup>S325D</sup>, pcDNA-Flag-GSK3β, pRK5-Flag-GSK3β, pRK5-Flag-GSK3β<sup>K85M</sup>. siRNA target sequences were described previously [17], and were as follows: mouse Osr1, 5′-GAUAUUCGAUUUGA AUUUA-3′; and mouse GSK3β, 5′-GAAAUGAACCCAAUAUA -3′. To differentiate Neuro2A cells, they were induced in serum-free DMEM with 10 μM retinoic acid for 24 h.

Immunoprecipitation

Neuro2A cells were transfected with indicated expression vectors. Then, lysates were prepared from transfected cells, and immunoprecipitated with indicated antibodies and Protein A/G PLUS-agarose (Santa Cruz). Immunoprecipitates were subjected to SDS-PAGE and western blotting, and bands were detected by the LAS-4000 mini (GE) image analyzer. For sequential immunoprecipitation, the anti-Flag antibody (M2, Sigma) was used and eluted with Flag peptides (Sigma). Eluates were divided and immunoprecipitated with anti-T7 antibodies and control mouse normal IgG.

RT-PCR analysis

Total RNA was isolated by TRIzol (Invitrogen). cDNA synthesis was carried out using Moloney murine leukemia virus reverse transcriptase (Invitrogen). GAPDH was used for the normalization of cDNA samples. Primer pairs were described previously [17], and were as follows: mouse GSK3β, 5′-GCAGCAAGGTAACCACAG TAGTGGC-3′ and 5′-TGGTGCCCT GTAGTACCAGAACAG-3′.

In vitro kinase assay

Neuro2A cells were transfected with Flag-WNK1, Flag-WNK1<sup>D368A</sup>, Flag-OSR1, Flag-OSR1<sup>K46M</sup>, or Flag-OSR1<sup>S325D</sup> expression plasmids. Lysates were prepared from transfected cells and immunoprecipitated with an anti-Flag M2 antibody (Sigma) and Protein A/G PLUS-agarose (Santa Cruz). Immunoprecipitates were incubated with bacterially-expressed GST fusion proteins (GST-GSK3β<sup>K85M</sup>) in kinase buffer containing 10 mM HEPES (pH 7.4), 1 mM DTT, 5 mM MgCl<sub>2</sub>, and 5 μCi [γ<sup>32</sup>P]-ATP at 30˚C. Phosphorylated substrates were subjected to SDS-PAGE, and bands were detected by the FLA3000 image analyzer (Fujifilm).

Antibodies

Antibodies used in this report were: mouse anti-Flag M2 (Sigma; 1:400 for immunoprecipitation), rabbit anti-Flag (Sigma; 1:1000 for western blotting), rat anti-HA (Roche; 1:1000 for western blotting), mouse anti-T7 (Merck; 1:2000 for immunoprecipitation), rabbit anti-T7 (MBL; 1:1000 for western blotting), anti-rabbit HRP-conjugated (GE; 1:10000 for western blotting), and anti-rat HRP-conjugated (GE; 1:10000 for western blotting).

Quantification and statistical analysis

Quantitative PCR was performed with an Applied Biosystems 7300 Real-Time PCR Cycler (ABI) using THUNDERBIRD SYBR qPCR Mix (TOYOBO). Primer sequences for Lhx8, Chat, Gad1, and GAPDH were described previously [17]. GAPDH was used for the normalization of
cDNA samples. Neurite lengths were measured using ImageJ software (NIH). Data were computed using Microsoft Excel (Microsoft) and StatPlus (AnalystSoft). Values and error bars represent the means and SDs, and are representative of at least three independent experiments.

Results

Shaggy is a novel candidate effector of the WNK signaling pathway

Overexpression of Drosophila WNK using hh-Gal4 driver resulted in an ectopic vein around vein 5 in the adult wing (Fig 1B compared with Fig 1A; [17]). As we showed previously, a heterozygous mutation of fray, which encodes a Drosophila homolog of SPAK/OSR1 that is a downstream effector of WNK, suppressed this phenotype [17]. Therefore, we performed screening to identify a new effector of the WNK signaling pathway using this system. We selected several mutants known to be components of other signaling pathways, such as the Wnt pathway, Notch pathway, TGFβ pathway, and the EGF pathway. We obtained several candidate suppressor genes, including sgg, which encodes the Drosophila homolog of mammalian GSK3β whereas some genes, including Akt1, did not show any interaction (Fig 1C and data not shown). Two independent sgg mutants (sgg1 and sggM11) suppressed the wing phenotypes by the overexpression of Wnk (Fig 1D and 1E), suggesting that sgg is a suppressor of the WNK signaling pathway and that this suppression is not an effect of the genetic background.

We further tested the interaction between sgg and fray. Ectopic expression of frayS347D (the constitutively active form of fray) resulted in a similar phenotype to that seen following the ectopic expression of Wnk (Fig 1F; [17]). The sgg1 mutant also repressed these phenotypes (Fig 1G) suggesting that sgg interacts with the WNK signaling pathway in Drosophila.

We next confirmed this genetic interaction between Wnk and sgg. Because WnkEY18 mutant clones led to abdominal developmental defects (Fig 1H; [17]), we attempted to rescue this phenotype by the overexpression of sgg. Using a combination of the FLP/FRT mosaic system and Gal80 suppression, we induced the local expression of sgg in WnkEY18 minute clones. As shown in Fig 1I, sgg overexpression partially rescued the abdominal phenotype of the WnkEY18 minute clones. These results suggest that sgg is a novel effector of the WNK signaling pathway, not only in wing development but also in abdominal development.

GSK3β functions as a positive effector downstream of WNK

Because GSK3β and the WNK pathway are highly conserved among many species [2, 3, 22], we next examined whether the interaction between WNK and GSK3β was also conserved in mammalian cells. In Neuro2A cells, WNK1 expression induced the expression of Lhx8 ([17]; see also Fig 2B lane 2). As shown in Fig 2, the expression of GSK3β also induced the expression of Lhx8 in Neuro2A cells (Fig 2A lane 2). However, the kinase dead form of GSK3β (GSK3βK85M) could not activate the expression of Lhx8, suggesting that GSK3β kinase activity is required for its expression (Fig 2A lane 3).

Next, we examined the epistatic interaction between WNK1 and GSK3β. The induction of Lhx8 was suppressed by the knockdown of GSK3β (Fig 2B lane 4). However, Lhx8 induction by GSK3β was not suppressed by the knockdown of both Wnk1 and Wnk4 (Fig 2C lanes 5 and 6), even though this knockdown did suppress Lhx8 induction by retinoic acid (RA) stimulation (Fig 2C lanes 3 and 4).

The expression of OSR1, a downstream molecule of WNK, and its constitutively active form (OSR1S325D) induced the expression of Lhx8 (Fig 2D lanes 3 and 5; [17]). This activation was also suppressed by the knockdown of GSK3β (Fig 2D lanes 4 and 6). In contrast, the induction of Lhx8 by GSK3β was not suppressed by the knockdown of Osr1 (Fig 2E lanes 5 and 6), although the induction of Lhx8 by WNK1 was suppressed by the knockdown of Osr1 (Fig 2E lanes 3 and 5).
lanes 3 and 4). These data suggest that the WNK–OSR1–GSK3β pathway is conserved not only in flies but also in mammals, and that GSK3β functions as a positive effector downstream of the WNK signaling pathway.

Fig 1. sgg is downstream of Wnk in the Drosophila wing vein and abdominal patterning. (A) Wild-type wing. (B) Wing from EY10165 (UAS-Wnk) fly driven by hh-Gal4. Additional veins around vein 5 (arrowhead) were observed. (C) Wing from fly overexpressing Wnk driven by hh-Gal4 with the Akt1^{34226} heterozygous mutant. (D) Wing from fly overexpressing Wnk driven by hh-Gal4 with the sgg{	extsuperscript{m11}} heterozygous mutant. (E) Wing from fly overexpressing Wnk driven by hh-Gal4 with the Akt1^{34226} heterozygous mutant. (F) Wing from UAS-fray{	extsuperscript{S347D}} fly driven by hh-Gal4. Additional veins around vein 5 (arrowhead) were observed. (G) Wing from fly overexpressing fray{	extsuperscript{S347D}} driven by hh-Gal4 with the sgg{	extsuperscript{m11}} heterozygous mutant. (H) Abdomen from adult fly with DWNK{	extsuperscript{EY18}} minute clones. Thin black lines indicate the clone border. (I) Abdomen from adult fly with DWNK{	extsuperscript{EY18}} minute clones and sgg overexpression. sgg was expressed only in DWNK{	extsuperscript{EY18}} minute clones using the Gal80 suppression technique. Thin black lines indicate the clone border (also the sgg expression area). Black arrowheads show rescued abdominal bristles. The detailed genotype is y w hsflp; arm-Gal4 / UAS-sgg; Wnk{	extsuperscript{EY18}} FRT2A / hsGFP hsCD2(y+) M(3)i55 Tub > Gal80 FRT2A. The numbers of wings or abdomina showing the phenotypes and of total observed wings or abdomina are indicated.

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WNK1 and OSR1 form a complex with GSK3β

We next investigated the biochemical interaction between WNK1 and GSK3β. We transiently expressed Flag-tagged GSK3β together with HA-tagged WNK1 or T7-tagged OSR1. When cell extracts were subjected to immunoprecipitation with anti-Flag, anti-HA (for WNK1), or anti-

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Fig 2. GSK3β is a positive effector downstream of WNK-OSR1. (A) Gene expression determined by RT-PCR or quantitative RT-PCR analysis was examined in Neuro2A cells overexpressing GSK3β or GSK3β ΔK85M. The value obtained from each sample was normalized to that of GAPDH. The value of Lhx8 from overexpressing GSK3β (lane 2) was set to 100. (B) Gene expression determined by RT-PCR or quantitative RT-PCR analysis was examined in Neuro2A cells overexpressing hWNK1 under GSK3β knockdown using siRNA. The value obtained from each sample was normalized to that GAPDH. The value of Lhx8 from overexpressing hWNK1 (lane 2) was set to 100. (C) Gene expression determined by RT-PCR or quantitative RT-PCR analysis was examined in Neuro2A cells stimulated by retinoic acid (RA) or overexpressing GSK3β under both Wnk1 and Wnk4 knockdown using siRNA. The value obtained from each sample was normalized to that of GAPDH. The value of Lhx8 from overexpressing GSK3β (lane 3) was set to 100. (D) Gene expression determined by RT-PCR or quantitative RT-PCR analysis was examined in Neuro2A cells overexpressing OSR1 or OSR1 ΔS324D (constitutively active form of OSR1) under GSK3β knockdown using siRNA. The value obtained from each sample was normalized to that of GAPDH. The value of Lhx8 from overexpressing OSR1 (lane 3) was set to 100. (E) Gene expression determined by RT-PCR or quantitative RT-PCR analysis was examined in Neuro2A cells overexpressing hWNK1 or GSK3β under Osr1 knockdown using siRNA. The value obtained from each sample was normalized to that of GAPDH. The value of Lhx8 from overexpressing hWNK1 (lane 3) was set to 100.
T7 (for OSR1) antibodies followed by immunoblotting, we found that GSK3ß interacted with WNK1 (Fig 3A lane 3), but not with OSR1 (Fig 3B lane 3). Because WNK1 bound to OSR1 [6, 7], we investigated whether the WNK1–OSR1 complex interacted with GSK3ß. As shown in Fig 3C, GSK3ß was immunoprecipitated after sequential IP by anti-Flag and anti-T7 antibodies, suggesting that GSK3ß forms a complex with WNK1 and OSR1.

GSK3ß is positively and negatively regulated by phosphorylation [24]. Because WNK1 and OSR1 are both Ser/Thr kinases [3, 25, 26], and WNK1–OSR1–GSK3ß forms the complex shown above, we examined whether WNK1 or OSR1 directly phosphorylated and regulated GSK3ß. To perform the in vitro kinase assay, we purified Flag-tagged WNK1 or OSR1 from cultured cell extracts, and produced a GST-fusion protein of the kinase dead form of GSK3ß (GST-GSK3ß K85M) in bacteria. We did not observe phosphorylation of GSK3ß by WNK1 or OSR1 (Fig 3D lanes 1–5). These results suggest that GSK3ß forms a complex with WNK1 and OSR1, but that the regulation of GSK3ß by the WNK signaling pathway does not depend on direct phosphorylation.

**GSK3ß is involved in neural specification**

As we showed previously [17], WNK plays an important role in neural specification through the regulation of Lhx8 expression. We examined whether GSK3ß was also involved in neural
GSK3β is involved in the neural specification downstream of WNK. Knockdown of GSK3β caused the shortening of neurites after RA stimulation (Fig 4B compared with Fig 4A, quantified in Fig 4C). Knockdown of GSK3β also decreased the expression of Lhx8 and the choline acetyltransferase gene (ChAT; a marker for cholinergic neuron) (Fig 4D lane 4). However, the gene expression of glutamic acid decarboxylase 1 (Gad1; a marker for GABAergic neurons) increased (Fig 4D lane 4). These

Fig 4. The WNK-OSR-GSK3β pathway is involved in the neural development. (A–B) siRNA-treated differentiated Neuro2A cells induced by RA for 24 h; (A) Control siRNA, (B) siGSK3β. (C) The average length of neurites in siRNA-treated differentiated Neuro2A cells induced by RA for 24 h, shown in A and B (Control siRNA (n = 93), siGSK3β (n = 91)). * p<0.0005 calculated by the Student’s t-test. (D) Gene expression determined by RT-PCR or quantitative RT-PCR analysis was examined in Neuro2A cells. Cells treated with siRNA against GSK3β (siGSK3β); (lanes 1 and 2) undifferentiated cells, (lanes 3 and 4) cells differentiated by RA for 24 h. The value obtained from each sample was normalized to that of GAPDH. The value of Lhx8, ChAT or Gad1 from differentiated cells under control siRNA treatment (lane 3) was set to 100. (E–H) Differentiated Neuro2A cells were transfected with various combinations of siRNAs and expression plasmids. (E) Control siRNA and control vector. (F) Control siRNA and GSK3β. (G) siWnk1 and siWnk4, and control vector. (H) siWnk1 and siWnk4, and GSK3β. (I) The average length of neurites in siRNA-treated differentiated Neuro2A cells induced by RA for 24 h, shown in E–H (Control siRNA and Control vector (n = 81), Control siRNA and GSK3β (n = 87), siWnk1 and siWnk4, and Control vector (n = 103), siWnk1 and siWnk4, and GSK3β (n = 77)). * p<0.0005 calculated by the Bonferroni correction. ns indicated non-significance. (J) Gene expression determined by RT-PCR or quantitative RT-PCR analysis was examined in Neuro2A cells. Cells were treated with various combinations of siRNAs and expression plasmids; (lanes 1–4) undifferentiated cells, (lanes 5–8) cells differentiated by RA for 24 h, (lanes 1–2 and 5–6) control siRNA, (lanes 3–4 and 7–8) siWnk1 and siWnk4, (lanes 1, 3, 5 and 7) control vector, (lanes 2, 4, 6 and 8) GSK3β. The value obtained from each sample was normalized to that of GAPDH. The value of Lhx8, ChAT or Gad1 from differentiated cells under the treatment of control siRNA (lane 5) was set to 100.

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results suggest that GSK3β is involved in neural specification, similar to that induced by the knockdown of both Wnk1 and Wnk4 as shown previously ([17]; see also Fig 4G and 4I).

We next examined whether the expression of GSK3β suppressed the neural specification phenotypes caused by the knockdown of Wnk. While the expression of GSK3β did not affect the elongation of neurites (Fig 4F compared with Fig 4E, quantified in Fig 4I), the expression of GSK3β induced Lhx8 and ChAT expression, and reduced Gad1 expression after RA stimulation in Neuro2A cells (Fig 4J lanes 5 and 6). Under conditions of both Wnk1 and Wnk4 knockdown, the expression of GSK3β partially rescued the elongation of neurites (Fig 4H compared with Fig 4G, summarized in Fig 4I), and Lhx8 expression (Fig 4J lanes 7 and 8); this in turn increased ChAT expression and decreased Gad1 expression (Fig 4J lanes 7 and 8). These results suggest that GSK3β is involved in neural development and functions downstream of the WNK signaling pathway.

**Discussion**

The WNK signaling pathway is involved in many biological processes, but the details of its components are unclear, except for in the kidney. Here, we screened candidate genes that genetically interact with the WNK signaling pathway in *Drosophila*. Among these, we identified shaggy, which encodes the *Drosophila* homolog of mammalian GSK3β (Fig 1). We showed that GSK3β activated Lhx8 expression and that GSK3β functions downstream of the WNK–OSR1 pathway by epistasis analysis (Fig 2). We also showed that GSK3β might form a tertiary complex with WNK1 and OSR1 (Fig 3). Furthermore, GSK3β was found to be involved in neural specification and neurite elongation (Fig 4), and GSK3β rescued the neural phenotypes induced by the knockdown of both Wnk1 and Wnk4 (Fig 4). However, we did not observe direct phosphorylation of GSK3β by WNK1 or OSR1 (Fig 3). This suggests that GSK3β functions as a positive downstream effecter in the WNK signaling pathway, although the regulation of GSK3β activity by the signaling pathway remains unclear and requires further study to elucidate how WNK–OSR1 transduces the signal to GSK3β.

GSK3β plays many roles in various signaling pathways. In the PI3K/AKT signaling pathway, AKT phosphorylates Ser-9 of GSK3β and inhibits GSK3β activity for cell proliferation [22]. Previous research has shown that the PI3K/AKT signaling pathway activates the WNK–OSR1–NCC pathway to regulate blood pressure [20]. However, GSK3 was reported to be a negative regulator for the destruction complex in the Wnt signaling pathway [22]. A recent study showed that WNK is a positive regulator of the Wnt signaling pathway [18]. Here, we found that GSK3β is a positive regulator downstream of the WNK–SPAK/OSR1 signaling pathway. These contradictions with regard to the regulation and role of GSK3β clearly indicate that the WNK–SPAK/OSR1–GSK3β signaling pathway for neural development is independent of PI3K/AKT or Wnt signaling pathways. The exact interaction between WNK and other signaling pathways remains to be determined and will require further analyses.

GSK3β is also known to be involved in the Notch signaling pathway in which the intercellular domain (ICD) of Notch directly regulates the transcription of target genes with several co-factors [27]. GSK3β was previously shown to bind and phosphorylate the Notch ICD which increased the transcriptional activity of the Notch ICD complex [28]. However, the mechanisms of GSK3β activation in the Notch signaling pathway are still unclear. Since our initial screening of *Drosophila* Wnk-related genes showed that Wnk has a weak genetic interaction with the Notch signaling pathway (data not shown), we hypothesize that the WNK pathway positively regulates the Notch signaling pathway through GSK3β in neural development. Further study will be required to prove this hypothesis, which is likely to be important for understanding the pathogenesis of HSAN2A.
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