Diabetes Impairs Wnt3 Protein-induced Neurogenesis in Olfactory Bulbs via Glutamate Transporter 1 Inhibition*

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Diabetes is associated with impaired cognitive function. Streptozotocin (STZ)-induced diabetic rats exhibit a loss of neurogenesis and deficits in behavioral tasks involving spatial learning and memory; thus, impaired adult hippocampal neurogenesis may contribute to diabetes-associated cognitive deficits. Recent studies have demonstrated that adult neurogenesis generally occurs in the dentate gyrus of the hippocampus, the subventricular zone, and the olfactory bulbs (OB) and is defective in patients with diabetes. We hypothesized that OB neurogenesis and associated behaviors would be affected in diabetes. In this study, we show that inhibition of Wnt3-induced neurogenesis in the OB causes several behavioral deficits in STZ-induced diabetic rats, including impaired odor discrimination, cognitive dysfunction, and increased anxiety. Notably, the sodium- and chloride-dependent GABA transporters and excitatory amino acid transporters that localize to GABAergic and glutamatergic terminals decreased in the OB of diabetic rats. Moreover, GAT1 inhibitor administration also hindered Wnt3-induced neurogenesis in vitro. Collectively, these data suggest that STZ-induced diabetes adversely affects OB neurogenesis via GABA and glutamate transporter systems, leading to functional impairments in olfactory performance.

Mammalian neurogenesis occurs in the subventricular zone (SVZ)‡4 of the lateral ventricles and the sub-granular zone of the dentate gyrus in the hippocampus (1–3). In the SVZ, self-renewing multipotent neural stem cells (NSCs) give rise to neuroblasts, which migrate through the rostral migratory stream into the olfactory bulb (OB) where they differentiate into multiple types of local interneurons. In addition to the sub-granular zone and SVZ, the OB core is another proposed source of NSCs (4–8). NSC cultures have been derived from the adult rodent and human OB (4, 5); thus, local NSCs in the OB may provide a valuable source for autologous transplantation in neurodegenerative disorders and other diseases. NSCs possess self-renewal and multipotency capacities and can differentiate into neurons, astrocytes, or oligodendrocytes (9, 10). Insulin supports the function of basic fibroblast growth factor (FGF2), which promotes maintenance of undifferentiated NSCs (11), as well as triggers the differentiation of multipotent NSCs into oligodendrocytes (12). Newly formed neurons are incorporated into the functional networks of the OB and dentate gyrus, demonstrating the substantial impact of adult neurogenesis on brain functions such as learning, memory processing, and odor discrimination (13–17).

Diabetes, one of the most common serious metabolic disorders in humans, is characterized by hyperglycemia that is caused by defects in insulin secretion, activity, or both (18). Diabetes is associated with cognitive decline and increased risks of Alzheimer disease and dementia (19–23). Cognitive deficits have also been reported in studies of rodent models of diabetes. The cytotoxic agent streptozotocin (STZ) selectively destroys insulin-producing β-cells of the pancreas by entering through the Glut2 glucose transporter. STZ-induced type 1 diabetic rodents exhibit deficits in behavioral tasks involving spatial learning and memory, such as performance in the Morris water maze and novel object recognition tests (24–26). The blood-brain barrier is devoid of Glut2 transporters (27), suggesting that STZ has limited direct effects on the brain. Impaired adult hippocampal neurogenesis is also observed in diabetic rodents, while abrogated long term potentiation, hippocampal neurogenesis, and cognitive deficits were restored in STZ-induced diabetic rats and db/db mice after adrenalectomy and corticosterone replacement at physiological concentrations (26). As such, glucocorticoids were believed to be responsible for the hindered neurogenesis and cognitive dysfunction in diabetes.

We recently demonstrated that adult hippocampal neurons and NSCs derived from the hippocampus and OB express insulin at detectable levels (28). In that study, NeuroD1 directly induced insulin (INS) gene expression in NSCs isolated from the adult hippocampus and OB. Wnt3 is an instructive factor secreted by astrocytes and promotes adult neurogenesis by stimulating NeuroD1 expression, a basic helix-loop-helix transcription factor (29). Thus, under Wnt3 activation, NeuroD1 promotes neurogenesis and insulin production in the adult OB

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4 The abbreviations used are: SVZ, subventricular zone; STZ, streptozotocin; OB, olfactory bulb; EAAT, excitatory amino acid transporter; GAT, GABA transporter; GFAP, glial fibrillary acidic protein; MBP, maltose-binding protein; GCL, granule cell layer; qPCR, quantitative PCR; GL, glomerular layer; NSC, neural stem cell; BDNF, brain-derived neurotrophic factor; HCN, hippocampal neural; DCX, doublecortin.
GAT1 Suppression Impairs Neurogenesis in OB in Diabetics

and hippocampal NSCs. Indeed, NSCs derived from the OB and hippocampus of adult diabetic rats retained their intrinsic capacity to produce insulin, making these cells a useful source for autologous cell transplantation in diabetes.

Dysfunctions in olfactory and OB neurogenesis have been observed patients with diabetes (30–32) and present clinically as altered olfactory-related behaviors. The characteristics of NSCs and their neurogenic niche in diabetes are critical elements of insulin function and neurogenesis; thus, it is important to understand the mechanism of diabetes-induced deficits in neuronal function. We previously demonstrated great similarities in the expression pattern of genes involved in diabetes and Wnt signaling between NSCs of the OB and hippocampus in diabetes. As such, we hypothesized that adult OB NSC neurogenesis and OB function are also affected by diabetes (33). In this study, we evaluated the morphology of the OB neurogenic niche. Most importantly, Wnt3-induced neurogenesis was inhibited in the OB of STZ-treated diabetic rats, which also exhibited several behavioral deficits, including loss of odor discrimination ability, cognitive dysfunction, and increased anxiety. Sodium- and chloride-dependent GABA transporter 1 (GAT1), which localizes to GABAergic terminals, was inhibited in the diabetic OB, and a GAT1 inhibitor suppressed Wnt3-induced neurogenesis in vitro. From these studies, we conclude that diabetes has an adverse effect on OB neurogenesis and function. Our findings provide evidence that insulin deficiency leads to attenuation of neurogenesis and GABA activity and glutamate neurotransmission systems in the OB, thereby causing functional impairment in olfactory performance. In addition, insulin may promote the neuronal differentiation of OB NSCs by controlling GABA uptake by GAT1.

Results

Strict Regulation of NSC Differentiation in the OB—To analyze the cell fate specification of NSCs in the OB, immunohistochemistry was performed to identify stem cells, neuronal progenitors, neurons, astrocytes, and oligodendrocytes in 7-week-old rats. The distribution of neural stem cells and mature neurons was first confirmed by immunostaining for Sox2 and NeuN, as markers for stem cells and mature neurons, respectively (Fig. 1A). NeuN was specifically expressed in the interneuron-rich granule cell layer (GCL) and partially in the glomerular layer (GL) but less in external plexiform layer. In contrast, Sox2, a multipotential NSC marker, was abundantly expressed in the GL. No co-localization between NeuN and Sox was observed in the OB. Thus, the localization of multipotent undifferentiated NSCs and mature neurons was distinct in the OB.

To investigate neuronal differentiation in OB, the distribution of Sox2-positive stem cells and the early stage neuronal differentiation marker NeuroD1 was assessed by immunohistochemistry. Notably, NeuroD1-positive cells did not co-localize with Sox2-positive cells in the GL (Fig. 1B, left). To detect the neuronal cells with further differentiation in the OB, the presence of immature neurons and mature neurons was further investigated with antibodies directed toward the immature neuronal markers TUJ1 and NeuN. TUJ1 (immature neuronal marker)-positive cells did not co-localize with NeuN-positive cells (Fig. 1B, right), indicating that the OB contained cells from each stage of neuronal differentiation, but NSCs and early stage neuronal cells, immature, and mature neurons did not co-localize with each other. The GL is suggested to be another source of NSCs in OB. In addition, neuronal differentiation from stem cells to neurons is strictly controlled, similar to that observed with hippocampal neuronal differentiation.

Because NSCs can also generate oligodendrocytes and astrocytes, we examined neuronal morphology and glial lineages in OB. Notably, positive cells for the immature oligodendrocyte marker Olig2 were observed in GL where NSCs and neural progenitor cells were found, but it did not co-localize with NeuN-positive cells (Fig. 1C, left). Alternatively, cells positive for the mature oligodendrocyte marker MBP were observed, but they did not co-localize with those expressing GFAP, a Wnt3-producing astrocyte marker, in GCL (Fig. 1C, right). GFAP-positive cells were also found in the GL, but these cells did not co-localize with NeuN-positive cells (Fig. 1D, left). Mature astocytes were also detected with S100β. Most S100β-positive cells were co-localized with GFAP-positive cells in the GCL. (Fig. 1D, right). Therefore, both mature and immature oligodendrocytes and astrocytes exist in OB. These results suggest that NSC differentiation into neurons, astrocytes, or oligodendrocyte is strictly regulated and that once a differentiation path has been determined, the cells no longer express markers of other lineages.

Diabetes Impairs Neuronal and Oligodendrocyte Differentiation—NSCs and other lineages were observed in the normal OB. Then, the expression of genes associated with each cell lineage was investigated in STZ-induced diabetic rats. Sox2 transcript levels in STZ-induced diabetic rats were comparable with those in the control rats (WT) 10 days after STZ treatment, but expression increased by day 30 in the diabetic versus WT rats (Fig. 2A, left). mRNA expression of another stem cell marker, Nestin, markedly decreased on day 10 but was comparable with that of the control at day 30 (Fig. 2A, right).

Expression of the immature neuronal marker β-tubulin III (Tubb3) and mature neuronal marker synapsin 1 (Syn1) was substantially lower in diabetic rats than WT rats on days 10 and 30 (Fig. 2B). Similar to Tubb3 and Syn1, we observed a substantial decrease in the expression of Mbp, a mature oligodendrocyte marker, in diabetic rats on days 10 and 30. The marker of immature oligodendrocytes Olig2 was comparable in WT and diabetic rats (Fig. 2C). Expression of the astrocyte markers GFAP and S100β markedly decreased in diabetic versus WT rats on day 10 and remained low on day 30 in both WT and diabetic rats (Fig. 2D). These data suggest STZ-induced diabetes is associated with a substantial reduction in the expression of neuronal and mature oligodendrocyte lineage-related transcripts in the OB over a relatively long period of time.

Western blot analysis was performed to examine the expression of lineage-specific markers in the OB on day 30 after diabetes induction. Sox2 protein expression was comparable in WT and diabetic rats (Fig. 3A, left). Nestin expression was also comparable in WT and diabetic rats (Fig. 3A, right). In contrast to the expression of stem cell markers, that of neuron-specific markers TUJ1 and SYN1 was substantially lower in diabetic than in WT rats (Fig. 3B). The expression of the oligodendro-
GAT1 Suppression Impairs Neurogenesis in OB in Diabetics

A

B

C

D
expression levels of the astrocyte marker S100 were markedly decreased in diabetic mice (34). These results suggest that the differentiation of neuron and oligodendrocyte lineages is impaired in diabetic progression. However, diabetes may not have a severe effect on the differentiation and maturation of astrocytes, because S100β characterizes a mature developmental stage in the astrocytic lineage (35), and its protein expression was similar in WT and diabetic rats on day 30. There was no correlation between gene and protein expression of several markers of NSCs, astrocytes, and oligodendrocytes such as Sox2, Olig2, and GFAP at 30 days after STZ treatment. Transcript levels do not necessarily correlate to the amount of expressed protein, because the translation of mRNAs into proteins is a highly regulated process. Although levels of some transcripts did not correlate with protein expression, it was clear that the expression of neuronal and mature oligodendrocyte markers was consistently reduced in diabetic rats at the transcriptional and post-transcriptional levels. These results suggest diabetes impairs neuronal and oligodendrocyte differentiation.

**Diabetes Impairs NSC Differentiation into Neurons**—To detect the proliferation and lineage specification rates of NSCs during diabetes progression, proliferating cells were labeled with BrdU on day 20 after STZ treatment. The percentage of Sox2+ BrdU-labeled new cells expressing the stem cell marker Sox2 was similar in WT and diabetic rats (Fig. 4, A and C); however, the percentage of newly dividing BrdU-labeled cells expressing the early neuronal differentiation marker NeuroD1 was remarkably lower in diabetic versus WT rats (Fig. 4, B and C). Less than 10% of BrdU-labeled cells expressed NeuroD1 in diabetic rats, whereas more than 40% of BrdU-labeled cells expressed this protein in WT rats (Fig. 4C). The number of BrdU-labeled cells expressing NeuN, a mature neuronal marker in diabetes, was reduced slightly but not significantly. No significant difference was observed between the percentage of BrdU-labeled cells expressing Olig2 and S100β in WT and diabetic rats (Fig. 4C). Thus, the newly dividing BrdU+ cells that expressed specific markers of early neuronal differentiation and mature neurons were partially suppressed in diabetic rats. Therefore, diabetes impaired NSC differentiation into neurons but had a minor influence on NSC proliferation and the differentiation of oligodendrocytes and astrocytes.

**Diabetes Reduces Wnt3 Expression in OB**—Wnt3 is required to promote NeuroD1 expression and neurogenesis in the adult hippocampus (29, 36, 37). The impairment of neuronal differentiation in diabetes (Figs. 2–4) prompted us to explore Wnt3 expression in the OB at 10 and 30 days after STZ treatment. We observed similar transcript and protein profiles of specific markers for stem cell and neuronal progenitors, neurons, astrocytes, and oligodendrocytes on days 20 and 30 in diabetic rats (data not shown); immunohistochemistry was performed on day 20 after STZ treatment. Although GFAP-expressing cells were present in both WT and diabetic rats, Wnt3-expressing

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**FIGURE 1. Morphological characteristics of stem cells, neurons, astrocytes, and oligodendrocytes in the adult OB.** A, distribution of neuronal stem cells and mature neurons. Left, representative merged image of Sox2 (red), NeuN (green), and DAPI-labeled nuclei (blue). Right, representative magnified image of GL. B, distribution of neurons in different stages. Left, representative merged image of NeuroD1 (red), Sox2 (green), and DAPI (blue). Right, representative image of TuJ1 (red), NeuN (green), and DAPI (blue). C, distribution of oligodendrocytes. Left, representative image of Olig2 (red), NeuN (green), and DAPI (blue). Right, representative image of MBP (red), GFAP (green), and DAPI (blue). D, distribution of astrocytes. Left, the representative image of NeuN (red), GFAP (green), with DAPI (blue). Right, representative image of S100β (red), GFAP (green), and DAPI (blue). EPL, external plexiform layer.

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**FIGURE 2. Expression levels of specific marker genes for stem cells, neurons, astrocytes, and oligodendrocytes in the adult OB of STZ-induced diabetes.** qRT-PCR analysis showing relative mRNA levels of specific marker genes for stem cells, neurons, astrocytes, and oligodendrocytes in the OB of wild-type (WT) and diabetic (DB) rats at 10 and 30 days (d) after STZ treatment. A, gene expression of the stem cell markers Sox2 and Nestin. B, gene expression of the neuronal markers β-tubulin3 (TUBB3) and synapsin1 (SYN1). C, gene expression of the astrocyte markers Olig2 and MBP. D, gene expression of the astrocyte markers GFAP and S100β. All data have been normalized to the levels of Gapdh mRNA as an internal control and indicated as the ratio relative to that in the WT at 10 days. White bars, WT; black bars, DB. Data are represented as mean ± S.D. (n = 4). *, p < 0.05, versus corresponding WT; **, p < 0.01 versus corresponding WT. Statistical significance was determined by Student’s t test.
cells seemed to be decreased in the GL and GCL regions of diabetic versus WT rats on day 20 (Fig. 5A). Indeed, the percentage of Wnt3+GFAP+ astrocytes is dramatically decreased in STZ-induced diabetes (Fig. 5B). It showed that Wnt3-expressing astrocytes in the OB neurogenic niche decrease severely in diabetes. In addition, a substantial reduction in Wnt3 transcript expression was observed in diabetic versus WT rats on day 30 after STZ treatment (Fig. 5C). The reduction of Wnt3 transcript expression in diabetes occurred over time after diabetes induction. Similarly, Wnt3 protein expression was remarkably inhibited on days 10 and 30 in diabetic rats (Fig. 5D). These results indicated that Wnt3 production was seriously inhibited in diabetic rats, although expression of the astrocyte-specific marker GFAP was observed in both WT and diabetic rats, suggesting inhibition of Wnt3 production in astrocytes of the OB neurogenic niche.

**Diabetes Inhibits Wnt Canonical Signaling and Its Downstream Targets in OB**—We explored the activation of Wnt signaling and Wnt target factors by real time qPCR and Western blotting. In the canonical Wnt signaling pathway, Wnt binds to its receptor Frizzled and a transmembrane protein called low-density lipoprotein receptor-related protein. Binding of Wnt to Frizzled and lipoprotein receptor-related protein triggers a series of events that disrupts the β-catenin destruction complex, resulting in stabilization and accumulation of β-catenin in the cytoplasm. In the absence of a Wnt ligand, cytoplasmic β-catenin is phosphorylated by phosphorylated GSK3β (p-GSK3β) and is targeted for degradation by the proteasomal machinery. The increased stability of β-catenin following Wnt activation enables translocation to the nucleus and binding to T cell-specific transcription factor/lymphoid enhancer-binding factor 1 transcription factors and mediates transcriptional
induction of target genes (38–40). GSK3β and p-GSK3β protein levels were reduced on days 10 and 30 in diabetic rats (Fig. 6A). In addition, the expression of β-catenin was reduced on day 30 after STZ treatment. These data suggest the reduction of Wnt3 leads to down-regulation of Wnt/β-catenin signaling in the OB.

NeuroD1 is a target of Wnt3 regulation and induces terminal neuronal differentiation in the OB (41). Transcript expression of NeuroD1 was suppressed on days 10 and 30 after diabetes induction and was accompanied by a reduction in protein levels on day 30, consistent with Wnt3 expression in diabetic rats (Fig. 6B). A neuronal precursor marker, doublecortin (DCX), is also a Wnt3 target (29, 42). Expression of DCX transcripts was reduced on day 10 and was maintained at low levels in WT and diabetic rats on day 30 (Fig. 6C). DCX protein expression was remarkably reduced in diabetic rats on day 30 after STZ treatment. These results suggest reduction of Wnt3 expression leads to a reduction in the transcript and protein expression of target genes.

Behavioral Alteration in STZ-induced Diabetes—Several lines of evidence suggest inhibition of Wnt signaling causes impairment of adult neurogenesis in the hippocampus and results in cognitive decline (42–44). Because the expression of Wnt3 and its target genes was reduced in the OB of diabetic rats (Fig. 6), behavioral alterations were evaluated using a hole-board test for odor discrimination, a novel object recognition test for recognition memory, and an elevated plus maze test for anxiety. The percentage of head-dips and time spent in the
food-scented hole was considerably reduced in diabetic rats on days 10 and 30 after STZ treatment (Fig. 7A). The results indicated poor odor discrimination and a severe loss of olfactory function in diabetes. During habituation in the novel object recognition test, all groups of rats spent equivalent time exploring the identical objects (left and right) (data not shown). In the trial, the discrimination index was significantly lower in diabetic than WT rats on days 10 and 30 (Fig. 7B). WT rats spent more time with the novel object, although diabetic rats could not discriminate between the novel and familiar objects on days 10 and 30, consistent with prior studies (26, 45, 46). In the elevated plus maze test, the percentage of entries and time spent in the open arms was significantly reduced in diabetic rats on days 10 and 30 (Fig. 7C). These data suggest diabetes contributes to the impaired olfactory-related exploratory behaviors, reduces learning and memory function, and increases anxious behaviors. Thus, the decrease in neurogenesis in the OB because of insulin deficiency may be involved in the functional impairment of olfactory performance.

**GAT1 Suppression Impairs Neurogenesis in OB in Diabetics**

**GAT1 Inhibition Impairs Wnt3-stimulated Neuronal Differentiation in STZ-induced Diabetes**—Recent studies suggest GABA may play a role in the regulation of adult neurogenesis through GABA<sub>A</sub> receptors (47–50). Alterations in GABAergic systems in the OB of diabetic rats were examined by analyzing the gene expression of GABA<sub>A</sub> receptor α2 (GABA<sub>A</sub>Rα2) and of GABA transporter (GAT) 1 and 3, which remove extracellu-
lar GABA after its release from the synaptic vesicle into the synaptic cleft. GAT1 transcript levels were substantially reduced on days 10 and 30 after diabetic induction. GAT3 transcript expression was reduced on day 10 in diabetic rats, but the difference was not significant until day 30. Transcript levels of EAAT1–3, vesicular glutamate transporter (VGLUT) 1, VGLUT2, and N-methyl-D-aspartate receptor R1. EAATs mediate uptake of glutamate from the neural cleft, although VGLUT transports glutamate into synaptic vesicles. The glutamatergic system was explored by assessing expression of EAAT1–3, vesicular glutamate transporter (VGLUT) 1, VGLUT2, and N-methyl-D-aspartate receptor R1. EAATs mediated uptake of glutamate from the neural cleft, although VGLUT transported glutamate into synaptic vesicles. In the diabetic rat group, transcript levels of EAAT1–3 were suppressed on day 10 and of EAAT2 and -3 were suppressed on day 30 after STZ treatment (Fig. 9A). In diabetic rats, VGLUT1 and -2 mRNA expression levels were markedly decreased on day 10, although these expression levels were significantly increased on day 30 in comparison with those for the WT (Fig. 9B, left and middle). Transcript expression of the glutamate receptor N-methyl-D-aspartate receptor R1 did not differ between WT and diabetic rats (Fig. 9B, right). Thus, diabetes also modulates the expression of glutamate transporters for the first 10 days after induction, suggesting diabetes-induced adverse effects may be seen in not only GABAergic but also glutaminergic transmission in the OB.

Because GAT1 expression was reduced on days 10 and 30, we evaluated neuronal differentiation after inhibiting GAT1 activ-
GAT1 Suppression Impairs Neurogenesis in OB in Diabetics

FIGURE 9. Modification of glutamatergic neurotransmitter systems in the OB. Relative mRNA levels of Eaat1, Eaat2, and Eaat3 (A) and of Vglut1, Vglut2, and Nmdar1 (B) in the OB of wild-type (WT, n = 4) and diabetic (DB, n = 4) rats at 10 and 30 days after STZ treatment, as determined by qRT-PCR analysis. All data were normalized to the levels of Gapdh mRNA as an internal control and indicated as the ratio relative to that in the WT at 10 days. White bars, WT; black bars, DB. Data are represented as mean ± S.D. (n = 3–6). *, p < 0.05; **, p < 0.01 versus corresponding WT.

FIGURE 10. GAT1 inhibition impairs Wnt3-induced neuronal differentiation in vitro. Quantification of relative mRNA levels of Wnt3, NeuroD1, and Tubb3 in NSCs after a 24-h treatment with the GAT1-selective inhibitor SKF89976A (1, 5, 10, 50, and 100 μM). *, p < 0.05 versus corresponding WT; **, p < 0.01 versus corresponding values for medium alone (GAT1 inhibitor = 0).

the decrease in neurogenesis. GFAP mRNA expressions were increased by the presence of both the GAT1 inhibitor and insulin at considerable levels. However, Wnt3 mRNA expressions were increased by insulin but decreased by GAT1 inhibitor. This suggests that GAT1 inhibition effects the Wnt3 production in astrocytes but that insulin could help to promote the production.

Discussion

The SVZ produces NSCs, which are also produced in the OB core (5–8). In addition to the OB core, this study demonstrated the expression of multipotent stem cell marker Sox2 in the GL of OB, suggesting that GL is the another source of NSCs in OB. In addition, NSCs, neurons, astrocytes, and oligodendrocytes in OB did not co-localize, suggesting the lineage commitment is strictly controlled.

Wnt3 is as an astrocyte-derived factor that promotes neuronal differentiation of adult hippocampal NSCs (29, 36). In this study, Wnt3 expression was observed in the rat OB, suggesting that Wnt3 regulates the adult neurogenesis there as it does in the hippocampus. We also provide evidence of the adverse effect of diabetes on neurogenesis in the OB and on OB function, and we identify the molecular mechanism by which neurogenesis is impaired in diabetes. STZ-induced diabetes had a particular effect on OB neurogenesis due to inhibition of Wnt3-mediated signaling and expression of its target genes, NeuroD1 and DCX. Down-regulation of NeuroD1 inhibits neural differentiation and reduces the number of mature neurons. In addition, STZ-induced diabetic rats showed several behavioral changes, including impaired olfactory function, deficits in learning and memory, and an increase in anxiety that can be attributed to the inhibition of GABA and glutamate transporters. Interestingly, inhibition of GAT1, which modulates local...
levels of GABA, suppressed Wnt3-regulated neuronal differentiation in vitro. These data suggest the alterations in GABAergic and glutamatergic neuronal systems could contribute to the down-regulation of Wnt3-induced neuronal differentiation in the OB of diabetic rats, thus leading to the impairment of olfactory function. Furthermore, our results indicated that insulin promotes the Wnt3-induced neurogenesis, even in the presence of the GAT1 inhibitor in vitro, supporting that insulin plays a supportive role in neurogenesis.

Growing evidence suggests GABA has a major role in survival, proliferation, migration, synapse formation, and integration of newly formed neurons into synaptic networks (50, 52–54). GABA is synthesized and released by neuroblasts (47, 49, 50). GABA<sub>A</sub> receptors are expressed in both neuroblasts and stem cells, and they are activated by local GABA (47–50). In the SVZ, GABA serves as a negative regulator for proliferation of NSCs and neuroblasts (50, 55) and migration (49). Thus, GABA activation is an important neurogenic niche signal to

FIGURE 11. Insulin promotes neurogenesis in vitro. A, quantification of relative mRNA levels of Wnt3, Neurod1, and Tubb3 in NSCs after 24-h treatment with insulin (0, 10, 100 ng/ml). *, p < 0.05; **, p < 0.01 versus corresponding values for medium alone (insulin = 0) Statistical significance was determined by Student’s t test. B, quantification of relative mRNA levels of Wnt3, GFAP, Neurod1 and Tubb3 after 24-h treatment with GAT1 inhibitor (0 and 50 μM) and Insulin (0 and 100 ng/ml). Statistical significance was determined by two-way analysis of variance with Bonferroni’s post hoc test. *, p < 0.05; **, p < 0.01 versus corresponding values in the medium without insulin treatment in the same GAT1 inhibitor treatment. #, p < 0.05; ##, p < 0.01 versus corresponding values in the medium without GAT1 inhibitor treatment in the same insulin treatment.
GAT1 Suppression Impairs Neurogenesis in OB in Diabetics

stop neuronal production. In the SVZ, the GABA transporter GAT3 is expressed in astrocyte-like NSCs and GAT1 in neuroblasts (49, 56). Electrophysiological studies have indicated that GAT1 and GAT3 in the SVZ reduce local GABA levels and limit GABA\_ receptor activation in astrocyte-like NSCs (50). These recent studies suggest that GATs regulate neurogenesis by maintaining ambient GABA levels. We also demonstrated that impairment of GAT1 in diabetic rats and a GAT1 inhibitor disturbs Wnt3-induced neuronal differentiation of NSCs in vitro. GAT1 and GAT3 are localized in the OB (57). The expression of these GATs and adult neurogenesis in the OB were impaired in STZ-induced diabetes in this study. Therefore, we suggest GABA uptake by GATs is critical for the maintenance of adult neurogenesis in the OB, and insulin regulates GAT1 activities. In addition, the correlation of GAT1 reduction with lower insulin expression was observed in OB, suggesting that insulin plays a crucial role in the maintenance of GAT1 function and GABA uptake. This study also showed that insulin stimulates Wnt3-induced neurogenesis and moderately inhibits the neurogenesis induced by the GAT1 inhibitor in vitro. Thus, it is suggested insulin may act as a supportive factor to promote the adult neurogenesis. The expression of GATs and the glutamate transporters EAATs and vGLUTs changed in diabetes in this study. Glutamate transporters may also be involved in the adult neurogenesis. Further studies are needed to elucidate the possible roles of GABA and glutamate transporters on the neurogenesis and insulin regulation of GATs and EAATs.

Diabetes impairs neurogenesis and synaptic neuronal plasticity, hampers hippocampal learning and memory, and increases anxiety-like behaviors in diabetic rats and mice (24–26). STZ-induced animals showed deficits in learning and memory in the Morris water maze and novel object recognition tests (26). Therefore, diabetes-associated cognitive deficits may be attributed to the reduction in hippocampal neurogenesis. In the hippocampus of STZ-induced diabetic rats, Wnt3 and insulin were reduced (28). Reduction in Wnt3-regulated neurogenesis was also observed in the OBs of STZ-induced diabetic rats; thus, Wnt3-induced neurogenesis in the OB and hippocampus of diabetic rats may occur in a similar fashion.

Wnt3-producing astrocytes are present only in the neurogenic niches. According to our results, it is evident that the number of Wnt3\_GFAP\_ astrocytes was dramatically reduced in STZ-induced diabetes, and GAT1 inhibition is involved in the decrease of Wnt3 production in astrocytes during neuronal differentiation. This suggests the involvement of the GAT1 inhibition in the decrease of Wnt3-producing GFAP\_ astrocytes in STZ-induced diabetes. In the study by Coleman et al. (59), insulin treatment prevented a decrease in GFAP expression in the hippocampus of untreated diabetic rats. This study also showed that insulin helped to increase Wnt3 and GFAP expression and recovery from the impaired neurogenesis by the GAT1 inhibition in vitro. Insulin may increase the number of Wnt3-secreting astrocytes in the neurogenic niches and activate Wnt3-inducing neurogenesis.

GFAP expression was shown to be severely decreased in the OB (34) and hippocampus in STZ-induced diabetes (59, 60). In this study, GFAP mRNA expressions were, however, increased by GAT1 inhibition in vitro, suggesting that diabetic conditions induce the decrease of GFAP expressions in the STZ-induced diabetic rats. It was reported that astrocytes are highly sensitive to acidic conditions (58), and the junctional communication among astrocytes is impaired in STZ-induced diabetes and cultured astrocytes (61). Accordingly, it is supposed that acidic conditions in diabetes alter GFAP expressions and astrocyte functions directly in STZ-induced diabetes.

It is also evident that glucocorticoids mediated the reduction of neurogenesis in dentate gyrus and SVZ in recent studies (62–64). Glucocorticoids were shown to decrease astrocyte proliferation (65, 66). Interestingly, glucocorticoid treatment in astrocyte culture showed a decrease in Wnt7 expression (66). Wnt7 is a Wnt family member, and it promotes neurogenesis similarly to Wnt3. Wnt3 expression in STZ-induced diabetes may also decrease in response to glucocorticoids. In addition to Wnt3, BDNF has also been implicated in adult neurogenesis, survival, and synaptic plasticity in the hippocampus (67, 68). Impairments of neurogenesis, spatial learning, and memory are observed in a BDNF-deficient animal model (69, 70). BDNF expression also decreased in the hippocampus of STZ-induced diabetic rats (71). It is reported that elevated levels of glucocorticoid reduce BDNF expression (72, 73). Thus, glucocorticoid-mediated inhibition of Wnt7a and BDNF might be involved in the suppression of neurogenesis in the OB of STZ-treated rats.

This study showed that the neurogenic niche of OB NSCs was mostly localized in the GL, although most mature neurons were present in the GCL. NSCs, neurons, astrocytes, and oligodendrocytes in the OB were morphologically distinct from each other, which suggests the lineage commitment from NSCs is strictly controlled. We also provide evidence that diabetes inhibits Wnt3-induced neuronal differentiation in the OB and alters neurotransmitter systems such as GABA and glutamate transporters. Diabetes led to several behavioral deficits, including impaired odor-mediated behavior, learning and memory, and increased anxiety. Thus, diabetes caused decreases in neurogenesis and malfunctions of the olfactory and other brain areas, perhaps contributing to these behavioral deficits. In addition, the impairment GATs and EAATs was found in the diabetic rats, and GAT1 inhibition disturbed Wnt3-induced neuronal differentiation of NSCs in vitro. Therefore, this suggests that alterations in GABA transporters lead to a decrease in Wnt3-induced neurogenesis and several behavioral deficits in diabetes. Not only GABA but also glutamate transporter systems were found to be altered in diabetes. It is presumed that the regulation of local GABA and glutamate neurotransmitter levels is important for the maintenance of adult neurogenesis in the OB and that insulin helps to maintain GABA and glutamate transporter activities. Thus, insulin is believed to be essential for the control of GABA and glutamate transporter activities, which in turn affect Wnt3-induced neurogenesis and olfactory functions. In addition, insulin was found to help promote neurogenesis and moderate the decrease in neurogenesis induced by GAT1 inhibition in vitro. As such, insulin may be an important factor for the maintenance of neurogenesis.
Experimental Procedures

Animals—Fisher 344 male rats (~4 months old) weighing 70–100 g were used in this study. All animals were maintained in a 12-h light/12-h dark cycle in a controlled temperature and relative humidity environment with standard rat chow and drinking water available ad libitum. To induce diabetes, animals were treated with a single intraperitoneal (i.p.) injection of 70 mg/kg STZ (Wako, Osaka, Japan) dissolved in saline (74, 75). Blood glucose levels were measured for 3 days after STZ administration. Rats with blood glucose levels greater than 300 mg/dl were considered diabetic. OBs were harvested from the rats at 10, 20, and 30 days after STZ injection. To label proliferating cells, BrdU (200 mg/kg, i.p.) was administered the STZ and analyzed in OBs isolated 20 days after diabetes induction.

All animals were anesthetized with pentobarbital sodium (100 mg/kg) and quickly perfused with saline. Collected Brain and OB samples were fixed in 4% paraformaldehyde solution. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Institute of Advanced Industrial Science and Technology.

Immunohistochemistry—Samples fixed with 4% paraformaldehyde overnight were placed in 30% sucrose solution until the tissues sank. Coronal sections (40 μm) were collected and stored in tissue collection medium (25% glycerin, 30% ethylene glycol, 0.05 m phosphate) at −20 °C. The immunohistochemical detection of BrdU, sections were incubated in 1N HCl at 37 °C for 15 min and then stored in ISOGEN solution at 20 °C. Extracted RNA was treated with DNase I (Life Technologies, Inc.) for 30 min at 37 °C to remove genomic DNA. cDNA synthesis was performed with SuperScript III (Life Technologies, Inc.) for 30 min at 37 °C.

RNA Extraction and qPCR—Isolated OBs were immediately stored in ISOGEN solution at −20 °C. Total RNA was isolated using ISOGEN (Wako) on ice according to the manufacturer’s instructions, dissolved in diethyl pyrocarbonate-treated water, and stored at −20 °C. Extracted RNA was treated with DNase I (Life Technologies, Inc.) for 30 min at 37 °C to remove genomic DNA. cDNA synthesis was performed with SuperScript III (Life Technologies, Inc.) or PrimeScript RT Master Mix (TAKARA Bio, Otsu, Japan) according to the manufacturer’s protocols. qPCR was performed with a Chromo4 system (Bio-Rad) and Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). All cDNAs were diluted 50-fold with diethyl pyrocarbonate-treated water. Primers were synthesized by Life Technologies, Inc., Japan (Table 1), and qPCR was performed as follows: 40 cycles each of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min.
extension at 60 °C for 40 s. Gapdh expression was used as the internal control. The ΔΔCt method was used to calculate the relative quantity of each target gene and normalized to Gapdh in each sample.

Western Blotting—Protein expression was analyzed as described previously (76). OBs were homogenized in lysis buffer (50 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10 mM NaF, 10 mM Na3P2O7, 2 mM Na2VO4, 1% Nonidet P-40, 1% sodium deoxycholate, 0.2% SDS) containing protease inhibitor mix (Nacalai Tesque, Kyoto, Japan) on ice (77). Samples were then centrifuged at 17,500 × g for 30 min at 4 °C and the supernatants collected. Lysate protein concentrations were quantified with a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) and diluted with loading sample buffer (62.5 mM Tris–HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 5% sucrose, and 0.005% bromphenol blue). Equal amounts of protein were separated in SuperSep gels (Wako) and transferred to PVDF membranes (Millipore). After blocking in 10% Blocking One (Nacalai Tesque) in 0.05% TBS-T for 1 h, blots were incubated with primary antibodies at 4 °C overnight. For immunodetection, the following primary antibodies were used: rabbit anti-GAPDH (1:5000; Santa Cruz Biotechnology); rabbit anti-SOX2 (1:1000; Millipore); mouse anti-Nestin (1:500; Pharmingen); mouse anti-TUJ1 (1:10,000; Promega); mouse anti-synapsin 1 (1:10,000; Transduction Laboratories); rabbit anti-Olig2 (1:1000; Millipore); mouse anti-MBP (1:1000; Abcam); rabbit anti-GFAP (1:10,000; DAKO); mouse anti-S100β (1:1000; Abcam), rabbit anti-Wnt3 (1:5000; Santa Cruz Biotechnology); rabbit anti-β-catenin (1:2000; Cell Signaling Technology); rabbit anti-phospho-GSK3β (1:2000; Cell Signaling Technology); mouse anti-NeuroD1 (1:500; Abcam); rabbit anti-Doublecortin (1:1000; Santa Cruz Biotechnology); and rabbit anti-GAT1 (1:1000; Abcam). After several washes with 0.05% TBS-T, immunoreactive bands were detected with HRP-conjugated secondary antibodies and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). The following HRP-conjugated secondary antibodies were used: donkey anti-rabbit and anti-mouse IgG (1:10,000, GE Healthcare). Protein bands were visualized with an LAS-3000 Imaging System (Fuji Film Corp, Tokyo, Japan) and quantified with ImageJ 1.32 software (National Institutes of Health, Bethesda, MD).

Behavior Analysis—Behavioral tests were performed in a gray acrylic box (60 × 60 × 60 cm) or the elevated plus maze and before the first trial. A discrimination index (time spent with object A − time spent with object B)/(total time exploring both objects) was calculated between the novel and familiar objects. The index varied between +1 and −1, where positive and negative scores indicate more or less time spent with the novel object, respectively. A discrimination index of 0 indicated equal exploration of both objects (79).

Elevated Plus Maze Test—The elevated plus maze apparatus consists of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm) extending from a central platform elevated 50 cm above the floor. The arms were connected with a central quadrangular area (10 × 10 cm) to form a cross. The rats were placed on the central quadrangular area and allowed to move freely on the arms for 10 min. The frequency of entry into the open or closed arms and the time spent on each arm were recorded (80). The percentage of time spent in the open arms was interpreted as an index of fear and anxiety. Rodent behavior was recorded by a digital camera placed above the apparatus. The percentage of time spent in the open arms (100 × open/total) and the number of entries into the open and closed arms were calculated. Each rat was tested only once and the apparatus was carefully cleaned with 70% ethanol between each trial and before the first trial.

Cell Cultures—The adult rat hippocampal neural (HCN) cell line from the adult rat hippocampus was cultured as described (81, 82). Briefly, NSCs were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium (DMEM/F-12, Life Technologies, Inc.) containing 1% antibiotic/antimycotic (Life Technologies, Inc.), 2 mM L-glutamine (Life Technologies, Inc.), 1% N2 supplement (Wako) and 20 ng/ml FGF-2 (Wako) in a 5% CO2 incubator at 37 °C. For neuronal differentiation, HCNs were cultured in DEME/F-12 containing 1 μM retinoic acid (Sigma) and 1 μM forskolin (Sigma). Cells were treated with the GAT1-selective inhibitor SKF89976A (Abcam; 0, 1, 5, 10, 50, and 100 μM) (83–86) or insulin (0, 10, and 100 ng/ml) during the neuronal differentiation for the indicated time periods and then collected for RNA isolation and qPCR analysis. To evaluate whether insulin rescued the neurogenesis hindered by the GAT1 inhibitor, HCNs were treated with insulin (0 or 100 ng/ml) after GAT1 inhibitor administration (0 or 50 μM) during neuronal differentiation. The next day, cells were collected for RNA isolation and qPCR analysis.
GAT1 Suppression Impairs Neurogenesis in OB in Diabetics

Statistical Analysis—For all animal experiments, the GAT1 inhibitor administration and insulin treatment experiments in culture were analyzed for statistical significance using Student’s t tests. All data are presented as mean ± S.D. p < 0.05 was considered to be significant. GAT1 inhibitor and insulin in vitro co-treatment results were analyzed for statistical significance by two-way analysis of variance and Bonferroni’s post hoc tests.

Author Contributions—T. K. conceived and coordinated the study and contributed to writing the manuscript. R. H. and T. W. designed, performed, and analyzed the experiments and wrote the manuscript. S. F. performed and analyzed the experiments. M. A. supervised and supported the research. All authors reviewed the results and approved the final version of the manuscript.

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