Activation of Wnt Signaling in Cortical Neurons Enhances Glucose Utilization through Glycolysis*

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The Wnt signaling pathway is critical for a number of functions in the central nervous system, including regulation of the synaptic cleft structure and neuroprotection against injury. Deregulation of Wnt signaling has been associated with several brain pathologies, including Alzheimer’s disease. In recent years, it has been suggested that the Wnt pathway might act as a central integrator of metabolic signals from peripheral organs to the brain, which would represent a new role for Wnt signaling in cell metabolism. Energy metabolism is critical for normal neuronal function, which mainly depends on glucose utilization. Brain energy metabolism is important in almost all neurological disorders, to which a decrease in the capacity of the brain to utilize glucose has been linked. However, little is known about the relationship between Wnt signaling and neuronal glucose metabolism in the cellular context. In the present study, we found that acute treatment with the Wnt3a ligand induced a large increase in glucose uptake, without changes in the expression or localization of glucose transporter type 3. In addition, we observed that Wnt3a treatment increased the activation of the metabolic sensor Akt. Moreover, we observed an increase in the activity of hexokinase and in the glycolytic rate, and both processes were dependent on activation of the Akt pathway. Furthermore, we did not observe changes in the activity of glucose-6-phosphate dehydrogenase or in the pentose phosphate pathway. The effect of Wnt3a was independent of both the transcription of Wnt target genes and synaptic effects of Wnt3a. Together, our results suggest that Wnt signaling stimulates glucose utilization in cortical neurons through glycolysis to satisfy the high energy demand of these cells.

Wnt signaling is essential for the development and function of the central nervous system (CNS), where it modulates several processes, such as adult hippocampal neurogenesis, formation of the synaptic cleft, and the regulation of mitochondrial dynamics (1–3). The deregulation of Wnt signaling by either a loss or gain of function is associated with the progression of various diseases, including fibrosis, cancer, and Alzheimer disease’s (AD) (4–6).

Wnt signaling can be fundamentally divided into two pathways: the canonical pathway (Wnt/β-catenin) and the non-canonical pathway. The canonical pathway begins with the binding of the Wnt ligand to its receptor, Frizzled (Fzd), thereby resulting in the downstream inactivation of the enzyme glycogen synthase kinase-3β (GSK-3β). The inactivation of GSK-3β results in the accumulation and translocation of β-catenin to the nucleus, where this protein stimulates the expression of Wnt target genes (7). However, in recent years, different cell type-specific functions of canonical Wnt ligands that are independent of the expression of target genes have been described, suggesting that Wnt signaling may also interact with other metabolic pathways (8–11).

Several reports have suggested a new role for Wnt signaling as a regulator of metabolic pathways. However, little is known about the effect of Wnt pathways on glucose metabolism in neurons (12–14). This role has been proposed based on the indirect effects of Wnt signaling on other regulators/sensors of glucose metabolism, including AMP-activated protein kinase (AMPK) or the Akt pathway (15–18). The brain is an avid consumer of glucose. In brain tissue, glucose is oxidized through glycolysis and oxidative phosphorylation to produce ATP, most of which is consumed by neurons during the recovery of ion gradients after synaptic transmission (19, 20). In several models

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2 The abbreviations used are: AD, Alzheimer’s disease; CaMKII, calcium/calmodulin-dependent protein kinase II; CaMKIV, calcium/calmodulin-dependent protein kinase IV; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; Cyt B, cytochalasin B; d-APV, d-(−)-2-amino-5-phosphonopentanoic acid; DCA, dichloroacetate; Dkk1, Dickkopf Wnt signaling pathway inhibitor 1; Fzd, frizzled; GLUT, glucose transporters; Glc-6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GSK-3β, glycogen synthase kinase-3β; HK, hexokinase; PPP, pentose phosphate pathway; 2-DG, 2-deoxyglucose; 2-[3H]DG, radioactive 2-deoxyglucose; rWnt3a, recombinant Wnt3a; qRT, quantitative RT; PET, positron emission tomography; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.
of neurological disorders, it has been shown that dysfunctional glucose utilization is a critical step in the onset and progression of disease (21, 22). Accordingly, enhancing glucose utilization in vivo induces significant improvements in cognitive functions, such as memory and learning (23–25). Despite the neuroprotective role of Wnt ligands, little is known about the effects of these ligands on glucose metabolism, a critical pathway for whole brain function and cell viability.

In the present study, we found that acute treatment with the Wnt3a ligand stimulated glucose uptake, hexokinase (HK) activity, and the glycolytic rate in neurons. The effect of Wnt3a on neuronal glucose metabolism was independent of both Wnt target gene transcription and the synaptic effects of the Wnt3a ligand. In addition, the effect of Wnt3a treatment was blocked by Wnt inhibitors and by an Akt pathway inhibitor. The effect of Wnt3a was also observed in a more complex system (mouse hippocampal slices). Together, our results provide evidence to support the concept that Wnt signaling has a role in regulating neuronal glucose metabolism.

**Results**

**Effects of the Acute Wnt3a Treatment on Neurons**—First, we used Western blot analysis to determine whether the acute rWnt3a treatment changed the expression of typical markers of canonical Wnt pathway activation, described previously for our laboratory (26, 27). The cultured cortical neurons were incubated with the rWnt3a ligand for 15, 30, and 90 min. We did not observe changes in the expression of β-catenin after rWnt3a treatment (Fig. 1A). However, after 30 min of treatment, we observed a 1.8 ± 0.1-fold increase in the levels of p-GSK3β-Ser9 (an inactivating phosphorylation) compared with the control. After 90 min of treatment, we observed a 2.2 ± 0.3-fold increase compared with the control (Fig. 1A). In addition, using qRT-PCR we did not observe changes in the mRNA expression of two Wnt target genes, c-jun and CaMK4, after the acute rWnt3a treatment. As a control, we detected an increase in the mRNA of both genes after 24 h of treatment with rWnt3a; this increase was blocked by co-incubation with the Wnt antagonist Dkk1 (Fig. 1B). Furthermore, we determined if rWnt3a treatment changed the expression of two important metabolic sensors in neurons, AMPK and Akt (4, 28, 29). We did not observe changes in the expression of p-AMPK-Thr172 (an activating phosphorylation) after rWnt3a treatment (Fig. 1C). However, after 15 and 30 min of rWnt3a treatment, we observed an important 2.10 ± 0.23-fold increase in the levels of p-Akt-Ser473 (active form of Akt) compared with the control. Interestingly, after 90 min, we detected a decrease in the activated form of Akt compared with the control condition (Fig. 1C).

**Activation of Wnt Signaling by Wnt3a Enhances Glucose Uptake in Cortical Neurons**—After establishing the timing of Wnt activation, we studied the effect of the Wnt3a treatment on glucose metabolism. We treated the cells with Wnt3a (conditioned media or recombinant) for 15 min and then measured glucose uptake (15–90 s). In these studies, we used a 0.5 mM glucose solution with traces of radioactive [2-3H]DG. Under control conditions, we observed the time-dependent uptake of [2-3H]DG, with a maximum of 5.6 ± 0.9 nmol/10⁶ cells at 90 s. After Wnt3a treatment, we observed a marked increase in [2-3H]DG uptake, with a maximum of 11.1 ± 1.3 nmol/10⁶ cells at 90 s. Co-incubation with Dkk1 blocked almost all increases in [2-3H]DG uptake. The presence of the GLUT inhibitor cytochalasin B (Cyt B, 20 μM) completely decreased the [2-3H]DG uptake to 1.11 ± 0.3 nmol/10⁶ cells at 90 s (Fig. 2A). Treatment with Wnt3a for 15 min triggered a 2-fold increase in [2-3H]DG uptake at 90 s, and this effect was abolished by the presence of Dkk1 (Fig. 2A). We found that the IC₅₀ value of the effect of Cyt B on the uptake of [2-3H]DG was 0.70 ± 0.06 μM. This finding is consistent with glucose transport through GLUTs (31) (Fig. 2A). Next, we tested the effects of different molecules on the Wnt3a-induced [2-3H]DG uptake. Under control conditions, the initial rate of [2-3H]DG uptake (measured at 30 s) was 3.2 ± 0.3 nmol/10⁶ cells. After Wnt3a treatment, the initial rate of [2-3H]DG uptake increased to 6.2 ± 0.3 nmol/10⁶ cells. This increase was blocked by co-incubation with Dkk1 and TCS183, and we observed similar results when we treated the cells with rWnt3a. In contrast, co-incubation with CNQX and d-APV, inhibitors of AMPA and NMDA receptors, respectively, did not alter the effect of Wnt3a on glucose uptake. However, co-incubation with an inhibitor of Akt pathway activation, AZD5363, blocked the increase in glucose uptake after rWnt3a treatment, suggesting that the effect of Wnt3a depends on Akt activation. Finally, we observed that glucose uptake was abolished in the presence of Cyt B or high concentrations of non-radioactive 2-DG as a control (Fig. 2A).

For a more comprehensive analysis of the effects of Wnt signaling, the kinetic parameters of glucose uptake were estimated. Under control conditions, we observed a Km value of 6.46 ± 1.18 mM and a Vmax of 4.85 ± 0.39 pmol/10⁶ cells/min (Fig. 3B). In cells that were treated with Wnt3a for 15 min, the Km and Vmax values were 3.67 ± 0.36 mM and 4.02 ± 0.24 pmol/10⁶ cells/min, respectively (Fig. 3B). These results showed that there was a significant decrease in the Km value after treatment with the Wnt3a ligand. There was no apparent change in the Vmax for transport, suggesting that there were no changes in the expression of GLUT transporters in the membrane.

**Wnt3a Treatment Enhances HK Activity and Stimulates the Glycolytic Rate**—We also monitored GLUT3 expression after treatment with rWnt3a for 15, 30, and 90 min by Western blotting. The rWnt3a treatment did not affect the levels of the GLUT3 transporter protein (Fig. 2A). The above results showed that treatment with Wnt3a enhances glucose uptake without changing the levels of GLUTs, suggesting intracellular effects. After uptake, glucose is phosphorylated by HK to generate glucose 6-phosphate (Glc-6P), which is mainly used in neurons for glycolysis or the pentose phosphate pathway (PPP).

We next studied the effect of Wnt signaling activation on HK activity. We measured HK activity after Wnt3a treatment for 15, 30, and 90 min. Under control conditions, we observed an activity of 0.76 ± 0.10 units/mg. We did not observe changes in the activity after 15 min of treatment. However, significant increases in HK activity, 1.50 ± 0.20 and 2.77 ± 0.30 units/mg, were observed after 30 and 90 min, respectively; the increase in HK activity induced by rWnt3a was blocked by co-incubation with Dkk1 (Fig. 3B). Furthermore, we observed that the effect of rWnt3a was independent of co-incubation with both d-APV
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A

Control | rWnt3a (min)
--------|----------------
        | 15 | 30 | 90 |

- **β-catenin**
- **p-GSK3β-Ser9**
- **GSK3β**
- **β-actin**

B

- β-catenin expression relative to β-actin (R.U. to control)

C

- p-AMPK-Thr172
- AMPK
- p-Akt-Ser473
- Akt
- β-actin

- p-AMPK-Thr172/AMPK ratio relative to β-actin (R.U. to control)

- p-Akt-Ser473/AKT ratio relative to β-actin (R.U. to control)
and CNQX. In contrast, the effect of rWnt3a was blocked by co-incubation with Dkk1, TCS183, and AZD5363. As a control, we also observed a strong inhibition of HK activity in the presence of high concentrations of 2-DG (Fig. 3B). The above results indicate that Wnt3a treatment increases glucose uptake and enhances HK activity.

Based on our results, we studied the effect of Wnt3a on the glycolytic rate in neurons. For this purpose, we used radiolabeled [3-3H]glucose (32). First, we measured the glycolytic rate after rWnt3a treatment for 15, 30, and 90 min. Under control conditions, the glycolytic rate was 0.56 ± 0.05 pmol/mg of protein. We did not observe significant changes after 15 min of treatment with the rWnt3a ligand. However, after 30 min, the glycolytic rate increased to 1.33 ± 0.16 pmol/mg of protein. The effect of rWnt3a was blocked by co-incubation with Dkk1 and was similar when the neurons were treated with LiCl (Fig. 3C). The presence of both CNQX and d-APV did not affect the increase in the glycolytic rate after rWnt3a treatment. However, the effect of rWnt3a on the glycolytic rate was blocked by co-incubation with Dkk1, TCS183, and AZD5363. As a control, we also observed a decrease in the glycolytic rate in the presence of 2-DG and DCA (Fig. 3C).

Previously, we described that treatment with Wnt3a increased the glycolytic rate in part by stimulating HK activity. We next determined whether Wnt3a treatment affected the activity of the bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB), the enzyme that catalyzes the synthesis of fructose 2,6-bisphosphate, to study other possible intracellular targets of Wnt3a. Fructose 2,6-bisphosphate activates phosphofructokinase, a rate-limiting enzyme in glycolysis. Research studies indicate that amino acids activate PFKFB2 through Akt-dependent phosphorylation at Ser^483 (33, 34). We observed that 15- and 30-min treatments with rWnt3a significantly increased the amount of the activated form of PFKFB by Western blotting; interestingly, after 90 min of treatment with rWnt3a, we observed a decrease in the activated form of the enzyme (Fig. 3D).

**Activation of Wnt Signaling Does Not Affect the Activity of Glucose-6-phosphate Dehydrogenase (G6PDH) or the PPP—**In neurons, Glc-6P is generated from glucose and may also be used by the PPP. We measured the activity of G6PDH, which catalyzes the most important regulatory step of the PPP to explore this pathway. After treatment with the rWnt3a ligand for 15, 30, and 90 min, we did not observe changes in G6PDH activity (Fig. 4A). Using Western blot analysis, we observed that treatment with rWnt3a did not change the levels of G6PDH protein (Fig. 4A). The activity of G6PDH is critical for the PPP. Therefore, PPP activity was measured. Under the control conditions, we observed that glucose oxidation through the PPP was 0.18 ± 0.02 nmol/min/mg of protein. We did not observe significant changes in PPP activity after rWnt3a treatment (Fig. 4B). The increase in glucose utilization through glycolysis must affect ATP generation by the mitochondria. Thus, we measured the levels of ADP, a substrate for the generation of ATP, to study this process. Under control conditions, we measured 53 ± 11 μM ADP, and after 30 min of treatment with rWnt3a, we measured an increase to 94 ± 11 μM. The increase in the ADP levels following treatment with rWnt3a was blocked by co-incubation with Dkk1 and by treatment with 2-DG (Fig. 4C).

**Wnt3a Treatment Induces Glucose Utilization in Brain Slices through Glycolysis—**We next studied whether the effects of Wnt3a observed in primary cortical cultures could be recapitulated in a more complex model to further analyze the effect of Wnt3a on glucose metabolism. For this purpose, we used cortical-hippocampal slices from mouse brains. First, we treated the slices with Wnt3a for different periods of times (i.e. 30, 60, and 90 min). We observed an increase in the accumulation of [2-3H]DG after 90 min (7.8 ± 0.9 nmol/10^⁶ cells) compared with the control condition (4.0 ± 0.6 nmol/10^⁶ cells) (Fig. 5A). This effect, which was observed after 90 min of treatment, was also observed in the presence of LiCl. The presence of Dkk1 and TCS183 blocked the effect of Wnt3a (Fig. 5A). The Wnt3a treatment (90 min) also caused a time-dependent increase in HK activity in the slices from 8.7 ± 0.8 units/mg of protein to 19.6 ± 3.0 units/mg of protein after 90 min of treatment. This effect was also blocked by Dkk1 and TCS183 (Fig. 5B). Furthermore, we measured the effect of Wnt3a treatment on G6PDH activity and did not observe changes after Wnt3a treatment (Fig. 5C). In addition, we measured the glycolytic rate in hippocampal slices. Under the control conditions, we measured a value of 3.5 ± 0.6 pmol/mg of protein. Following Wnt3a treatment (90 min), we observed an increase in the glycolytic rate to 4.3 ± 0.5 pmol/mg of protein; this increase was blocked by Dkk1 (Fig. 5D). Moreover, we measured PPP activity using radioactive glucose and did not observe changes compared with the control conditions. Under control conditions, we observed a decrease in PPP activity in the presence of the inhibitor 2-DG (Fig. 5E).

**Discussion**

In the present study, we analyzed the relationship between Wnt signaling and energy metabolism in cortical neurons. We report that acute Wnt3a treatment stimulates glucose uptake without significantly changing the expression and function of GLUT3, but with an increase in the affinity of the transporter. These effects of Wnt3a occurred independently of the transcription of Wnt target genes and of the synaptic effect of Wnt3a. The acute effect of Wnt3a included an increase in the activity of two control steps of glycolysis (HK and PFKFB2); these changes were directly correlated with an increase in glucose uptake, the glycolytic rate, and the utilization of ADP. In addition, we excluded the possibility that acute Wnt3a treatment involved changes in G6PDH activity and the activity of the PPP (Fig. 6).
FIGURE 2. Effect of Wnt3a treatment on glucose uptake. A, Wnt3a treatment stimulated 2-DG uptake in a time-dependent manner. The effect of Wnt3a treatment was significant after 15 s of exposure, and the time-dependent effect was blocked by co-incubation with Dkk1. The IC50 value was similar to the values reported for GLUTs. The effect of Wnt3a was independent of both transcription and synaptic effects of Wnt3a and was blocked by co-treatment with Dkk1, AZD5363, and TCS183. B, the initial uptake of trace amounts of 2-DG (at 15 s) was measured in the presence of increasing concentrations of unlabeled glucose (0–30 mM) under the control conditions and following Wnt3a treatment (15 min). The data represent the mean ± S.E. of n = 5 experiments, each performed in triplicate. The continuous lines in each plot correspond to the best non-linear regression fit of a rectangular hyperbola to the data using SigmaPlot 12 software.
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A

[Image of Western blot with GLUT3 and β-actin bands showing time points (15, 30, 90 min) and control condition.]

[Bar graph showing GLUT3 expression relative to β-actin (R.U. to control) across different time points (15, 30, 90 min).]

B

[Graph showing hexokinase activity (units/mg protein) for rWnt3a and rWnt3a+Dkk1 treatment groups over time (15, 30, 90 min).]

[Column graph comparing hexokinase activity (units/mg protein) across various treatments including Control, rWnt3a, CNQX, D-APV, TCS183, Dkk1, AZD5363, and 2-DG.]

C

[Graph showing rate of 3H2O production from (3-3H)glucose (pmol/mg protein) for rWnt3a and rWnt3a+Dkk1, and LUC treatment groups over time (15, 30, 90 min).]

[Bar graph comparing rate of 3H2O production (pmol/mg protein) across various treatments including Control, rWnt3a, CNQX, D-APV, TCS183, Dkk1, AZD5363, and 2-DG.]

D

[Image of Western blot with PFKFB2, p-PFKFB2-S483, and β-actin bands showing time points (15, 30, 90 min) and control condition.]

[Bar graph showing p-PFKFB2, Ser,483 ratio relative to β-actin (R.U. to control) across different time points (15, 30, 90 min).]
The activity of the Wnt signaling pathway is essential for the development and function of central synapses that modulate several processes, such as adult hippocampal neurogenesis, the establishment of synapses, neuronal firing activity, neuronal plasticity, nerve transmission, dendritic spine formation, and mitochondrial dynamics (2, 35–39). The deregulation of Wnt activity by loss or gain of function has been associated with the progression of various diseases, including cancer, diabetes mellitus type II, fibrosis, and AD (6, 30, 40, 41). However, the relationship between this signaling pathway and glucose metabolism in the brain has not been clearly established (4).

Glucose is the main energy substrate of the adult brain. It has been estimated that glucose supplies ~63% of the necessary energy for the brain of 7-day-old rats, and this value increases with age (42). The energy requirement of the brain varies considerably between different cell types, as well as between the control and activated states. Glucose metabolism in the brain is partly controlled by astrocytes through their proximity to blood vessels and neurons, thereby controlling blood flow and regulating the blood-brain barrier (43). Therefore, astrocytes are optimal for glucose uptake from the blood. Approximately 50% of the glucose from capillaries is taken up directly by astrocytes.
The amount of glucose that is later transformed for energy consumption is higher in neurons, suggesting that astrocytes transfer an intermediate substrate to neurons or store glucose as glycogen, supporting a metabolic coupling between astrocytes and neurons (44–47). Despite the importance of glucose metabolism in neurons little is known about the regulation of this process, this was the central focus of this work.

Most of the energy that is produced in the brain is used for the propagation of action potentials and for the maintenance of the membrane potentials that are required for neuronal transmission (19, 48). It has been reported that various peripheral pathologies that affect glucose levels lead to alterations in brain functions, including functions related to neuronal plasticity, memory, and learning (22, 49). In Drosophila, it has been described that increase of the glucose transport induces a decrease in the neuronal damage induced by amyloid β (50). In AD patients, a decrease in glucose utilization has been reported in various regions of the brain that are directly involved in cognitive functions, such as memory and learning. Accordingly, previous studies indicated a close relationship between glucose consumption and cognitive function (51, 52).

The involvement of Wnt signaling in the regulation of glucose metabolism has regained importance in recent years, due to studies in humans in which several components of the Wnt pathway have been identified as risk factors for metabolic diseases, including diabetes mellitus type II and age-related dementia; however, the ultimate effect depends on whether the canonical or non-canonical Wnt pathway is affected (4, 14, 53–55). Furthermore, activation of the Wnt/β-catenin pathway in vivo promotes a decrease in the plasma glucose level, which modulates the localization and expression of GLUT4 in adipocytes and increases glucose uptake in these cells (56). It has also been proposed that these pathways could have an important role in the control of energy intake and food behavior, modulating the energy balance throughout the body (57). Despite these antecedents, little is known about the effect of Wnt ligands on glucose metabolism at the cellular and molecular level.

FIGURE 5. Wnt pathway activation stimulates 2-DG uptake in hippocampal slices. A, in slices, Wnt3a treatment induced a time-dependent increase in the intracellular accumulation of 2-DG. This effect was blocked by Dkk1 and TCS-183. B, Wnt3a treatment increased HK activity. C, we did not observe changes in the activity of G6PDH in hippocampal slices after Wnt3a treatment. D, Wnt3a treatment induced a large increase in the glycolytic rate. E, treatment with Wnt3 did not affect the activity of the PPP in hippocampal slices. The data represent the mean ± S.E. of n = 5 experiments, each performed in triplicate. *, p < 0.01; **, p < 0.005, Bonferroni’s test.
In our model, we observed that short treatments with Wnt3a did not affect the expression or localization of the major GLUT transporter described in neurons (i.e. GLUT3) (58). However, after 30 min of treatment, we observed an increase in the affinity of the transporter for the substrate. Because we did not observe changes in the $V_{\text{max}}$ values, we discounted an increase in the other isoforms of GLUT transporters in the membrane. The increase in the affinity of the transporter was correlated with a strong increase in glucose uptake after Wnt3a treatment. The effect of Wnt3a on glucose uptake was independent of the synaptic effects of Wnt3a, but dependent on Akt activation.

Regarding the downstream effects, we observed that Wnt3a treatment increased HK activity in neurons, which is important because HK activity is the first regulatory step in glucose metabolism and is responsible for generating Glc-6P (59 – 61). Glc-6P is a critical substrate because it is the intracellular substrate of several metabolic pathways, including glycolysis, PPP, and glycogen synthesis (45). Also, it has recently been described that changes in the expression or localization of HK in brain cells could be correlated with the progression of AD in specific brain areas such as the frontal cortex, suggesting this enzyme has a new therapeutic target (62).

Glycolysis is the main pathway that uses glucose in the mammalian brain. We observed a robust increase in glucose utilization through this pathway after Wnt3a treatment. The effect of Wnt3a was independent of the synaptic effects of Wnt3a, but dependent on Akt activation, because an inhibitor of this protein was able to block the Wnt3a-induced increase in the glycolytic rate. The control of glycolytic flux is distributed between several enzymes, including HK and PFKFB2. We observed that Wnt3a significantly increased the activity of the HK enzyme, as well as levels of the activated form of PFKFB2, suggesting that Wnt3a treatment activated neuronal glucose metabolism at several cellular levels. We also reported that

**FIGURE 6. Schematic representation of the effect of Wnt3a on glucose metabolism in cortical neurons.** Wnt3a stimulates glucose uptake and HK activity. Downstream, Wnt3a increases the glycolytic rate. The increase in the glycolytic rate is correlated with an increase in the generation of ADP, without changes in PPP activity. The effects of Wnt3a over the glucose metabolism was independent of the synaptic effect of Wnt3a, because the co-incubation both D-APV and CNQX does not block the metabolic effect. The mechanism of action of Wnt3a is dependent of activation of the metabolic sensor Akt, suggesting a complex network between the metabolic pathways.
Wnt3a enhanced glucose utilization through glycolysis. The increase in the glycolytic rate must be proportional to the increase in the pyruvate level. Furthermore, pyruvate can translocate into the mitochondria due to the production of ATP. We observed that treatment with the Wnt3a ligand increased the levels of the major substrate for ATP synthesis, ADP, thereby suggesting that this pathway activates the oxidative metabolism of glucose in neurons (37, 63). Meanwhile, we did not observe changes in the regulatory enzyme G6PDH and in the overall PPP. Although the neuronal utilization of glucose for glycogen synthesis has been suggested for years (64), neurons have only recently been shown to use glucose to generate glycogen. Neuronal glycogen may be important for promoting neuronal survival under pathological conditions, such as oxidative stress and hypoxia (65). However, the neurons could be able to use but not accumulate glycogen because this process has been related with neuronal death in some pathologies (66, 67). The effect of the Wnt3a ligand on glycogen synthesis in neurons requires further study.

One remarkable aspect of our model was that the effect of Wnt3a on the glycolytic pathways in neurons was apparently independent of the synaptic effects described for the canonical ligands, as these effects were not affected in the presence of inhibitors of AMPA and NMDA receptors, both of which are involved in glutamatergic transmission (68–72). An interesting aspect of this issue is that despite the reports that described the importance of Wnt signaling in neuronal plasticity, the effect on glucose metabolism could involve interactions with other signaling pathways, such as AMPK and Akt signaling (30). Previously, it has been described that the canonical Wnt ligand could activate the Akt pathway after acute treatment; however, little is know about the molecular mechanism (18, 73). Both AMPK and Akt act as metabolic regulators to modulate the activity of several metabolic effectors, including PFKFB2, eNOS, GSK-3β, glycogen synthase, and PGC1α. Interestingly, several of these effectors also interact with the Wnt pathway at different cellular levels, suggesting a complex network of pathways that lead to the final cellular effect (10, 15, 30, 74, 75). Furthermore, our results suggest that the effect of Wnt3a treatment was dependent of the activity of GSK-3β because an inhibitor of GSK-3β inhibition (TCS183) blocks the effect of Wnt3a. The GSK-3β is involved in several metabolic pathways and represent an interesting field of study.

In our model, we observed that the pharmacological activator of Wnt signaling by LiCl increased the glycolytic rate and glucose uptake. However, positron emission tomography (PET) studies of humans have shown that the long term administration of lithium decreases glucose utilization in the cerebral and hippocampus of older adults (76). However, it is difficult to correlate the PET results with our model because our model used an acute treatment with Wnt activators. Meanwhile, we do not know the specific response of each cell type in the PET studies. Given that the native context of the brain includes other cells that have a critical role in regulating neuronal metabolism (77–79), such as astrocytes, we tested 2-DG uptake in the more complex system of hippocampal slices. Here, we observed results that were similar to the in vitro studies, thereby suggesting that the effects of Wnt3a are mainly localized in neurons; however, further studies are required to confirm this hypothesis.

In conclusion, our data suggest a novel function of Wnt signaling as an activator of glucose metabolism in neurons. This novel role of Wnt signaling in neuronal physiology may be an interesting topic in the search for new therapies against neurological disorders.

**Experimental Procedures**

**Animals and Ethical Standards**—Slices were prepared from 2-month-old male C57BL/6j mice. The animals were housed at the Animal House Facility of the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, in accordance with the Guide for the Care and Use of Laboratory Animals (NIH-USA Publication 86-23).

**Primary Neuronal Cell Culture**—Cortical neurons were obtained from the forebrains of 17-day-old rat embryos, as previously described (32, 80). Briefly, the cells were seeded in poly-D-lysine-coated culture dishes at a density of 5 × 10^5 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., MA). After 30 min, the culture medium was changed to Neurobasal medium (NeuB) (Thermo Fisher Scientific Inc.) supplemented with the B27® serum-free supplement for neural cell culture (Thermo Fisher Scientific Inc.), 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2.5 mg/ml of Fungizone (Thermo Fisher Scientific Inc.). The cell cultures were incubated in 5% CO₂ in a humidified environment at 37 °C. For all experiments, the neuronal cells were used after 7 days of in vitro culture.

**Generation of Control and Wnt3a-conditioned Media**—Control and Wnt3a-conditioned media were prepared from L control (ATCC: CRL-2648) and L-Wnt3a (ATCC: CRL-2647) cells. The cells were cultured according to the protocol described by the American Type Culture Collection (Manassas, VA). Briefly, the cells were grown to 70% confluence in modified Eagle's medium (MEM) (Invitrogen) supplemented with 10% FBS and 0.4 mg/ml of the antibiotic G-418 (Thermo Fisher Scientific Inc.). Then, the cells were grown in MEM in the absence of G-418 and FBS for 4 days. The media containing the Wnt3a ligand was recovered (batch 1), and this process was repeated with the same cells after an additional 4 days (batch 2). Then, batches 1 and 2 of the conditioned media were combined (82). In the present study, we also used recombinant Wnt3a ligand (rWnt3a) at a concentration of 300 ng/ml (7.5 nM) (R&D Systems, Minneapolis, MN).

**Cell Treatment**—Before treatment, the neurons were maintained in NeuB without B27® for 30 min and then incubated several times with control media, Wnt3a media, or rWnt3a (15, 30, and 90 min). All media were prepared in NeuB without B27. The neurons were also treated with inhibitors or activators of Wnt signaling, and the inhibitors/activators were co-incubated with the corresponding Wnt ligand. The inhibitors of canonical Wnt signaling that were used included Dickkopf 1 (Dkk1, 30 ng/ml; an agonist of Wnt ligands) (83) and TCS183 (300 μM; a peptide used as a competitive inhibitor of GSK-3β (Ser9) phosphorylation) (84, 85). We used LiCl (10 mM, which is a classic inhibitor of GSK-3β) as an activator of canonical Wnt signaling.
Glucose Uptake Analysis—After Wnt3a (15, 30, and 90 min) treatment in the presence or absence of Wnt activators or inhibitors, the neurons were carefully selected under the microscope to ensure that only plates showing uniform neuronal growth were used. After incubation with the Wnt ligand, the cells were washed with incubation buffer (15 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, and 0.8 mM MgCl2) supplemented with 0.5 mM glucose (32). Uptake was measured at room temperature by the addition of 1–1.2 μCi of 2-deoxy-
D-[1,2-3H]glucose ([2-3H]DG) at a final specific activity of 1–3 disintegrations/min/pmol (~1 mCi/mmol). Uptake was arrested by washing the cells with ice-cold PBS supplemented with 1 mM HgCl2. The incorporated radioactivity was assayed by liquid scintillation counting. For the pharmacological experiments, the glucose metabolism inhibitors were incubated with a radioactive substrate for 10 min. For the slice experiments, the slices were incubated with [2-3H]DG, after which we followed a standard methodology, as described above. Kinetic values were determined using a single rectangular hyperbola of the form

\[
V = \frac{V_{\text{max}} \cdot [\text{Glc}] \cdot (K_{\text{m}} + [\text{Glc}])}{K_{\text{m}} \cdot (K_{\text{m}} + [\text{Glc}])}
\]

which was adjusted to the data by non-linear regression using SigmaPlot 12 software (95).

[2-3H]DG was purchased from PerkinElmer Life Sciences.

Quantification of HK Activity—After treatment with Wnt3a, the cells were washed with PBS, treated with trypsin/EDTA, and centrifuged at 500 × g for 5 min at 4 °C. Then, the cells were resuspended in isolation medium (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 2 mg/ml of aprotinin, 1 mg/ml of pepstatin A, and 2 mg/ml of leupeptin) at a 1:3 dilution, sonicated at 4 °C, and then centrifuged at 1,500 × g for 5 min at 4 °C. Finally, the HK activity of the supernatant was quantified. For the assay, the purified fraction was mixed with the reaction medium (25 mM Tris-HCl, 1 mM DTT, 0.5 mM NADP/Na+, 2 mM MgCl2, 1 mM ATP, 2 units/ml of G6PDH, and 10 mM glucose), and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 10% trichloroacetic acid (TCA), and the generation of NADPH was measured at 340 nm (96).

Determination of G6PDH Activity—After treatment with the appropriate compound, the cells were washed with PBS, collected by detaching the cells with 0.25% trypsin, 0.2% EDTA (w/v), and pelleted. Then, the cells were resuspended in isolation medium (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 2 mg/ml of aprotinin, 1 mg/ml of pepstatin A, and 2 mg/ml of leupeptin) at a 1:3 dilution, sonicated at 4 °C, and then centrifuged for 5 min at 1,500 × g and 4 °C. Subsequently, the pellet was discarded, and the supernatant was further separated by centrifugation at 13,000 × g for 30 min at 4 °C. Finally, the supernatant was used to quantify the G6PDH activity in a reaction buffer containing 1 mM ATP and 10 mM glucose 6-phosphate (Glc-6P) for 30 min at 37 °C. The reaction was stopped by the addition of 10% TCA. Then, the generation of NADPH was measured at 340 nm (96).

Measurement of Glucose Oxidation through the PPP—Glucose oxidation via the PPP was measured as previously described based on the difference in 14CO2 production from [1-13C]glucose (decarboxylated in the 6-phosphogluconate dehydrogenase-catalyzed reaction and in the Krebs cycle) and [6-14C]glucose (only decarboxylated in the Krebs cycle). After
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rWnt3a treatment (with or without inhibitors or activators), the medium was removed, and the neurons were washed with ice-cold PBS and collected by trypsinization. Cell pellets were resuspended in O2-saturated Krebs-Henseleit buffer (11 mM Na2HPO4, 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH2PO4, 1.2 mM MgSO4, and 1.3 mM CaCl2, pH 7.4), and 500 µl of this suspension (~106 cells) was placed in Erlenmeyer flasks with another 0.5 ml of the Krebs-Henseleit solution containing 0.5 µCi of D-[1-14C]glucose or 2 µCi of D-[6-14C]glucose and 5.5 mM d-glucose (final concentration). The Erlenmeyer flasks were equipped with a central well containing an Eppendorf tube with 500 µl of benzethonium hydroxide. The flasks were flushed with O2 for 20 s, sealed with rubber caps, and incubated for 60 min in a 37 °C water bath with shaking. The incubations were stopped by the injection of 0.2 ml of 1.75 M HClO4 into the main well, although shaking was continued for another 20 min to facilitate the trapping of 14CO2 by benzethonium hydroxide. Radioactivity was assayed by liquid scintillation spectrometry (97, 98). Both [1-14C]glucose and [6-14C]glucose were purchased from PerkinElmer Life Sciences.

**Determination of the Glycolytic Rate**—The glycolytic rates were determined using previously described methods (32, 99). After treatment with rWnt3a, the neurons were placed into tubes containing 5 mM glucose and then washed twice in Krebs-Henseleit solution (11 mM Na2HPO4, 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH2PO4, 1.2 mM MgSO4, and 1.3 mM CaCl2, pH 7.4) containing the appropriate concentration of glucose. After equilibration in 0.5 ml of Hanks’ balanced salt solution/glucose at 37 °C for 10 min, 0.5 ml of Hanks’ balanced salt solution containing various concentrations of [3-3H]glucose was added, with a final specific activity of 1–3 disintegrations/min/pmol (~1 mCi/mmol). Aliquots of 100 µl were then transferred to another tube, placed inside a capped scintillation vial containing 0.5 ml of water, and incubated at 45 °C for 48 h. After this vapor-phase equilibration step, the tube was removed from the vial, a scintillation mixture was added, and the H2O content was determined by counting over a 5-min period. The [3-3H]glucose was obtained from PerkinElmer Life Sciences.

**Slice Preparation**—Hippocampal slices were prepared according to previously described standard procedures (30, 100). Briefly, transverse slices (350 µm) from the dorsal hippocampus were sectioned in cold artificial cerebrospinal fluid (ACSF, in mM: 124 NaCl, 2.6 NaHCO3, 3 d-glucose, 2.69 KCl, 1.25 KH2PO4, 2.5 CaCl2, 1.3 MgSO4, and 2.60 NaHPO4) using a Vibratome (Leica VT 1000s, Germany) and incubated in ACSF for 1 h at room temperature. After this incubation, the slices were treated with Wnt3a and analyzed at several times, 30, 60, and 90 min, for the different metabolic experiments.

**ADP Content**—After rWnt3a treatment, we measured the ADP levels in the whole-cell lysates of primary neurons using an ADP Assay Kit (ab83359, Abcam, UK), according to the manufacturer’s instructions (81).

**Statistical Analysis**—All experiments were performed 5 times, with triplicates for each condition in each experimental run. The results are expressed as mean ± S.E. The data were analyzed by one-way or two-way analysis of variance, followed by Bonferroni’s post hoc test; *p ≤ 0.05 and **p ≤ 0.01 were considered significant differences. Statistical analyses were performed using Prism software (GraphPad, La Jolla, CA).

**Author Contributions**—P. C., L. F. B., and N. C. I. conceived and designed the experiments; P. C., C. S.-A., and P. S. performed the experiments; P. C., L. F. B., and N. C. I. analyzed the data; N. C. I. contributed reagents/materials/analysis tools; and P. C., L. F. B., and N. C. I. wrote the manuscript.

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