Glucose transporter 2 (GLUT2; gene name SLC2A2) has a key role in the regulation of glucose dynamics in organs central to metabolism. Although GLUT2 has been studied in the context of its participation in peripheral and central glucose sensing, its role in the brain is not well understood. To decipher the role of GLUT2 in brain development, we knocked down slc2a2 (glut2), the functional ortholog of human GLUT2, in zebrafish. Abrogation of glut2 led to defective brain organogenesis, reduced glucose uptake and increased programmed cell death in the brain. Coinciding with the observed localization of glut2 expression in the zebrafish hindbrain, glut2 deficiency affected the development of neural progenitor cells expressing the proneural genes atoh1b and ptf1a but not those expressing neurod. Specificity of the morphant phenotype was demonstrated by the restoration of brain organogenesis, whole-embryo glucose uptake, brain apoptosis, and expression of proneural markers in rescue experiments. These results indicate that glut2 has an essential role during brain development by facilitating the uptake and availability of glucose and support the involvement of glut2 in brain glucose sensing.

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INTRODUCTION

Glucose represents the main energy source for many organisms and its cellular uptake is facilitated by passive diffusion glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices with the amino and carboxyl termini exposed to the cytosol. Each GLUT isoform has a specific role in glucose metabolism as determined by its pattern of tissue expression, substrate specificity, transport kinetics, and regulated expression under different physiological conditions. Particularly, the intestine, endocrine pancreas, kidney, and liver, tissues that have key roles in carbohydrate metabolism, express GLUT2, a low-affinity transporter for glucose, fructose, mannose, and galactose, that is also a high-affinity transporter for glucosamine. The ability of GLUT2 to transport different types of hexoses at a wide range of concentrations ensures fast equilibration of glucose between the extracellular space and the cell cytosol. GLUT2 participates in the intestinal and renal absorption of glucose, the stimulation of insulin secretion by glucose in β-pancreatic cells and the entry and output of glucose by the liver. Mutations in the GLUT2 gene cause hepatorenal glycosgen accumulation, nephropathy, and defects in glucose homeostasis in humans (i.e., Fanconi–Bickel syndrome; FBS). In addition, GLUT2 is also expressed in the central nervous system, particularly in neurons and glial cells of brain areas known to participate in the central glucose sensing system that regulates glucose homeostasis and food intake. In glial cells, GLUT2 is involved in the detection of hypoglycemia and has an important role mediating the counter-regulatory response to glucose deficit. Evidence for the important role of GLUT2 in the regulation of feeding comes from observations indicating that individuals from two Canadian populations harboring a single-nucleotide polymorphism in the GLUT2 gene (Thr119ile) show abnormal sugar intake. Similarly, abnormal feeding behavior has been observed by blocking GLUT2 intracerebroventricularly in rats, in GLUT2- null mice, or in GLUT2-SDD mice. Unfortunately, to date, the neuronal functions of GLUT2 are poorly understood and the importance of GLUT2 during brain development is not known.

In the present study, we have used the developing zebrafish embryo model to examine in vivo the physiological role of glut2. The ease of genetic manipulation, rapid development, and optical transparency of the zebrafish embryo provide a unique opportunity to unravel the mechanisms following the perturbation of glucose homeostasis associated with loss of function of this transporter. Our findings indicate that abrogation of zebrafish glut2 in vivo results in severe abnormalities in the development of the brain and particularly in neural progenitor cells. These alterations are associated with impaired glucose uptake and a significant increase in cell apoptosis in the brain of morphant embryos. Our results support the notion of an important role of GLUT2 in the development of the brain, particularly in regions involved in glucose sensing.
MATERIALS AND METHODS

Zebrafish Maintenance

Wild-type zebrafish of the AB/Tl and Casper strains were handled following a procedure approved by the Ethical Committee of Animal Experimentation of the University of Barcelona and maintained according to standard protocols (http://zfin.org). Embryos were grown at 28.5 °C in egg water (i.e., water used to raise young embryos; 60 μg/mL Instant Ocean Sea Salts, Blacksburg, VA, USA).

Imaging

Embryos were examined with a Leica MZ16 FA fluorescence stereomicroscope and images were acquired with a DFC420C camera (Leica, Wetzlar, Germany) and Leica Application Suite 3.8 (LAS) Microscope Software. Pictures were analyzed using ImageJ 1.45 software (National Institutes of Health). TUNEL-positive cells were analyzed and quantified using Icy 1.2.4.1 bio-imaging software (www.bioimagemanalysis.org).

In Situ Hybridization and Immunohistochemistry

Antisense probes were generated for zebrafish glut22 and for preproinsulin, atoh1b, atoh1c, ptf1a, and neurod that were amplified by PCR and subcloned into pGEM-T Easy vector (Promega, Barcelona, Spain). glut2 was linearized with SpeI and atoh1b, atoh1c, ptf1a, and neurod were linearized with Sall and used as template for the generation of riboprobes using the DIG and Fluorescin labeling kits (Roche, Mannheim, Germany).

For whole-mount immunostaining, zebrafish embryos were fixed in 4% paraformaldehyde and washed with PBS (pH 7.4) containing 1% dimethyl sulfoxide (Merck, Darmstadt, Germany) and 0.3% Triton X-100 (Sigma-Aldrich, Barcelona, Spain; phosphate-buffered saline-dimethyl sulfoxide-Triton X100 (PBS-DTx)) at room temperature. Embryos at 24 and 48 hours post fertilization (hpf) were digested with collagenase type IA (Sigma-Aldrich) diluted in PBS-DTx (1 mg/mL) at 37 °C for 10 and 20 minutes, respectively. Next, after 2 to 5 hours of incubation in blocking solution (PBS-DTx with 5% sheep serum) the specimens were incubated with an antibody against acetylated tubulin (Sigma-Aldrich) diluted (1:200) in blocking solution during 16 hours at 4 °C under slow stirring (30 to 50 rpm). Embryos were then washed thoroughly with PBS-DTx and incubated with the secondary antibody, goat anti-mouse Alexa-conjugated 488 diluted (1:500) in blocking solution for 12 to 24 hours at 4 °C. After extensive washing with PBS-DTx (pH7.4), the specimens were stored in PBS.

Morpholino Design and Injections

To knockdown zebrafish glut2 expression, we designed antisense morpholinos targeting the translational start site (5′-ACGTCTCTCATTGTTGATG AAGT-3′) and the splice acceptor site of exon 6 (5′-AGCATCCGAGAC AACAGGACACC-3′). Morpholinos were reconstituted in RNAse-free water according to manufacturer’s instructions (Gene Tools LLC, Philomath, OR, USA). Morpholinos targeting the translational start site (ATG MO) and the splice acceptor site (splice MO) were titrated at doses of 2.2 to 8.4 ng into...
Generation of a Zebrafish glut2 Expression Construct and Transient Transfection of MIN6 Cells
The full-length sequence of the zebrafish glut2 cDNA was amplified by PCR and subcloned in pcDNA3 vector containing enhanced GFP (green fluorescent protein). Approximately 1 × 10⁵ cells/well were plated in 12-well plates and transfected 24 hours later at a confluence of 70 to 80% with Lipofectamine 2000 (Life Technologies) following the manufacturer’s indications. Cells were stimulated and/or lysed at 48 hours post transfection. Overexpression experiments were performed by transfecting 2 μg/well of zfglut2-GFP construct. Cells were lysed 24 hours post transfection. Mock controls were transfected only with lipofectamine and cultured in the same conditions as other transfected cells.

In Vitro Glucose Uptake Assays
MIN6 cells were washed twice with HEPES-buffered saline and incubated with HEPES-buffered saline containing 50 mM glucose/2.0 mCi/ml 2-[3H]-deoxyglucose (2-[3H]-DG) for 30 minutes at room temperature. After this period, the transport solution was removed and cells were rinsed three times with ice-cold PBS containing 50 mM D-glucose. Finally, cells were lysed with 0.05 N NaOH, and the radioactivity was determined by scintillation counting using a β-counter (Packard Bioscience, Meriden, CT, USA). Nonspecific uptake was carried out in the presence of 50 μM D-glucose in the transport solution, and these values were subtracted from all other values. Glucose uptake was measured in triplicate, normalized to total protein and expressed as fold induction with respect to nonstimulated cells.

Glucose Measurements
Glucose measurements were performed using a fluorescence-based enzymatic detection kit (Biovision Inc., Milpitas, CA, USA).

Statistical Analysis
Results are expressed as mean ± S.E. Statistical differences were analyzed by Kruskal–Wallis and Mann–Whitney nonparametric tests and considered to be significant at P < 0.05.

RESULTS
Zebrafish glut2 is Expressed in the Developing Brain, Liver, Pronephric Tubules, and Endocrine Pancreas
We first determined the localization of the expression of zebrafish glut2 during early development by in situ hybridization (ISH). At 24 hpf, glut2 expression was localized in the telencephalon, eyes, hindbrain, and pronephric duct (Figures 1A and 1B). By 48 hpf, strong expression of glut2 was observed in the head and in the pronephric duct (Figure 1C). At 72 and 120 hpf, glut2 appeared expressed in the liver, pronephric tubules, anterior intestine, and endocrine pancreas (Figures 1E, 1E', 1F, 1F', 1H, 1H', 1I, 1I'), as demonstrated by the colocalization of preproinsulin, a specific marker for this tissue (Figure 1I, inset). At 72 and 120 hpf, glut2 was also expressed in the hindbrain, specifically in the cerebellum and medulla oblongata (Figures 1D, 1D', 1G, 1G').

Knockdown of Zebrafish glut2 Disrupts Brain Development
To study the function of glut2 during the early development in zebrafish, we investigated the effects of abrogation of glut2 by using two different antisense morpholinos, one to inhibit translation at the start methionine (ATG MO) and a second one designed to interfere with splicing between exons 5 and 6 (splice MO) that code for transmembrane helix 6, an important structure for the glucose transport activity of class I GLUTs. Embryos injected with the ATG MO showed a severe delay in the development of the embryo, mainly in the brain area. Morphant embryos at 24 hpf showed less developed eyes and a dense mass was observed in the brain area instead of the hindbrain ventricle, causing the loss of the midbrain/hindbrain structures (Figures 2D and 2E). Embryos injected with the splice MO appeared to be a phenocopy of the ATG morphants, confirming the specificity of the morpholino (Figures 2G and 2H). By 48 hpf, morphant embryos displayed a defective formation and enlargement of the hindbrain ventricle associated with anterior displacement of the telencephalon (Figures 2F and 2I). Morphant embryos did not survive past 96 to 120 hpf. The incidence of the morphant phenotype was 95.9 ± 1.4% (n = 765) in embryos injected with the ATG MO and 93.4 ± 1.2% (n = 536) in embryos injected with the splice MO. Furthermore, we confirmed the extent of the splice blocking activity of the splice MO at different stages throughout development in glut2 morphants. Analysis of glut2 mRNA transcripts by reverse transcription PCR from embryos injected with the splice MO revealed the appearance of aberrant splice products from 24 hpf until 96 hpf (Supplementary Figure 1) although the efficiency of the morpholino appeared to decrease at 96 hpf. To further demonstrate the specificity of the glut2 morphant phenotype, we performed rescue experiments by co-injecting the ATG MO or splice MO with the rat Glut2 or zebrafish glut2 mRNA, respectively, lacking the morpholino target sequences (Figure 3). Our results show that rat Glut2 mRNA was able to rescue the phenotype in ATG morphant embryos (Figures 3A, 3B, 3D, 3F, 3G, and 3I) and that zebrafish glut2 mRNA was able to rescue the phenotype in splice morphant embryos (Figures 3A, 3C, 3E, 3F, 3H, and 3J). To demonstrate the functionality of the zebrafish glut2 mRNA in the rescue of the splice morphants, we examined glucose uptake under basal conditions in MIN6 cells transfected with a zebrafish glut2 expression construct. MIN6 cells overexpressing zebrafish glut2 showed a significant increase in glucose uptake compared with control cells (Supplementary Figure 2). Taken together, these results show that glut2 knockdown affects brain development in zebrafish embryos.

Defective Glucose Uptake in glut2-Deficient Embryos
To evaluate the functional consequence of glut2 abrogation on glucose metabolism, we performed glucose uptake experiments using 2-NBDG, a non-metabolizable fluorescently labeled glucose analog. We observed a significant decrease in 2-NDBG uptake in the head and body in ATG morphant embryos, with most of the glucose remaining inside the yolk (Figures 4d, 4e, 4g, and 4h). In contrast, ATG morphants rescued with rat Glut2 mRNA recovered glucose uptake as evidenced by the significant increase in the amount of fluorescent glucose signal in the head and the body and by the significant decrease in fluorescence in the yolk when compared with ATG morphant embryos, yielding a similar 2-NDBG distribution than control embryos (Figure 4B). In addition, to further characterize the defect in glucose homeostasis, we measured free glucose levels in glut2-deficient embryos. Our results show that ATG morphant embryos presented lower levels of free glucose at 24 hpf (Supplementary Figure 3). These results strongly suggest that glut2 morphant embryos may be experiencing hypoglycemia due to the observed reduction in glucose uptake from the yolk.

Knockdown of glut2 Influences Brain Development in Zebrafish by Affecting Cerebellar Progenitor Cells
In view of the severe alterations in the hindbrain structure as a result of glut2 abrogation, we set out to further characterize this phenotype in the central nervous system (CNS) by performing immunohistochemical analyses using an antibody against neuron-specific acetylated tubulin. In control embryos, a basic axon scaffold had formed in the embryonic zebrafish brain by 24 hpf, consisting of two bilaterally symmetric longitudinal tracts connected by commissures, providing a template for subsequent development (Figures 5i and 5i'). In contrast, the neuronal architecture of glut2 morphants was clearly altered, revealing thinner, poorly fasciculated longitudinal tracts (Figures 5ii and 5ii'). ATG morphant embryos co-injected with rat Glut2 mRNA
recovered the brain structure similar to control embryos (Figures 5iii and 5vii). Surprisingly, control and ATG morphant embryos showed no significant differences in axonal structure at 48 hpf (data not shown).

To assess the effects of glut2 abrogation in the hindbrain region, we performed ISH for various proneural genes: ptf1a, atoh1b, atoh1c, and neurod. ptf1a is a marker of progenitor cells of GABAergic neurons in the ventricular zone. To confirm glut2 expression in endocrine pancreas, double in situ hybridization showing pre-proinsulin expression was performed (I, inset). (D’–I’) Schematic representations of D–I highlighting with different colors the regions where glut2 is expressed. Black dotted contours outline the hindbrain region (D’ and G’) and the pancreas (I’). Anterior intestine (ai), corpus cerebelli (cce), endocrine pancreas (ep), hindbrain (hb), liver (li), medulla oblongata (mo), pronephric duct (pd), pronephric tubule (pt), telencephalon (t).

Figure 1. Localization of the expression of glut2 in zebrafish. Whole-mount in situ hybridization showing expression of zebrafish glut2 mRNA at 24 hours post fertilization (hpf; A and B), 48 hpf (C), 72 hpf (D–F), and 120 hpf (G–I). Left (A–C, E, and H), right lateral views (F and I), and dorsal views of the hindbrain region (D and G) are shown. To confirm glut2 expression in endocrine pancreas, double in situ hybridization showing pre-proinsulin expression was performed (I, inset). (D’–I’) Schematic representations of D–I highlighting with different colors the regions where glut2 is expressed. Black dotted contours outline the hindbrain region (D’ and G’) and the pancreas (I’). Anterior intestine (ai), corpus cerebelli (cce), endocrine pancreas (ep), hindbrain (hb), liver (li), medulla oblongata (mo), pronephric duct (pd), pronephric tubule (pt), telencephalon (t).
are markers of progenitor cells of glutamatergic neurons whereas neurod is a marker of immature and mature granule cells. Embryos injected with ATG MO lacked expression of ptf1a at 24 hpf and their expression pattern at 48 hpf was similar to that in control embryos at 24 hpf (Figures 5A–5F). Similarly, atoh1b showed a marked delay in its pattern of expression in ATG morphants at 24 and 48 hpf (Figures 5G–5L). In contrast, ATG morphants showed no significant alteration in neurod expression at 24 and 48 hpf (Figures 5M–5R). At 72 hpf, however, the pattern of expression of atoh1b, neurod, and atoh1c, first expressed at this stage, was not different between control and ATG morphants (Supplementary Figure 4). Moreover, ATG morphant embryos rescued with rat Glut2 mRNA recovered normal expression patterns of atoh1b and ptf1a (Supplementary Figure 5).

Loss of glut2 Leads to an Increase in Apoptotic Cell Death

On the basis of the observed expression of glut2 in the hindbrain and on the consequences of glut2 abrogation in this brain region, we hypothesized that the loss of glut2 in zebrafish embryos could affect the incidence of cell death. Examination of cell death in ATG morphant embryos at 24 hpf using the vital dye acridine orange showed a significant increase (1.6-fold, P < 0.05) in cell death, primarily in the brain area, over control embryos (Figures 6A–6C). ATG morphants co-injected with rat Glut2 mRNA presented similar levels of cell death as control embryos (Figures 6D and 6E). To determine whether the observed increase in cell death in glut2 morphant embryos corresponded to apoptotic cell death, we performed TUNEL assays and observed a significantly higher number of apoptotic cells present in the hindbrain of glut2 morphants compared to control embryos at 24 hpf (Figures 6E, 6F, and 6H).

Transcriptome Profiling of glut2 Morphants Evidences Changes in the Expression of Genes Involved in Neural Processes and Apoptosis

To study the effects of glut2 abrogation on gene expression in zebrafish embryos, we performed a transcriptome analysis of zebrafish ATG morphant embryos at 72 hpf and compared it with control embryos. Microarray analysis was performed setting significance cutoffs at 1.5-fold change at P < 0.05 (sample t-test). A total of 1,912 genes (DEGs) were found to be regulated in glut2 morphant embryos: 1,025 upregulated and 887 downregulated genes. A set of 11 selected DEGs were validated by quantitative PCR (Supplementary Table 2). Next, to better characterize the annotated DEGs, we performed a gene ontology analysis for functional classification (Supplementary Table 3). Analysis of Gene Ontology-Biological Process revealed a significant enrichment in functional categories involved in neural processes (e.g., neuron projection, neurotransmitter metabolic process, and visual perception), programmed cellular death (e.g., apoptosis and cell death), patterning, muscle development, immune processes, and response to hypoxia/oxygen levels in glut2 morphant embryos. Among DEGs involved in neural development, several genes known to participate in the organization and maintenance of the mid-hindbrain boundary (eng2b, fgf13a, her8.2, pax2b), as well as in glutamate and glycine neurotransmission (grin1b and glra4a) were downregulated in glut2 morphant embryos (Table 1). Furthermore, marker genes for GABAergic neurons (pvalb7, aldca),
glutamatergic neurons (atoh8, zic2a, tbr1b), and neural stem cells (sox2) were upregulated in glut2 morphant embryos. Also coincident with the increased apoptosis in glut2 morphant embryos, several pro-apoptotic genes (aifm1, bnip3lb, badb, cideb, cdip1, dram) were found to be upregulated. A number of genes related to insulin/IGF-I signaling were also downregulated (e.g., insr, iris1, ifg1rb, ifg2bp1, mtor, mapk1, mapk14b, pik3r2) in glut2 morphant embryos. In addition, the expression of genes involved in the bone morphogenetic protein/wnt pathways (bmp4, tgfβ1a, fstb, fstb2, dvl1a, dvl3a, wnt16, and wif1) were significantly altered in embryos lacking glut2. Not surprisingly, the mRNA expression levels of glut2 (slc2a2) as well as that of transferrin (tfn) and pdx1, marker genes for the glut2-expressing tissues liver and endocrine pancreas, respectively, were significantly decreased in glut2 morphant embryos (Table 1, Supplementary Table 2).

**DISCUSSION**

In this study we describe a vertebrate model of GLUT2 deficiency. Using a reverse genetic approach, we have knocked down glut2 expression in zebrafish embryos causing disorganization of the hindbrain, severe mispatterning of axonal scaffolds, and alterations in the development of the neural progenitor cells. Furthermore, we have related these observed functional consequences of glut2 depletion to a reduction in glucose uptake and availability and, consequently, to an increase in programmed cell death in the brain region.

Expression analysis of zebrafish embryos by ISH showed that glut2 is expressed in the liver, pronephric tubules, anterior intestine, endocrine pancreas, and importantly, in the hindbrain, particularly in the corpus cerebelli and medulla oblongata. In mammals, GLUT2 expression has been reported in the cerebellum, brain nuclei, hypothalamic nuclei, neurons, glial cells, and astrocytes, where GLUT2 is believed to be expressed in glucose sensing neurons that regulate feeding behavior, energy metabolism, and glucose homeostasis. Therefore, the similar neural localization of the expression of glut2 in zebrafish is indicative of the existence of a glucose sensing region in the zebrafish brain. Despite current data strongly linking GLUT2 to central glucose sensing in the mammalian brain, little is known on the importance of GLUT2 in the development of the CNS. In the present study, we show that abrogation of glut2 expression during early development in zebrafish had critical consequences in the formation of the CNS. The glut2 morphant embryos showed severe alterations in the formation of the hindbrain ventricle, affecting the midbrain-hindbrain structures. In view of the coinciding neurodegenerative morphant phenotype and the localization of glut2 expression in the hindbrain at early developmental stages, we hypothesized that abrogation of glut2 may have affected the development of neural progenitor cells. In zebrafish, as in mammals, neurons are classified into two major groups: the excitatory glutamatergic, and the inhibitory GABAergic neurons. In the mouse, glutamatergic neurons derived from progenitor cells located in the upper rhombic lip express the proneural gene Atoh1, whereas the glutamatergic immature and mature granule cells express the proneural gene NeuroD that is required for their generation and differentiation. On the other hand, murine GABAergic neurons are derived from progenitor cells expressing the proneural gene Ptf1a. Recently, it has been shown that the neurogenic processes of both glutamatergic and GABAergic neurons are conserved between mammals and zebrafish and that the above-mentioned proneural genes are also specifically expressed in progenitor cells of the hindbrain region in zebrafish. Here, we show that abrogation of glut2 caused important alterations in the expression...
pattern of proneural marker genes in the hindbrain region during early development in zebrafish. First, glut2 morphant embryos showed a significant delay in the expression pattern of ptf1a, indicating that abrogation of glut2 may have affected the development of ptf1a-expressing cells from the ventricular zone, reported in mice to be the source of all GABAergic neurons in the cerebellum.19 Second, glut2 morphant embryos showed altered expression pattern of atoh1b as well as an alteration of the upper rhombic lip region at 24 and 48 hpf. Altered expression of ionotropic NMDA glutamate and glycine receptors (grin1b and glra4a, respectively) in glut2 morphant embryos further suggest that the connectivity of ptf1a- and atoh1b-expressing neurons could also be affected. Interestingly, abrogation of glut2 induced the expression of known marker genes of Purkinje (pvalb7 and aldca), granule (atoh8, zic2a and tbr1b), and neural stem cells (sox2), suggesting that a compensatory mechanism to the alterations in hindbrain structure may have taken place at 72 hpf. Furthermore, the observed alteration of the hindbrain structure in glut2 morphant embryos was related to a severe alteration of the neural scaffold, as evidenced by acetylated tubulin immunostaining, and to the downregulation of the expression of genes that participate in the establishment of the mid-hindbrain boundary and the patterning of the hindbrain (eng2b, pax2b, fgf13a, and her8.2)16 at 24 hpf. Therefore, abrogation of glut2 disrupted hindbrain development and, specifically, the development of progenitors for GABAergic and glutamatergic

Figure 4. Knockdown of glut2 results in inhibition of glucose uptake in vivo. (A) (a–i) Bright field (upper line, a–c), fluorescent (middle line, d–f), and overlay (bottom line, g–i) pictures of control, ATG morphants, and rescued embryos at 24 hpf. (B) Measurement of fluorescent signal in embryos injected with 2-NBDG. Control injected embryos (a, d, and g) displayed significant amounts of fluorescent glucose throughout the embryo; in contrast, ATG morphants (b, e, and h) showed very minimal fluorescent glucose visible at 60 minutes after injection. Embryos injected with ATG MO+rat GLUT2 mRNA (ATG MO Rescued) recovered glucose uptake to levels similar to Con MO (c, f, and i). * indicates significant differences compared with the Con MO injected embryos (*P < 0.05; **P < 0.01; ***P < 0.001). # indicates significant differences compared with ATG MO injected embryos (##P < 0.01; ###P < 0.001).
neurons in the zebrafish hindbrain. Overall, these results clearly indicate that glut2 is an important factor for neurodevelopment in the zebrafish embryo.

Here, we also show that abrogation of glut2 expression in zebrafish embryos resulted in a significant reduction in whole-body glucose uptake and in a decrease in cell viability due to apoptotic cell death mainly in the brain region. It is known that the entry and utilization of glucose in cells can act as important mediators of cell survival, linking glucose availability with cellular viability, as is well described in cancer cells.20

Figure 5. glut2 abrogation causes hindbrain disorganization and affects the expression of cerebellar proneural genes. To study the consequences of glut2 knockdown in the hindbrain structure we performed immunostaining using an antibody against acetylated tubulin in con MO, ATG MO, and ATG MO+rat GLUT2 mRNA rescued embryos at 24 hpf (i, ii, iii). At this stage, morphant embryos showed disorganized axon tracts. Rescued embryos showed a hindbrain structure similar to control injected embryos. Lateral longitudinal fascicles (llf); medial longitudinal fascicles (mlf). To further study the consequences of glut2 abrogation in the neural progenitor cells we performed ISH for the proneural genes ptf1a (A–D), atoh1b (G–J), neurod (M–P) in control injected embryos at 24 hpf (A, G, and M), and 48 hpf (C, I, and O), and in ATG morphants at 24 hpf (B, H, and N) and 48 hpf (D, J, and P). To better illustrate the effects caused by the abrogation of glut2, immunostained medial longitudinal fascicles have been outlined (i’, ii’, iii’). The expression patterns observed by ISH of the proneural genes are represented with diagrams overlapping the expression patterns in control and ATG morphants at 24 hpf and 48 hpf of ptf1a (E and F), atoh1b (K and L), and neurod (Q and R). A, anterior; L, left; R, right.
In neurons, glucose deprivation induces apoptosis that can be reverted by the enhancement of GLUT1 expression by IGF-1,21 an important neural survival factor22 that blocks the activity of the pro-apoptotic factor Bad through the PI3K/Akt pathway.23 Recently, a link between glucose homeostasis and apoptosis has been established in glut1-deficient and akt2-deficient zebrafish embryos, two in vivo models of altered glucose homeostasis that showed increased apoptosis and an almost identical neurodegenerative phenotype that could be rescued by abrogation of bad.24,25 In the present study, we provide evidence supporting the hypothesis that the decrease in glucose availability caused by glut2 abrogation resulted in the upregulation of the expression of bad and several other pro-apoptotic factors. Interestingly, a number of components of the insulin/IGF-I signaling pathway were downregulated in glut2 morphant embryos, suggesting that this important survival pathway was suppressed as a result of glucose deprivation due to glut2 abrogation. Furthermore, the expression of igfbp1a, a marker of decreased glucose availability26 that is transcriptionally repressed by insulin and that causes defects in brain development when overexpressed27, was upregulated in glut2 morphant embryos, supporting the hypoglycemic phenotype. Therefore, we propose that the decrease in glucose uptake and availability in glut2 morphant zebrafish embryos may have led to a reduction in the production of survival signals through the insulin/IGF-I signaling pathway and/or the production of glucose metabolites and, consequently, to an increase in apoptotic cell death. Our results support the notion that glucose is essential for cell survival in the CNS in zebrafish and that glucose transporters...
| Gene name | Description | FC | P   |
|-----------|-------------|----|-----|
| aldaco    | Aldolase C, fructose-bisphosphate, a | 2.12 | 0.044 |
| atoh8     | Atonal homolog 8 | 4.52 | 0.000 |
| cadh1     | Cadherin 1, epithelial | 2.01 | 0.002 |
| eng2b     | Engrailed 2b | −1.58 | 0.048 |
| fgf13a     | Fibroblast growth factor 13a | −2.01 | 0.003 |
| foxb3     | Forkhead box A3 | −1.52 | 0.012 |
| foxb1b    | Forkhead box B1b | −1.79 | 0.000 |
| grn1b     | Glutamate receptor, ionotropic, NM2A 1b | −2.55 | 0.017 |
| gln4a     | Glycin receptor, alpha 4a | −2.11 | 0.013 |
| her2b     | Hairy-related 8.2 | 2.03 | 0.005 |
| her9      | Hairy-related 9 | 1.51 | 0.010 |
| hoxa13a   | Homeo box A13a | 1.66 | 0.048 |
| hoxb1a    | Homeo box B1a | 1.72 | 0.025 |
| hoxb3a    | Homeo box B3a | 2.12 | 0.037 |
| hoxb5b    | Homeo box B5b | 1.51 | 0.002 |
| hoxb6a    | Homeo box B6a | 1.56 | 0.046 |
| hoxb6b    | Homeo box B6b | 1.96 | 0.026 |
| hoxb9a    | Homeo box B9a | −1.58 | 0.028 |
| hoxc1a    | Homeo box C1a | −1.51 | 0.029 |
| hoxc8a    | Homeo box C8a | −1.50 | 0.044 |
| hoxd1a    | Homeo box D11a | 1.63 | 0.043 |
| hoxd13a   | Homeo box D13a | −1.82 | 0.000 |
| mgm       | Neuregin, neureoutgrowth-associated | 1.58 | 0.024 |
| nrxn1a    | Neurexin 1a | −1.82 | 0.008 |
| nrxn2b    | Neurexin 2b | −2.01 | 0.026 |
| nos1      | Nitric oxide synthase 1 (neuronal) | −3.77 | 0.016 |
| pax2b     | Paired box gene 2b | −1.94 | 0.012 |
| pax3a     | Paired box gene 3a | 1.54 | 0.019 |
| ppp1b7    | Parvalbumin 7 | 2.26 | 0.004 |
| sox2      | SRY-box containing gene 2 | 1.99 | 0.001 |
| tbr1b     | T-box, brain, 1b | 2.09 | 0.014 |
| elf4ebp3  | Eukaryotic translation initiation factor 4E BP3 | 1.72 | 0.006 |
| insa      | Insulin receptor a | −1.54 | 0.005 |
| inr1      | Insulin receptor substrate 1 | −1.77 | 0.003 |
| igfr1b    | Insulin-like growth factor 1b receptor | −1.63 | 0.006 |
| igf2bp1   | Insulin-like growth factor 2 mRNA binding protein 1 | −1.60 | 0.044 |
| igf2bp1a  | Insulin-like growth factor 2 mRNA binding protein 1a | 1.58 | 0.009 |
| mtor      | Mechanistic target of rapamycin | −1.98 | 0.007 |
| map3k7    | Mitogen activated protein kinase, kinase kinase 7 | −1.70 | 0.034 |
| mapk1     | Mitogen-activated protein kinase 1 | −1.52 | 0.026 |
| mapk14b   | Mitogen-activated protein kinase 14b | −1.73 | 0.023 |
| mapk8     | Mitogen-activated protein kinase 8b | −1.92 | 0.011 |
| map2k6    | Mitogen-activated protein kinase 4b | −1.69 | 0.019 |
| map2k5    | Mitogen-activated protein kinase 5 | −1.63 | 0.039 |
| map2k6    | Mitogen-activated protein kinase 6 | −1.76 | 0.024 |
| pkd1      | Pancreatic and duodenal homeobox 1 | −1.64 | 0.008 |
| pik34r    | Phosphoinositide-3-kinase, regulatory subunit 4 | 1.50 | 0.002 |
| pik3r2    | Phosphoinositide-3-kinase, regulatory subunit 2 | −1.77 | 0.007 |
| prkcbb    | Protein kinase C, beta b | −1.64 | 0.011 |
| prkq      | Protein kinase C, theta | 11.3 | 0.031 |
| socsa1    | Suppressor of cytokine signaling 1a | 3.10 | 0.000 |
| tfrb1b    | Transferrin receptor 1b | −1.60 | 0.002 |
| tfla      | Transferrin-a | −1.74 | 0.003 |

Control and morphant embryos were used for gene expression analysis using a zebrafish oligonucleotide microarray (GPL13390). Data are shown as fold change (FC). P: P value.

have a key role in this process. In addition to Glut2 and Glut1, that is expressed astrocytes and in the blood–brain barrier allowing the entry of glucose into the brain from the circulation, the mammalian brain expresses primarily Glut3, a high affinity GLUT that is expressed in neurons, but also Glut4, Glut5, and Glut8 at lower levels.1,28 in view of the complexity of the GLUT system in the brain, further studies will be required to elucidate the specific role of the different GLUTs expressed in the brain.
Given that abrogation of glut2 resulted in increased apoptosis primarily in the brain region, we hypothesize that glucose deprivation-induced apoptosis in the hindbrain of glut2 morphants may have been responsible for the observed disruption of hindbrain organization and, specifically, for the alterations in the development of progenitors for GABAergic and glutamatergic neurons in the embryonic zebrafish hindbrain. At the present time, we cannot ascertain if glut2 abrogation may have induced apoptosis specifically in GABAergic and glutamatergic precursors nor if these effects may have been direct or indirect through other glut2-expressing cells. This leads to the important question of where specifically glut2 is expressed in the zebrafish brain, a question that has not been completely resolved even in mammals. In the mammalian brain, GLUT2 is expressed in glucose sensitive neurons in the hypothalamus and in the brainstem, but the exact nature of GLUT2-expressing neurons (i.e., GABAergic or glutamatergic) in the central glucose sensing system has been poorly described to date, although there is evidence indicating that GABA release in neurons is regulated by glucose. GABAergic neurons in the ventromedial hypothalamus are inactivated when glucose levels decrease under hypoglycemic conditions, enhancing the counterregulatory response to hypoglycemia. These observations suggest the possibility that GLUT2-expressing neurons in structures belonging to the central glucose sensing system could be, at least in part, GABAergic neurons. Supporting these observations, Glut2 has recently been shown to be expressed in a homogenous glucose-sensing subpopulation of GABAergic neurons in the nucleus of the tractus solitarius in mice. Unfortunately, our attempts at localizing glut2 in ptf1a- and atoh1b-expressing cells were unsuccessful due to the low levels of expression of glut2 in the zebrafish brain, as in mammals. Approaches involving genetic labeling of glut2-expressing cells will be required to identify the nature of cells that express glut2 in the zebrafish brain in future studies.

It is worth noting that the functional consequences of abrogating glut1 or glut2 (this study) in zebrafish are both severe and very similar, particularly when considering that the effects of glut2 abrogation occur in the absence of changes in the expression of glut1 (data not shown). A possible explanation for the apparently similar function of glut1 and glut2 in early zebrafish development may lie in possible differences in their pattern of expression that would allow for transporter-specific glucose availability in different key brain areas during early development. Recently, it was reported that morpholinos could cause non-specific apoptosis and changes in the expression of proneural marker genes by activation of p53. However, our data on the ability of rat Glut2 mRNA to rescue the apoptotic morphant phenotype (Figure 6), on the similar phenotype generated by the two different morpholinos used (Figures 2 and 3) and on the lack of changes in the mRNA expression levels of p53 and its downstream targets mdm2 and p21 in glut2 morphant embryos (data not shown), strongly argue against the possibility of off-target effects of the morpholinos in our study.

Interestingly, glut2 morphant embryos showed altered expression of several checkpoint (leptin (lept) and NPY (npy)) and pygmb), as well as of uncoupling proteins 1 and 3 (ucp1, ucp3), suggesting that some of the factors known to participate in the GLUT2-mediated control of feeding and thermoregulation in mammals are also dependent on the presence of a functional glut2 gene in zebrafish. Although ucp1 is obviously not involved in thermoregulation in zebrafish but may instead reduce ATP production, these observations add to those on the localization of glut2 in the zebrafish hindbrain in support of the hypothesis that a central glucose sensing mechanism involving glut2 may be present in the zebrafish brain, providing a novel and useful experimental model for investigating the role of GLUT2 in glucose sensing in the brain. Furthermore, consistent with the expression of GLUT2 in liver and endocrine pancreas, glut2 abrogation decreased the expression of tfa and pdx1, two well-known marker genes for these important tissues in glucose metabolism. In particular, pdx1 is necessary for the proper regulation of the glucose-dependent insulin secretion by β-cells and for pancreas development in zebrafish since pdx1-null zebrafish lack this organ. Hence, the downregulation of pdx1 expression suggests that the glucose-responsive regulation of insulin synthesis in endocrine pancreas could be affected as a consequence of the abrogation of glut2 in zebrafish. Interestingly, genetic inactivation of Glut2 specifically in the nervous system was recently shown to reduce pancreatic β-cell mass and proliferation and suppress first-phase insulin secretion due to decreased parasympathetic activity in mice, supporting the notion of a central function for GLUT2 in glucose homeostasis. At this time, it is not known if the decrease in the expression of pdx1 in zebrafish glut2 morphant embryos could be the result of the abrogation of the expression of glut2 in the pancreas, or in the brain, or in both.

To summarize, in the present study we provide evidence for the physiological role of glut2 in glucose homeostasis. Importantly, we demonstrate that glut2 is essential for the development of neural progenitors for GABAergic and glutamatergic neurons, suggesting the existence of a glucose-sensing region in the zebrafish brain. We propose that the lack of glut2 in specific brain areas in the zebrafish embryo results in glucose deprivation causing increased apoptotic cell death that we believe is the underlying cause for the observed alterations in brain development. Importantly, the observed phenotype in glut2 morphant embryos shows certain similarities with FBS patients. Like glut2 morphants, FBS patients that are diagnosed during their infancy exhibit growth delay and impaired glucose homeostasis that is characterized by fasting hypoglycemia and hypergalactosemia. In some cases, hepatomegaly is also observed as a consequence of glycogen accumulation, a process that becomes exacerbated during infancy. Unfortunately, we were not able to assess whether abrogation of glut2 in zebrafish also affected liver glycogen levels because we failed to detect glycogen in the liver in zebrafish embryos up to 72 hpf (data not shown). It is possible that the developmental stage of the studied embryos may have been too early to be able to appreciate glycogen accumulation in the liver, resembling the human situation. However, the mRNA expression levels of glycogen phosphorylase (pygmb), an enzyme involved in glycogen breakdown, were decreased in glut2 morphant embryos, suggesting that glycogen metabolism may have been altered in zebrafish embryos as a result of glut2 abrogation. This is supported by reports indicating that deficiency of this enzyme in the liver is responsible for glycogen storage disease type VI, characterized by hepatomegaly and growth retardation. On the other hand, cases diagnosed with FBS include patients with delayed psychomotor development. Interestingly, psychomotor delay has been associated with alterations in the cerebellar development in humans at early ages. Our findings that glut2 is expressed in the zebrafish cerebellum and that the development of the cerebellum is altered in glut2 morphant embryos indicates that our glut2-deficiency model recapitulates yet another phenotype of FBS patients. In conclusion, our study demonstrates the physiological importance of glut2 in glucose uptake and availability during brain development and provides a novel model system for the study of diseases derived from GLUT2-deficient states, representing an attractive tool for the development of new drug or genetic therapies for the treatment of FBS.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.
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