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ARTICLE

Investigating the role of an immediate early gene FOS as a potential regulator of autophagic response to hypoglycemia in embryonic hypothalamic neurons

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Abstract

Hypoglycemia-associated autonomic failure (HAAF) is a well-established complication of diabetes. Although HAAF has serious outcomes such as recurrent morbidity, coma, and death, the mechanisms of HAAF and its pathological components are largely unknown. Our previous studies have revealed that hypoglycemia is associated with the upregulation of an immediate early gene – FOS. In addition, it is documented that glucose deprivation activates neuronal autophagic activities. Therefore, the present study aimed to identify the role of FOS and one of the core components of the autophagy pathway, Beclin-1 (encoded by the BECN1 gene), in the regulation of autophagic mechanisms in embryonic hypothalamic neurons in response to hypoglycemic conditions. Embryonic Mouse Hypothalamic Cell Line N39 (mHypoE-N39 or N39) was cultured in reduced concentrations of glucose (2000, 900, 500, and 200 mg/L). Gene and protein expression, as well as immunofluorescence studies on autophagy were conducted under different reduced glucose concentrations in N39 hypothalamic neurons with and without FOS and BECN1 gene knockdowns (KD). The outcomes of the present study have demonstrated a significant increase in autophagosome formation and subsequent lysosomal degradation in the hypothalamic neurons in response to reduced glucose concentrations. This hypoglycemic response appears to be lowered to a similar extent in the FOS KD and BECN1 KD cells, albeit insignificantly from the negative control, is indicative of the involvement of FOS in the autophagic response of hypothalamic neurons to hypoglycemia. Moreover, the KD cells exhibited a change in morphology and reduced cell viability compared with the control cells. Our findings suggest that reduced FOS expression could potentially be associated with impaired autophagic activities that are dependent on BECN1, which could lead to decreased

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or blunted hypothalamic activation in response to hypoglycemia, and this, in turn, may contribute to the development of HAAF.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Despite its serious consequences, the pathogenesis of hypoglycemia-associated autonomic failure (HAAF) remains largely unknown. Previous findings from our group revealed vital association of hypoglycemia with upregulation of an immediate early gene – FOS. Furthermore, glucose deprivation, as in HAAF, may lead to activation of neuronal autophagic responses.

WHAT QUESTION DID THIS STUDY ADDRESS?

Our present data suggest a functional increase in autophagy in the hypothalamic neurons in response to reduced glucose concentrations. However, this response appears to be lowered in the FOS- and BECN1-knocked down cells, suggesting the involvement of FOS in the autophagic response of hypothalamic neurons to hypoglycemia.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

Our findings also suggest reduced FOS expression to be associated with impaired autophagy, which together could lead to decreased hypothalamic activation in response to hypoglycemia, and this in turn may contribute to the development of HAAF.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Further functional studies are essential to understand the relevance of FOS-mediated regulation of autophagy in the functional consequences of HAAF. In-vivo studies would also provide a better insight into the interaction between FOS and autophagy mechanisms in HAAF and would help in investigating the clinical relevance of modulating autophagy to treat diabetic neuropathy.

INTRODUCTION

Diabetic neuropathy (DN) is a chronic complication of both type 1 and type 2 diabetes mellitus (DM) with an incidence that could range up to 60%.[1,2] It is characterized by diverse clinical presentations, and therefore establishing appropriate management plans for DN is very challenging.[3] The two subtypes of DN (peripheral and autonomic) usually coexist; however, autonomic neuropathy has far-reaching adverse effects given that many organ systems are involved, including gastrointestinal, cardiovascular, and the central nervous system.[4]

The hypothalamus is one of the most important and integrative regions within the central nervous system which is involved in the regulation of a wide range of critical physiological functions including awareness of hypoglycemia. The metabolic processes in the brain are dependent on peripheral glucose and any reduction in glucose beyond the physiological levels leads to the activation of a robust counter-regulatory response.[5] This response involves a group of neurons within the lateral hypothalamus and the rostral ventrolateral medulla (RVLM). The activation of the neuronal pathway between the hypothalamus and the RVLM in hypoglycemic conditions stimulates the sympathetic drive to the adrenal gland leading to hypoglycemia awareness.[6–8]

In patients with DM, this counter-regulatory response is either reduced or absent leading to a condition called hypoglycemia-associated autonomic failure (HAAF).[9] The latter has been recognized as an alarming condition since it is associated with serious outcomes, including coma and death. Given that in DM the responses of the pancreatic islets to hypoglycemia are disabled, the body mainly relies on the autonomic adrenomedullary regulatory system to correct the hypoglycemic conditions.[10] Although the dramatic outcomes of HAAF are well-documented in human and animal studies, the precise pathogenesis of this disorder is yet to be identified. Several hypotheses have been suggested to explain the etiology of HAAF. These include neuroinflammation,

oxidative stress, hypothalamic and medullary abnormalities, autonomic shift to adrenal glands, and abnormalities in regulation of neurotransmitters that are involved in these pathways.[11–14]

Recently, several lines of evidence have indicated the involvement of disrupted autophagy mechanisms in the pathogenesis of various neurodegenerative disorders including diabetic peripheral neuropathies.[2] Autophagy is a critical process which is involved in multiple homeostatic processes including development, differentiation, tissue remodeling, and neuronal survival and death.[15] This process consists of sequential steps of sequestration of cytoplasmic cargo, transport to lysosomes, lysosomal degradation, and utilization of degradation products. These steps are monitored and regulated by a distinct group of autophagy-related genes (Atg) and protein complexes. Some of the important markers of autophagy include Beclin-1, conjugate marker ATG5-ATG12, ATG16L, and microtubule-associated protein 1A/1B light chain 3B (LC3B).[16] Of these, Beclin-1 (encoded by the BECN1 gene) is an important mediator of autophagy that, through its interaction with several autophagy proteins, forms class III phosphoinositide 3-kinase (PI3K) complexes that initiate the nucleation of the phagophore.[17] As such, the BECN1 gene is involved early in the induction of autophagy. LC3B is one of the most commonly used markers for autophagy as it integrates into the autophagosomal membrane and hence is used to understand the process of autophagosome formation.[18] While the lysosome-associated membrane protein 2A (LAMP2A) marker serves to examine autophagosome–lysosomal fusion, LC3B and LAMP2A together provide an indication of cellular autophagic activity.[19,20]

Autophagy within the brain has special features since the structure of the neurons is unique and complex compared with other cells. It is characterized by a high level of compartmentation where there is a great deal of variation between autophagic vesicles in the cell bodies, axons, and synapses.[21] The key role of autophagy in maintaining neuronal homeostasis has been demonstrated by previous reports which have shown the presence of neurodegeneration, aggregation of intracellular proteins, severe degenerative phenotypes, and neonatal lethality in Atg-knockout mice.[22–24]

Neuronal autophagy can be triggered by several factors including starvation. The classic assumption is that the brain senses the reduction in the nutrient levels and this induces autophagy which, in turn, degrades proteins to produce amino acids.[25] Further investigation of this concept has shown that food restriction causes upregulation of autophagy in neurons within certain regions in the brain including the hypothalamus.[26–28] The mechanisms of autophagy involved in regulating hypothalamic functions are unknown, and given that neuronal activity is the key property of the brain, investigating the effects of neuronal manipulation (stimulation/inhibition) will yield more physiologically relevant results. Several studies have supported the hypothesis that the involvement of autophagy in DN has answered crucial questions and helped in the identification of novel therapeutic targets.[2,29] This was further supported by the finding that sera from diabetic patients induced autophagic activities in cultured neuronal cells.[30] Despite the emerging body of evidence, the role of autophagy in the progression of DN due to glucose variability is still controversial with a need for further studies. Therefore, it is of great interest to investigate the potential regulators of autophagic response to hypoglycemia.

FOS is an immediate early gene that is rapidly and transiently induced in response to extracellular stimuli and has been implicated in long-term adaptation.[31] We have previously demonstrated an increase in FOS expression in response to hypoglycemia suggesting the involvement of FOS in hypoglycemic response.[32] In addition, different regions of the ventromedial nucleus of the hypothalamus contribute to glycemic control through their differential regulation of FOS expression, further highlighting the complexity and the need for better understanding of neuronal FOS signaling in response to hypoglycemia.[33] We and others have studied and identified the neurophysiological properties of different neuronal circuits within the brain that are involved in sensing and regulating the glycemic response via direct influence on endocrine glands including the pancreas and the adrenal gland.[8,34,35] Considering these findings, we hypothesized that the neuronal circuits in the hypothalamus and RVLM that are usually responsive to hypoglycemia are regulated by FOS-mediated autophagy mechanisms. Therefore, the present study aimed to identify the role of FOS and the autophagy-related protein, BECN1, in the regulation of autophagic mechanisms in embryonic hypothalamic neurons (mHypoE-N39) in response to hypoglycemic conditions. Outcomes from this study could help unravel important clues about the pathogenesis of HAAF which, until now, have remained elusive. Furthermore, identifying autophagy as a potential mechanism could help develop targeted therapies for better clinical outcomes for patients with HAAF.

METHODS

Cell culture and hypoglycemia treatment

Embryonic Mouse Hypothalamic Cell Line N39 (mHypoE-N39 or N39) was obtained from Cedarlane (Burlington, Ontario, Canada) and was cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich, St. Louis, MO,

TABLE 1 Specific primers used for gene expression analysis of LC3B, LAMP2A, FOS, BECN1, and GAPDH by quantitative real-time polymerase chain (qPCR) reaction.

Gene	Forward primer (5t–3t)	Reverse primer (5t–3t)	Product size (bp)
MAP1LC3B	GTCCTGGACAAGACCAAGTTCC	CCATTCACCAGGAGGAAGAAGG	119
LAMP2A	GAGCAGGTGCTTTCTGTGTCTAG	GCCTGAAAGACCAGCACCAACT	162
FOS	CCCAAACTTCGACCATGA	GGCTGGGAATGGTAGTAGG	115
BECN1	AGCTGGAGTTGGATGACGAA	TGATTGTGCCAAACTGTCCG	138
GAPDH	GAAATCCCATCACCATCTTCCAGG	GAGCCCCAGCCTTCTCCATG	120

Abbreviation: bp, base pairs.

USA) containing 4500 mg/L glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Aldrich) and 1% penicillin-streptomycin (P/S; Sigma Aldrich) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were cultured in 75 cm^2 cell culture flasks (VWR, Radnor, PA, USA), and when the cells reached 80% confluency they were harvested using 0.25% trypsin-EDTA solution (Sigma Aldrich).

Treatment conditions included culturing the cells in reduced concentrations of glucose to mimic hypoglycemia. Four different conditions of low glucose were established by seeding the N39 cells at a density of 1.5×10^6 in 100 × 15 mm petri dishes with DMEM containing 2000, 900, 500, and 200-mg/L glucose which were obtained by mixing 22.2, 10, 5.6, and 2.22 mL DMEM containing 4500 mg/L glucose with 27.8, 40, 44.4, and 47.78 mL DMEM without glucose, respectively. Cells were maintained under these treatment regimens for 24, 48, and 72 h, and were harvested at the end of each of the three timepoints, respectively.

Gene expression analysis

The cells harvested from different glucose conditions and timepoints were subjected to RNA isolation using Easyblue™ Total RNA extraction kit (Intron Biotechnology, Burlington, MA, USA) following the manufacturer's protocol. The RNA was subsequently quantified using the Nanodrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the standard A260/A280 absorption ratio was used to determine the purity. RNA (0.5 µg) was used to reverse transcribe to cDNA in a final reaction volume of 20 µL containing Multiscribe™ reverse transcriptase in 10× RT buffer, 100 mM dNTP mix, and 10× RT random primers from the High-Capacity cDNA synthesis kit (Applied Biosystems, Foster City, MA, USA). The reaction mix was subjected to 25°C for 9 min and 46 s followed by 37°C for 120 min and 85°C for 5 min in a Veriti thermal cycler (Applied Biosystems).

Quantitative real-time polymerase chain reaction (qPCR) was performed using Quantstudio™ 3 Real time PCR system (Applied Biosystems) with Maxima SYBR green/ROX qPCR Mastermix (2x) (Applied Biosystems). The total reaction volume taken for each reaction was 10 µL. The cycling parameters followed were as per the manufacturer's protocol with UDG pretreatment 50°C for 2 min, 1 cycle; initial denaturation 95°C for 10 min, 1 cycle; denaturation 95°C for 15 s, annealing 60°C for 30 s, and extension 72°C for 30 s for a total of 40 cycles. To confirm the purity of the PCR products obtained, a melt curve involving sequential heating at 95°C for 15 min, 60°C for 1 min, and 95°C for 15 s was done. The specific primer sequences used for the amplification of each of the genes tested in this study are listed in Table 1. The relative fold change in gene expression was derived by using the $2^{(-\Delta\Delta Ct)}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene.

Protein expression analysis

The cells were lysed using the M-PER™ mammalian protein extraction reagent (Thermo Fisher Scientific) in combination with the Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific) at a ratio of 1:1000. The extracted proteins were quantified using the Biorad Protein Assay Dye Reagent Concentrate (Biorad Laboratories, Hercules, CA, USA). Loading volumes of the protein lysates corresponding to 15–30 µg were calculated for sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a gel composition of 12% and subsequent Western blotting was performed. The protein bands thus obtained on the gels were transferred to nitrocellulose membranes (Biorad Laboratories) using the semi-dry transfer method in the Transblot Turbo transfer system (Biorad Laboratories). The membranes were subsequently incubated for 1 h at room temperature with blocking buffer (5% Skimmed Milk Powder in 1× Tris-buffered saline with 0.1% of Tween 20 [TBST]; Sigma Aldrich) and were subjected to overnight incubation at

4°C with primary antibodies diluted in blocking buffer (1:000 dilution), which included anti-LC3B antibody (ab48394; Abcam, Cambridge, UK) and anti-LAMP2A antibody [EPR4207(2)] (ab125068; Abcam). Anti-β actin [RRID: AB_476692] (1:2000; Sigma Aldrich) was used as housekeeping control to normalize the proteins for analysis. The following day, the blots were subjected to washing with 1× TBST and were treated with the respective primary antibody-compatible HRP-labeled secondary antibodies, anti-rabbit IgG [7074S], and anti- mouse IgG [7076S] (Cell Signaling Technology, Danvers, MA, USA) [RRID:AB_476692] at 1:1000 dilution in blocking buffer. The visualization of the blots was done using the Clarity Western ECL substrate reagent (Biorad Laboratories) on the ChemiDoc™ Touch Gel and Western Blot Imaging System (Bio-Rad). The images of bands thus obtained were quantified for analysis using the Biorad Image Lab Software (Version 6.1).

Immunofluorescence study on autophagy

The N39 cells were grown on coverslips in DMEM containing 4500 mg/L glucose supplemented with 10% FBS and 1% P/S in a humidified atmosphere of 5% CO_2 at 37°C until 50%–70% confluence was reached. The medium was removed, and the cells were treated with medium containing reduced concentrations of glucose (DMEM containing 2000, 900, 500, and 200 mg/L glucose; 4500 mg/L was taken as control). The cells were stained for autophagosomal detection using the autophagy detection kit (Abcam, Waltham, MA, USA) following the manufacturer's protocol. The live cells thus processed were subjected to imaging by fluorescence microscopy (Olympus IX53, Tokyo, Japan).

FOS and BECN1 gene knockdown in N39 cells

For gene expression studies, N39 cells were seeded at a density of 50,000 cells per well in a 24-well plate (Jet Biofil, Guangzhou, China) with DMEM containing 4500 mg/L glucose supplemented with 10% FBS and 1% P/S in a humidified incubator with 5% CO_2 at 37°C. The medium was replaced after 24 h with antibiotic- and serum-free 1× Opti-MEM® medium (Gibco, Life Technologies Corporation, Grand Island, NY, USA) containing glucose concentrations of 4500, 900, and 200 mg/L. The reduced glucose conditions were obtained by mixing 10 mL (for 900 mg/L) and 2.22 mL (for 200 mg/L) DMEM containing 4500 mg/L glucose with Opti-MEM (1×) medium to a final volume of 50 mL (stock). Prepared stock (1 mL) was used for C (control), NC (negative control), FOS, and BECN1 knockdown (KD) treatments. After 24 h, the respective wells were subjected to siRNA treatment using Silencer® Select Negative control (5 nmol) (Cat. #4390843) (Ambion™ Thermo Fisher Scientific Baltics, Vilnius, Lithuania), FOS Silencer® Select Pre-designed siRNA (5 nmol) (Cat. #4390771, ID: s66198) (Ambion™ Thermo Fisher Scientific Baltics), and BECN1 Silencer® Select Pre-designed siRNA (5 nmol) (Cat. #4390771, ID:s80166) (Ambion™ Thermo Fisher Scientific Baltics) in combination with Lipofectamine® RNAiMAX reagent (13778-150) (Life Technologies Corporation, Carlsbad, CA, USA). The cells thus treated were incubated for 48 h at 37°C with 5% CO_2 in a humidified incubator, harvested, and subjected to RNA extraction followed by cDNA synthesis and gene expression analysis as described in 'Gene expression analysis'. The primers used are listed in Table 1.

For protein expression studies, N39 cells were seeded at a density of 300,000 cells per well in a six-well plate with DMEM containing 4500 mg/L glucose supplemented with 10% FBS and 1% P/S in a humidified incubator with 5% CO_2 at 37°C. The medium was replaced after 24 h and transfection was performed across the hypoglycemic conditions with glucose concentrations of 4500 and 200 mg/L as described earlier. The cells treated with siRNA were incubated for 48 h at 37°C with 5% CO_2 in a humidified incubator, harvested, and subjected to protein extraction, SDS-PAGE, and Western blotting with subsequent imaging and analysis of the proteins following the methods described in 'Protein expression analysis'.

Statistical analysis

All data are represented as mean ± standard error of the mean (SEM) and were analyzed by two-way ANOVA (GraphPad Prism 9, RRID:SCR_002798) followed by Tukey's test for multiple comparisons when two variables were compared between more than two groups. Probability values of $p < 0.05$ were considered statistically significant.

RESULTS

Induction of autophagy in hypothalamic neurons in response to low glucose conditions

Low glucose conditions are known to induce the initiation of autophagy as a cellular survival mechanism. Therefore, we investigated the autophagy levels in embryonic hypothalamic neurons, N39 cells, when exposed to hypoglycemia under glucose concentrations of 4500,

2000, 900, 500, and 200 mg/L over a period of 24–72 h. To assess autophagy, we first measured the mRNA and protein expression of the autophagosomal marker LC3B. Our results indicate that there was no significant difference in the mRNA expression of LC3B across the tested conditions (Figure 1a). However, LC3B lipidation, the ratio of LC3BII to LC3BI signifying the formation of autophagosomes, showed a significant increase at the lowest glucose concentration of 200 mg/L ($p < 0.0001$) at 72 h when compared with the glucose concentrations of 4500, 2000, 900, and 500 mg/L at 72 h (Figure 1b). Similarly, the protein expression of LC3BII, the functional form which localizes to autophagosome membrane, also showed a similar pattern of increase at the lowest glucose concentration of 200 mg/L ($p < 0.01$) at 72 h when compared with the glucose concentrations of 4500, 2000, and 900 mg/L at 72 h.

We then measured the mRNA and protein expression of LAMP2A, a lysosomal membrane protein implicated in lysosomal stability and autophagy, that is indicative of autophagic degradation (Figure 1c). Interestingly, although a reducing trend was observed in the mRNA expression of LAMP2, an opposite pattern was observed

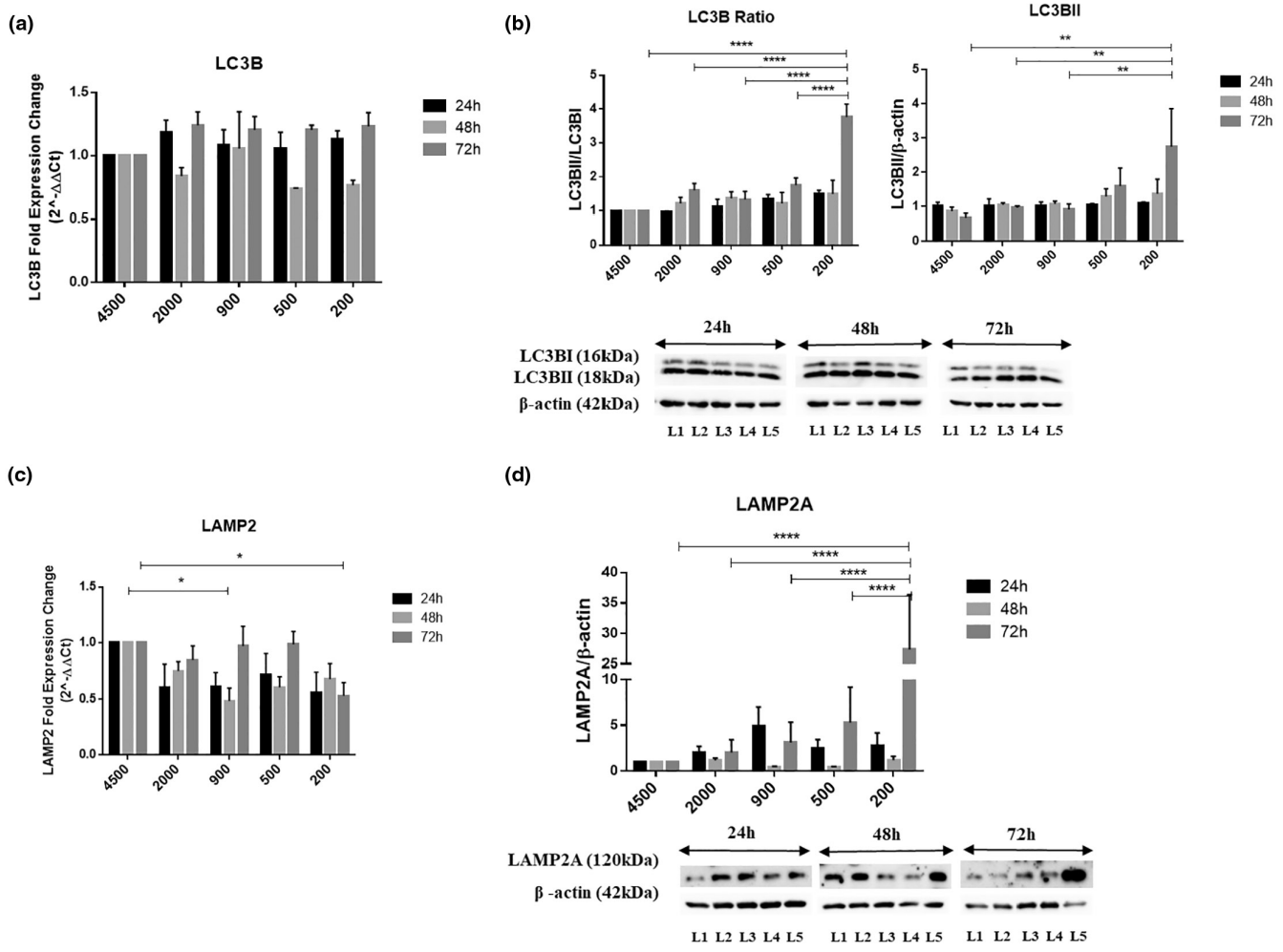

FIGURE 1 Induction of autophagy in hypothalamic neurons in response to hypoglycemic conditions. N39 cells were exposed to glucose concentrations of 4500, 2000, 900, 500, and 200 mg/L for 24, 48, and 72 h. The relative fold change in the gene expression levels of (a) LC3B and (c) LAMP2 in N39 cells as compared with the gene expression levels at the glucose concentration of 4500 mg/L, which is considered as the reference with a value of 1, and determined by using the quantitative real-time polymerase chain reaction (qPCR) process. (b) Shows the representative estern blot indicating the protein expression of LC3BI (16KDa) and LC3BII (18KDa) at 24, 48, and 72 h across decreasing glucose concentrations[#] and the corresponding densitometric analysis of LC3B lipidation represented as the ratio of LC3BII to LC3BI as well as LC3BII normalized to β-actin. (d) Shows the representative Western blot indicating the protein expression of LAMP2A (120 KDa) at 24, 48, and 72 h across decreasing glucose concentrations[#] and the corresponding densitometric analysis of LAMP2A expression. The protein expression of β-actin (42 KDa) at 24, 48, and 72 h at decreasing glucose concentrations[#] was used as loading control. All experiments were repeated at least three times and the graphical data are represented as mean ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ determined using two-way ANOVA. [#]Glucose concentrations: L1 = 4500 mg/L, L2 = 2000 mg/L, L3 = 900 mg/L, L4 = 500 mg/L, L5 = 200 mg/L.

in the protein expression (Figure 1d). N39 cells exposed to hypoglycemia under reducing glucose concentrations of 2000, 900, 500, and 200 mg/L showed a significant decrease in the gene expression of LAMP2A at 900 mg/L at 48 h (*$p < 0.05$) and 200 mg/L at 72 h (*$p < 0.05$) when compared with the reference level at 4500 mg/L (Figure 1c). With N39 cells exposed to decreasing glucose concentrations, LAMP2A protein expression was however noted to increase at both 24 and 72 h across the tested hypoglycemic conditions in a dose-dependent manner (Figure 1d). A statistically significant change was noted at 72 h at the lowest glucose concentration of 200 mg/L when compared with the respective expression at 4500, 2000, 900, and 500 mg/mL glucose concentrations ($p < 0.0001$) (Figure 1d). Intriguingly, an oscillating pattern of LAMP2A expression was noted across 24, 48, and 72 h at each of the lower glucose concentrations of 2000, 900, 500, and 200 mg/L in comparison to their respective values at 4500 mg/L. Raw images of the blots are shown in Figures S1 and S2.

Since increased formation of autophagosomes was indicated by Western blot, we next assessed the autophagosomal levels in live N39 cells exposed to glucose concentrations of 4500, 2000, 900, 500, and 200 mg/L by fluorescent microscopy. N39 cells showed a consistent and notable increase in the accumulation of autophagosomes with decreasing glucose concentrations of 2000, 900, 500, and 200 mg/L, particularly at 48 h (Figure 2). At 48 h the autophagosomes were detected in the perinuclear space in small numbers at 4500 mg/L while the number of autophagosomes progressively increased at 2000, 900, and 500 mg/L, with the maximum number being detected at the lowest glucose concentration of 200 mg/L, indicating an increase in autophagosome formation with decrease in glucose levels and with time (Figure 2).

Effects of low glucose conditions on FOS and BECN1 gene expression in N39 cells with FOS and BECN1 gene knockdowns

Our previous studies have demonstrated an upregulation in FOS expression in response to hypoglycemia,[32] therefore. we next knocked down the expression of FOS in N39 cells by transient siRNA transfection. In addition, since BECN1 has been reported to play a role in neuronal viability and has been implicated in multiple neurodegenerative diseases, we also knocked down BECN1 in N39 cells to address the association between FOS and autophagy, as cells lacking BECN1 demonstrate a deficiency in autophagy.[35–37] To validate the knockdown of FOS in FOS KD cells, FOS gene expression was compared between the negative control (NC) and FOS KD cells at 4500 mg/L glucose concentration, and a 60% drop in the gene expression of FOS was observed (Figure 3a). Similarly, a 90% reduction in the gene expression of BECN1 was observed in BECN1 KD cells in comparison to NC at 4500 mg/L glucose concentration (Figure 3b). With successful knockdown of FOS and BECN1 gene expression in the respective KD cells, we then exposed these cells to hypoglycemic conditions under glucose concentrations of 900 and 200 mg/L to better understand the association between FOS and BECN1 in regulating the autophagic response of N39 cells to hypoglycemia. While the mRNA expression of FOS was impeded in the FOS KD cells at 4500 mg/L,

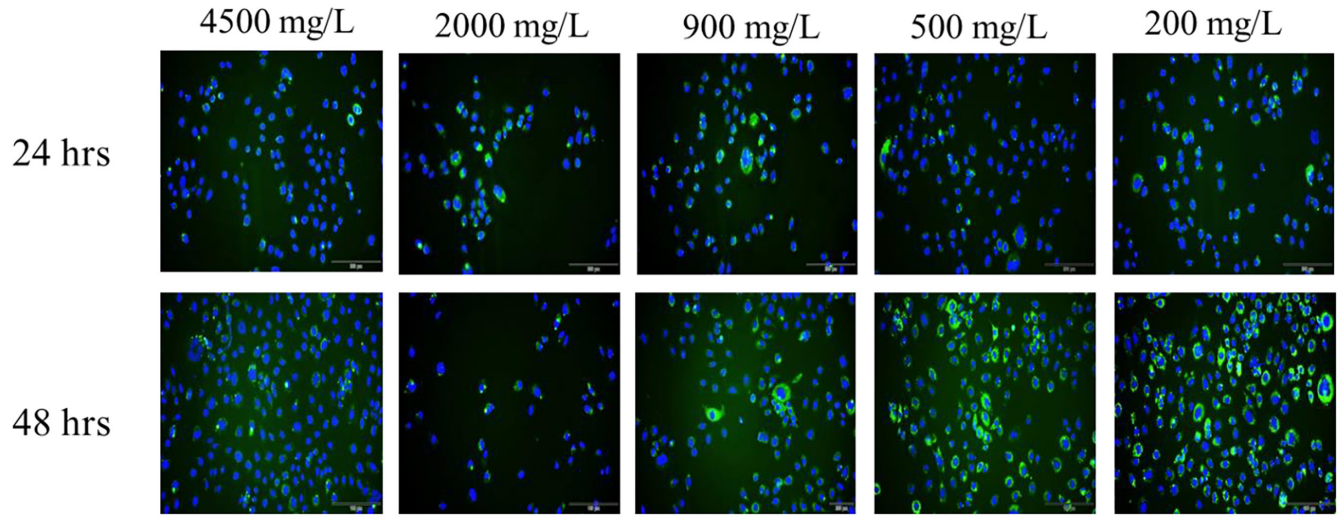

FIGURE 2 Fluorescence microscopy images of live N39 cells exposed to various glucose concentrations for 24 and 48 h depicting the levels of autophagosomes (green) and nuclei (blue with DAPI stain). Scale bar 200 μM.

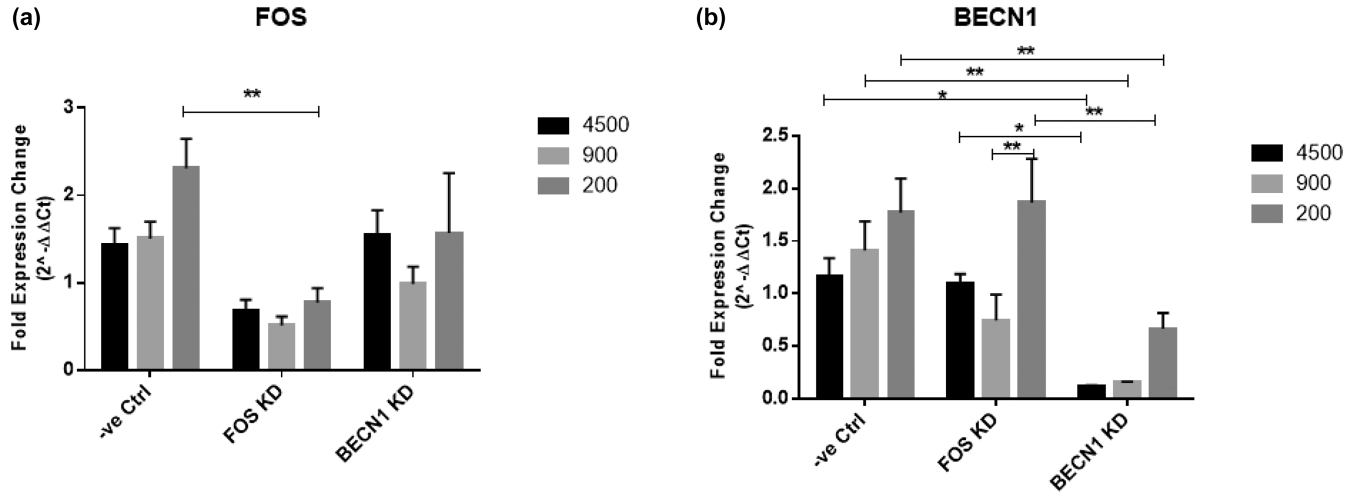

FIGURE 3 Effects of low glucose conditions on FOS and BECN1 gene expression in N39 cells with FOS and BECN1 gene knockdowns. N39 cells were treated with siRNA negative control, FOS siRNA, and BECN1 siRNA, and were exposed to various concentrations of glucose (4500, 900, and 200 mg/L) for 48 h post-siRNA transfection. The relative fold change in the mRNA expression levels of (a) FOS and (b) BECN1 in FOS knockdown (FOS KD) and BECN1 knockdown (BECN1 KD) cells were compared with the mRNA expression of negative control cells at the corresponding glucose concentrations of 4500, 900, and 200 mg/L, as determined using the quantitative real-time polymerase chain reaction (qPCR) process. All experiments were repeated at least three times and the graphical data are represented as mean ± standard error of the mean (SEM). $*p < 0.05$, $**p < 0.01$ determined using two-way ANOVA.

it remained low in response to low glucose conditions (Figure 3a). The FOS gene expression was retained in BECN1 KD cells and showed an oscillating pattern with reducing glucose conditions. Similarly, BECN1 expression was significantly reduced in the BECN1 KD cells and an increase in BECN1 expression was noted in the BECN1 KD cells at the lowest glucose concentration of 200 mg/L (Figure 3b). Nevertheless, this increase was significantly lower than that observed in the negative control and FOS KD cells at 200 mg/L. At the same time, BECN1 gene expression was retained in FOS KD cells and showed an oscillating pattern with reducing glucose conditions. Given the variability in these experiments and lack of statistical significance, these oscillations could also be attributed to background noise.

Effects of low glucose conditions on the expression of LC3B and LAMP2A in N39 cells with FOS and BECN1 gene knockdowns

The mRNA and protein expression levels of LC3B and LAMP2A were then examined in N39 cells subjected to FOS and BECN1 gene knockdowns and exposed to glucose concentrations ranging from 4500 to 200 mg/L. At 4500 mg/L glucose concentration, LC3B gene expression showed a weak drop in FOS KD cells and a more visible drop in BECN1 KD cells when compared with the NC cells at that concentration. A significant increase in LC3B gene expression was, however, observed in FOS KD cells at 200 mg/L concentration of glucose in comparison to untransfected control at 200 mg/L (Figure 4a). This may be attributed to high experimental variability at 200 mg/L in control cells as demonstrated by the error bar. The LC3B gene response to hypoglycemic conditions in FOS KD cells, however, was similar to that observed in NC cells. Despite the KD of BECN1 gene, a dose-dependent increase in LC3B expression was noted in BECN1 KD cells without statistical significance. The conversion of LC3BI to LC3BII did not show a notable difference between the treatment groups at 4500 mg/L (Figure 4b). In comparison to our observation of increased LC3B lipidation in untransfected N39 cells at 200 mg/L versus 4500 mg/L, the increase in LC3B lipidation was noted to be reduced in NC cells and impeded in FOS KD cells in response to low glucose conditions. Since autophagy mechanisms are known to be impeded in BECN1 KD cells, no change was observed in LC3B lipidation in these cells in response to hypoglycemic conditions. In comparison to the NC cells, albeit statistically insignificant, the LC3B lipidation at 4500 and 200 mg/L glucose concentrations appeared to show a reducing trend across FOS KD cells and BECN1 KD cells, with the lowest levels observed in BECN1 KD cells.

The mRNA expression of LAMP2 significantly decreased in control N39 cells with reducing glucose concentrations ($*p < 0.05$) (Figure 4c). While a similar pattern was observed across all groups, a statistically significant change was also observed in FOS KD cells. Conversely,

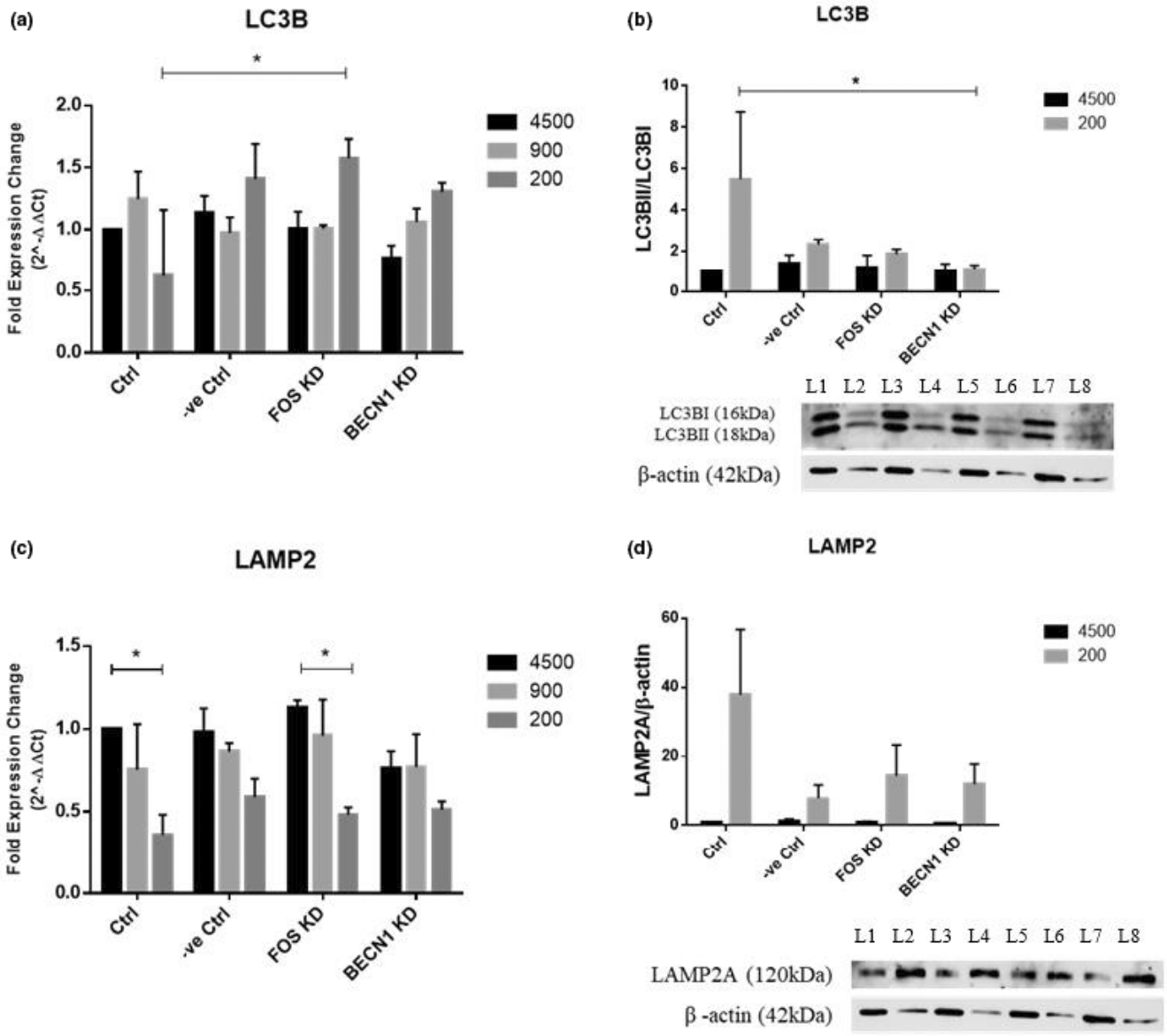

FIGURE 4 Effects of low glucose conditions on the expression of LC3B and LAMP2A in N39 cells with FOS and BECN1 gene knockdowns. N39 cells (Ctrl), N39 + siRNA negative control (−ve Ctrl), N39 + FOS siRNA (FOS KD), and N39 + BECN1 siRNA (BECN1 KD) cells were exposed to various concentrations of glucose (4500, 900, and 200 mg/L) for 48 h post-siRNAs transfection. The relative fold change in the mRNA expression levels of (a) LC3B and (c) LAMP2 in these cells were compared with the mRNA expression of Ctrl cells at the glucose concentration of 4500 mg/L, which is considered as the reference with a value of 1, and determined using the quantitative real-time polymerase chain (qPCR) reaction. (b) Representative Western blot indicating the protein expression of LC3BI (16 KDa) and LC3BII (18 KDa) in these cells at glucose concentrations of 4500 and 200 mg/L and the corresponding densitometric analysis of LC3B lipidation represented as the ratio of LC3BII to LC3BI. (d) Representative Western blots indicating the protein expression of LAMP2A (120 KDa) in these cells at glucose concentrations of 4500 and 200 mg/L and the corresponding densitometric analysis of LAMP2A expression. The protein expression of β-actin (42KDa) was used as loading control. All experiments were repeated at least three times and the graphical data are represented as mean ± standard error of the mean (SEM). *$p < 0.05$, ****$p < 0.0001$ determined using two-way ANOVA. Representation of lanes in images for Western blots: L1 = control (4500 mg/L glucose), L2 = control (200 mg/L glucose), L3 = negative control (4500 mg/L glucose), L4 = negative control (200 mg/L glucose), L5 = FOS KD (4500 mg/L glucose), L6 = FOS KD (200 mg/L glucose), L7 = BECN1 KD (4500 mg/L glucose), L8 = BECN1 KD (200 mg/L glucose). Glucose concentrations are represented in milligrams/liter (mg/L).

in line with our observation in Figure 1d, a marked increase in LAMP2A protein expression was noted at 200 mg/L in comparison to 4500 mg/L glucose in control N39 cells (Figure 4d). The protein expression of LAMP2A in response to hypoglycemic conditions was impeded in NC cells in comparison to untransfected control cells. The FOS KD and BECN1 KD cells also exhibited an increase in LAMP2A protein expression in response to low glucose

conditions; however, this increase was comparable to the response seen in NC cells. Raw images of the blots are shown in Figures S3 and S4.

Modulation of autophagy in FOS and BECN1 KD N39 cells in response to low glucose conditions

In the next group of experiments, the autophagosomal levels in FOS and BECN1 KD N39 cells in response to low glucose conditions were assessed using fluorescence microscopy (Figure 5). As expected, control N39 cells exposed to reducing glucose concentrations showed a notable increase in the accumulation of autophagosomes in a dose-dependent manner. In contrast, the N39 cells with FOS and BECN1 knockdowns showed feeble autophagosomal levels in the perinuclear space at 4500 mg/L concentration of glucose and the levels remained low across the reducing glucose concentrations, indicating an inhibition in autophagosomal formation in the absence of FOS and BECN1 genes. Another noteworthy observation was the change in the morphology and viability of the KD cells compared with the control cells. While control cells appeared with normal morphology, KD cells appeared stressed and showed reduced cell viability. Taken together, our results suggest that the FOS and BECN1 genes are directly involved in the regulation of autophagy in hypothalamic neurons exposed to hypoglycemia.

DISCUSSION

Continuous supply of glucose to the brain is essential to maintain its metabolic drive. Multiple counter-regulatory mechanisms, such as secretion of adrenaline and other glucocorticoids, exist in the body to ensure a timely response to hypoglycemia. However, iatrogenic hypoglycemia in patients with DM which is associated with defects in these counter-regulatory hormone responses has severe

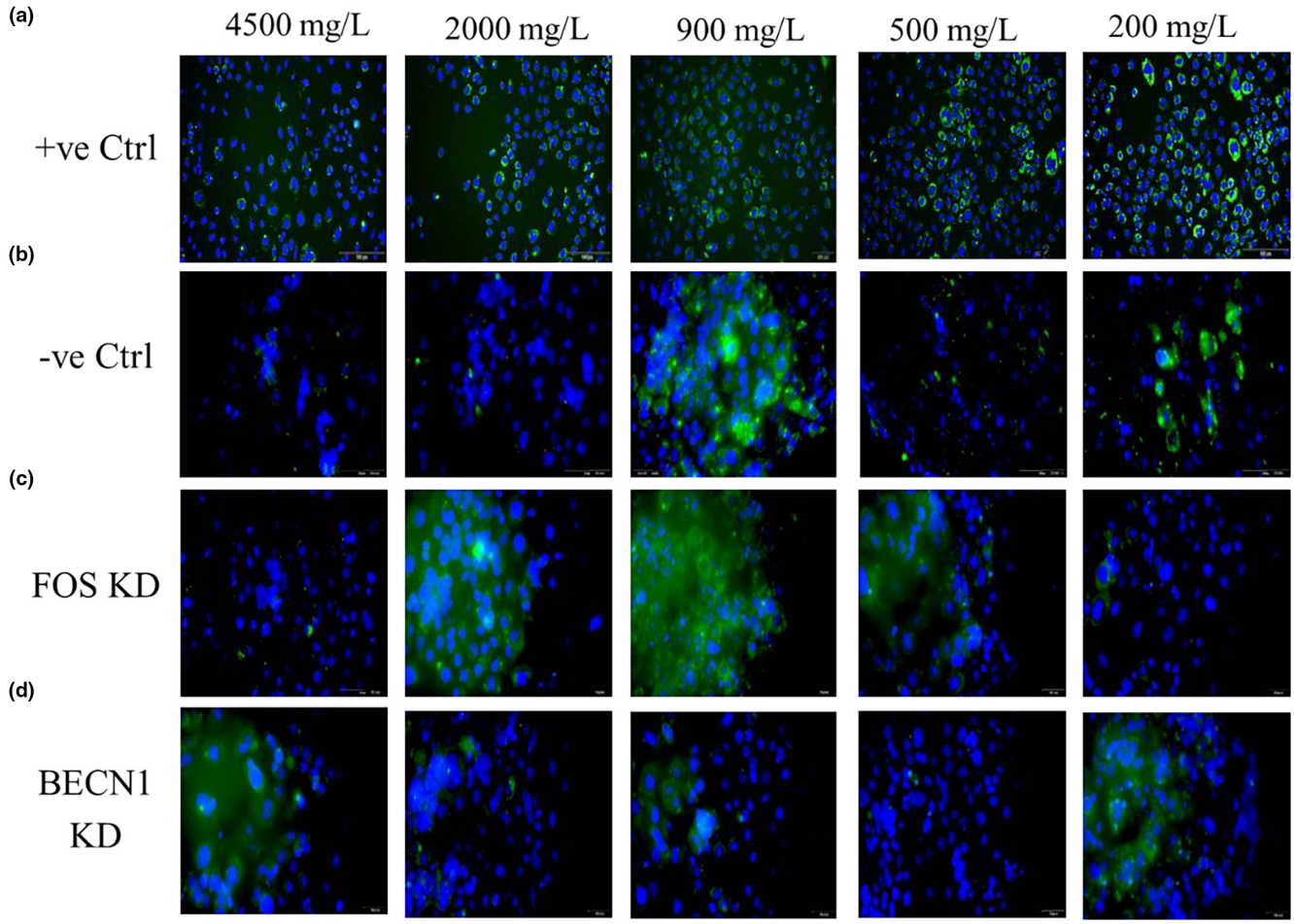

FIGURE 5 Fluorescence microscopy images of (a) N39 cells without siRNA treatment (positive control), (b) N39 with siRNA negative control, (c) FOS knockdown (KD) N39 cells, and (d) BECN1 KD N39 cells at different glucose conditions (4500, 2000, 900, 500, and 200 mg/L). The levels of autophagosomes are depicted in green and nuclei in blue with DAPI stain. Scale bar 200 μM.

consequences, including neuronal death. Thus, neuronal sensing and response to hypoglycemia is a critical part of the physiological counter-regulatory response.[38] Glucose variability (GV) has been a well-established contributor to the microvascular and macrovascular complications of DM.[39] GV exerts its deteriorating effects through multiple biochemical, cellular, and molecular events, including GV-induced disruption in autophagic flux, which may lead to reduced viability and activation of apoptosis pathways.[39] The present study therefore aimed to test the hypothesis that autophagy is one of the critical mechanisms in the hypoglycemic response of hypothalamic neurons and this mechanism might be mediated by the hypothalamic regulatory protein, FOS, along with BECN1.

For the first time, the present study has demonstrated that the hypothalamic neurons respond to hypoglycemia by increasing autophagosome formation and subsequent lysosomal degradation. Since autophagosome formation involves the conversion of soluble LC3BI to lipid-bound LC3BII, we measured the ratio of LC3BII to LC3BI as an indicator of the autophagosomal levels in the cells. Although no significant increase in the mRNA expression of LC3B was observed with reducing glucose concentrations or increasing time, a dose-dependent increase in LC3B lipidation, indicative of increased autophagosome formation, was noted with reducing glucose concentrations and increasing time. This was further confirmed by the observation of increased fluorescent staining for autophagosomes in live N39 cells under conditions of reduced glucose. The autophagosomes further accumulated in cells under the lower glucose conditions of 900, 500, and 200 mg/L glucose with longer time (48 h).

Autophagy has been implicated as an important regulator of neuronal health, particularly under stress.[40–42] Cellular processes, such as autophagy and ciliogenesis, go hand in hand in the development of hypothalamic neurons undergoing axonogenesis.[43] The dynamic regulation of autophagy across the different neuronal compartments in a spaciotemporal manner facilitates specialized neuronal functions.[44] In a study conducted in microglia, the cells were exposed to an acute drop from a high glucose state to a normal glucose state, and this was found to be accompanied by increased LC3BII expression.[45] The findings of this study indicated that increased metabolic stress can pave the way to neuroinflammation and neurodegeneration. Similarly, an AMPK-induced increase in autophagy was reported in hypothalamic cell lines and astrocytes subjected to low glucose availability.[37,46] Under low glucose conditions or glucose deprivation, the cells are stressed, and the balance between autophagy and apoptosis, which are key players in maintaining cellular homeostasis, is dysregulated. Under such stressful conditions, the cells resort to autophagy as a survival mechanism.[47] However, due to a drop in glucose to critically low levels, autophagy mechanisms may undergo a defect or may not be sufficient to meet the excessive demand and the cells can no longer withstand the nutrient stress and ultimately resort to apoptosis. Our previous study demonstrated a dose-dependent decrease in the viability and increased apoptosis of hypothalamic N39 cells in response to hypoglycemia.[32] A similar observation was made in photoreceptor cells under low glucose conditions, where they were shown to undergo autophagy and apoptosis through the AMPK/RAPTOR/mTOR and BCL2/BAX pathways, respectively.[46] The autophagy defect in this study was mainly attributed to the failure in the fusion of autophagosomes to lysosomes due to a decrease in LAMP2A protein expression at such extreme low levels of glucose.[46]

Interestingly in our study, the lysosomal marker LAMP2A, which is involved in the fusion between autophagosomes and lysosomes, showed a decline in mRNA expression at a glucose concentration of 900 mg/L at 48 h and 200 mg/L at 72 h. Nevertheless, the protein expression of LAMP2A significantly increased at 200 mg/L at 72 h (Figure 1d). This discrepancy in the mRNA and protein expression of LAMP2A may be explained by the varying turnover times of LAMP2 mRNA and protein, particularly in a dynamic pathway such as autophagy.[48] Moreover, since qPCR and Western blotting measure the steady-state levels of mRNA and protein, respectively, factors such as post-transcriptional modifications, rate of translation, translational efficiency, mRNA, and protein stability are not reflected in the measurements, resulting in non-proportional mRNA and protein levels. Furthermore, LAMP2A also showed an oscillating pattern of protein expression across 24, 48, and 72 h under all the tested hypoglycemic conditions (Figure 1d). Under the different conditions of glucose starvation, while an increase in LAMP2A protein expression was noted at 24 h, it demonstrated a drop at 48 h followed by a rise again at 72 h. This may be attributed to the slow turnover rate of LAMP2A, as the study by Cuervo and Dice reported that LAMP2A in total cell lysates to have a half-life of approximately 46 h.[49] Therefore, while LAMP2A appears to increase at 24 h in response to the hypoglycemic conditions, the lysosomal degradation of LAMP2A may contribute to its decline at 48 h and its subsequent restoration by 72 h in response to the low glucose levels.

Nevertheless, our finding of increased LC3B lipidation and LAMP2A protein expression thus suggests a functional increase in autophagy in N39 cells upon exposure to reducing glucose concentrations that mimic a hypoglycemic environment. Despite the reduced viability of N39 cells at 200 mg/L glucose, their ability to increase autophagy at this concentration suggests a functional response to achieve euglycemia.[29] However, persistent hypoglycemic

attacks could result in defective autophagic mechanisms which may contribute to increased metabolic stress and subsequent neurodegeneration. In the study by Gerónimo-Olvera et al.,[50] while autophagosome formation increased during glucose deprivation, glucose replenishment could not rescue cortical neurons from cell death.[50] However, in this instance, autophagy inhibition was found to be beneficial for neuronal survival and this response was found to be associated with calpain activity. Hypoglycemic damage induced calpain activation, which in turn led to lysosomal dysfunction and subsequent impairment in autophagy pathway in the presence of glucose deprivation causing neuronal death. Autophagy, therefore, appears to play a multifaceted role in maintaining neuronal health during hypoglycemic stress.

The hypothalamus is a key component of the brain glucose sensing network, and it is composed of different nuclei, such as the ventromedial nucleus and paraventricular nucleus, that control the sympathoadrenal counter-regulatory responses to hypoglycemia.[51,52] Selected populations of neurons within the hypothalamus monitor the plasma glucose levels and undergo activation in response to decreased glucose availability.[53] Alternative substrates to glucose such as β-hydroxybutyrate could serve as energy fuels in the brain during hypoglycemia to prevent neuronal cell death. When rats were subjected to severe hypoglycemia, autophagosomal accumulation was observed in the cortex and the hippocampus regions of the brain, which was associated with an impairment in autophagic degradation.[41] Administering β-hydroxybutyrate in these rats modulated autophagy dynamics during severe hypoglycemic stress stimulating autophagy flux and improving cell viability.

Neuronal FOS expression reportedly signifies the activation of neurons by different stimuli.[54] The differential expression of FOS in distinct populations of neurons suggests a distinct separation of glycemic control functions within the different parts of the hypothalamus, reinforcing the heterogeneity and inherent complexity of the hypothalamic region.[33] FOS expression was induced in ventromedial and lateral arcuate nucleus of the hypothalamus (ARH) during fasting and short-term refeeding in mice.[55] Previously, we demonstrated an increase in FOS expression with reducing glucose concentrations, suggesting a protective role of FOS against hypoglycemia.[32] Since very few studies have investigated the molecular events regulating hypoglycemic control in embryonic neurons within the hypothalamus, we next sought to better understand the role of FOS in their autophagic response to hypoglycemia. To understand the association between FOS and BECN1 in regulating the autophagic response of hypothalamic neurons to low glucose conditions, we used siRNA-based transfection to transiently knockdown the expression of these genes and then assessed the autophagic response of FOS KD and BECN1 KD cells to hypoglycemic conditions. While FOS mRNA expression was hindered in the FOS KD cells across glucose conditions of 4500, 900, and 200 mg/L, BECN1 expression stayed low in the BECN1 KD cells at concentrations of 4500 and 900 mg/L but then showed an increasing trend at 200 mg/L, signifying the increased induction of autophagy through BECN1 expression at this concentration. The increasing BECN1 expression in FOS KD cells with reducing glucose levels may suggest a functional BECN1 mRNA response to low glucose conditions in FOS KD cells.

A reduction in LC3BII conversion and autophagosome formation was observed following BECN1 knockdown in ipsilateral thalamus.[56] Interestingly, this was accompanied by a decrease in neuronal loss, gliosis, and apoptosis in the ipsilateral thalamus. LAMP2 expression was further attenuated when BECN1 was knocked down in neuroendocrine cells.[57] In our study, the LC3B lipidation at both 4500 mg/L and 200 mg/L glucose concentrations demonstrated a declining trend across FOS KD cells and BECN1 KD cells in comparison to NC cells. The FOS KD cells showed a weak LC3B response to hypoglycemia but the induction of LC3BI to LC3BII conversion was negligible in BECN1 KD cells in response to hypoglycemia. Since autophagy activation is reported to be impaired in BECN1 KD cells,[25,37,56] the comparable protein expression of LC3B and LAMP2A in FOS KD and BECN1 KD cells in response to low glucose conditions indicates an association between FOS and BECN1 in the regulation of autophagy when neuronal cells are exposed to low glucose conditions. The similar pattern of LC3B lipidation and LAMP2A protein expression in FOS KD and BECN1 KD cells highlights the progression of autophagy to be affected similarly in these cells.

It is, however, important to note that LC3B lipidation and LAMP2A protein expression was also affected in NC cells in comparison to control N39 cells. This could be attributed to the nature of NC siRNA which despite having no homology to known mammalian genes, could still cause off-target specificity owing to transfection procedures or the toxicity of siRNA itself. This is a well-recognized pitfall of siRNA transfection.[58] Therefore, while the FOS and BECN1 mRNA expression deteriorated significantly using the corresponding siRNAs in comparison to the NC, the observation that the LC3B and LAMP2A protein response to hypoglycemic conditions in FOS KD and BECN1 KD cells was not significantly different to the NC cells needs to be interpreted with caution. A recent study investigated the role of hypothalamic mTORC1 signaling in agouti-related protein (AgRP) neurons and its involvement in nutrient sensing.[55,59] The

authors of this study demonstrated that AgRP neurons with disrupted mTORC1 signaling showed no statistically significant change in FOS expression across the hypothalamic areas. Therefore, mechanistic studies are essential to develop our understanding of the interaction between FOS and autophagy in regulating hypothalamic neurons.

The absence of increased autophagosomal staining in FOS KD and BECN1 KD cells with reducing glucose conditions in comparison to the positive and negative controls further highlights the autophagy activity to be hindered in these KD cells. This is in line with the findings from a previous study that reported a pivotal role of FOS-mediated BECN1 upregulation for activation of autophagy in dopaminergic receptors.[60] This study identified a FOS binding sequence in the promoter region of the BECN1 gene whereby in response to a stimulus, an increase in FOS expression led to increased transcription of BECN1 and subsequent autophagic activity. This may offer a potential mechanistic explanation for the impaired autophagic response observed in the FOS KD cells when exposed to hypoglycemic conditions (Figure 4). Although the use of siRNA transfection has greatly helped in determining the outcomes of the present study, its toxic effects were also observed on the morphology and viability of KD cells. Though this may affect the interpretation of results, the benefits of using siRNA transfection outweigh the risks.

Taken together, these findings point towards autophagy being one of the potential mechanisms contributing to the pathogenesis of HAAF. As a mediator of autophagic responses in hypoglycemia, our results are also suggestive of the role of FOS as a potential biomarker for patients with HAAF, which warrants further investigation.

AUTHOR CONTRIBUTIONS

R.K.R., A.S., R.R., and B.M.M wrote the manuscript. B.M.M. designed the research. R.K.R., A.S., and R.R. performed the research and analyzed the data. S.A. and B.M.M. reviewed the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declared no competing interests for this work.

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SUPPORTING INFORMATION

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