

Long Term Effects of Neonatal Hypoglycemia on Muscarinic Receptor Function in the Cerebellum

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Abstract

Neonatal stress conditions like hypoglycemia cause brain damage by affecting various signaling pathways thereby causing long term effects on brain functions. A proper understanding of the signaling pathways affected by this stress will help to devise better neonatal care. The focus of the current study was to evaluate the effect of neonatal hypoglycemic insult on cerebellar metabotropic cholinergic receptor function in one month old rats. The receptor analysis of cholinergic muscarinic receptors were done by radioreceptor assays and gene expression was analysed using Real Time PCR. Neonatal hypoglycemia significantly reduced ($p < 0.001$) the cerebellar muscarinic receptor density with a down regulation ($p < 0.001$) of muscarinic M3 receptor subtype gene expression in one month old rats. Both muscarinic M1 and M2 receptor subtype expression were not significantly altered. The catabolic enzyme in acetyl choline metabolism- acetylcholine esterase – showed a significant ($p < 0.001$) up regulation with no significant change in the anabolic enzyme – choline acetyl transferase, signifying a change in the turnover ratio. Targeting these pathways at different levels can be exploited to devise better treatment for neonatal stress management and also for diseases with impaired insulin secretion such as diabetes.

Keywords: Muscarinic receptor, ChAT, AChE, Real Time PCR, Radioreceptor assay.

Introduction

Neonatal hypoglycemia is a common complication among preterm infants, small-for-gestational-age infants, and infants of diabetic mothers. Currently, there has been in-depth understanding of diagnosis and clinical intervention of neonatal hypoglycemia, but the extent of neonatal hypoglycemic brain injury remains undefined. Neonatal hypoglycemia may cause irreversible neurological sequelae [1]. Persistent and recurrent hypoglycemia can severely impair brain growth and its function [2, 3]. Studies showed that if neonatal hypoglycemia is not timely and properly treated, the infants may develop permanent brain injury like neonatal hypoglycemic encephalopathy [4]. Many earlier studies reported that the cerebellum is relatively resistant to hypoglycaemia. Recent studies using imaging techniques pointed out the hypoglycemia-induced cerebellar injury. Cerebral blood flow increases during hypoglycemia; opposite to the other parts, however, glucose utilization in the occipital lobe and cerebellum decreases, resulting in the vulnerability of these areas. The study by Kim et al, reported the cerebellar dysfunction caused by hypoglycemia using quantitative dynamic PET study, thus suggesting that cerebellum is not invariably resistant to hypoglycemia [5]. Sherin et al, 2010 reported that cerebellar cholinergic neurotransmission is impaired during hyperglycemia

and hypoglycemia and hypoglycemia is causing more prominent imbalance in cholinergic neurotransmission which is suggested to be a cause of cerebellar dysfunction associated with hypoglycemia [6]. Acetylcholine (ACh), the major neurotransmitter of the parasympathetic nervous system, can enhance glucose-stimulated insulin secretion from pancreatic beta-cells with muscarinic M3 receptors playing an essential role for maintaining proper insulin secretion and glucose homeostasis [7]. The actions of ACh on peripheral tissues that are innervated by parasympathetic nerves are mediated by muscarinic ACh receptors (mAChRs), which have five molecularly distinct members (M1–M5 mAChRs) [8]. The release of ACh is controlled by its metabolism catalyzed by acetyl choline esterase (AChE) and choline acetyl transferase (ChAT).

Taking into account of the fact that cerebellar cholinergic receptors are affected by hypoglycaemia, we focussed on the long term effect of neonatal hypoglycaemic insult on cholinergic receptor function in the cerebellum. To study this, one month old rats exposed to neonatal hypoglycaemia was evaluated for the expression of cholinergic receptors and the enzymes involved in its metabolic pathways by receptor analysis and Real Time PCR studies.

Materials and Methods

Animals

Wistar neonatal rats (postnatal day 7, P7) weighing 10.0–12.0 g were used for all experiments. All groups of neonatal rats were

maintained with their dams under optimal conditions—12 h light and 12 h dark periods and were fed standard food and water ad libitum. Each group consist 4 - 6 rats and standard error mean was obtained from 4-6 separate experiments. All animal care procedures were in accordance with Institutional, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and National Institute of Health guidelines.

Induction of hypoglycemia in neonatal rats Wistar neonatal rats used for the experiments were grouped into two as follows: (i) control (C) - neonatal rats injected with saline, subcutaneously for ten days starting from P7; (ii) Hypoglycemic (H) - Hypoglycemic rats received human regular insulin (Actrapid) at a dosage of 10 IU/kg body weight subcutaneously and fasted for 240 min [9]. After confirmation of euglycemia (blood glucose >50 mg/dL) and normal activity, neonatal rats were reunited with their dams. Hypoglycemia was induced for ten days starting from P7. All the experimental rats were maintained at optimum conditions for one month period. The effect of the hypoglycaemic stress was determined on postnatal day 30 (one month old rat), in which the weaning period terminates and the rat enter into physiological adulthood [10].

Control and hypoglycemic rats were sacrificed by cervical dislocation and cerebellum was dissected out quickly over ice according to the procedure of Glowinski and Iversen, and stored at -80°C for various experiments [11].

Estimation of Blood Glucose Concentration

Blood glucose was estimated in control and experimental rats using a blood glucose meter (Accu-Chek® Compact, Roche Diagnostics, Indianapolis, IN) and the results were expressed in mg/dl of blood.

Total muscarinic Receptor Binding Assay

The total muscarinic receptor binding assay in the cerebellum was done according to the modified procedure of Yamamura and Snyder [12]. Total muscarinic binding assay was done using 0.1-2.5nM of [3H] Quinuclidinylbenzilate (QNB) with 100-150µg protein concentration. Bound radioactivity was counted with cocktail-T in a Perkin Elmer Tri Carb 2810 TR liquid scintillation counter. Protein was measured by the method of Lowry et al [13].

Linear regression analysis of receptor binding for Scatchard plots:

The receptor binding parameters were determined using Scatchard analysis [14]. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on x-axis and bound/free on y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

Gene expression studies of muscarinic M1, M2, M3 receptor subunits; cholinergic markers - ChAT and AChE using Real Time PCR

RNA was isolated from cerebellum using Tri reagent. Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit. Real-Time PCR assays were performed in 96-well plates in an ABI 7300 Real-Time PCR instrument (Applied Biosystems, Foster

City, CA, USA). PCR analyses were conducted with gene-specific primers and fluorescently labeled Taq probe for muscarinic M1, M2, M3 receptor subunits, choline acetyl transferase and acetyl choline esterase designed by Applied Biosystems. Endogenous control (β-actin) labeled with a reporter dye was used as internal control. All reagents were purchased from Applied Biosystems. The real-time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate. The ΔΔCT method of relative quantification was used to determine the fold change in expression.

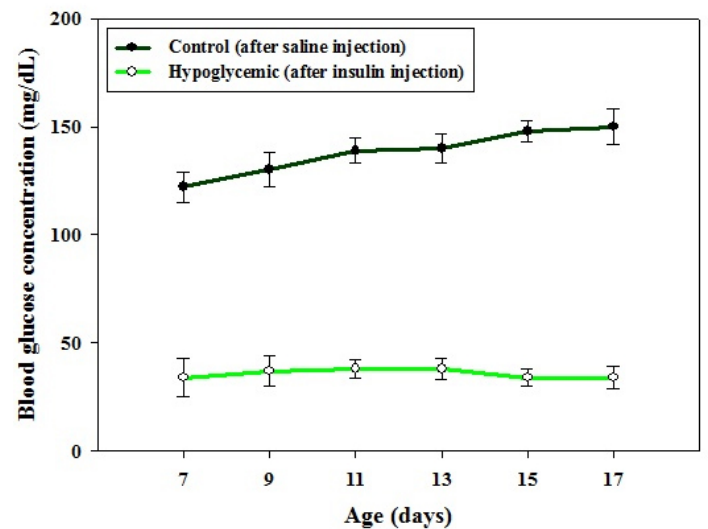
Statistical Analysis

The equality of all the groups was tested by the analysis of variance (ANOVA) technique for different values of p. Further the pair wise comparisons of all the experimental groups were studied using Students-Newman-Keuls test at different significance levels. The testing was performed using GraphPad Instat (Ver. 2.04a, San Diego, USA) computer program.

Results

Blood glucose concentration in control and experimental group

Hypoglycemia was confirmed by reading the blood glucose level after induction. The blood glucose concentration during postnatal period from P7 to P17 varies significantly with glucose concentration reaching towards hypoglycemic range by P13 (50.0±5 before insulin injection; 38±5 after insulin injection). By P17, the rats overcome the endocrine regulation and become hypoglycemic with a glucose concentration of 38.0±2 before insulin injection; 34.0±5 after insulin injection. (Figure-1). In control rats, the blood glucose level from P7 to P17 varies significantly which is in accordance with the usual glucose level variation observed during the initial post natal period. In hypoglycemic group, the glucose concentration remained <40 mg/dL from P7 to P17 after insulin injection even though hypoglycemia was reached by P13.



Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.

Figure 1: Blood glucose level from postnatal day 7 to postnatal day 17 in control and hypoglycemic rats: Control rats showed normal blood glucose level where as the experimental group become hypoglycemic by postnatal day 13.

Receptor analysis for muscarinic receptors in the cerebellum of control and experimental rats

Scatchard analysis of [3H] QNB binding against atropine to total muscarinic receptors in the cerebellum of one month old rats exposed to neonatal hypoglycemia showed a significant decrease ($p < 0.01$) in Bmax and Kd compared to control. The decreased Bmax indicates a significant reduction in muscarinic receptor number in the cerebellum of hypoglycemic group. (Table-1, Figure- 2).

Table 1: Scatchard analysis of muscarinic receptor subtypes in the cerebellum

Experimental groups	Total muscarinic receptor	
	Bmax (fmol/mg protein)	Kd (nM)
Control	168.55 ± 5.50	7.30 ± 0.50
Hypoglycemic	130.50 ± 4.00 ^a	7.22 ± 0.65

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.

^a $p < 0.001$ when compared to control.

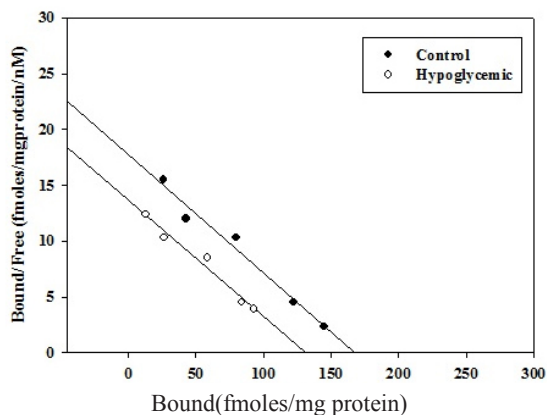
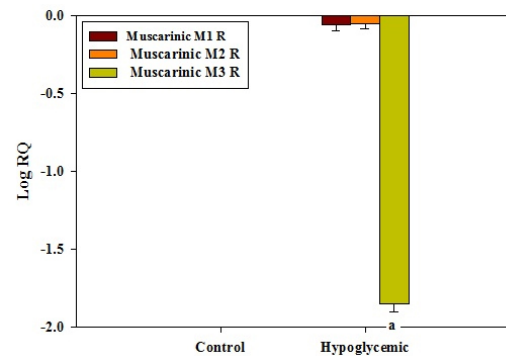


Figure 2: Representative Scatchard plot for linear regression analysis of total muscarinic receptors in the cerebellum. Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4 - 6 rats.

Real Time PCR analysis of muscarinic M1, M2 and M3 receptor subunits in the cerebellum Real Time PCR analysis showed that muscarinic M3 receptor subunit mRNA was significantly down regulated ($p < 0.001$) in the cerebellum of one month old rats exposed to neonatal hypoglycemia compared to control. Muscarinic M1 and M2 receptor mRNA showed no significant change when compared to control. This indicates that the decreased muscarinic receptor function in the cerebellum of one month old rats exposed to neonatal hypoglycemia can be mainly attributed by muscarinic M3 receptor subtype. (Figure- 3).



Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. ^a $p < 0.001$ when compared to control.

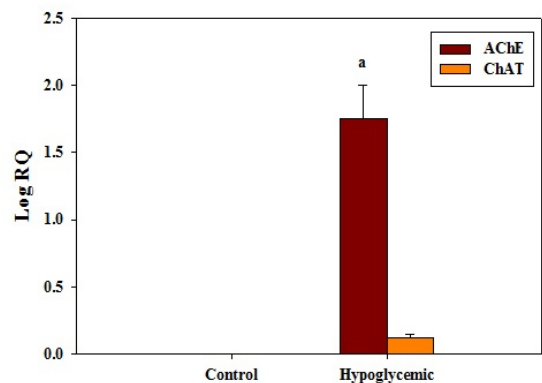
Figure 3: Gene expression of muscarinic M1, M2 and M3 receptor subtypes were done in the cerebellum using Real Time PCR analysis. The $\Delta\Delta\text{CT}$ method of relative quantification was used to determine the fold change in expression. PCR analyses were conducted with gene-specific primers and fluorescently labeled Taq probe Rn 00589936_s1 muscarinic M1 receptor, Rn 02532311_s1 muscarinic M2 receptor and Rn 00560986_s1 muscarinic M3 receptor.

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4 - 6 rats.

^a $p < 0.001$ when compared to Control.

Real Time PCR analysis of cholinergic markers - acetyl choline esterase and choline acetyl transferase

The gene expression of acetyl choline esterase showed a significant up regulation ($p < 0.001$) with no significant change in the expression of choline acetyl transferase in the cerebellum of hypoglycemic group compared to control (Figure- 4). This is an indication of synthesis of more acetyl choline esterase, the rate limiting enzyme in the catabolic pathway of acetyl choline, thereby increasing its breakdown.



Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. ^a $p < 0.001$ when compared to control.

Figure 4: Gene expression of acetyl choline esterase and choline acetyl transferase were done in the cerebellum and $\Delta\Delta\text{CT}$ method of relative quantification was used to determine the fold change in expression. PCR analyses were conducted with gene-specific primers and fluorescently labeled Taq probe Rn 00596883_m1

acetyl choline esterase and Rn 01453446_m1 choline acetyl transferase.

Values are Mean \pm S.E.M of 4-6 separate experiments. Each group consist 4 - 6 rats.

a $p < 0.001$ when compared to Control

Discussion

We observed that the control rats showed normal blood glucose levels consistent to its developmental periods. In hypoglycemic rats, the glucose level reached hypoglycemic range by postnatal day 13 (P13). Glucose-dependent insulin secretion is modulated by several neurotransmitters released from peripheral autonomic nerves [15-17]. The major neurotransmitter of the peripheral parasympathetic nervous system, acetylcholine, is known to facilitate the release of insulin in a glucose-dependent fashion [16,17]. This activity has been shown to be mediated by activation of muscarinic acetylcholine receptors located on the pancreatic β cells [15-17]. The data from the current study showed a significant decrease in muscarinic acetyl choline receptor density in the cerebellum of one month old rats exposed to neonatal hypoglycaemia. The gene expression studies pointed that the reduction might be mainly mediated via decreased muscarinic M3 receptor subtype.

Earlier studies from our laboratory reported alteration in muscarinic receptor function in both neonatal and adult hypoglycemia in the cerebral cortex [9]. We observed a significant decrease in total muscarinic receptor density in the cerebellum of one month old rats exposed to neonatal hypoglycemia. The gene expression of muscarinic M3 receptor subtype showed a significant decrease with alterations in the key metabolic enzymes in cholinergic pathway. Reduction in muscarinic receptors was reported under hypoglycaemic and hyperglycaemic situations, which contributes to defective insulin signalling, neurodegenerative and cardiovascular complications [18-20].

The overall decrease in metabotropic cholinergic receptors in one month old rats signifies the impact of an early life hypoglycemic shock in the brain function and insulin signalling pathways. Parasympathetic stimulation via muscarinic receptors of pancreatic islets augments glucose-stimulated insulin secretion [19]. Phosphorylation of the M3-muscarinic receptor plays an important mechanistic role in facilitating insulin release from pancreatic islets and M3- receptor has been identified as the bona fide receptor responsible for the cholinergic regulation of glucose-induced insulin release [21]. The reduction in this receptor subtype gene expression observed in our study might have very significant role in regulating the overall muscarinic receptor function and glucose homeostasis in hypoglycemic condition.

AChE plays a very important role in the ACh-cycle, including the release of ACh [22]. The duration of action of ACh at the synaptic clefts is critically dependent on AChE activity [23]. We observed elevated AChE mRNA expression in the cerebellum of one month old rats exposed to neonatal hypoglycemia, indicating the disruption in acetyl choline catabolism. The decreased cholinergic function mainly mediated through muscarinic M3 receptor subtype observed in one month old rats exposed to neonatal hypoglycemia lead to altered glucose homeostasis and insulin signaling, which can trigger the early onset of conditions like diabetes. The regulation of muscarinic function at a later stage of life due to

neonatal hypoglycemia need to be considered seriously due to the significance of brain muscarinic receptors in glucose regulation and other cognitive functions.

Conclusion

We focused on understanding the cholinergic muscarinic receptor regulation in one month old rats exposed to neonatal hypoglycemia in the current study. We observed a reduction in overall muscarinic receptor density with a significant decrease in M3 receptor subtype in one month old rats exposed to neonatal hypoglycemia. This alteration is coupled with increased expression of rate limiting enzyme in acetyl choline degradation – acetyl choline esterase, without any change in its anabolic rate limiting enzyme, choline acetyl transferase. This signifies a faster degradation of the acetyl choline from the synapse with no compensation by its increased synthesis. The reduction in the cholinergic receptors with the lesser ACh can negatively affect the glucose regulation and other higher functions. Targeting these pathways at different levels can be exploited to devise better treatment for neonatal stress management and also of diseases with impaired insulin secretion such as diabetes.

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Conflict Of Interest Disclosure

The authors have no conflicts of interest.

Authors' Contributions

The experiment was carried out and manuscript was prepared by Anju T R. Anju T R and Joy K P are involved in experimental design and editing the manuscript.

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