Synopsis of the thesis on

Cellular and Molecular Aspects of Insulin Resistance in Brain

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Introduction

Insulin is a peptide hormone secreted from pancreas which crosses the blood brain barrier (BBB) through receptor mediated trans-cytosis to bind to its cognate receptors which are widely but unevenly distributed throughout the brain regions (Havrankova *et al.*, 1978). The orchestrated interplay of insulin signaling with that of other neural circuits is vital for the central regulation of several brain functions (Porte *et al.*, 2005). The most investigated action of brain insulin signaling is the hypothalamic appetite regulation in response to nutritional and peripheral signals by its crosstalk with neuropeptides and neurotransmitters (Sato *et al.*, 2005). Also, there is substantial amount of immuno-reactive insulin and INSR in the strategic areas for cognition as well as motor skills where it acts as neuroprotector and neuromodulator (Rhoads *et al.*, 1984; Boyd *et al.*, 1985; Lozovsky *et al.*, 1985; Shuaib *et al.*, 1995).

Apart from region specific actions, insulin exerts varied cellular responses in different brain cells (Marks *et al.*, 1990; Wozniak *et al.*, 1993). The initial focus of the neurobiologists has been neuronal insulin signaling which is known to promote survival as well as neurotransmission, but lately it has been established that other brain cells such as astrocytes, oligodendrocytes as well as resident neural stem cells (NSCs) are also depended on insulin (van der Pal *et al.*, 1988; Muhic *et al.*, 2015). Neurons are the units of brain function; and since neuronal regeneration as well as function are dependent on NSCs and astrocytes respectively; the modulation of fate of these cells in response to insulin needs a special attention and can help to elucidate brain function in healthy and diseased conditions.

Concluding that, since brain insulin signaling is very specific to region, cell type as well as the micro environment; its alteration can have undesirable neurological outcomes. Deregulation of insulin signaling culminating into insulin resistance is the key feature of diabetes (Reaven, 1988). Interestingly, diabetes has also been associated with mild to moderate alterations in brain functions. There are several mechanistic explanations evolved till date to explain diabetes induced neurological dysfunctions (Duarte, 2015). An increasing number of epidemiological and experimental investigations have demonstrated a condition of insulin resistance in brain (also termed as TYPE3 diabetes) that is prevalent not only in diabetic conditions but also in stress disorders, Alzheimer's disease, Parkinson's diseases etc (Aviles-Olmos *et al.*, 2013; Willette *et al.*, 2015). However, there still remain several inconclusive discrepancies regarding the co- occurrence of diabetes and brain insulin resistance accounting

for the reasons such as factors causing diabetes, its duration as well as the age of onset. Chronic stress resulting in elevated levels of glucocorticoid (GCs) is one among several reasons accounting for the etiology of diabetes (Rafacho *et al.*, 2014). GCs are known to oppose insulin action which is well confirmed in the cells of peripheral tissues; but there are very few studies involving GC induced insulin dysfunction in brain. Since, many of the neurological consequences observed in diabetes are strikingly similar to those observed following chronic stress suggesting that glucocorticoids (GC) may be the common mechanistic mediators for the onset of brain insulin resistance. However, the exact contribution of insulin resistance on brain functions is yet to be determined.

Hypothesis: The defects in brain insulin signaling, also known as brain insulin resistance are manifested at cellular, regional, physiological as well as psychological level. There always has been a pathological co-relationship between brain dysfunctions to that of diabetes and stress conditions (=elevated level of glucocorticoids). However, the exact mechanistic phenomenon linking diabetes and stress to that of brain insulin resistance is not clearly understood. Hence, we hypothesized that glucocorticoid induced diabetes could lead to brain insulin resistance. Also, there might be adverse effect of insulin resistance on NSCs and astrocytes which are the key players involved in proper brain functioning.

Significance: Understanding the mechanism underlying the link between brain insulin resistance and peripheral insulin resistance (or diabetes) can aid in designing therapeutic strategies to overcome several neurodegenerative diseases. Also, identification of cellular players involved in brain insulin resistance can help in targeting the brain cells for regeneration therapy for replacing the lost neural cells under diseased condition and for modulation of the brain micro-environment.

Specific Objectives:

1] Regional and neurobehavioral study of brain insulin resistance in glucocorticoid induced diabetic rat model.

2] To elucidate the role of insulin resistance and glucocorticoid on metabolism of astrocytes: An *in vitro* study.

3] To elucidate the role of insulin resistance and glucocorticoid on the fate of NSCs: An *in vitro* study.

Objectives 1: Regional and neurobehavioral study of brain insulin resistance in glucocorticoid induced diabetic rat model.

a) Assessment of region specific alterations in insulin signaling in glucocorticoid induced diabetic rat model and its correlation to neurobehavior.

Glucocorticoid induced diabetes was established by injecting dexamethasone (dexa), a synthetic glucocorticoid (3mg/kg b.w./day for 28 days) in Female Charles Foster rats (Belani *et al.*, 2014). The development of diabetes was confirmed by Fasting Insulin Resistance Index (FIRI) and were further analysed for a battery of different neurobehavioral tests as well as regional assessment of brain insulin signaling; and compared to that of control rats (Normal saline injected).

The results demonstrated that dexa treatment did not display any alteration in hippocampal insulin signaling as well as its function of memory formation and exploratory behavior. Likewise, there was no change in expression of insulin signaling in cortex of dexa group; however cortical motor functions were compromised in dexa rats as demonstrated in grip strength test and gait analysis. These motor dysfunctions can be attributed to the other factors such as hyperglycemia prevalent during dexa induced diabetic condition. There was also no change in the cerebellar insulin signaling as well as performance of dexa rats in balance beam test. However, strikingly decreased hypothalamic insulin signaling was observed in dexa treated rats as compared to control rats signifying that hypothalamus is the prime region for onset of dexamethasone induced brain insulin resistance. Furthermore, hypothalamic insulin resistance can be accounted for depression as well as appetite change in dexa rats as compared to control. Thus, concluding that there was a region specific alteration in dexamethasone induced diabetic model.

The appropriate mechanism involving the role of hypothalamic insulin resistance on appetite loss in dexa induced diabetic model is not well understood. Since appetite regulation is mediated by the cross talk of insulin signaling to that of other neural circuit such as neuropeptides, neurotransmitters, nutrient sensors etc., these candidate players were assessed in hypothalamus after dexa treatment. The results demonstrated that the gene expression of *Obrb* which relays the signal of leptin to modulate the expression of neuropeptides remained unchanged because of dexa treatment. However, there was a remarkable upregulation of orexic signals -*Agrp* and *Npy* with downregulation of the anorexic signals- *Pomc* and *Mc4r*.

Besides neuropeptides, different lines of investigations suggested that disturbances of eating behavior are associated with hypothalamic neurotransmission as well as nutrient sensors. Thus neurotransmitters were estimated and a significant decrease was obtained in the levels of appetite stimulating neurotransmitters such as glutamate, GABA and dopamine. Also significant reduction was observed in the expression of glutamate transporter 1. Hypothalamic insulin resistance and hyperglycemia resulted in remarkable reduction of glucose sensors - GLUT1 as well as in SirT1 in dexa rats. Absence of brain PPARγ results in an increase in energy expenditure and a decrease in food consumption even when fed with high fat diet and thus a drastic decrease in PPARγ expression observed in present study justified the reduction in food intake and weight loss in this model. Also dexa mediated hypothalamic insulin resistance resulted in reduced brain glycogen stores thereby further disrupting metabolic and neurotransmitter homeostasis.

Extrapolating the results from current model, it can be postulated that the appetite and weight loss observed during stress as well as during diabetes is because of the multifaceted interaction of hypothalamic insulin signaling, glucocorticoid levels, appetite regulating neuropeptides and neurotransmitters.

b) Impact of glucocorticoid induced maternal insulin resistance on hypothalamic appetite regulating circuitry of the neonatal brain.

The interwoven projections of hypothalamic appetite regulation are laid as early as at the embryonic stage and thus, the study of effect of maternal insulin resistance caused by glucocorticoid (dexamethasone) on the circuitry of appetite regulation in foetus hypothalamus was assessed. It was seen that there was no significant change in the physiological and biochemical parameters of the pups born to dexa dams as compared to the control dams. However, a sexual dimorphic change was seen in the expression of insulin signaling proteins and neuropeptide genes in the hypothalamus of male and female pups. There was an insulin resistance like condition in female pups born to dexa dams with down regulation of *Pomc* and *Mc4r* genes. However, in case of dexa born male pups there was no prominent change in insulin signaling proteins but there was up regulation of *Pomc* and *Mc4r* genes. These results concluded that the intrauterine conditions of the insulin resistant mothers programmed the appetite regulation in brain of male and female pups differently.

Objective 2: To elucidate the role of glucocorticoids and insulin resistance on metabolism of astrocytes: An *in vitro* study.

Primary cultures of astrocytes were prepared from the post-natal day (PND) 0–2 forebrains of neonatal rats as per the standard protocol (McCarthy & de Vellis, 1980). These cells were characterized for astrocytic markers and more than 95% of the cells obtained were positive for GFAP and vimentin while negative for neuronal marker MAP2 and oligodendrocytic marker O1.

a) Impact of insulin resistance on astrocyte metabolism.

Primary cultures of astrocytes were treated with PI3K inhibitor- wortmannin which resulted in decreased expression of p-Akt confirming the interference of the insulin signaling pathway. Inhibition of insulin stimulated PI3K pathway lead to a significant decrease in the expression of GFAP, an intermediate filament responsible for astrocytic morphology as well as reduced the GLUT 1 as well as glycogen content in primary astrocytes. There were prominent alterations in the gene expression of Astrocytic Neuronal Lactate Shuttle (ANLS) transporter proteins– GLAST and GLT 1 as well as the lactate transporters – MCT 1 and MCT 4.

To further confirm the effect of insulin resistance, insulin receptor gene was silenced in primary astrocyte cultures using lipofectamine mediated shRNA transfection method. These cells were cultured under the selection pressure of hygromycin for 10 days and harvested for further analysis. The immunoblotting of INSR confirmed that the transfection efficiency was ~ 60 %. There was a significant reduction in the viability of the INSR knock-down (KD) cells as assayed by MTT when compared to control cells. Also there was a drastic reduction in the number of cells present in the synthesis phase as demonstrated by cell cycle analysis. Hampering of insulin signaling resulted in the reduced expression of GFAP, GLUT1 as well as glycogen content. Although there was no change in the lactate secretion in INSR KD cells as compared to control cells, but when induced with glutamate to mimic the condition of excitotoxicity, there was a decrease in the lactate levels in conditioned media of INSR KD astrocytes as compared to control. Further gene expression of ANLS glutamate and lactate transporters need to be assessed in INSR KD cells.

Thus, *Insr* gene silencing provides direct evidence that insulin signaling plays a noteworthy role in the regulation of astrocytic metabolism.

b) Impact of glucocorticoid on insulin signaling of astrocytes.

After the confirmation of role of insulin signaling on astrocyte metabolism, the aim was to establish if glucocorticoid can interfere with insulin signaling in these cells. Thus primary astrocyte cultures were treated with the glucocorticoid receptor (GR) -selective synthetic agonist dexamethasone (1 μM) as well as GR receptor antagonist – RU486 and then were induced with insulin. Protein expression of candidate insulin signaling proteins were studied and the results demonstrated that there was significant reduction in the downstream signaling protein expression of PI3K and activated AKT in the dexamethasone pre-treated astrocytes induced with insulin as compared to untreated control astrocytes. This reduced insulin signaling because of dexamethasone was restored to control levels when they were pre-treated with glucocorticoid receptor agonist (RU486). Since activated AKT can lead to phosphorylation of GSK 3β and thus resulting in its inactivation; decreased phosphorylation of GSK was evident in the groups where astrocytes were pre-treated with dexamethasone along with a decrease in glycogen content which could be restored upon treatment with RU486. One of the important functions of astrocytes is the glutamate uptake and lactate release at the synapses to protect the neurons from excitotoxicity and there were alterations in gene expression of glutamate and lactate transporters when pre-treated with dexamethasone. Thus, glucocorticoids can impair insulin signaling in astrocytes, thereby affecting its metabolism which in turn will have negative consequences on neuronal function.

Objective 3: To elucidate the role of glucocorticoids and insulin resistance on the fate of NSCs: An *in vitro* study.

Neural stem cells (NSCs) were isolated from forebrain of PND 0 rat pup as per the protocol by Pacey KK et al., 2006. The cells were cultured in non-tissue culture treated plates in the form of neurospheres. The clonogenic property of NSCs i.e. formation of neurosphere from single cell was assessed during passaging. Characterization of NSCs was done using immunocytochemistry where these cells were positive for classical neural stem cell marker - nestin and CD133. Characterized NSCs were assessed for purity using anti-nestin antibody by flow cytometric analysis where more than 70 % of the cells were nestin positive. Multipotency of NSCs to differentiate into oligodendrocytes, astrocytes and neurons was studied using specific differentiation media. These NSCs successfully differentiated as was

confirmed by the presence of specific cell markers for oligodendrocytes, astrocytes and neurons by immuno staining against O1, GFAP and MAP2 respectively.

a) Exploring the role of insulin in determination of NSC fate.

The aim of this objective was to determine the role of insulin action in influencing the neural stem cell fate. NSCs were incubated with different concentrations of insulin and assayed for viability using MTT assay. Further these cells were allowed to differentiate into astrocytes, oligodendrocytes and neurons by withdrawal of mitotic growth factors (EGF and bFGF) from NSC media in presence of varying insulin concentration. Changes in insulin signaling and the percentage of all the three differentiated cells types were monitored and were compared with that of control cells differentiated in presence of optimum amount of insulin. The results suggested that concentration of insulin affected NSC survival. The optimum and high concentration of insulin was essential for NSC maintenance (as shown by nestin) and neurogenesis (as shown by MAP2). However, astrocytes differentiation was favoured in low insulin concentration (as shown by GFAP). These results were also confirmed using cytometric analysis and the result indicated decreased number of GFAP positive cells (=astrocytes) with increased concentration of insulin. Thus, it indicated that insulin levels regulate neural stem cell plasticity *in vitro*.

b) Impact of insulin resistance on NSCs.

To further confirm the effect of insulin resistance, plasmid constructs having shRNA against insulin receptor with hygromycin resistance gene was transfected in NSCs. The knock down efficiency was more than 60%. There was a remarkable decrease in the cell survival as well as proliferation after *Insr* gene KD. The INSR KD NSCs as well as control NSCs were differentiated into astrocytes, oligodendrocytes and neurons. The expression of candidate markers for stem cell maintenance, lineage determination, differentiated neural cells (viz. neurons, astrocytes and oligodendrocytes) as well as metabolism was assessed using TaqMan real time analysis before and after differentiation of NSCs. The results demonstrated alteration in the key genes involved in neurogenic as well as astrogenic differentiation along with alteration in the expression of important metabolic genes.

Thus, insulin resistance in NSCs can have fatal effects on survival, proliferation as well as differentiation fate, and thus can exacerbate neurodegeneration in brain.

c) Impact of glucocorticoid on insulin signaling in NSCs.

To establish the effect of glucocorticoid (GR) on insulin signaling in NSCs *in vitro*, primary stem cell cultures were treated with the GR-selective synthetic agonist dexamethasone (1 μM) as well as GR receptor antagonist – RU486 and then were induced with insulin. Protein expression of candidate insulin signaling proteins were studied and the results demonstrated that there was a reduction in the downstream signaling protein expression of PI3 kinase and activated AKT in the dexamethasone pre-treated NSCs induced with insulin as compared to untreated control NSCs. This reduced insulin signaling because of dexamethasone was restored to control levels when they were pre-treated with glucocorticoid receptor agonist (RU486).

Conclusion

- There exists a regional difference in the onset of TYPE 3 diabetes/ Brain insulin resistance and its corresponding behavioral functions in response to glucocorticoid induced diabetes.
- The prime target of glucocorticoid induced brain insulin resistance is the hypothalamus leading to depression and appetite change.
- The interactions of hypothalamic insulin resistance with that of other neural circuits is responsible for the reduction in appetite in glucocorticoid induced diabetic model.
- There is a sexual dimorphic modification in hypothalamic appetite regulation in response to maternal insulin resistance induced by glucocorticoid.
- Insulin resistance can alter the regulation of astrocytic metabolism which in turn can disrupt lactate shuttling to neurons.
- Insulin resistance modulates the fate of neural stem cell survival, proliferation and differentiation.

Thus, overall concluding that glucocorticoid (stress) apart from inducing diabetes can extend its effect to central nervous system resulting in brain insulin resistance. This study can be extrapolated to understand the unexplained mood disorders as well as appetite change because of stress and diabetes. Since insulin signaling is indispensable for astrocytes and neural stem cells; cellular targeting of these cells to ameliorate insulin resistance can be used as therapeutic interventions for neuro-degenerating diseases.

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