CHAPTER 6

TO ELUCIDATE THE ROLE OF INSULIN RESISTANCE AND GLUCOCORTICOID ON THE FATE OF NSCs: AN *IN VITRO* STUDY

TO ELUCIDATE THE ROLE OF INSULIN RESISTANCE AND GLUCOCORTICOID ON THE FATE OF NSCS: AN IN VITRO STUDY

Neural stem cells are the multipotent cells with self-renewal capacity in brain (Gage and Temple, 2013). The multipotentiality of NSCs is defined by its competence for differentiation into three main cell types of brain namely neurons, astrocytes and oligodendrocytes. During embryonic period, neural stem cells receive positional and temporal information generated from gradient of signaling molecules (such as shh, BMP, noggin, FGFs, etc.) for patterning of anterior-posterior and dorsal-ventral axes of brain (Urban and Guillemot, 2014). These cells undergo asymmetric cell divisions influenced by neurogenic wave followed by gliogenic wave. Thus, neurogenesis (formation of neurons) is an early embryonic event, while gliogenesis (formation of glia) begins during late embryogenesis and continues even during postnatal stages at low rate. As the development advances, the production of restricted progenitors and differentiated cells leads to decline in the population of NSCs rendering postnatal brain with a small pool of bona fide NSCs. In postnatal brain, two potential neurogenic niche with active neurogenesis has been identified, namely subventricular zone (SVZ) near lateral ventricle and subgranular zone (SGZ) of hippocampus (Stolp and Molnar, 2015). Apart from these two niches, non-neurogenic areas such as hypothalamus, cortex, olfactory lobe, and so on have also demonstrated to have resident neural stem/progenitor cells that remain quiescent throughout the adult brain (Lie *et al.*, 2002; Chipperfield *et al.*, 2005). The functional outcome of adult neurogenesis especially that of hippocampus has been linked to learning and memory, raising the possibility for participation of new neurons in the formation or integration of new memories (Aimone *et al.*, 2014). Also, environmental changes have shown to directly affect adult neurogenesis implying two-way relationship between the brain and behaviour (Yau *et al.*, 2015; Wakabayashi *et al.*, 2016).

NSCs have been characterized with specific cellular markers for identification. These cells are known to express Nestin and GFAP- an intermediate protein filament, CD133- a transmembrane cell surface protein, Sox2 - a transcription factor of Sox family, LeX - a glycan motif, PCNA and Ki67- proliferating marker (Zhang and Jiao, 2015). Recent notion also claims that astrocytic parenchymal cells can have the potential to reacquire

stem cell traits to contribute to de novo neurogenesis following injury (Gonzalez-Perez and Quinones-Hinojosa, 2012). Differentiation of these NSCs and their commitment to a specific lineage can be assessed by signature cellular markers as shown in the Fig 6.1.

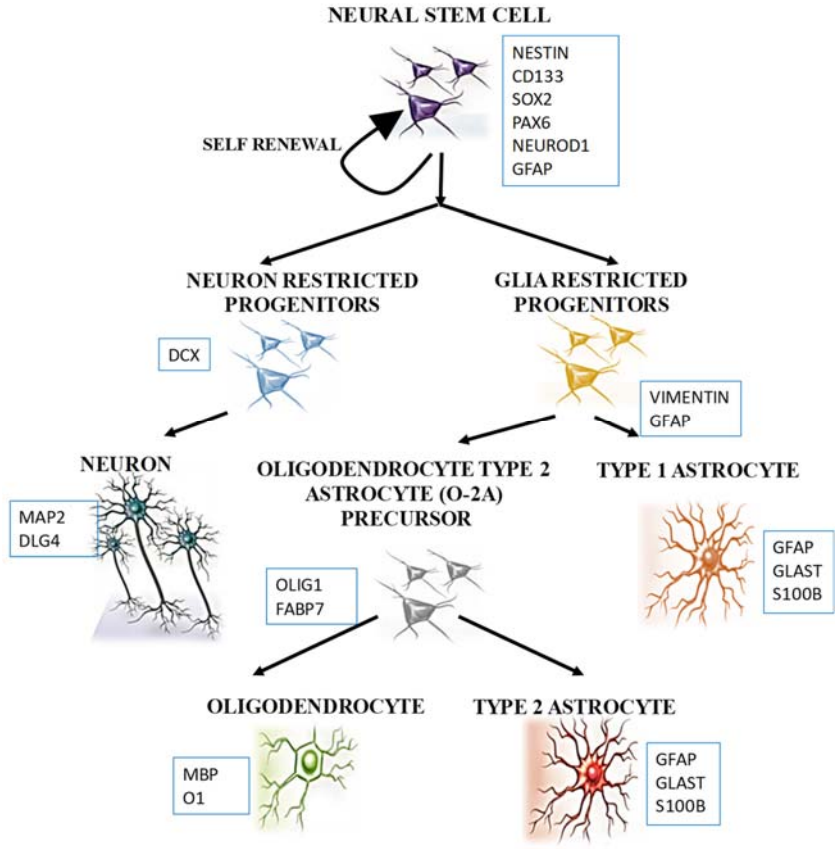


Figure 6. 1 Expression of candidate cellular markers during NSC differentiation.

Insulin is amongst the peripheral peptide hormone that influences NSC fate. Its interaction with several other signaling pathways (EGF, Notch, Wnt) are known to balance NSCs quiescence, activation as well as differentiation phase in the adult nervous system. It stimulates proliferation in embryonic rat neurospheres *in vitro* even in the absence of EGF and bFGF (Erickson *et al.*, 2008). Also, insulin withdrawal from hippocampal NSCs resulted in autophagic cell death (Yu *et al.*, 2008; Baek *et al.*, 2009; Ha *et al.*, 2017). In human as well as in rat brain, intensity of insulin receptor is high in neurogenic niche such as SVZ and hippocampus (Fernandez and Torres-Aleman, 2012; Heni *et al.*, 2015). Thus, these *in vitro* studies marked insulin as a cardinal factor for the survival and self-renewal of NSCs. Correspondingly in diseases such as diabetes (both T1DM and T2DM), alteration in the insulin level demonstrated significantly compromised neurogenesis (Beauquis *et al.*, 2008; Yi *et al.*, 2009). Similarly, in obese high fat diabetic model, high fat diet accelerated impairment in hippocampal

neurogenesis (Lindqvist *et al.*, 2006; Purkayastha and Cai, 2013). This observation has been parallelly made in human subjects, where diabetics show declined memory functions, an attribute of dysfunctional neurogenesis (Saedi *et al.*, 2016). Thus, conceptualizing that it might be insulin resistance in these stem cells that contribute to alteration in its fate. In order to explore this phenomenon *in vitro, Insr* gene silencing was attempted to mimic insulin resistance in NSCs.

Apart from insulin, one of the contributing factor for NSC regulation is glucocorticoids (GCs). Synthetic GC (dexamethasone) treated adult NSCs showed impaired differentiation towards the neuronal phenotype, whereas corticosterone-treated mouse hippocampal NSCs were driven toward oligodendrogenesis at the expense of neurogenesis (Chetty *et al.*, 2014). The inhibitory role of glucocorticoids on NSCs can be established from the adrenalectomy model where lack of GCs increased proliferation (Chen *et al.*, 2000; de Celis *et al.*, 2016). Long-term exposure to high levels of corticosterone is known to disrupt learning in animals (Oitzl *et al.*, 1998) while maintaining physiologic levels of corticosterone is shown to enhance neurogenesis leading to restoration of LTP and reversal of learning deficits in type-2 diabetic mice (Stranahan *et al.*, 2008).

Elevated GC levels can lead to diabetes by induction of insulin resistance in several peripheral cell types. Our *in vivo* results clearly demonstrated that dexamethasone induced diabetic rat model had reduced neural stem cell pool in hippocampus. Also, from our second objective, it is clearly evident that insulin signaling is also impaired in astrocytes because of glucocorticoid exposure. Hence, we further wanted to speculate if the ill effect of glucocorticoid on NSCs is because of induction of insulin insensitivity in these cells. Therefore, involvement of insulin and glucocorticoid in modulating NSC fate of survival and the balance between neurogenesis and gliogenesis may present a new perspective for neuro-restoration, especially in diseases such as diabetes, stress, Alzheimer's, etc.

Thus, the key questions of this objective were:

1) Does insulin concentration regulate survival and differentiation in postnatal NSCs?

2) Does shutting down insulin signaling by *Insr* knockdown in NSCs affect its fate and differentiation?

2) Does glucocorticoid induce insulin resistance in NSCs as that in peripheral cells?

6.1 EXPLORING THE ROLE OF INSULIN IN DETERMINATION OF NSC FATE.

6.1.1 PLAN OF WORK

Primary neurosphere cultures of neural stem cells were prepared from the postnatal day (PND) 0 forebrains (including hippocampus) of neonatal rats as per the protocol BY Pacey *et al.,* 2006. After third passage, cells were characterized for stem cell markers using immunostaining and flow cytometry. Simultaneously, cells were assessed for multipotency by its differentiation into astrocytes, neurons and oligodendrocytes using specific differentiation media. Initial 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 [(no insulin, 0.04μM insulin (low insulin), 0.22 μM insulin (optimum insulin) and 4.3 μM insulin (high insulin)] and assayed for viability using MTT assay. The doses were adapted from Rhee et al where they clearly demonstrated stated the optimum dose of insulin for rat NSCs (Rhee *et al.*, 2013). Further these cells were allowed to spontaneously 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 plan of work is as shown in Fig 6.2.

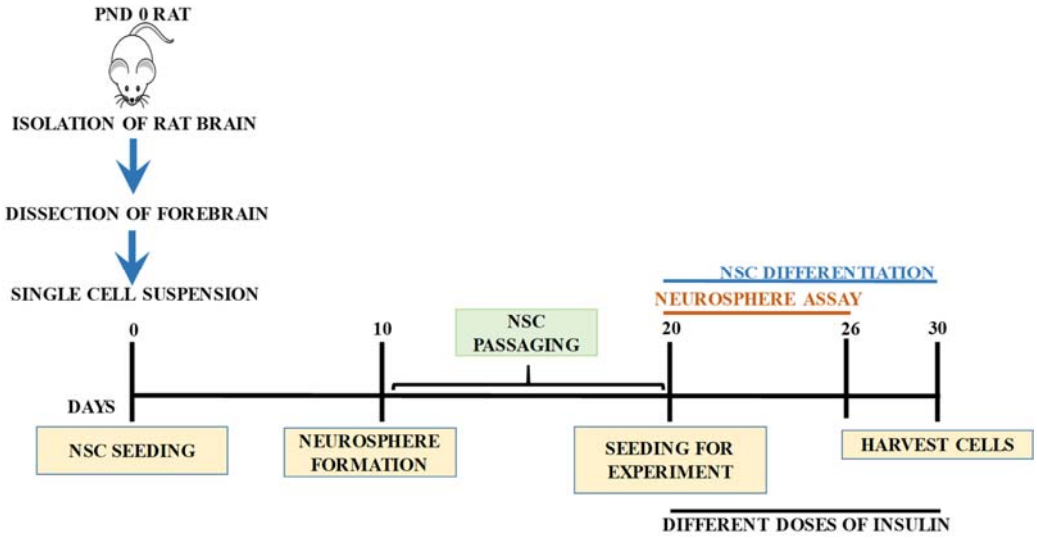


Figure 6. 2 Plan of work for dose dependent effect of insulin on NSC fate.

6.2.2 RESULTS

CHARACTERIZATION OF NEURAL STEM CELLS

Neural stem cells (NSCs) were successfully isolated from forebrain of PND 0 rat brain and cultured in the form of neurospheres. The clonogenic property of NSCs i.e. formation of neurosphere from single cell was assessed during passaging as shown in figure 6.3A. Immunostaining with CD133 and GFAP revealed that isolated cells were positive for both the stem cell markers as shown in Fig 6.3B, further confirming that isolated cells were NSCs. Also, flow cytometric analysis affirmed the purity of NSCs to be around 98% as shown in Fig 6.3C.

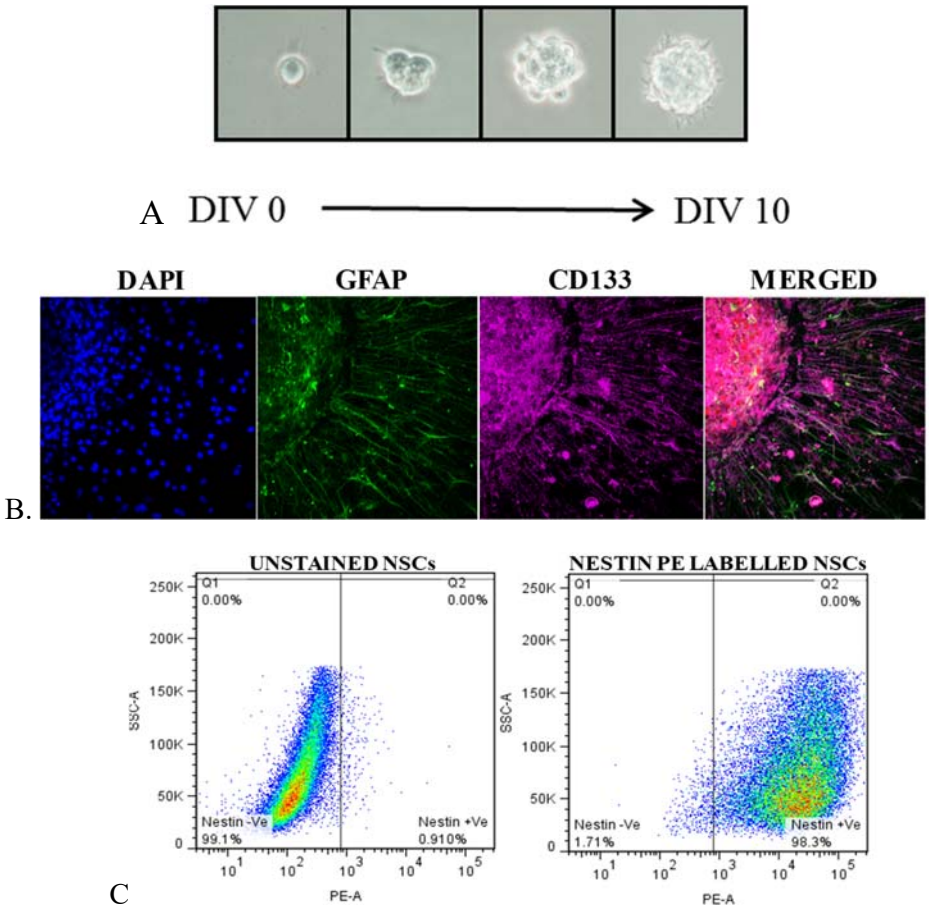


Figure 6. 3 A. Representative phase contrast image of formation of neurosphere. B. Immunostaining of neurospheres with GFAP (as shown in green) and CD133 (as shown in pink). Dapi was used to counter stain nucleus. C. Flow cytometric analysis of NSC with PE labelled nestin.

Multipotency of NSCs to differentiate into oligodendrocytes, astrocytes and neurons was studied by subjecting NSCs to specific differentiation media for 10 days. The media for Oligodendrocyte differentiation was neurobasal media with B27 supplement, glutamine and T3; for astrocyte differentiation, the media was DMEMF12 with N2

supplement, glutamine and 1% serum while for that of neuronal differentiation, the media used was neurobasal media with B27 supplement and glutamine. Differentiation was confirmed by the presence of specific cell markers for oligodendrocytes, astrocytes and neurons by immunostaining against O1, GFAP and MAP2 respectively as shown in Fig 6.4.

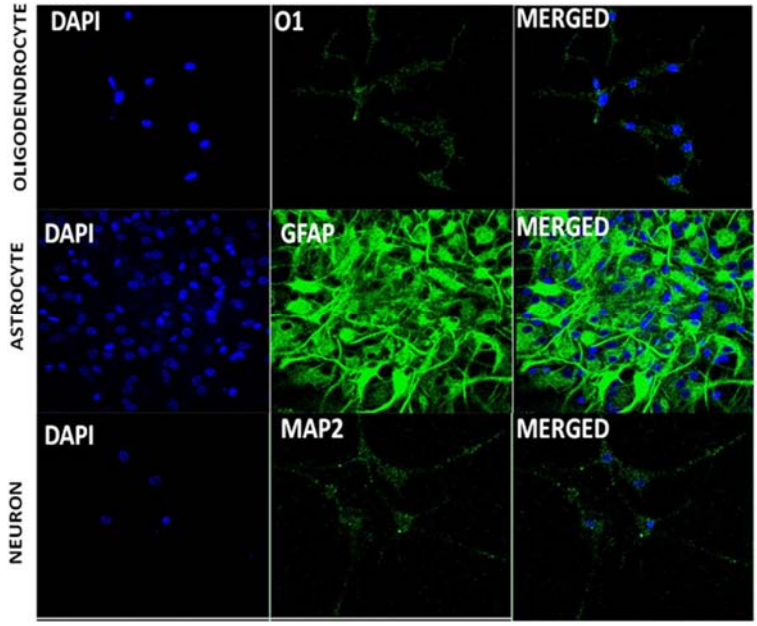


Figure 6. 4 NSCs were differentiated into different types of neural cells – oligodendrocytes (as shown by O1-green), astrocytes (as shown by GFAP-green) and neurons (as shown by MAP2-green) using specific differentiation media.

SURVIVAL AND DIFFERENTIATION WAS AFFECTED BY INSULIN IN DOSE DEPENDENT MANNER

To assess the role of different concentration of insulin on NSC survival, they were incubated with different concentrations of insulin, and assayed for viability using MTT assay. Results demonstrated that incubation for long time with both low as well as high insulin concentration affected survival in NSCs as shown in Fig 6.5. However, for 24 hours, not much difference was observed. Also, morphometric analysis of neurosphere cloned from single NSC for 6 days demonstrated that 0.22 and 4.3uM concentration had significantly higher number of larger neurospheres as compared to 0 and 0.04 uM insulin as shown in Fig 6.6.

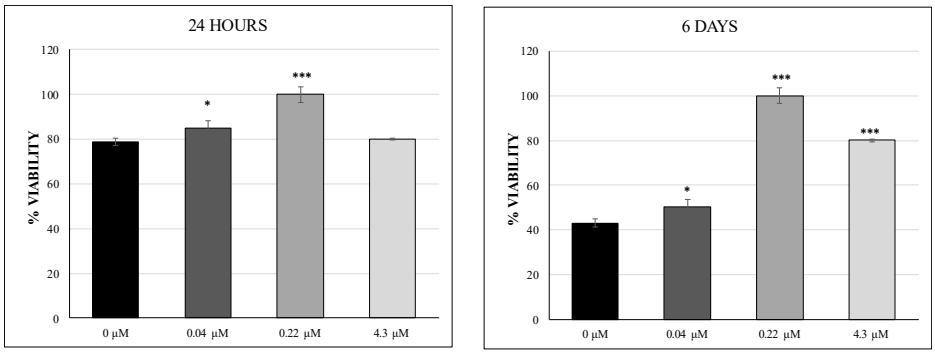


Figure 6. 5 NSCs were incubated in different concentration of insulin for 24 hours (left) and 6 days (right) and % viability was assessed by MTT assay. Data presented as Mean ± SEM of n=3. * p value <0.05, ** p value <0.01; *** p value < 0.001 as compared to no insulin group.

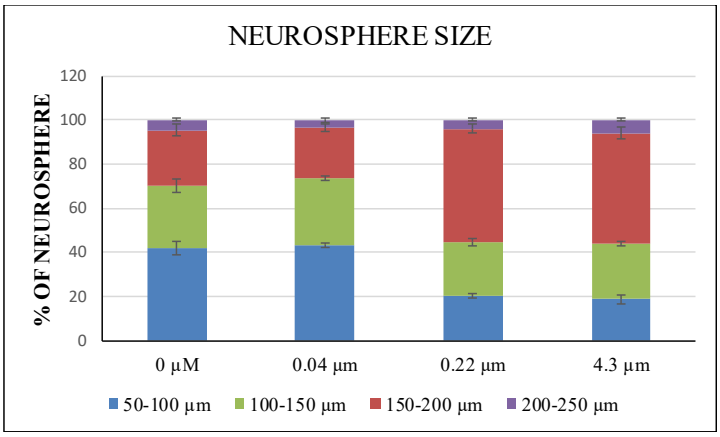


Figure 6. 6 Morphometric analysis of neurospheres formed upon incubation with different concentration of insulin for 6 days. Data presented as Mean ± SEM of n=3.

Neural stem cells were then differentiated spontaneously with varying concentration of insulin in differentiation media and the percentage of all the three differentiated cell types were monitored. Increasing the concentration of insulin lead to an increase in the activation of candidate downstream molecules in dose dependent manner. This increase in insulin signaling was further correlated with the differentiation fate of NSCs. The optimum and high concentration of insulin was essential for NSC maintenance (as shown by Nestin) and neurogenesis (as shown by MAP2) as shown in Fig 6.7. However, astrocytes differentiation was favored in low insulin concentration (as shown by GFAP). The above result was confirmed using cytometric analysis and the result indicated decreased number of GFAP positive (Nestin negative) cells (=astrocytes) with increased concentration of insulin. Thus, it indicated that insulin levels regulate neural stem cell plasticity *in vitro*.

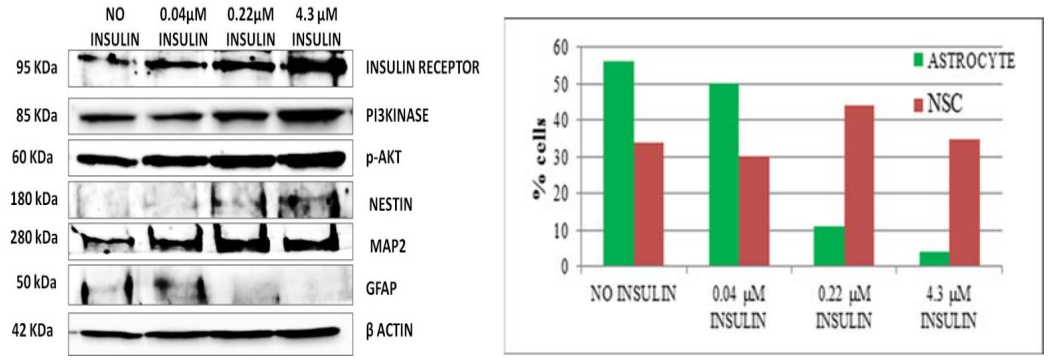


Figure 6. 7 (left) Immunoblotting of candidate insulin signaling proteins and brain cell markers in NSCs differentiated in varying concentration of insulin. (right) Graph demonstrating the cytometric analysis of NSCs after differentiation for astrocytes and NSCs.

6.2 IMPACT OF INSULIN RESISTANCE (*Insr* KNOCKDOWN) ON NSCS

6.2.1 PLAN OF WORK

Neural stem cells were transfected with plasmids for shRNA against insulin receptor along with control plasmids procured from Qiagen (Sure silencing shRNA plasmid Hygromycin KR44532H Cat. No. 336312) using lipofectamine 2000 (Invitrogen). Before transfection, cells were shifted to antibiotic (penicillin and streptomycin) free media for 24 hours. Transfection was performed in OptiMEM media with DNA to lipofectamine concentration as per manufacturer's instruction. After incubation with DNA and lipofectamine for 8 hours, cells were shifted to NSC media. Post 24 hours of transfection, cells were subjected to hygromycin pressure for 8 days for removal of non-transfected cells. These cells were then harvested, confirmed for *Insr* silencing. For assessment of differentiation fate, NSCs were allowed to adhere on poly-l-lysine coated plates, and transfected. Post 48 hours of transfection, cells were shifted to differentiation media, and allowed to differentiate for 10 days. These cells were then harvested and assessed for their differentiation fate. The diagrammatic representation for plan of work is as shown below in Fig 6.8.

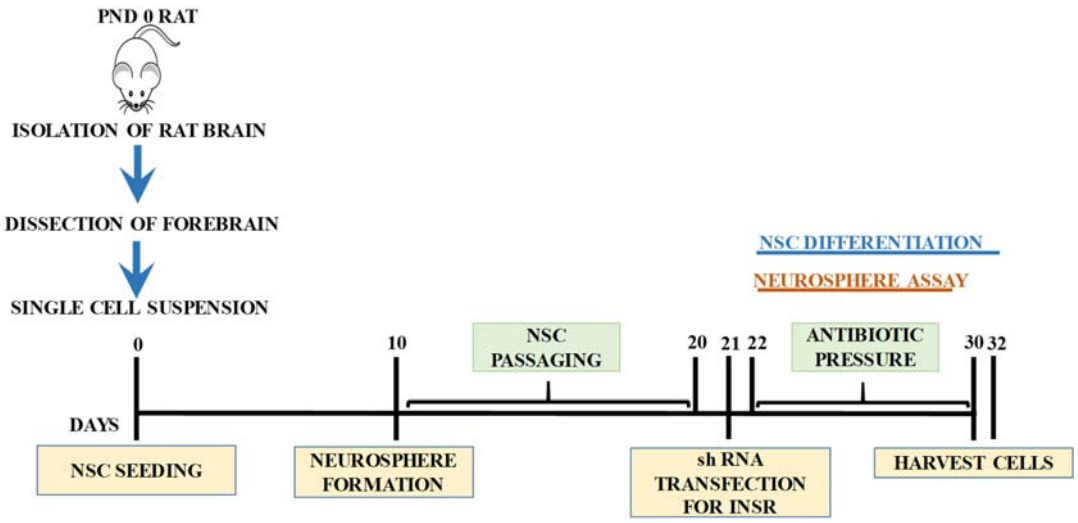


Figure 6. 8 Plan of work for *Insr* gene silencing in NSC for determination of impact of insulin signaling on NSC fate.

INSULIN RECEPTOR GENE KNOCKDOWN (KD) IN NSC

NSCs were transfected with plasmid clone 4 containing shRNA against insulin receptor. Transient transfection was performed and after 7-8 days of continuous hygromycin pressure, cells were assessed for transfection efficiency. As shown in Fig 6.9 (A-B), around 60% transfection was observed as compared to transfected control cells.

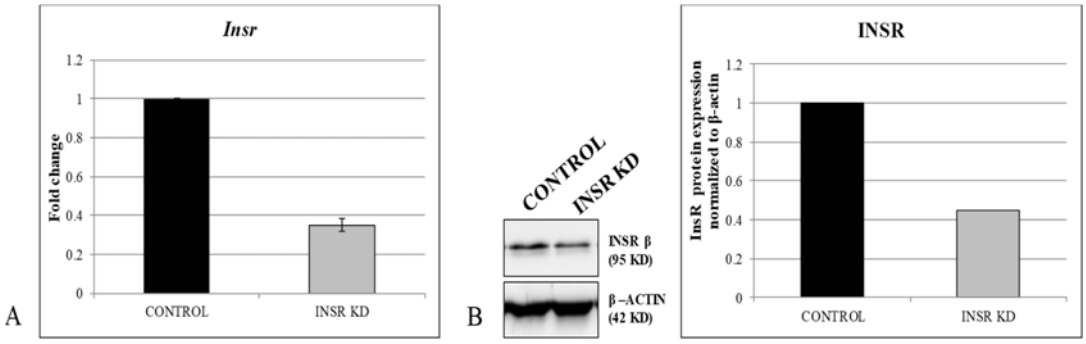


Figure 6. 9 A. Gene expression by real time PCR for *Insr* gene to access gene knockdown efficiency (n=2). B Immunoblotting of INSR protein to confirm the knockdown of *Insr* gene post transfection in NSCs (n=1).

SURVIVAL AND PROLIFERATION IS COMPROMISED IN INSR KD NSC

Survival was remarkably decreased to 50 % in INSR KD as shown in Fig 6.10A as measured by MTT assay. Proliferation was reduced post insulin receptor gene silencing as observed by neurosphere size (Fig 6.10B). Similar, observation was made in cell

cycle analysis where as compared to transfected control, there was a prominent rise in the number of apoptotic cells with a drop-in cell number in the synthesis phase as shown in Fig 6.11.

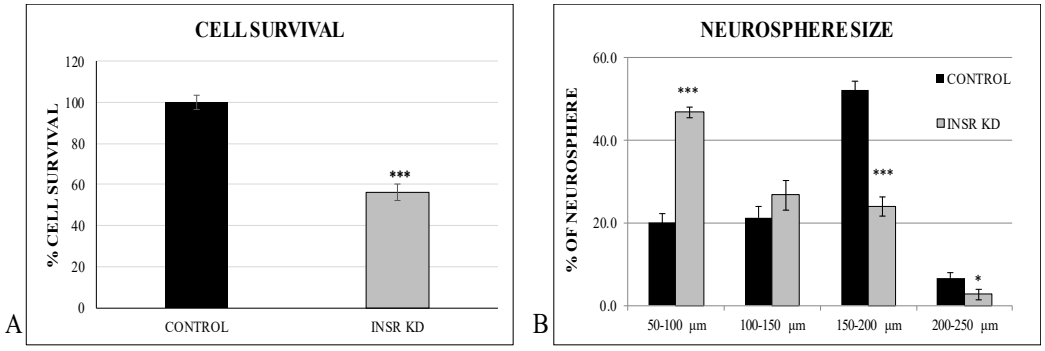
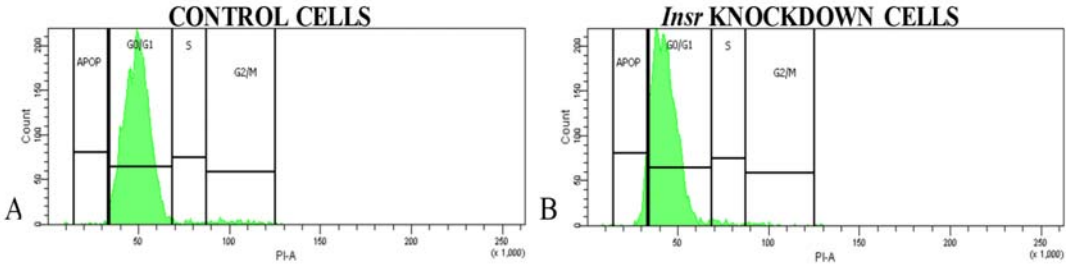


Figure 6. 10 A MTT assay demonstrated 50% reduction in cell survival in insulin receptor knockdown (INSR KD) NSCs as compared to transfected control (control) cells. B. Neurosphere size was assessed post transfection in control and INSR KD NSCs. Data presented as Mean ± SEM of n=3. * p value <0.05, *** p value < 0.001 as compared to control cells.



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POPULATION	% PARENT	
	CONTROL CELLS	*Insr* KNOCKDOWN CELLS
G0/G1	95.7	86.2
SYNTHESIS	1.1	3.0
G2M	2.4	1.2
APOPTOTIC	0.4	6.0

Figure 6. 11 Cell cycle analysis by flow cytometry in NSCs in *Insr* KD condition (B) as compared to transfected control cells (A). Table (C) denoted percent parent population for different stages of cell cycle.

UNDIFFERENTIATED AS WELL AS DIFFERENTIATED INSR KD NSCS HAS ALTERATION IN CANDIDATE STEMNESS GENES

Undifferentiated as well as differentiated *Insr* gene KD NSCs were assessed for candidate stem cell marker, and compared with that of control transfected cells. Undifferentiated INSR KD NSCs demonstrated a reduction in the expression of *Nestin*, *Pax6*, *Fabp7*, *NeuroD1* and *Vimentin*. During differentiation, these stem cell genes

decreased leading to an increase in the expression of more cell specific differentiation markers. Similar observation was made where there was decrease in these markers in both control and INSR KD differentiated cells as shown in Fig 6.12.

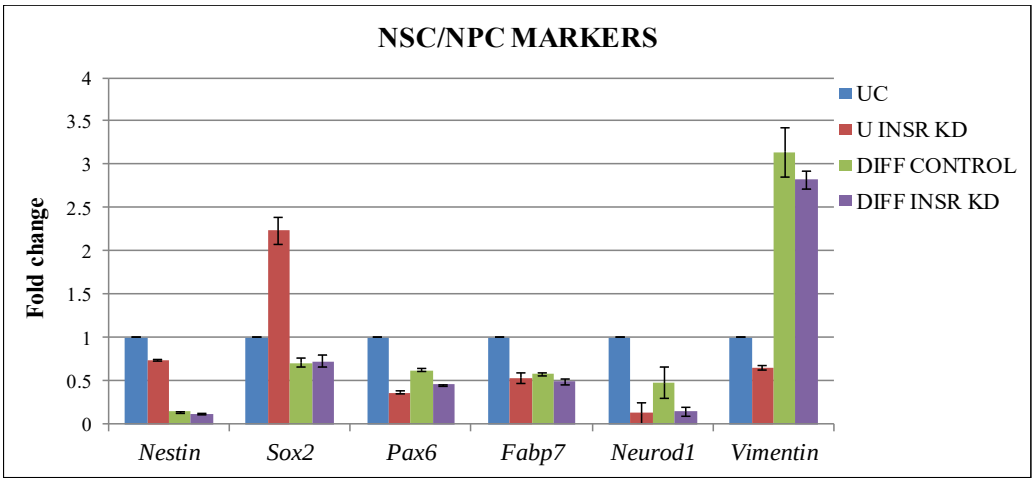


Figure 6. 12 Taqman Real time gene analysis of signature markers for NSC maintenance after *Insr* gene silencing in undifferentiated control NSCs (UC), undifferentiated NSCs with InsR knockdown (U INSR KD), differentiated NSC control (DIFF CONTROL) and Differentiated NSCs with InsR knockdown (DIFF INSR KD). Error bars represent standard deviation for n=2.

INSR KD ALTERS DIFFERENTIATION FATE IN NSCS

Post differentiation in *Insr* gene KD NSCs, there was a remarkable decrease in the neurogenic gene expression, demonstrating that insulin signaling is important for neurogenesis (Fig 6.13A). However, when assessed for astrocytic (Fig 6.13B) and oligodendrocytic markers (Fig 6.13C), there was a slight reduction in these markers post differentiation. Thus, insulin signaling is indispensable for neurogenesis but might not be that important for astrocytic and oligodendrocytic differentiation. Also, differentiated INSR KD NSCs had decreased expression of glucose transporters as shown in Fig 6.13D, thus further marking the major role of insulin signaling in their regulation in differentiated cells rather than undifferentiated NSCs.

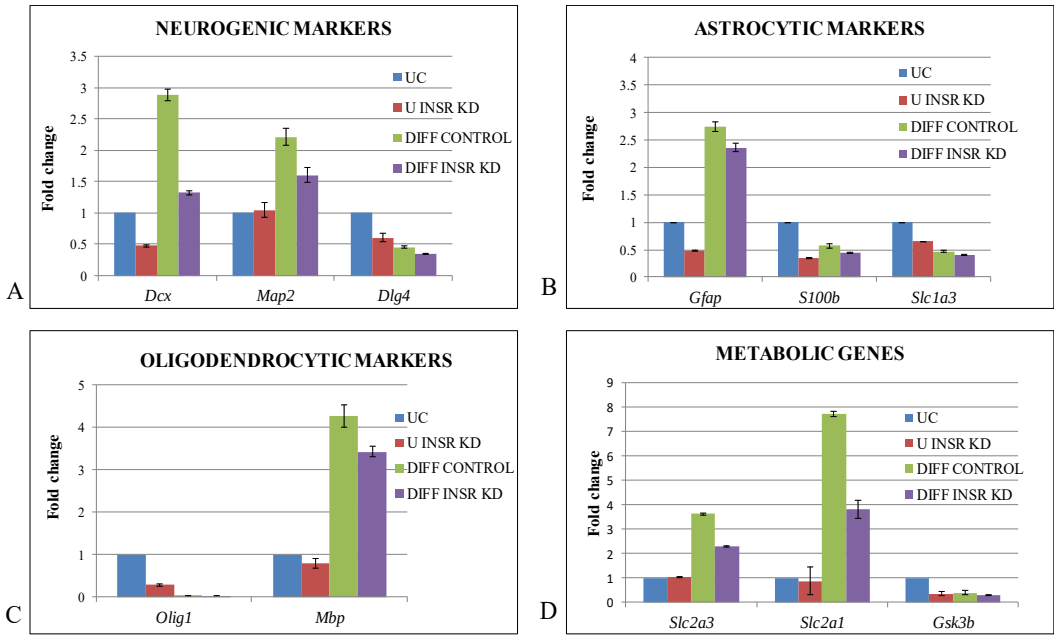


Figure 6. 13 Taqman Real time gene analysis of candidate differentiation markers for neurons (A), astrocytes (B) and oligodendrocytes (C) along with metabolic genes (D) after *Insr* gene silencing in undifferentiated control NSCs (UC), undifferentiated NSCs with *InsR* knockdown (U INSR KD), differentiated NSC control (DIFF CONTROL) and Differentiated NSCs with *InsR* knockdown (DIFF INSR KD). Error bars represent standard deviation for n=2.

6.3 IMPACT OF GLUCOCORTICOID ON INSULIN SIGNALING IN NSCS.

6.3.1 PLAN OF WORK

Primary NSCS were incubated with 1 µM dexamethasone treatment for 24 hours. While in groups where glucocorticoid receptor (GR) inhibitor i.e. 3 µM RU486 was used, pre-treatment was given for 1 hour for the prior occupancy of GR so that dexamethasone cannot act. For assessment of insulin signaling by western blotting of candidate molecules, cells were induced with 50nM insulin for 30 min, and then harvested. For analysis of outcome of insulin signaling on cell survival and cell cycle, cells were harvested post 180 min. For assessment of differentiation fate, cells were kept under the continuous exposure of dexamethasone, RU486 and insulin for 8 days. Media was replaced every third day. The plan of work is as depicted in Fig 6.14.

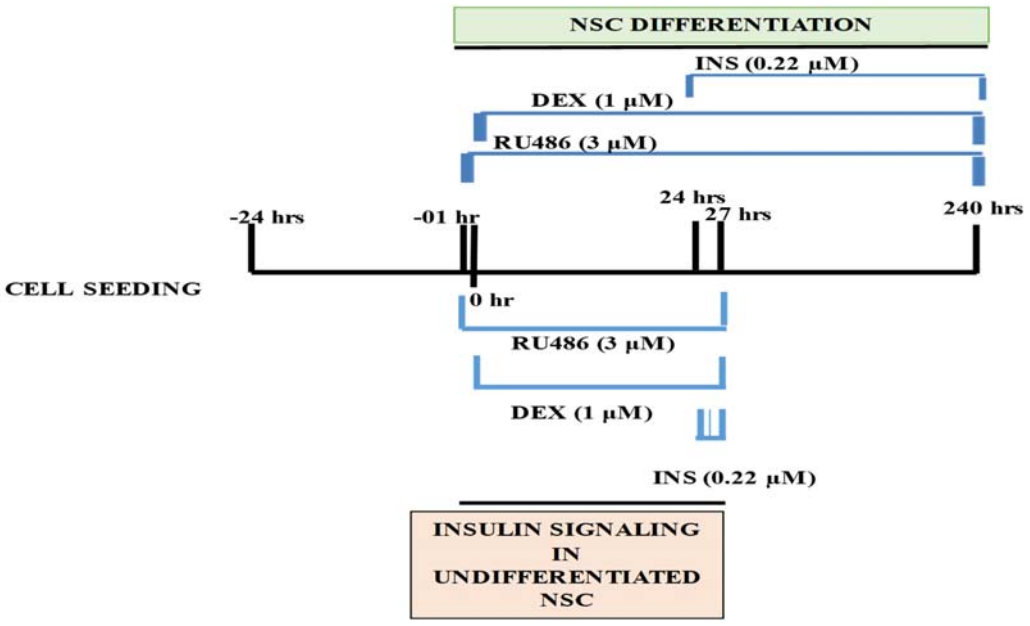


Figure 6. 14 Plan of work for assessment of impact of dexamethasone on insulin signaling and differentiation in NSC.

6.3.2 RESULTS

SURVIVAL AND PROLIFERATION OF NSCS ARE AFFECTED BY DEXAMETHASONE TREATMENT

Survival was assessed by MTT assay after the treatment period and the results exhibited a significant decrease as observed in dexamethasone treated group when compared to other groups (Fig 6.15). Subsequently, cell cycle analysis was performed on these cells from each group, and apoptosis was evident in dexamethasone alone treated group as shown in Fig 6.16. Induction with insulin could lower the number of apoptotic NSCs post dexamethasone treatment. Thus, dexamethasone treatment did not interfere with survival and proliferation of NSC induced by insulin.

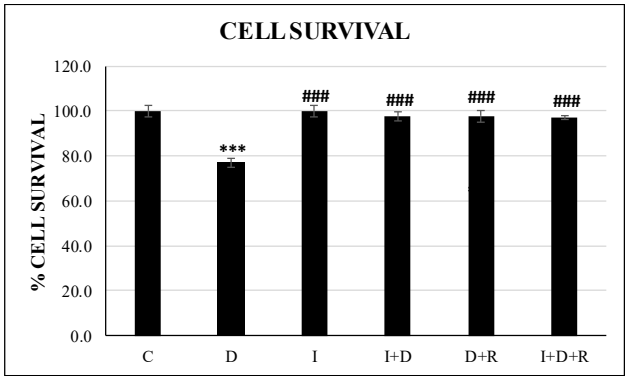
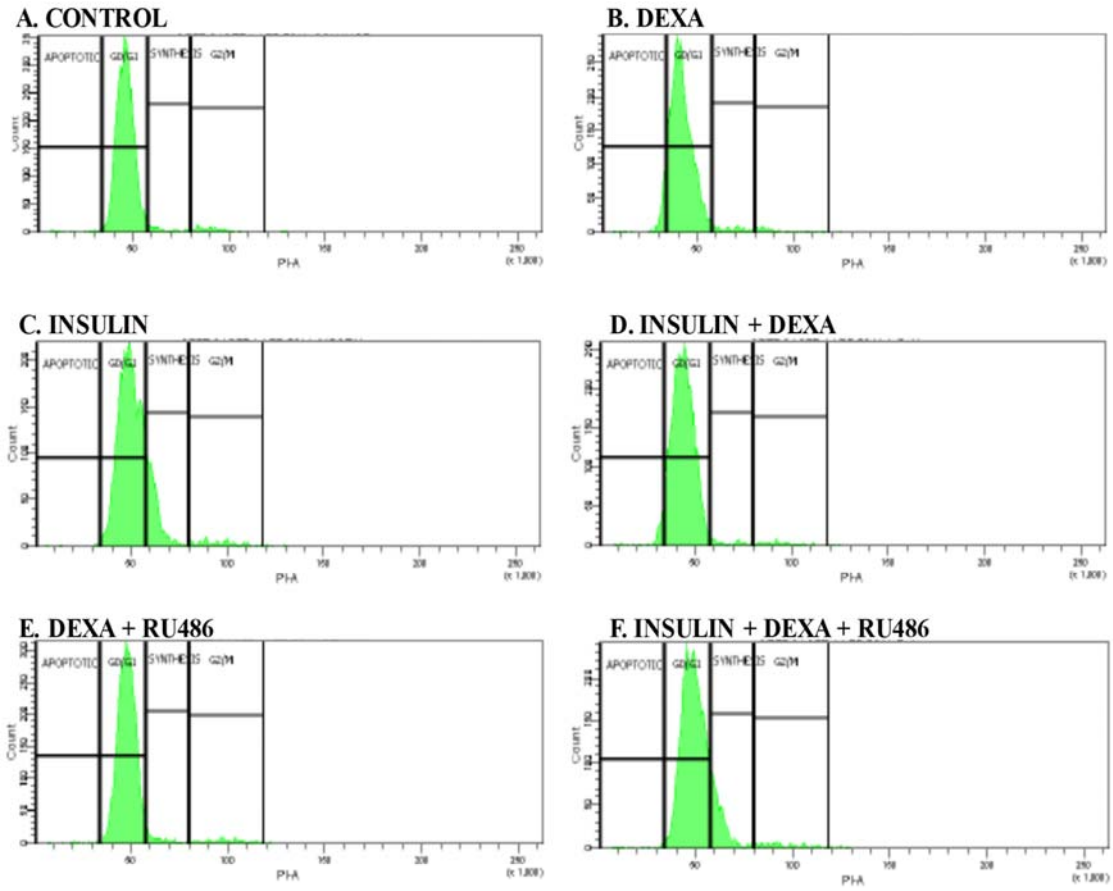


Figure 6. 15 Cell survival as demonstrated by MTT assay after treatment. Data presented as Mean ± SEM of n=3. ***p value≤0.001 as compared to control; ### pvalue ≤ 0.001 as compared to dexa. C: Control, D: Dexamethasone, I: Insulin, R: RU486.



G.

POPULATION	% PARENT					
	C	D	I	I+D	D+R	I+D+R
G0/G1	94.9	87.3	78.5	91.6	92.8	81.2
SYNTHESIS	2.0	2.3	16.2	1.8	3.0	13.4
G2M	2.5	1.6	2.7	1.9	2.9	2.0
APOPTOTIC	0.6	7.2	0.2	2.0	0.4	0.5

Figure 6. 16 Cell cycle analysis by flow cytometry in control (A), dexamethasone treated (B), insulin treated (C), insulin induced dexamethasone treated (D), RU486 and dexamethasone treated (E), and insulin induced dexamethasone +RU486 treated (F) NSCs. Table (G) denoted percent parent population for different stages of cell cycle.

DEXAMETHASONE TREATMENT REDUCED INSULIN SENSITIVITY IN NSCS

To establish the effect of glucocorticoid (GR) on insulin signaling, primary NSC 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 for 30 min. Protein expression of candidate insulin signaling proteins were studied and the results demonstrated a slight decrease in the protein expression of activated INSR (Fig 6.17 B) and PI3K (Fig 6.17 C). However, insulin induced activation of AKT (Fig 6.17 D) was impaired in dexamethasone treated NSCs.

This reduced AKT activation because of dexamethasone was restored to control levels when they were pre-treated with glucocorticoid receptor antagonist (RU486).

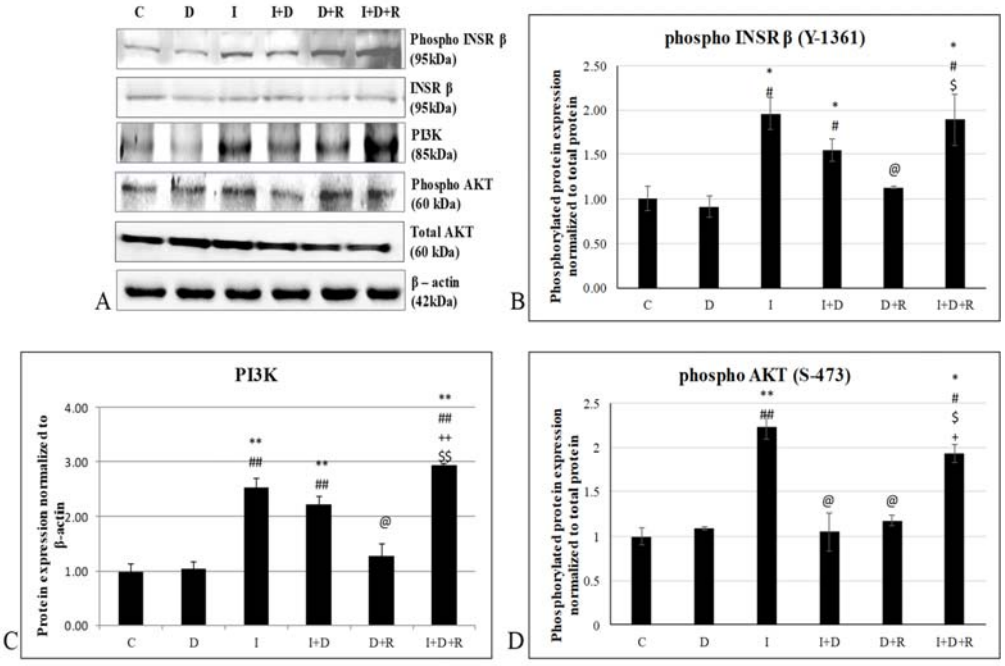


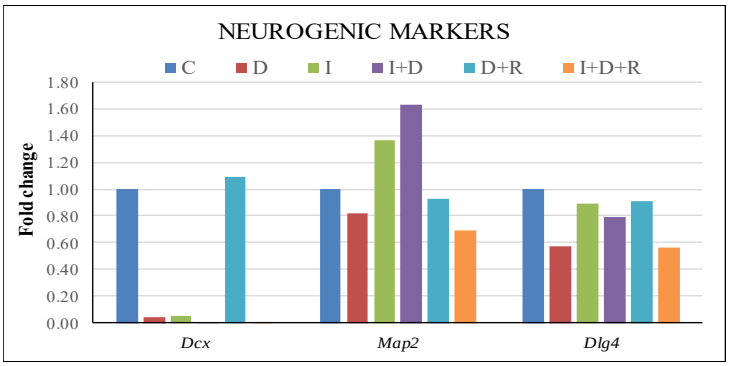
Figure 6. 17 A. Representative images of immunoblotting of phospho INSRβ Y-1361, Total INSRβ, PI3Kinase, phospho AKT S-473 and Total AKT keeping β-actin as endogenous control. B-D: Graphs represents densitometric analysis done using Image J software. Data presented as Mean ± SEM of n=2-3. *p value≤0.05 and **pvalue ≤ 0.01 as compared to control; #p value ≤ 0.05 and ## pvalue ≤ 0.01 as compared to dexa; @ p value ≤ 0.05 and @@ pvalue ≤ 0.01 as compared to insulin; +p value ≤ 0.05 and ++ pvalue≤0.01 as compared to insulin+dexa. $ p value ≤ 0.05 and $$ pvalue ≤ 0.01 as compared to dexa + RU486.

INSULIN AND DEXAMETHASONE ALTER TRANSCRIPTOME PROFILE DURING DIFFERENTIATION

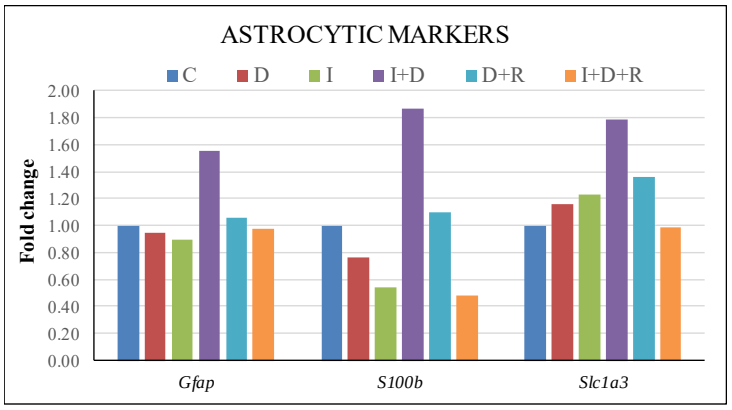
Insulin and dexamethasone individually decreased the expression of stem cell maintenance genes during 10 days of differentiation. Insulin increased the expression of neurogenic gene i.e., MAP2 significantly. Dexamethasone treatment reduced the expression of MAP2, however insulin co-exposure could bring back the expression of MAP2. Thus, signifying that insulin could play an important role in restoring neurogenesis affected during glucocorticoid exposure as shown in Fig 6.18A. However, when assessed for astrocytic markers, neither insulin nor dexamethasone alone changed their expression. The astrocytic markers viz. *Gfap*, *S100b* and *Slc1a3* were significantly high in co-treated group with insulin and dexamethasone as shown in Fig 6.18B. On the contrary, oligodendrocytic markers were increased in alone insulin and

dexamethasone treated groups, but decreased in co-exposed group as shown in Fig 6.18C.

A



B



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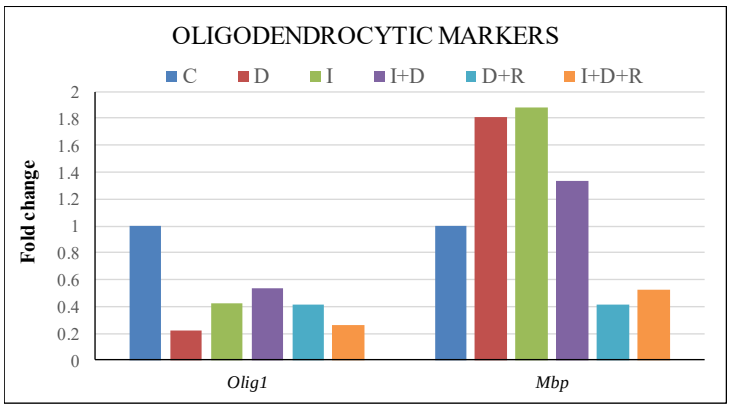

Figure 6. 18 Taqman Real time gene analysis of candidate differentiation markers for neurons (A), astrocytes (B) and oligodendrocytes (C) after the treatment period. C: Control, D: Dexamethasone, I: Insulin, R: RU486.

6.4 DISCUSSION

The discovery of NSCs in the postnatal brain opened a new avenue for the replacement of damaged cells in the central nervous system in any neurodegenerative diseases. However, there still lacks systematic framework to illustrate the exact code followed by these cells for their maintenance and differentiation. Majority of the research comes from the NSCs isolated from embryonic brain, which being exposed to different niche and signals that may not always replicate in NSCs from adult brain. This complicates the estimation of survival, proliferation as well as differentiation fate of stem cells in adult born neurodegenerative diseases. Thus, for the study of neuro-regeneration in adult brain, postnatal neural stem cells may serve as a better model. Recently, great emphasis has been shifted in the elucidation of brain defects observed in diabetic patients amongst which cognitive impairments is most frequently observed. The function of cognition as well as learning is associated with adult neurogenesis in hippocampus which has substantial pool of NSCs even in adult brain (Yau *et al.*, 2015). Depletion of stem cells and decline in neurogenesis is clearly established from the studies in diabetic rodent models of T1DM and T2DM displaying altered levels of insulin (Beauquis *et al.*, 2008; Stranahan *et al.*, 2008; Ramos-Rodriguez *et al.*, 2014; Dorsemans *et al.*, 2017). Hence, NSCs were isolated from PND0 rat brain and successfully cultured *in vitro*. They were confirmed by neurosphere forming capability and by immunostaining with Nestin, GFAP and CD133. They were multipotent, and differentiated well into all the three neural cell types – astrocytes, oligodendrocytes and neurons.

Our foremost query was whether insulin played a role in fate determination of neural stem cells. To understand the decisive role of insulin on NSCs, they were exposed to varying concentration of insulin, 0 µM, 0.04 µM (low), 0.22 µM (optimum) and 4.3 µM (high). Our data represented that low as well as high concentration of insulin is crucial for cell survival and proliferation. Thus, replicating the results by Rhee that NSC maintenance is dependent on critical insulin concentration (Rhee *et al.*, 2013). Further, these cells were subjected to spontaneous differentiation by withdrawal of mitogens. Low insulin level diverted the differentiation towards astrocyte differentiation while significantly reduced neurogenesis. However, higher insulin doses drove the NSCs towards neurogenesis. This observation was also made by Han et al 2008 where high concentrations of insulin induces neuronal differentiation of postnatal NSCs (Han *et*

al., 2008). Further, Arsenijevic and Weiss, 1998 proved that culturing of embryonic NSCs with both insulin and IGF-1 leads to a greater production of neurons during differentiation compared to cultures stimulated by IGF-1 alone (Arsenijevic and Weiss, 1998). These results if extrapolated to developmental production of neurons and glia, it can be said that the higher insulin level during embryonic stage in brain might be one of the driving force for neuronal differentiation. Also, we speculate that decreasing insulin levels as observed in post embryonic, aged as well as diabetic brain, renders microenvironment more suitable for gliogenesis. This is in accordance with several other published literatures where STZ induced T1DM model as well as Zucker diabetic fatty T2DM model showed decreased number of proliferating cells in hippocampus as well as fewer number of new born neurons (Jackson-Guilford *et al.*, 2000; Yi *et al.*, 2009). However, to the contrary, Goto-Kakizaki (GK) rat's dentate gyrus demonstrated increased proliferation of NSC/NPCs however newly formed cells had reduced survival (Lang *et al.*, 2009). Thus, extent of metabolic derangement in diabetes might decide the consequences on neurogenesis in *in vivo* systems.

In vitro culturing of NSCs from T2DM rats, exhibited reduced responsiveness to mitogens (Lang *et al.*, 2009). Thus, we hypothesized that as in peripheral cells, insulin resistance at cellular level in NSCs can be the causal factor. Thus, to understand this phenomenon, *in vitro* model of insulin resistant NSCs were established by *Insr* gene silencing. Transient transfection with gene silencing efficiency of around 60 % was attained successfully. As observed in the dose dependent experiment, here also hampering of insulin signaling severely compromised the survival. Thus, further affirming the report stating that insulin signaling in conjunction with EGF receptor stimulation is necessary for cell-cycle progression (Alagappan *et al.*, 2014). Insulin is important for the maintenance of NSCs in the undifferentiated state via promoting the function of FGF-2 (Adepoju *et al.*, 2014). Thus, claiming that in spite of other mitogens such as EGF, FGF, etc in culture media, insulin is an irreplaceable factor crucial for NSC survival. Similar observations have been made in stem cells from other organs where insulin signaling regulated proliferative function which has been hampered during diabetes (Andres *et al.*, 2013; Li *et al.*, 2017).

Assessment of candidate markers responsible for stemness demonstrated that INSR KD resulted in the decrease in the transcript levels of Nestin, Pax6, NeuroD1, Fabp7 and Vimentin except for Sox2. Nestin is an intermediate filament, frequently used as a

marker for NSCs which represents a cohort of stem/progenitor cells involved in active proliferation (Dahlstrand *et al.*, 1995). Its expression is fundamental for survival and self-renewal property of NSCs (Park *et al.*, 2010). Similarly, Pax6 modulates expression of several molecules involved in maintaining the balance between proliferation as well as differentiation. Pax6 is generally expressed in early neuronal progenitors along with other factors like DLX2, DCX, etc. to specify the neuronal subtype from stem/progenitors (Curto *et al.*, 2014). NeuroD1 is indispensable for triggering neuronal differentiation in hippocampal NSCs and the decline in NeuroD1 expression will directly affect neuronal differentiation and influence mature neuronal genes (Pataskar *et al.*, 2016). In accordance with our results, Hidaka et al., 2013 demonstrated a marked reduction in nestin and neuroD1 levels in dentate gyrus of STZ induced T1DM diabetic brain (Hidaka *et al.*, 2013). Further, reduction in Fabp7 as well as vimentin expression represented diminished commitment (or pool) of glial progenitors towards glial fate (Sancho-Tello *et al.*, 1995; Zhang and Jiao, 2015). Thus, our results clearly imply that insulin signaling is involved in the stem cell multipotency, and thus could be one of the reason for reduced proliferation as well as differentiation efficiency.

Apart from playing its part in maintenance, insulin is found to promote differentiation of neuron, astrocyte as well as oligodendrocyte. Thus, neurogenic markers were evaluated in differentiated INSR KD NSCs where sharp decrease was observed in expression of DCX and MAP2. DCX and MAP2 being neuronal markers, clearly demonstrated that neurogenesis was altered in insulin resistant condition. Similar observation was made by Han et al., where inhibition of insulin induced phosphorylation of mTorSer2448 and of pS670K results in prevention of neuronal differentiation from embryonic stem cells (Han *et al.*, 2008). Also, insulin mediated Akt phosphorylation at Ser473 and Thr308 contributes to the neurogenesis of mouse olfactory bulb stem cells (OBSCs) (Otaegi *et al.*, 2006). Further, in our study, there was also a decrease in the expression of mature oligodendrocytic marker MBP, however to the contrary only slight reduction was observed in the expression of astrocytic markers. These results were hand in hand with our dose dependent differentiation experiment with insulin, where insulin proved to be more important for neurogenesis rather than astrogliogenesis. Therefore, extrapolating to diabetic brain, insulin resistance in NSCs might be the culprit leading to diminished NSC proliferation and neurogenesis.

Interestingly, the level of major glucose transporters- GLUT1 and GLUT3 were not affected in insulin resistant condition in undifferentiated NSCs, but these cells when differentiated had decreased level of glucose transporters. Thus, alteration in insulin signaling in the stem cell stage will program metabolic derangement towards reduced glucose uptake when differentiated.

Comparable observations have been made in chronic stress models with elevated GC levels where NSC survival, proliferation and differentiation are affected (Schoenfeld and Gould, 2012). Stress and diabetes has always been related where chronic elevated GC levels can lead to diabetes by inducing insulin resistance, and vice versa elevated GC level is observed during diabetes. Thus, we speculated that there might be some common factor negatively affecting NSCs. In our *in vivo* model of dexamethasone induced diabetes, we could clearly demonstrate a decrease in NSC markers in hippocampus, and also the *in vitro* assessment established that GCs can induce insulin resistance in astrocytes. Thus, connecting the dots, we were interested in understanding whether GC (dexamethasone) exposure can render these cells insulin resistance as in astrocytes.

Dexamethasone exposure to NSCs severely affected the survival and proliferation. Decreased cell proliferation might be caused because of dexamethasone induced G1 arrest or decrease in cyclin D1 as demonstrated in murine-derived multipotent neural stem cell line, C17.2 and embryonic rat NSC respectively (Sundberg *et al.*, 2006; Mutsaers and Tofighi, 2012). However, insulin as well as GC receptor antagonist treatment displayed rescue of NSCs from cell death and cell cycle arrest. Thus, signifying the protective role of insulin signaling in restoring proliferation in NSCs post stress episodes. Further, assessment of insulin signaling machinery after dexamethasone treatment revealed remarkable reduction in insulin mediated AKT activation. Thus, chronic GC exposure can compromise the insulin sensitivity and as demonstrated by INSR KD NSCs might have altered fate. This to the best of our knowledge, will be the first report displaying insulin unresponsiveness in neural stem cells.

Further, differentiation fate was determined in neural stem cells exposed to dexamethasone. Alone dexamethasone treatment decreased the expression of MAP2, a marker for mature neurons. However, in insulin co-exposure group this decrease was

restored. It is reported that chronic corticosterone treatment as well as dexamethasone administration has shown to decrease the hippocampal neurogenesis in rats (Yu *et al.*, 2004). Paradoxically, there are reports also which are associated with increased rates of adult neurogenesis in spite of elevated glucocorticoid levels in situations like physical exercise (Mirescu and Gould, 2006; Masahiro *et al.*, 2015). Thus, suggesting that glucocorticoid might be cross talking with other signaling pathways to exert the final outcome on neurogenesis. The assessment of astrocytic markers exhibited that individually neither insulin nor dexamethasone increased astrogliogenesis. Similar effect by cortisol has been established where high cortisol inhibited neurogenesis without affecting astrogliogenesis (Anacker *et al.*, 2013). Nevertheless, co-exposure of insulin and dexamethasone remarkably increased astrocytic markers viz *Gfap*, *S100b* and *Slc1a3*. In case of oligodendrocytic differentiation, alone treatment with dexamethasone and insulin increased MBP (mature oligodendrocytic marker), but together antagonised their effect on oligodendrogenesis when treated together. In similar line, Chetty *et al*., showed that *in vitro* exposure to GC induced a pro-oligodendrogenic transcriptional program in hippocampal NSC and resulted in an increase in oligodendrogenesis with a decrease in neurogenesis (Chetty *et al.*, 2014). The antagonistic effect of insulin and dexamethasone on the same phenomenon is difficult to explain and it would further require detailed analysis. Thus, this study gave the insight on the complex nature of hormonal regulation on the differentiation fate of NSCs. This might be one of the reason for the lack of reproducibility on the NSC studies in different animal models of diabetes.

Therefore, this objective shed light on the importance of critical role of insulin on decision of NSC fate. It can be clearly established that insulin signaling is indispensable for NSC survival and proliferation. The mitotic effect of insulin could not be replaced by any other mitogens say EGF, FGF etc. Also, if rendered insulin resistance because of any adverse conditions such as GC exposure, neurogenesis and gliogenesis will be severely affected disrupting the ideal neuron to glia ratio for proper brain functioning. These studies will further be instrumental in deciding pathology as well as stem cell and insulin based therapy for diabetes as well as stress related neurodegenerative threats.

Thus, the outcome of this objective can be pictorially summarized as Fig 6.19.

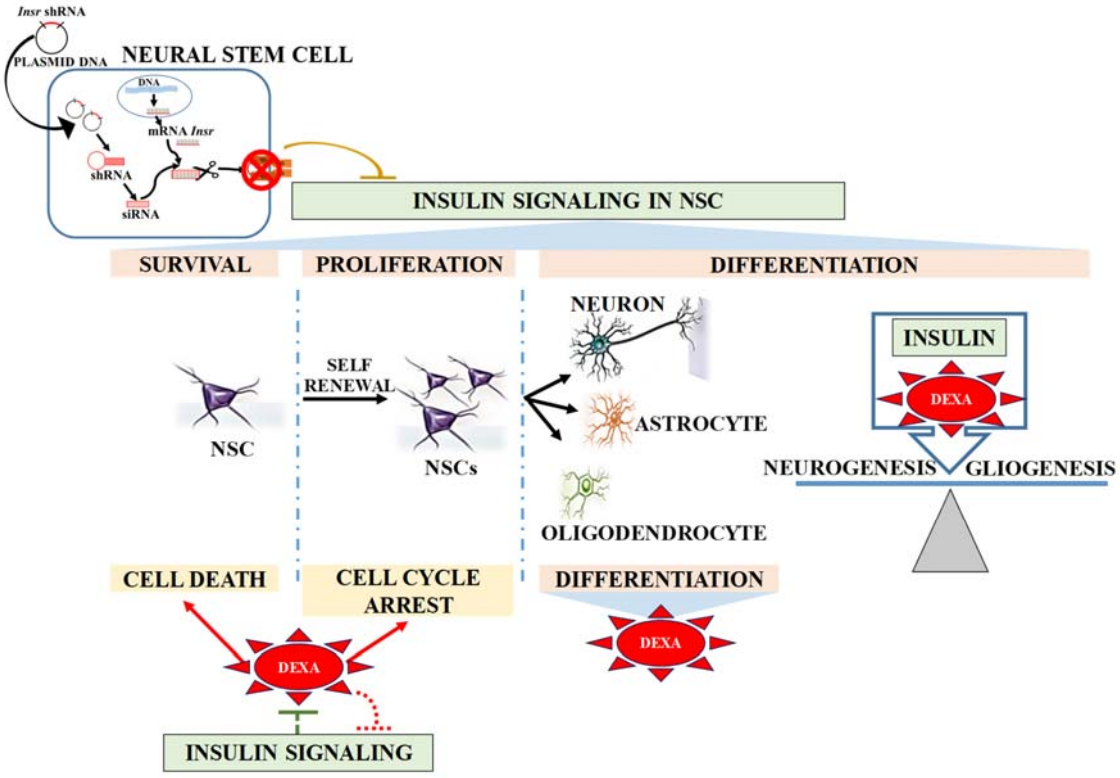

Figure 6. 19 Summary of "The role of insulin resistance and glucocorticoid on the fate of NSCs".

1. *Insr* gene silencing demonstrated the vital role of insulin signaling in survival, proliferation and differentiation of neural stem cells (NSCs).
2. Insulin could rescue the negative effect (as shown by red lines) on cell survival and proliferation induced by dexamethasone (Dexa-synthetic glucocorticoid). Dexa exposure impaired insulin mediated AKT activation but did not affect insulin action on NSC survival and proliferation.
3. Insulin and dexa acted differently when treated alone and in combination on the differentiation of NSCs.