CHAPTER 5

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

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Astrocytes are specialized glial cells that tile the entire CNS in an orderly and well-organized manner. The term astrocyte derives from a combination of a Greek word for star (astron; plural, astra) and the scientific word for cell (cyte, which is in turn derived from the Greek word kytos, meaning vessel). They are one of the most important glial cell for maintenance of the neural milieu and support of other brain cells. Friede demonstrated that there is a corresponding increase in neuron to astrocyte ratio with an increase in phylogeny, which is further concomitant with an increase in brain complexity (Friede and Van Houten, 1962; Friede, 1963). For instance, the glia/neuron ratio (also known as glial index) in the cerebral cortex of mouse is 0.36 while that of man is 1.48. Also, human astrocytes are larger, structurally more complex, and more diverse than astrocytes in subordinate species (Herculano-Houzel, 2014). Thus, announcing their indispensable role in the brain.

Immuno -histochemical techniques have enabled the identification and characterization of astrocytes using specific molecular markers. The glial fibrillary acid protein (GFAP), an intermediate filament is the most widely used prototypical marker for identification of astrocytes. Other molecular markers include glutamine synthetase (GS), S100 calcium-binding protein B (S100B), aldehyde dehydrogenase 1 family member L1 (ALDH1L1), etc (Khakh and Sofroniew, 2015). Regardless of the fact that gliogenesis occur post neurogenesis during development, astrocytes are important for demarcation of gray and white matter, formation of molecular boundaries for cell migrations, guiding axonal formations and pruning, myelination as well as adult neurogenesis (Sofroniew and Vinters, 2010). The 'tripartite synapse' hypothesis further posits that astrocytes are essential for information processing by neural circuits (Kim *et al.*, 2017). Astrocytes have multiple bidirectional interactions with blood vessels and contributes to CNS metabolism by furnishing energy metabolites such as glucose from blood vessels to neural elements (Obermeier *et al.*, 2013).

Ever since Pellerin and Magistretti discovered that astrocytes provide lactate as metabolic fuel for neurons, a concept termed as Astrocyte Neuron Lactate Shuttle (ANLS), there has been much interest in the metabolism of astrocyte (Pellerin and Magistretti, 1994). According to ANLS as shown in Fig 5.1, the conditions of high

neuronal activity where neurons require a fast supply of energy is sensed by the astrocytes by taking up excitatory neurotransmitter like glutamate via glutamate transporters, Excitatory Amino Acid Transporters (EAATs). During firing, glutamate (Glu) is released at the synapse which is taken up by astrocytes via excitatory amino acid transporters (EAATs) together with 3 Na+ ions. This Na+ is extruded by the action of the Na+/K+ ATPase, consuming ATP. This trigger non-oxidative glucose utilization in astrocytes and glucose as well as glycogen stores are converted to lactate by lactate dehydrogenase 5 (LDH5). This lactate is shuttled to neurons through monocarboxylate transporters (MCT) to be used as an energy substrate following its conversion to pyruvate by LDH1. Concomitantly, astrocytes participate in the recycling of synaptic glutamate by converting to glutamine (GLN) via glutamine synthetase (GS) and shuttled to neurons through amino acid transporters (AATs) where it is converted back to glutamate by glutaminases (GLS). Astrocytes are also instrumental in secretion of trophic factors to neurons and other brain cells and maintenance of blood brain barrier.(Magistretti and Pellerin, 1996; Katsu-Jimenez *et al.*, 2017). They are also the only storage sites of glycogen granules in the CNS for sustained energy needs (Waitt *et al.*, 2017).

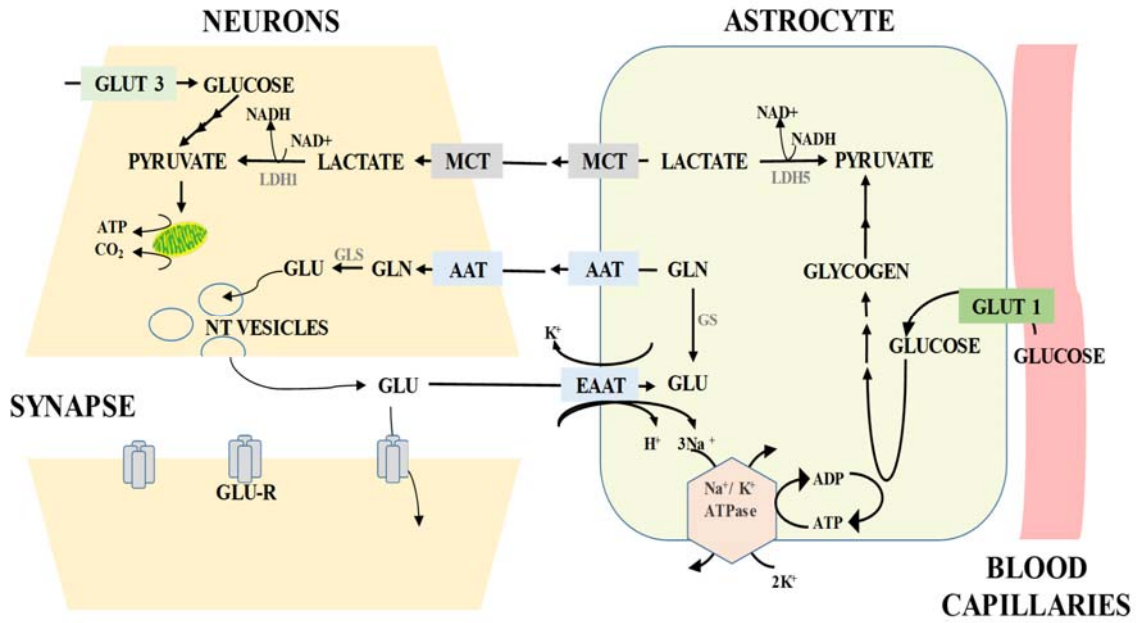


Figure 5. 1: Astrocyte-Neuron Lactate Shuttle. GLUT, Glucose transporter; LDH, Lactate dehydrogenase; MCT, Monocarboxylate transporter; GLU, Glutamate; GLN, Glutamine; NT, Neurotransmitter; GLS, Glutaminases; GLU-R, Glutamate receptor, EAAT, Excitatory Amino Acid Transporters; AAT, Amino acid transporters; GS, Glutamine synthetase.

Central and peripheral signals during healthy and diseased condition modulate the efficiency of astrocytes. In this thesis, emphasis has been made on a very important peripheral hormone i.e. insulin which is known to be involved in several pathological conditions including diabetes. In mammalian brain, two INSR types viz INSR -A (neuron specific type) and INSR-B (peripheral type) are present where astrocytes predominantly express INSR isoform B. Insulin plays a role in astrocytic proliferation, differentiation and metabolism. A three-fold increase in the astrocytic glycogen content was reported when treated with insulin (Swanson and Choi, 1993; Heni *et al.*, 2011). Also, it is reported that the expression of glutamate transporter in astrocyte is under the influence of insulin (Ji *et al.*, 2011). Reactive astrogliosis (an abnormal increase in the number of astrocytes) with an increase in GFAP content is reported in diabetic murine models with prominent hyperinsulinemia (Saravia *et al.*, 2002). These reports make it evident that astrocytes are insulin responsive, however there are still several unknown facets of insulin on astrocytes that need to be explored. Thus, in first part of this objective we attempted to silence *Insr* gene, to mimic the phenomenon of insulin resistance in astrocytes. Although, several studies are there reporting role of insulin on astrocytes, there has always been uncertainty because of co-activation of IGF receptors after insulin stimulations. Thus, *Insr* knockdown model will unveil the effect of insulin signaling as well as insulin resistance *per se* in these cells.

Stress is one of the causal factor for the onset of diabetes by induction of peripheral insulin resistance and recent evidences suggest its role in induction of morphological and functional disturbances in astrocytes (Tynan *et al.*, 2013; Mayhew *et al.*, 2015). Elevated glucocorticoid (GC) level is the characteristic feature of stress response and numerous reports has confirmed that it can affect astrocytic fate (Yu *et al.*, 2011; Longden *et al.*, 2014; Zuo *et al.*, 2015). Carter *et al.* also demonstrated temporal patterning of GCs mediated cell specific transcription regulation of several candidate genes in astrocytes (Carter *et al.*, 2012; Carter *et al.*, 2013). It is well established fact that GCs can induce insulin resistance in peripheral cells. Few of the distinctive features of insulin dependent peripheral cells such as - glycogen storage as that of hepatocytes; lactate synthesis as that of myocytes; metabolic dependence on insulin and abundance of insulin receptor B isoform as that of hepatocyte, myocyte, adipocyte, and so on are shared by astrocytes. Also, the results from our first objective clearly demonstrated that there were remarkable modifications in astrocytic components in hippocampus and

hypothalamus of dexamethasone treated rats as compared to that of control. Thus, we predict that GCs might lead to insulin resistance in astrocytes as designed in present chapter.

Thus, the key questions of this objective were:

1) Does shutting down insulin signaling by *Insr* knockdown in astrocytes affect its fate and function?
2) Does glucocorticoid induce insulin resistance in astrocytes as that in peripheral cells?
3) Is there any crosstalk between insulin and dexamethasone in astrocytes?

5.1 IMPACT OF INSULIN RESISTANCE (*Insr* GENE KNOCKDOWN) ON ASTROCYTE METABOLISM.

5.1.1 PLAN OF WORK

Primary cultures of astrocytes were prepared from the postnatal day (PND) 0–2 forebrains of neonatal rats as per the protocol (McCarthy and de Vellis, 1980) (refer Chapter 3 Materials and methods). After third passage, cells were characterized for astrocytic markers using immunostaining and flow cytometry following which the cells were scaled up and seeded for experiments. Plasmids for shRNA against insulin receptor along with control plasmids were procured from Qiagen (Sure silencing shRNA plasmid Hygromycin KR44532H Cat. No. 336312) and the transfection was carried out using lipofectamine 2000 (Invitrogen). Before transfection, astrocytes were allowed to reach ~70% confluency and then were shifted to antibiotic (penicillin and streptomycin) free complete media for 24 hours. Transfection was performed in antibiotic and serum free 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 complete 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, and then proceeded for analysis of several parameters. The diagrammatic representation of plan of work is as shown below.

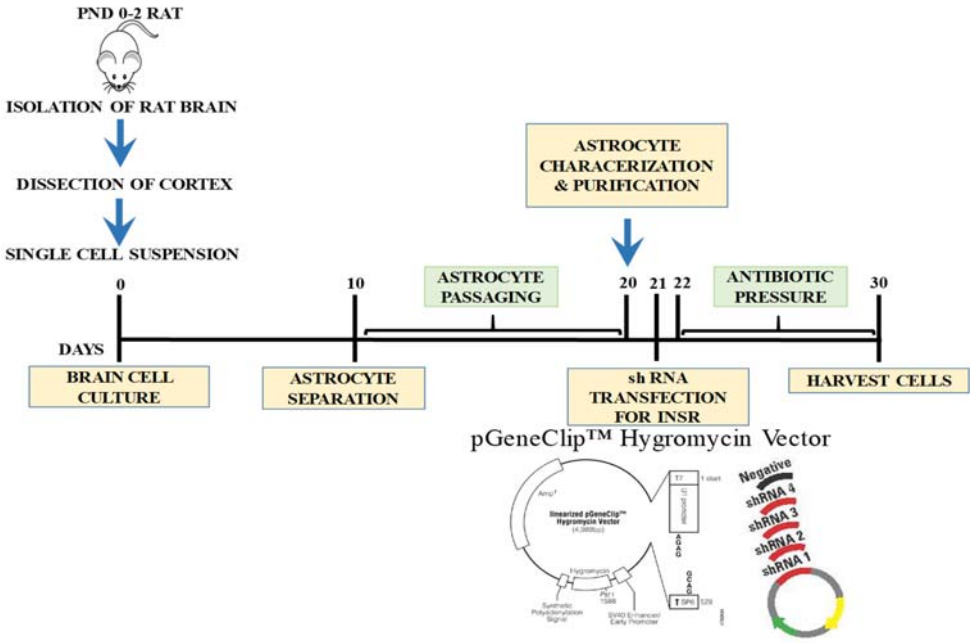


Figure 5. 2 Plan of work for *Insr* gene silencing in primary rat astrocytes.

5.1.2 RESULTS

CHARACTERIZATION OF ASTROCYTES

Initially seeded brain cells were allowed to reach confluency, subsequently which microglia, and oligodendrocytes were removed by differential shaking from the bed of astrocytes. These astrocytes were further passaged, and then characterized using astrocyte specific marker. Immunostaining demonstrated that these cells were positive for GFAP and vimentin marker (Fig 5.3). Further, percentage purification was assessed by flow cytometry where 98% cells were positive for GFAP staining while they were negative for neuronal (MAP2) and oligodendroglial markers (O1) as shown in Fig 5.4 A-D. Thus, further confirming that these cells were astrocytes.

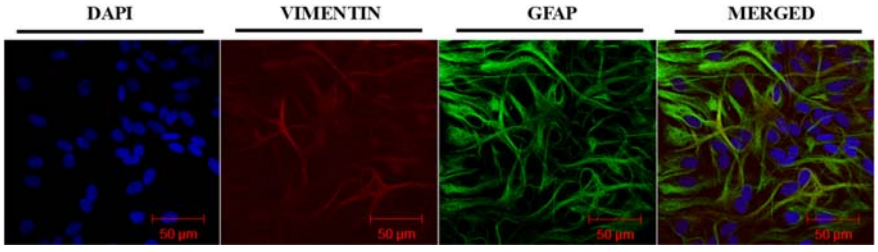


Figure 5. 3 Characterization of astrocytes by immunocytochemistry where they were positive for Vimentin (as shown in red), and GFAP (as shown in green). Dapi (as shown in blue) was used to counterstain nuclei. Scale bar denote 50 µm.

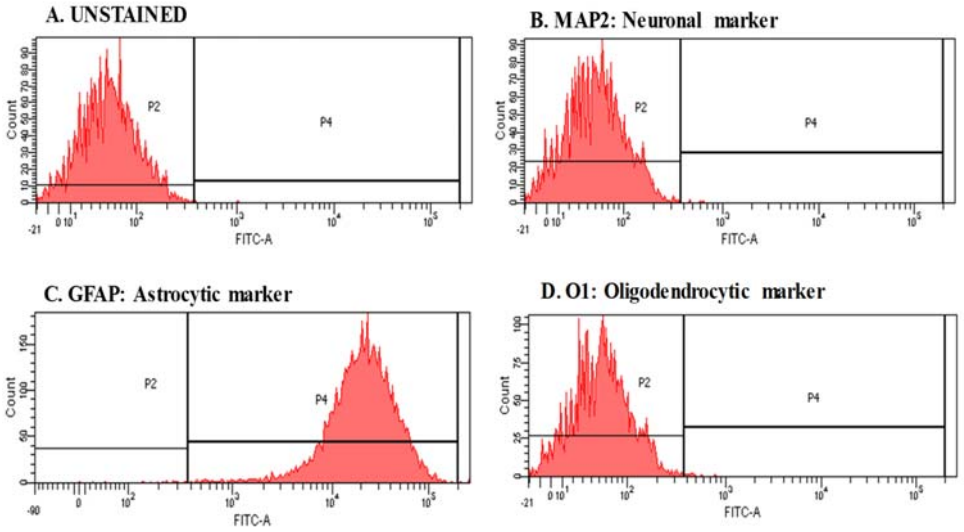


Figure 5. 4 Flow cytometric analysis of astrocytes. More than 98% cells were positive for GFAP (C)- an astrocytic marker, while negative for neuronal (B), and oligodendrocytic marker (D) against unstained cells (A).

INSULIN RECEPTOR GENE SILENCING (KD) IN ASTROCYTES

Astrocytes were transfected with four different plasmid clones with 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 5.5, more than 60% transfection was observed with clone 3 and 4 as compared to control cells and other plasmid clones. For all further experiments clone 4 was used.

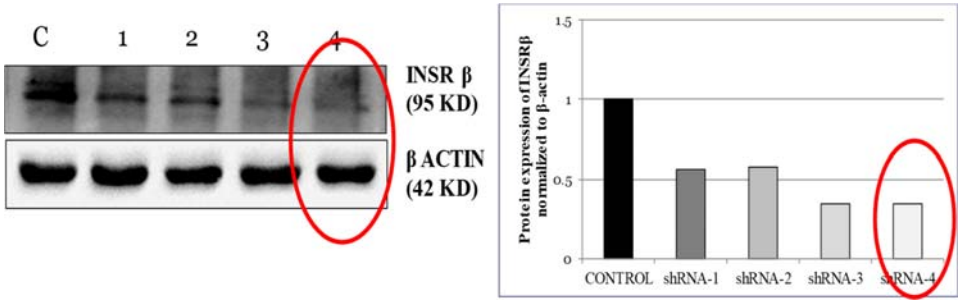


Figure 5. 5 Insulin receptor silencing was confirmed by immunoblotting of INSRβ. Highest efficiency of gene silencing was obtained with plasmid clone 3 and 4 shRNA as evident from blot (left) as well as densitometric analysis (right) after normalization to internal control.

MORPHOLOGY, SURVIVAL AND PROLIFERATION IS COMPROMISED IN INSR KD ASTROCYTES

Astrocyte morphology was slightly altered with evident autophagic vacuoles as observed in phase contrast microscopy in INSR KD astrocytes as compared to control (Fig 5.6A). GFAP is important for the morphological stability of the astrocytes However, neither increase or decrease was observed in the expression of GFAP post

Insr gene silencing (Fig 5.6B). Survival was remarkably decreased to 40 % in INSR KD as shown in Fig 5.7 as measured by MTT assay. 8% cells were observed to be in sub G1 phase indicating cell death in *Insr* KD cells in flow cytometric cell cycle analysis (Fig 5.8 A-C). There was also decrease in the expression of GLUT1 protein (Fig 5.9A-B) along with a steep decrease in the glycogen content (Fig 5.9C) in INSR KD cells.

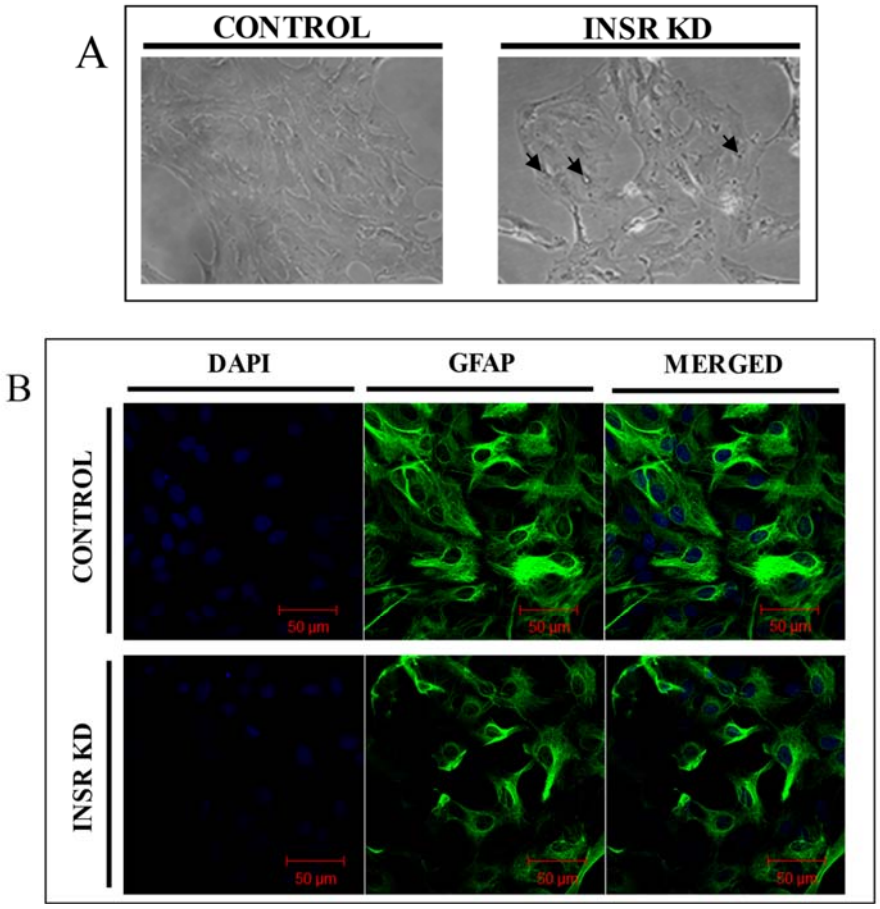


Figure 5. 6 A: Phase contrast imaging demonstrated that autophagic vacuoles (as shown by black arrows) were prominent in INSR KD astrocytes as compared to control cells. **B: Immunostaining for GFAP expression** in INSR KD astrocytes as compared to control cells. Scale bar represents 50µm.

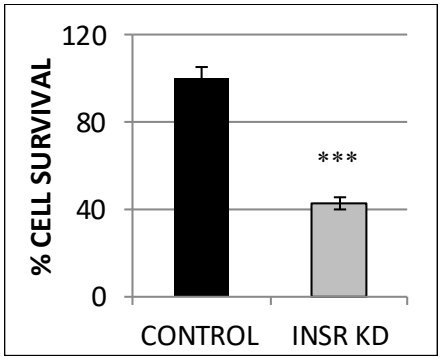
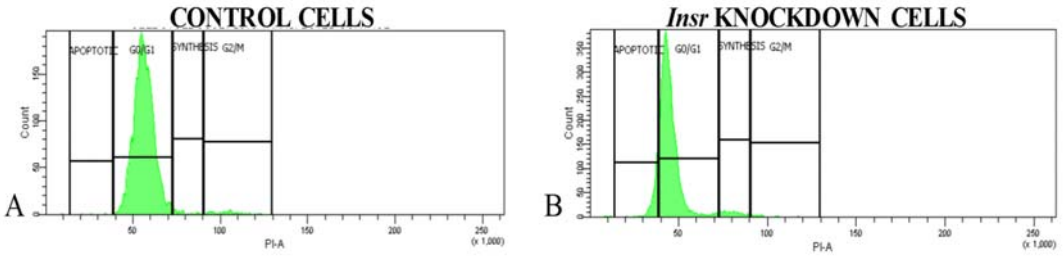


Figure 5. 7 MTT assay demonstrated 50% reduction in cell survival in insulin receptor knockdown (INSR KD) astrocyte as compared to transfected control (control) cells. Data represents MEAN± SEM for n=3. *** pvalue < 0.001 as compared to control.



C

POPULATION	CONTROL CELLS % PARENT	*Insr* KNOCKDOWN CELLS % PARENT
G0/G1	95.9	84.7
SYNTHESIS	1.5	3.6
G2M	2.3	0.6
APOPTOTIC	0.2	8.9

Figure 5. 8 Cell cycle analysis by flow cytometry. Apoptosis was observed in *Insr* KD condition (B) as compared to transfected control cells (A) in propidium iodide stained cytometric analysis of cell cycle. Table (C) denoted percent parent population for different stages of cell cycle.

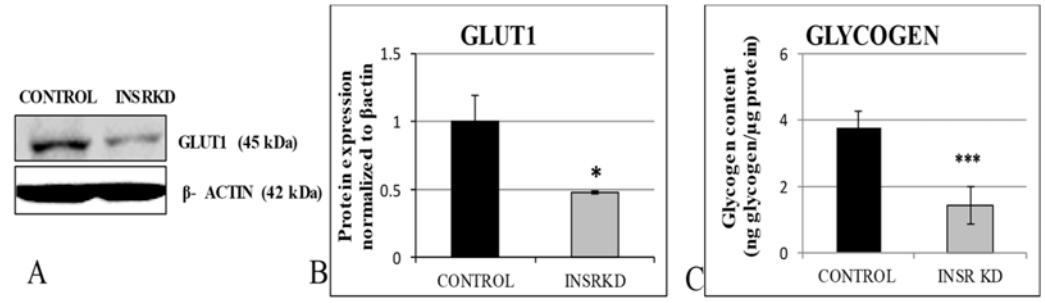


Figure 5. 9 A: Immunoblotting of GLUT1 in cells post transfection. B: Graph represents densitometric analysis done using Image J software keeping β-actin as endogenous control. C: Glycogen levels in INSR KD astrocytes as compared to transfected control cells. Data presented as Mean ± SEM of n=2-3 for control and INSR KD group. * p value <0.05, *** p value <0.001 as compared to control.

ASTROCYTIC COMPONENTS OF ASTROCYTE NEURON LACTATE SHUTTLE (ANLS) ARE ALTERED IN INSR KD CELLS

One of the most important feature of astrocyte is ANLS. Thus, further we assessed the dependence of astrocytes on insulin for secretion of lactate to provide alternate source

of energy to neurons. The excess glutamate as observed during neuronal firing was mimicked in the *in vitro* condition by addition of 100 μM glutamate for 3 hours prior to cell harvest. Neurotransmission is sensed by the astrocytes with the help of glutamate transporters (Glast and Glt1) which take up glutamate and initiate conversion of glucose and glycogen to lactate. As shown in Fig 5. 10 A-B, there was remarkable upregulation of *Glast* while there was down regulation of *Glt1* in *Insr* KD astrocytes. Lactate is shuttled through monocarboxylate transporters and as shown in Fig 5.10 C-D, there was upregulation of *Slc16a1* with downregulated *Slc16a3* expression levels in *Insr* KD astrocytes. The level of lactate released in response to glutamate challenge was also drastically reduced in INSR KD cells Fig 5.10 E. Glycogen is mobilized during high energy needs and to cope with it astrocytes continuously replenish the stores. Since insulin promotes the glycogen formation in astrocytes and as shown in result Fig 5.10 F, glycogen synthesis was significantly decreased of *Insr* KD cells, indicating its inability to restore cellular glycogen levels. Thus, these results are indicative that astrocytes become metabolically compromised in insulin resistant condition leading to disturbed ANLS.

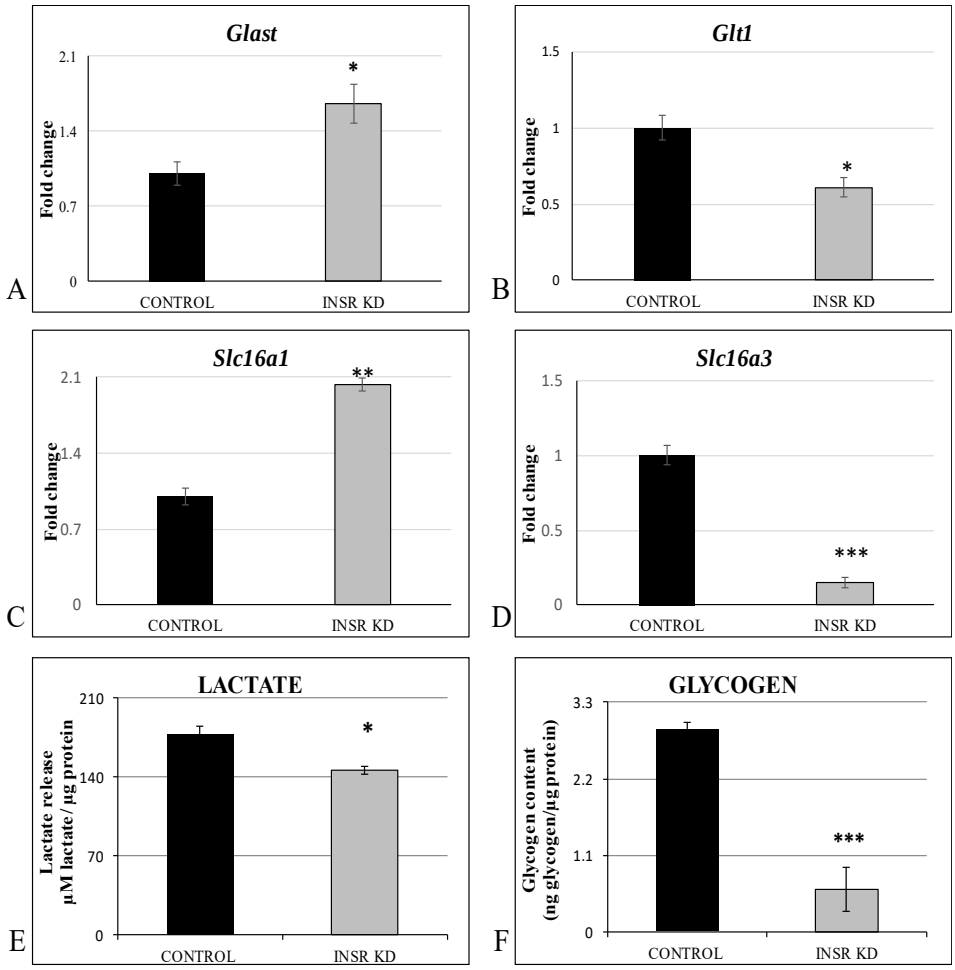


Figure 5. 10 Analysis of astrocytic components of ANLS post glutamate challenge. Quantitative real-time PCR analysis of *Glast* (A), *Glt1* (B), *Slc16a1* (C), and *Slc16a3* (D) genes against endogenous control *Actb* from INSR KD astrocytes as compared to transfected control cells. Lactate estimation (E), and glycogen content analysis (F) from INSR KD astrocytes as compared to transfected control cells. Mean ± SEM of n=2-3 for control and INSR KD group. * p value <0.05, ** p value <0.01, and *** p value <0.001 as compared to control.

5.2 IMPACT OF GLUCOCORTICOID ON INSULIN SIGNALING OF ASTROCYTES.

5.2.1 PLAN OF WORK

As observed in chapter 3 of objective 1, *in vivo* exposure of dexamethasone demonstrated variation in astrocytic components in hippocampus and hypothalamus of brain. Hence, we further wanted to analyzed the impact of dexamethasone under *in vitro* condition to delineate its direct effect and/or its indirect effect mediated by interference with insulin signaling. Thus, primary astrocyte cultures were incubated with DMEMF12 containing 0.5 % charcoal stripped FBS to limit residual serum steroid / insulin effects before 48 hours of glucocorticoid treatment. 1 µM dexamethasone

treatment was given for 24 hours. While in groups where glucocorticoid receptor (GR) inhibitor i.e. 3 µM RU486 (Sigma) was used, pre-treatment was given for 1 hours for the prior occupancy of GR so that dexamethasone cannot act [dose and timepoint for dexamethasone and RU486 adapted from (Unemura *et al.*, 2012)]. 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 such as changes in survival, proliferation, protein or gene expression, cells were harvested post 180 min (3 hours) of insulin induction [dose and timepoint for insulin adapted from (Heni *et al.*, 2011)]. For assessment of ANLS components of astrocytes, cells from all the groups were incubated with glutamate for 3 hours to mimic neuronal firing [dose of glutamate adapted from (Vaarmann *et al.*, 2013)]. Thus, the groups were as follows:

C	Untreated control cells	
D	Cells treated with 1 µM dexamethasone (D) for 24 hours	
D+R	Cells pretreated with 3 µM RU486 (R) for 1 hour and then incubated with 1 µM dexamethasone	
I	Only insulin induced cells	Induced with 50nM insulin (I) for 30 or 180 min
I+D	Cells treated with 1 µM dexamethasone for 24 hours	
I+D+R	Cells pretreated with 3 µM RU486 for 1 hour and then incubated with 1 µM dexamethasone	

The diagrammatic representation of plan of work as in Fig 5.11.

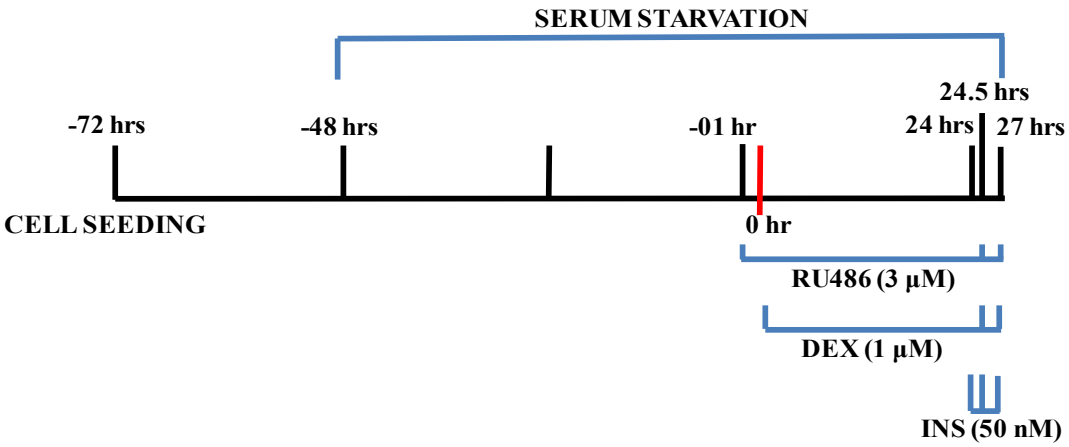


Figure 5. 11 Plan of work for the elucidation of impact of dexamethasone on insulin signaling and function of astrocytes.

5.2.2 RESULTS

SURVIVAL AND PROLIFERATION OF ASTROCYTES WAS NOT AFFECTED BY DEXAMETHASONE TREATMENT

Survival was assessed by MTT assay after the treatment period and as reflected from the results, no significant difference was observed in any of the groups as shown in Fig 5.12. Subsequently, cell cycle analysis was performed on these cells from each group by propidium iodide staining through flow cytometry. Individual insulin and dexamethasone treatment increased the proliferation of astrocytes, where their co-treatment had a synergistic effect as shown in Fig 5.13 (A-G).

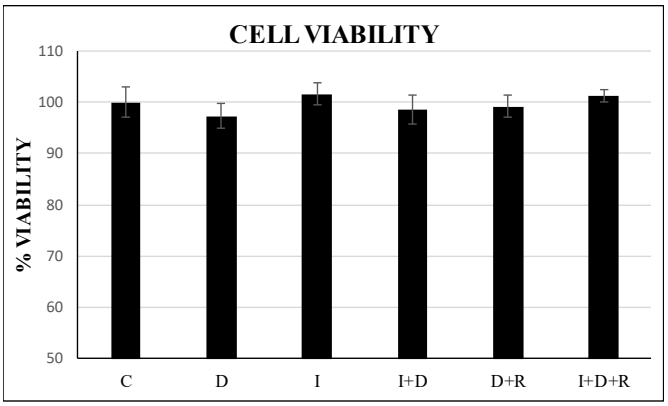
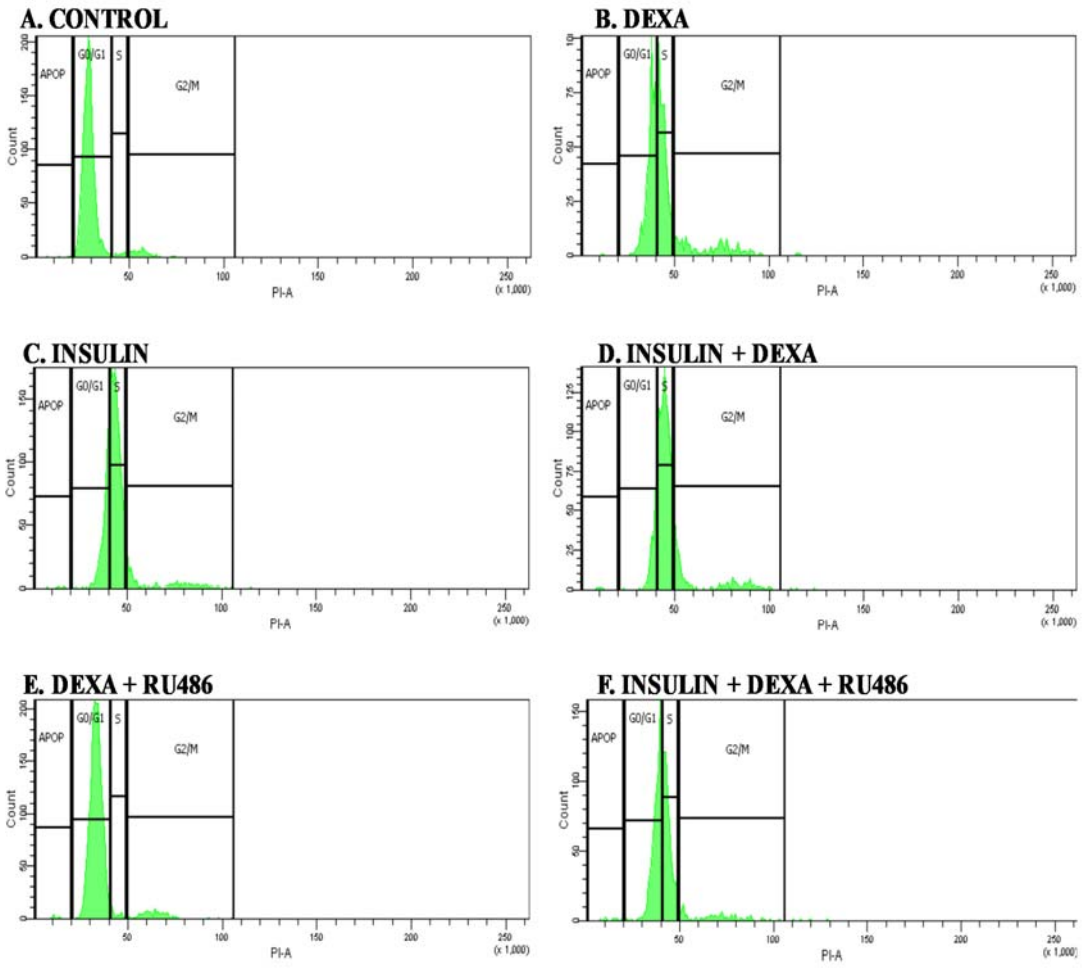


Figure 5. 12 No change was observed in cell survival as demonstrated by MTT assay. Mean ± SD of n=3 for all the groups. C: Control; D: dexamethasone treated; I: insulin; R:RU486.



G.

POPULATION	% PARENT					
	C	D	I	I+D	D+R	I+D+R
G0/G1	92.5	40.0	23.6	12.0	90.9	45.4
SYNTHESIS	1.6	41.5	60.3	64.7	2.1	40.4
G2M	5.3	13.8	9.6	16.1	6.0	8.0
APOPTOTIC	0.3	0.2	0.3	0.4	0.5	0.7

Figure 5. 13 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) astrocytes. Table (G) denoted percent parent population for different stages of cell cycle.

DEXAMETHASONE TREATMENT INDUCED REDUCED INSULIN SENSITIVITY IN ASTROCYTES

Insulin is known to promote astrocytic proliferation while dexamethasone has detrimental effect on astrocytes. To establish the effect of glucocorticoid (GR) on insulin signaling in astrocytes *in vitro*, primary astrocyte 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 for 30 min. Protein expression of candidate insulin signaling proteins were studied and the results demonstrated that although no change was observed in INSR levels (Fig 5.14 B), there was significant reduction in the downstream signaling protein expression of PI3 kinase (Fig 5.14 C) and activated AKT (Fig 5.14 D) had reduced response to insulin in dexamethasone treated astrocytes. This reduced insulin signaling because of dexamethasone was restored to control levels when they were pre-treated with glucocorticoid receptor antagonist (RU486).

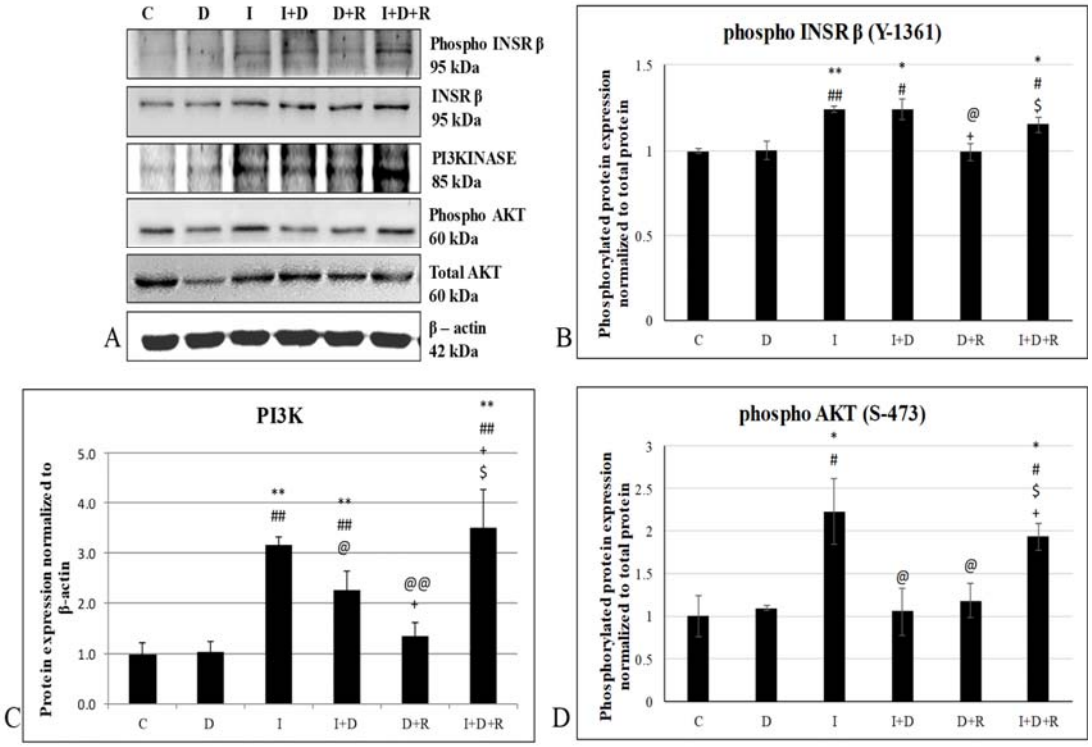


Figure 5. 14 A. Representative images of immunoblotting of phospho INSRβ Y-1361, Total INSRβ, PI3Kinase, phospho AKT S-473 and Total AKT keeping β-actin as endogenous control. B-D: Graphs represents densitometric analysis done using Image J software. Data presented as Mean ± SEM of n=2-3. *p value≤0.05 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.

GFAP, GLUT1, AND GLYCOGEN LEVELS WERE ALTERED POST DEXAMETHASONE TREATMENT IN ASTROCYTES

GFAP expression was assessed in astrocytes, where only dexamethasone treated group demonstrated decreased expression of GFAP. This reduction in expression was rescued by the induction with insulin or RU486 as shown in Fig 5.15. Further analysis of GLUT1 and glycogen levels demonstrated a decrease in insulin stimulated GLUT1 and glycogen content in

dexamethasone treated astrocytes as shown in Fig 5.16. The negative effects of dexamethasone were restored upon treatment with RU486.

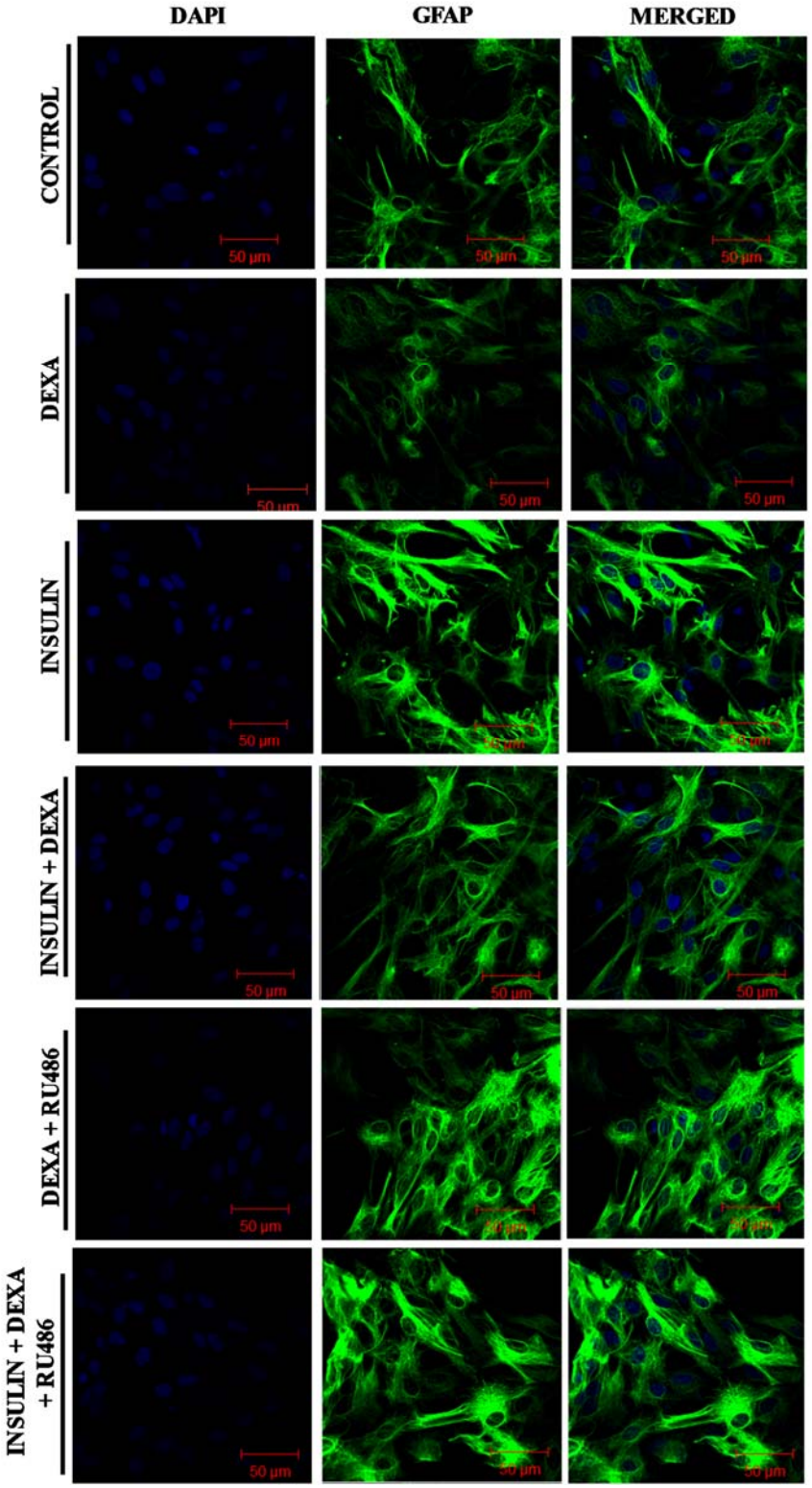


Figure 5. 151 Representative images of immunostaining for GFAP in astrocytes post treatment. Scale bar represents 50µm.

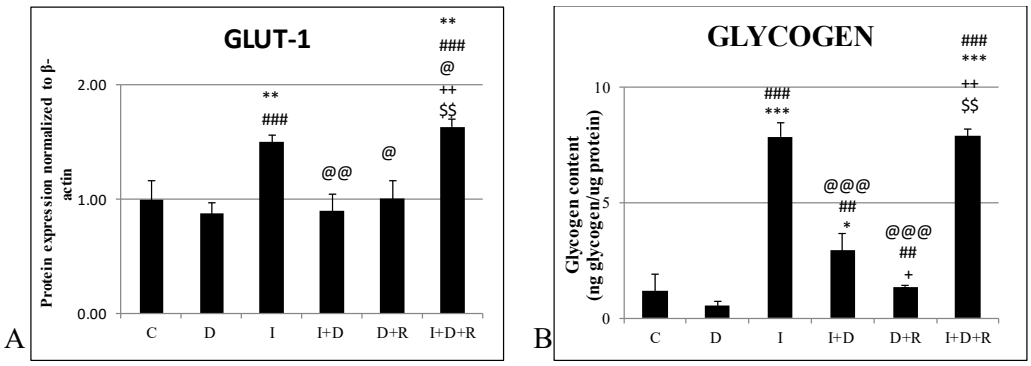


Figure 5. 16 GLUT1 protein expression (A) and Glycogen levels (B) estimated in various groups post treatment. Data presented as Mean ± SEM of n=2-3. **pvalue ≤ 0.01 as compared to control; ## pvalue ≤ 0.01 and ### pvalue ≤ 0.001 as compared to dexa; @ p value ≤ 0.05, @@ p value ≤ 0.01 and @@@ pvalue ≤ 0.001 as compared to insulin; ++ pvalue≤0.01 as compared to insulin+dexa. $$ pvalue ≤ 0.01 as compared to dexa + RU486.

ASTROCYTIC COMPONENTS OF ANLS ARE DISTURBED POST DEXAMETHASONE TREATMENT IN ASTROCYTES

The key astrocytic components of ANLS were assessed in response to dexamethasone and insulin in astrocytes post glutamate induction. *Insr* silencing results demonstrated that MCT4 (Slc16a3) and GLT1 might be under the transcription regulation of insulin signaling. And since dexamethasone impaired insulin signaling in astrocytes, we wanted to assess whether it is reflected in ANLS astrocytic components or not. However, our results demonstrated that dexamethasone independently modulated the expression of glutamate and lactate transporter. Although dexamethasone as well as insulin both increased the expression of *Glast* and *Glt1*, when they were introduced together, there was a decline in the mRNA levels as shown in Fig 5.17 A-B. Further, the assessment of expression of monocarboxylate transporters required for the lactate release also revealed that individual induction with both dexamethasone and insulin raised the level of MCT1(Slc16a1) and MCT4, however in insulin induced dexamethasone group, we could not find any additive effect as shown in Fig 5.17 C-D. Lactate release post glutamate was maximum in insulin induced group as shown in Fig 5.17 E while in dexamethasone treatment there was a decline in lactate release in spite of rise on MCT levels. Glycogen stores were recovered in insulin treatment group but remained low in insulin induced dexamethasone group as shown in Fig 5.17F.

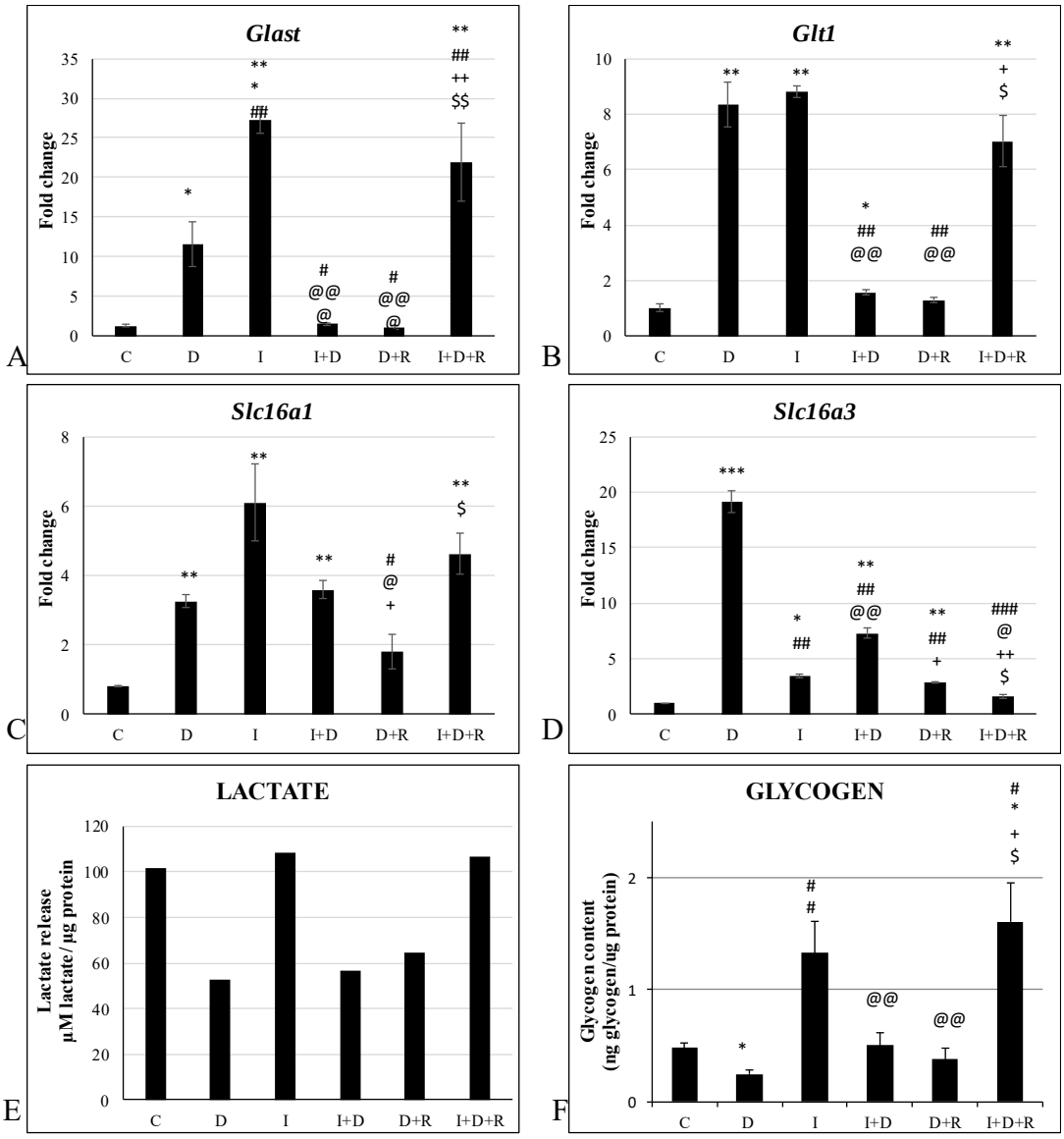


Figure 5. 172 Analysis of astrocytic components of ANLS post glutamate challenge. Quantitative real-time PCR analysis of *Glast* (A), *Glt1* (B), *Slc16a1* (C), and *Slc16a3* (D) genes from astrocytes from all the groups. Lactate estimation (E), and glycogen content analysis (F) from all the groups. Mean ± SEM of n=2-3. *p value ≤0.05; **pvalue ≤0.01 and ***pvalue ≤0.001 as compared to control. #p value ≤0.05; ##pvalue ≤0.01 and ### pvalue ≤0.001 as compared to dexa. @ p value≤0.05; @@ pvalue ≤ 0.01 and @@@ pvalue ≤0.001 as compared to insulin. +p value ≤0.05; ++pvalue ≤0.01 and +++pvalue ≤0.001 as compared to insulin+dexa. $pvalue ≤0.05; $$ pvalue ≤0.01 and $$$ pvalue ≤0.001 as compared to dexa + RU486.

5.3 DISCUSSION

Glia has always been overlooked as the submissive element of the nervous system over the neurons for brain functions. Among these glial cells, astrocytes are the most functionally versatile, imperative cells for the neural networking and processing. They are irreplaceable, beginning with their role in development and extending through physiological regulation to a variety of neurodegenerative disorders. Their regulation of brain micro-environment by shuttling of metabolites are among several of their important roles. This metabolic regulation is channelized by interplay with several intracellular and extracellular signaling components derived from central as well as peripheral system. Insulin is one such important peptide hormone secreted from pancreas that reaches brain where investigations has certified it being crucial for astrocytes. Also, several diseased conditions such as Alzheimer's, Parkinson's, diabetes, and so on claimed to develop insulin resistant brain, however the contribution of astrocytic insulin resistance is yet unstated. Thus, speculating that metabolism in astrocytes will be different in insulin responsive and resistant state.

For simplification, this hypothesis has been addressed in the *in vitro* system of primary astrocytes cultured from forebrain of postnatal 0-3 day rats. The protocol proposed by McCarthy & de Vellis, 1980 was followed and more than 96% pure astrocytic cultures as evident from GFAP immunostaining were established. Further, i*n vitro* model of insulin resistance was established by silencing astrocytic *Insr* using shRNA technology. Insulin signaling being a vital component, and owing to limited passaging in primary cultures which lead to serious reduction in cell survival, there were struggles in the clonal expansion from individual insulin resistant astrocytes. Thus, after transient transfection under continuous antibiotic pressure, the cells were harvested and analysed for *Insr* knockdown. There was more than 60% reduction in the *Insr* post transfection. Thus, the model for assessment of insulin resistance in astrocytes was successfully established.

Amongst numerous cell system including brain cells, supplementation of insulin has been known to aid in survival (van der Pal *et al.*, 1988; Ang *et al.*, 1992; Jonassen *et al.*, 2001; Garwood *et al.*, 2015). In accordance with this, our results also established that insulin resistance compromised the survival instinct in astrocytes, and thus

resulting in cell death. This lead to speculation that loss of astrocytes in diabetic brain might be one of the contributing factor towards neurodegenerations.

GLUT1 is the major glucose transporter present on astrocytes for glucose uptake. GLUT1 is a partially insulin dependent glucose transporter where insulin can up regulate its gene expression via either activation of AKT (in hepatoma cells) (Barthel *et al.*, 1999) or via pp70 S6 kinase (as shown in myocytes) (Taha *et al.*, 1995). Although, brain cells are recognized to be insulin independent in terms of glucose uptake, insulin resistant astrocytes had two-fold reduction in GLUT1 protein expression. Thus, current study confirms the role of insulin signaling in GLUT1 expression and glucose uptake even in astrocytes.

Astrocytes being the sole cell type having energy stores in form of glycogen in brain, glycogenesis becomes a key event that needs to be orchestrated appropriately (Brown and Ransom, 2015). In peripheral glycogen storing cell i.e., hepatocytes, glycogenesis is known to be completely dependent on insulin. Similar studies on astrocytes by Muhic *et al*., clearly demonstrated that insulin (1µM and not 100nM) without affecting glucose flux, stimulates increase in astrocytic glycogen stores via PI3K/p-AKT pathway (Muhic *et al.*, 2015). These studies corroborated with the current study where *Insr* KD astrocytes had decreased glycogen content as compared to insulin responsive astrocytes.

Astrocytes sense the neuronal firing with advent of their neurotransmitter transporters to mobilize their glycogen stores for neuron in the form of lactate, a phenomenon known as ANLS. They also sense and uptake neurotransmitters from the synapses to protect the neurons from excitotoxicity. Glutamate is well studied over other neurotransmitters for the modelling of ANLS. Hence, in the present study, insulin resistant astrocytes were exposed to exogenous glutamate *invitro* to mimic neurotransmission. Of the several subtypes of glutamate transporter, GLT1 and GLAST are restricted to astrocytes which are expressed in different proportions in different regions of the brain (Lehre *et al.*, 1995). Insulin exposure lead to an increase in expression of GLT1 in cultured astrocytes (Ji *et al.*, 2011). However, in our study, post glutamate challenge, there was reduction in the expression of *Glt1* in *Insr* KD astrocytes. This reduction in GLT1 in insulin resistant condition clearly suggest its regulation by insulin. On the contrary, there was an increase in GLAST expression in

Insr KD astrocytes. The plausible explanation could be the compensatory mechanism to cope up with a decrease in one subtype by increasing the synthesis of other.

Glutamate sensing is followed by conversion of glycogen to lactate. This lactate, thus produced is shuttled through monocarboxylate transporters viz MCT1 and MCT4 exclusively present on astrocytes in the brain parenchyma (Pierre and Pellerin, 2005). Insulin is known to induce the expression of monocarboxylate transporters (MCT2) in neuronal cells (Chenal and Pellerin, 2006), thus we predicted that insulin resistance might downregulate monocarboxylate transporters in astrocytes. Among both astrocytic MCTs, MCT4 have high Km (about 30 mmol/L). Thus, MCT4 will not be saturated, and the transport rate will therefore increase with the lactate concentration signifying its importance in lactate efflux. We observed an increase in level of MCT1 and a decrease in the level of MCT4 post glutamate exposure. Although, this increase in lactate transporter was not accompanied with an increase in lactate release as evident from conditioned media of *Insr* KD astrocytes. An immediate response towards mobilized glycogen to glucose will be initiation of glycogenesis for the replenishment of these stores, but reduced recovery in glycogen levels was observed in insulin resistant condition. However, we cannot deny that some of the changes in expression of lactate transporter and lactate level might be an outcome of reduced glucose uptake and glycogen content instead of direct insulin action. Still, our results made it utmost clear that insulin resistance negatively altered astrocytic metabolism especially its function in ANLS.

Once established the role of insulin in astrocytic survival, proliferation, and function, it was interesting to know the effect of glucocorticoid (GCs) on insulin signaling in astrocytes. This speculation for the involvement of astrocyte was generated because of the development of brain insulin resistance in dexamethasone induced diabetic rat. Glucocorticoids (GCs), a class of steroid hormones are vital components of neuroendocrine system, and are indispensable for coordinating the adaptive responses to basal and stress related homeostasis (Myers *et al.*, 2017). This paved way to the extensive use of synthetic glucocorticoids in several therapeutic interventions. Despite their long-standing use at clinical level; they are associated with a large number of potential side effects as well as complications. Insulin resistance is of particular concern amongst the common side effects observed due to chronic high levels of GC (Di Dalmazi *et al.*, 2012). The mechanism of chronic stress / steroid induced peripheral

insulin resistance is a well-established mechanism known to involve liver, muscle, adipose tissue, ovary etc. However, not much has been explored in defining the role of GCs in the onset of insulin resistance essentially with reference to astrocytes.

Thus, in this study cultured astrocytes were treated with dexamethasone, a synthetic GC to assess its involvement in affecting insulin sensitivity. Although survival was not affected and there was an increase in proliferation of astrocytes, the results displayed an impaired post insulin receptor cascade involving AKT in astrocytes in dexamethasone treated group that could be reversed by the pre-treatment with glucocorticoid receptor antagonist. Similar observation has been made in myocytes and adipocytes where dexamethasone plays a role in inhibiting insulin signaling via reduction in the levels of insulin receptor substrate 1 (IRS1) protein and PI3K/AKT activation (Sakoda *et al.*, 2000; Son *et al.*, 2015). This impaired insulin signaling can alter several of the important astrocytic function mainly glucose uptake and glycogen synthesis. As evident from our *in vitro Insr* silencing in astrocytes, GLUT1 is dependent on insulin signaling. This was also reflected in dexamethasone treated group where impaired insulin signaling reduced the level of GLUT1. Similar comments have been made regarding the role dexamethasone/GCs in the peripheral cells such as adipose tissue and 3T3L1, where decrease in GLUT1 expression was observed (Chu *et al.*, 1990; Sakoda *et al.*, 2000). However, there do exist some contradictory reports where an increase in GLUT1 was seen in L6 skeletal cells post dexamethasone treatment (Ewart *et al.*, 1998). While in other isoform i.e. GLUT4 present in adipose tissue, there exists synergism between insulin and GC on its gene transcription mediated through promoter activation, thus increasing its expression (Hajduch *et al.*, 1995; Hernandez *et al.*, 2003). This lead us to conclude that effect of GC and insulin signaling on glucose transporter is unique to its isoform and target cell.

As mentioned earlier glycogen in the brain is localized exclusively in astrocytes. Even though the glycogen content in astrocytes is relatively low compared with that in the liver and skeletal muscle; any alteration in the glycogen level can lead to neuronal energy drain during firing. In adipocytes, dexamethasone directly influenced the insulin mediated pathway of glycogen synthesis (Anil Kumar and Marita, 2000). However, in hepatocytes, it is proved that the modulation of glycogen synthesis is dependent on dose and duration of dexamethasone, where high short term dose lead to glycogen depletion (Zheng *et al.*, 2009). Also, Allaman *et al* demonstrated that GCs can have the

modulatory effect on long term glycogen synthesis induced by nor-adrenaline (Allaman *et al.*, 2004). Since there was no reduction in glycogen content in dexamethasone alone treated astrocytes, it was clear from our results that glycogen reduction was because of inhibition of insulin signaling by dexamethasone. These studies corroborated with the *in vivo* study from objective 1 where in dexamethasone induced diabetic rat model, there was a drastic decrease in glycogen content in hippocampus as well as hypothalamus.

Insulin signaling modulated glutamate uptake and lactate release. Thus, similar aspect was studied with exposure to dexamethasone and induction with glutamate. Cultured astrocytes demonstrated alterations in the level of glutamate and lactate transporters when pre-treated with dexamethasone and then induced with glutamate. As observed in case of glucose transporter and glycogen levels, dexamethasone did not display inhibitory effect of insulin action on glutamate and lactate transporters on astrocytes. In case of *Glast* and *Glt1*, insulin as well as dexamethasone individually raised its expression level, however when together inhibited this upsurge. Alone dexamethasone treatment (100nM for 72 hours) provoked a marked increase of GLT-1 transcription and protein levels in cortical astrocytes (but not in cerebellar and midbrain astrocytes), accompanied by an enhanced glutamate uptake in a study conducted by Zschocke et al., 2005 (Zschocke *et al.*, 2005). This promoting effect of dexamethasone on GLT-1 gene expression was abolished by the GR antagonist RU486 similar to that of our study. Also, Perisic T *et al* discovered epigenetically adaptive DNA element of the GLT-1 promoter responding to dexamethasone being decisive for brain region specific activity and reactivity (Perisic *et al.*, 2012). Rauen and Wiessner (2000) had demonstrated that glucocorticoid hormone cortisol upregulated GLAST gene and protein expression in retinal glia cells (=Muller cells), they suggested that transcriptional regulation of glial proteins may impact on transmitter clearance (Rauen and Wiessner, 2000). However, we could not find any report mentioning any mechanism that might answer the decrease in *Glt1* or *Glast* in combinatorial exposure of insulin and dexamethasone. Further dissection of genomic as well as nongenomic effects of both hormones will be required to justify our results.

Further, the assessment of expression of monocarboxylate transporters required for the lactate release also revealed that individual induction with both dexamethasone and insulin raised the level of MCT1 and MCT4, however in insulin induced

dexamethasone group, we could not find any additive effect. We could not find any published results that could shed light on effect of insulin or dexamethasone on MCTs. Hence, to the best of our knowledge, ours could be the first result demonstrating the expression level of MCTs in response to insulin and dexamethasone post glutamate challenge in astrocytes. Nevertheless, the level of lactate secreted in conditioned media corresponded more to glycogen content rather than MCT levels, where insulin group had more lactate secretion as compared to other groups. Thus, the increase in MCT expression might lead to the depletion of the astrocytic energy stores in dexamethasone treated groups against insufficient glucose uptake and glycogen synthesis. In long run, this might end up in astrocytic metabolic deregulation and improper supply of metabolites to neurons.

Thus, the current *in vitro* study establishes that insulin signaling is playing a noteworthy role in maintaining the astrocytes. Also, astrocytes are very susceptible to glucocorticoid induced impaired insulin signaling. The results accentuate that there are adverse effects of glucocorticoid and insulin resistance on astrocytes, negatively affecting its metabolism. Our results conclude that astrocytes are susceptible to developing insulin resistance which might be the case in several diseased conditions such as diabetes, stress, Alzheimer's, Parkinson's, etc. causing irreparable damage. Being the modulator of multiple crucial functions in the central nervous system, deregulation of astrocytic function might end up in neuronal death, and thus cause neurodegenerative diseases. Although, we could establish the effect of GC on insulin mediated regulation of glucose transporter expression and glycogen levels, further study needs to be conducted to understand the crosstalk between insulin and glucocorticoid in the regulation of astrocytic components of ANLS. This will further be instrumental in deciding pathology as well as therapy for diabetes as well as stress related neurogenerative threats.

In nut shell, the outcome of this objective can be pictorially summarized as Fig 5.18.

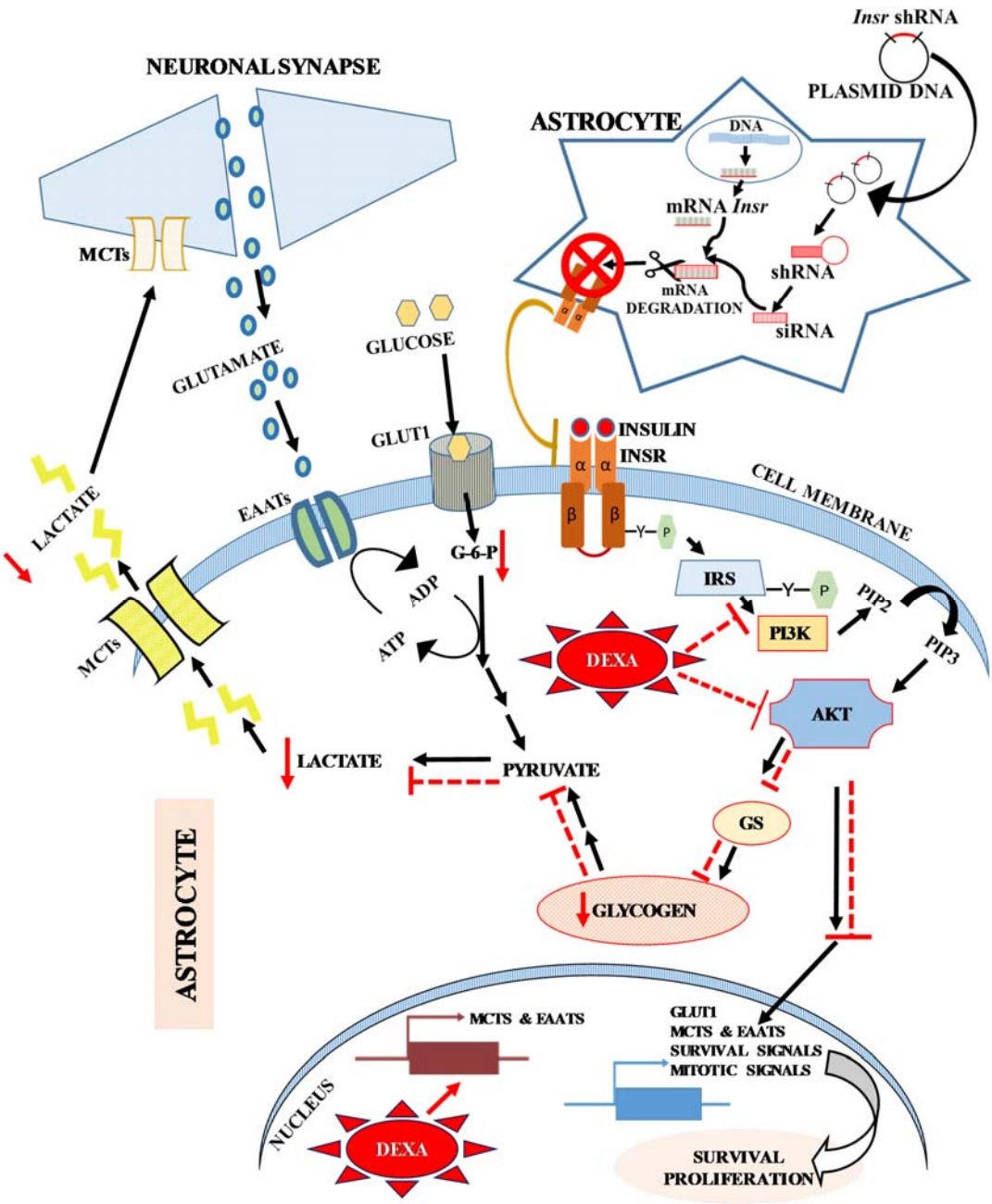


Figure 5. 18 Summary of "The role of insulin resistance and glucocorticoid on metabolism of astrocyte".

1. *Insr* gene silencing demonstrated the vital role of insulin signaling in survival, proliferation and metabolism of astrocytes. Key astrocytic functions regulated by insulin are glycogen and lactate synthesis along with transcription regulation of glucose, glutamate and lactate transporters.
2. Dexamethasone (Dexa-synthetic glucocorticoid) exposure impairs insulin signaling, thus hampering insulin actions on astrocytes (as shown by red dashed lines).