CHAPTER 4

REGIONAL AND NEUROBEHAVIORAL STUDY OF BRAIN INSULIN RESISTANCE IN GLUCOCORTICOID INDUCED DIABETIC RAT MODEL

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Insulin signaling in different brain regions crosstalk with several neural and peripheral cues to generate appropriate responses (Blazquez *et al.*, 2014). Also, brain insulin signaling is reported to work in collaboration with insulin signaling in peripheral tissues (Stanley *et al.*, 2016). Thus, it can be well correlated that disconcerted peripheral insulin signaling / insulin resistance as observed in pathological conditions such as Type 2 diabetes mellitus (T2DM) can outspread negative effects on brain insulin signaling. However, there exists discrepancy on the neurological outcomes in T2DM both at preclinical and clinical studies, owing to the differences in genetic/metabolic trigger and environmental contributing factors. Thus, we hypothesize that the root cause of these differences can be attributed to the specificity in the brain region affected as well as the cause and duration of T2DM.

Several models have been developed for mimicking T2DM condition in animals such as obese monogenic (db/db mice, ob/ob mice) / polygenic models (KK mice, NZO mice), non-obese diabetic models (GK rat, Tori rat), chemically induced diabetic models (Streptozotocin, Goldthioglucose), dietary induced models (high fat/ fructose induced), surgically manipulated models (VMH lesion model), knockout animal models (INSR or GLUT4 knockout mice models), and so on (Srinivasan and Ramarao, 2007). Although providing useful insights, drawback with genetic, knockout, and chemical models is that the aetiology do not resemble involvement of multiple gene defects, lifestyle as well as environmental factors as observed in T2DM in humans. Also, majority of the models are obesity induced which renders investigation of T2DM development in absence of obesity very difficult.

Thus, for the dissociation of confounding obesity factors such as leptin dependence, and to mimic stress induced T2DM model, chronic exposure of glucocorticoid (GCs) was given to rats. Glucocorticoids are steroid hormones that relay the signals of stress, which at high concentration for chronic period are known to induce diabetes by generating insulin resistance in peripheral tissues (Vegiopoulos and Herzig, 2007). GCs induced peripheral insulin resistant model demonstrate hyperglycaemia, hyperinsulinemia as well as altered lipid profile. These metabolic alterations can in turn

again exert disparity in insulin actions in brain which will be further reflected at structural, physiological and psychological levels. However, very few studies are available in context to the peripheral insulin resistance induced by long-term effects of circulating elevated GCs on brain insulin signaling (Piroli *et al.*, 2007; Osmanovic *et al.*, 2010). In light of this, T2DM rat model was developed by chronic exposure of dexamethasone (a synthetic GCs) in rats. This T2DM model has lean phenotype with pronounced peripheral insulin resistance, and had been an established diabetic model previously exploited in our lab to understand ovarian insulin resistance (Belani *et al.*, 2014). Thus, we aimed to assess brain insulin signaling and its neurobehavioral upshots in dexamethasone treated rat model in this objective.

Several roles of insulin in brain has been explored where the most studied functional avenues are neurogenesis and appetite regulation in hippocampus and hypothalamus respectively (Bateman and McNeill, 2006; Yu and Kim, 2012). Insulin might affect hippocampal neural stem cells (NSCs) by its crosstalk with signaling pathways such as Wnt and Notch, which are crucial for maintenance and fate commitment of NSCs. Similarly, insulin in hypothalamus interacts with Npy/AgRP and Pomc neurons which plays decisive role in satiety and hunger. Thus, maintaining energy uptake and expenditure.

Alteration in neurogenesis as well as appetite is one of the feature reported both in diabetes as well as stress (Mirescu and Gould, 2006; Ho *et al.*, 2013; Yau and Potenza, 2013). Thus, we hypothesized that glucocorticoid induced diabetes might disturb hippocampal and hypothalamic insulin signaling and function. This question has been systematically addressed in the first part of this objective where we have observed remarkable changes in the hypothalamic insulin signaling with change in appetite behavior.

From developmental point of view, appetite-mediated ingestive behavior is programmed *in utero* depending on intrauterine conditions, thus preparing for new-born and adult ingestive behavior (El-Haddad *et al.*, 2004; Cripps *et al.*, 2005). Also, neurogenesis is reported to take place between embryonic days 14 to 18 in the rat hypothalamus, while the blood brain barrier forms between embryonic days 11 and 17 (Risau and Wolburg, 1990; MacKay and Abizaid, 2014). So, during this time, the hypothalamus is highly vulnerable to any changes that take place in its immediate

environment, like the intrauterine conditions. Thus, owing to the fact that the density of insulin receptor is remarkably high in brain during embryonic period as well as the circuit for appetite regulation begins to be programmed since developmental stage, it would be intriguing to know if maternal insulin resistance leads to an altered hypothalamic signaling in neonates. Hence, in second part of this objective, we analysed the programming of the fetal hypothalamus in response to glucocorticoid induced maternal insulin resistance.

Thus, the key questions that we have addressed were:

1) Whether all the brain regions respond similarly to chronic dexamethasone induced T2DM and develop insulin resistance?

2) Would there be any evident neurobehavioral alterations in dexamethasone induced T2DM?

3) Does weight loss is dexamethasone induced T2DM be correlated to hypothalamic circuit of appetite regulation involving insulin signaling?

4) Whether alteration in *in utero* condition because of maternal insulin resistance will affect the programming of hypothalamic appetite regulation in fetus?

4.1 ASSESSMENT OF REGION SPECIFIC ALTERATIONS IN INSULIN SIGNALING IN GLUCOCORTICOID INDUCED DIABETIC RAT MODEL AND ITS CORRELATION TO NEUROBEHAVIOR.

4.1.1 PLAN OF WORK

3 mg of dexamethasone (a synthetic glucocorticoid) per kg of body weight was subcutaneously injected for 28 days in female young Charles Foster rats to develop diabetic rat model. During the treatment period, food intake, water intake, and body weight were monitored at regular interval in both the groups. After treatment period, confirmation of peripheral insulin resistance in dexamethasone (dexa) treated rats was done using serum insulin, glucose, and corticosterone levels which were compared to that of control rats (injected with equivalent dose of sterile normal saline). Different sets of control and diabetic animals were kept for different parameters viz. protein expression studies, histological studies and behavior studies. For protein expression studies, different regions of brain – hypothalamus, hippocampus, cortex and cerebellum was dissected out and assessed for candidate insulin signaling proteins by western blotting. For histological studies, rat's brain was fixed using transcardial perfusion of formaldehyde followed by cryo-sectioning. These sections were then proceeded for

haematoxylin-eosin staining or immunostaining. For behavior studies, rats were moved to the testing area in their home cages and allowed to adapt to the new environment for at least 1 h before testing. After acclimatization, they were made to perform a battery of neuro-behavior tests. Also, hypothalamus and hippocampus were analysed in depth for their respective functions. Please refer to Chapter 3 for detailed materials and methods. The schematic plan of work for this objective is as shown in Fig 4.1.

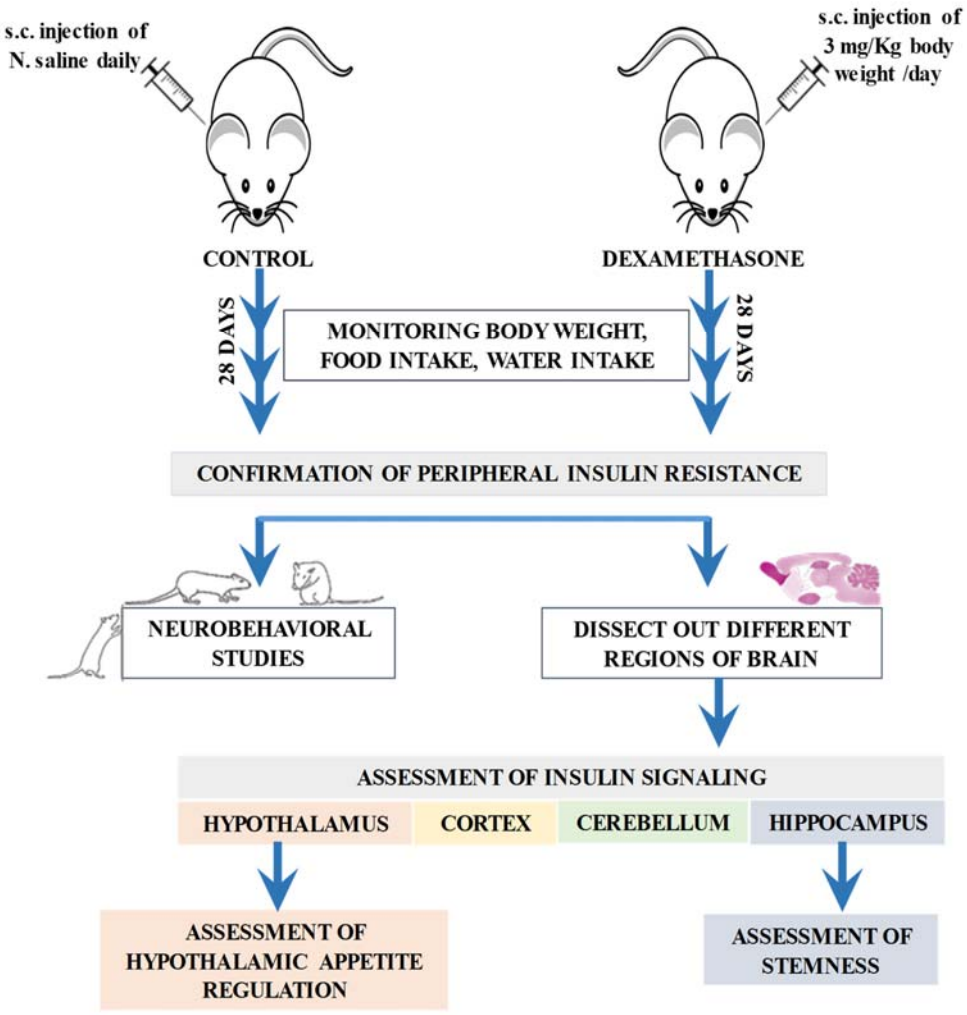


Figure 4. 1 Plan of work for the *in vivo* assessment of brain insulin resistance and its outcome in dexamethasone induced diabetic rat model.

4.1.2 RESULTS

CONFIRMATION OF DEXAMETHASONE INDUCED DIABETIC RAT MODEL

The alterations in food intake and body weight during the length of 24 h at a regular interval of 3 days were monitored in rats treated with N. saline or dexamethasone for 28 days. A statistically significant reduction in body weight (Fig 4.2A) and food intake

(Fig 4.2B) was observed in rats following dexamethasone treatment for 28 days as compared to saline-treated control rats (control). Also, there was marked increase in water intake in dexamethasone treated group as compared to control as shown in Fig 4.2C. At the end of the treatment period, dexamethasone treated group demonstrated mild fasting hyperglycaemia with significant intolerance to oral glucose as shown in Fig 4.2D. The fasting glucose levels, insulin levels and Fasting Insulin Resistance Index (FIRI) positively correlated with an insulin resistant phenotype in dexamethasone treated rats as shown in Table: 4.1. Chronic treatment with dexamethasone suppressed the endogenous corticosterone level as shown in Table: 4.1. Thus, dexamethasone treatment resulted in reduced appetite, body weight and endogenous corticosterone levels with a marked rise in glucose intolerance and FIRI.

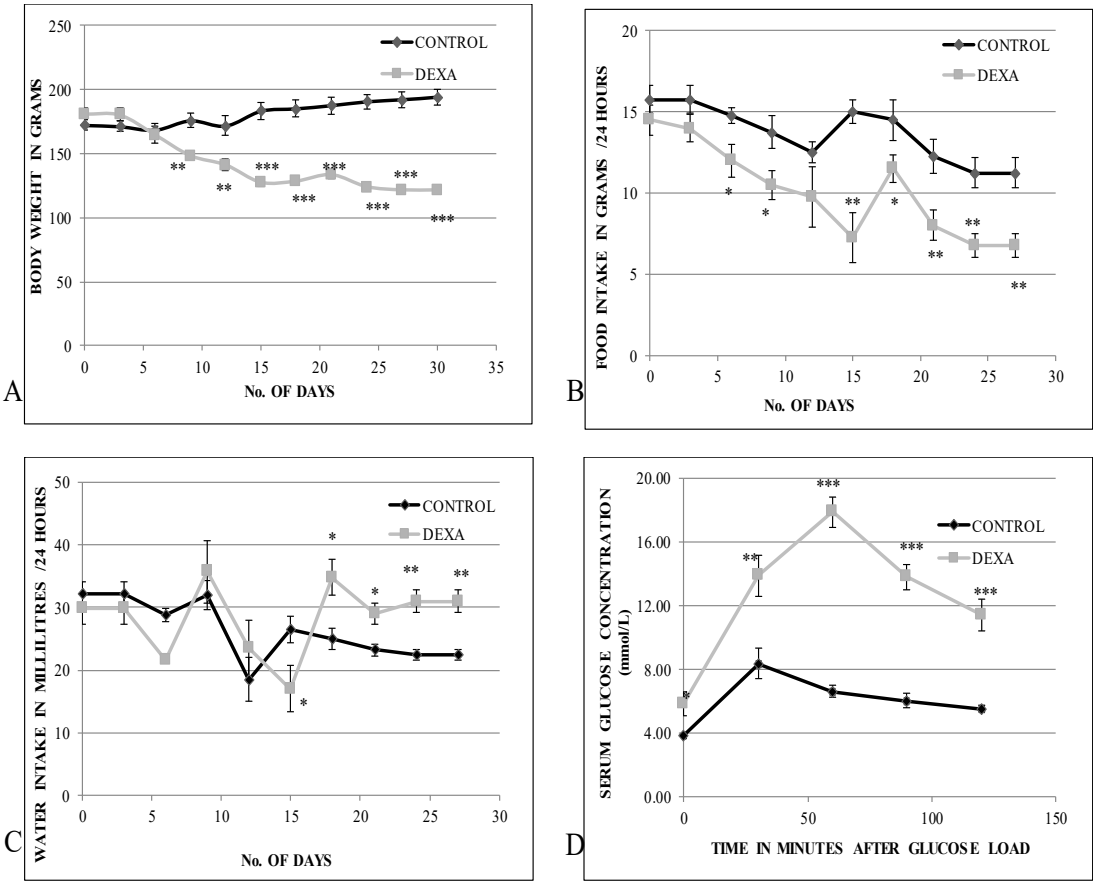


Figure 4. 2 Assessment of bodyweight (in grams) (A), food intake (in grams/24 hours) (B), water intake (in ml/24 hours) (C), and oral glucose tolerance (D) in dexamethasone (dexa) treated rats and control rats. Data presented as Mean ± SEM of n=6 for control and dexa group. * p value <0.05, ** p value <0.01, and *** p value <0.001 as compared to control.

Table 4. 1: Biochemical parameters of dexamethasone treated rats (Dexa) as compared to control rats.

	CONTROL	DEXA
Fasting Serum Insulin (µIU/ml)	4.59 ± 1.71	58.21±9.9***
Fasting Serum Glucose (mmol/L)	2.55 ± 0.86	3.56± 0.9*
Fasting Insulin Resistance Index (FIRI)	0.4 ± 0.27	8.3 ± 2.7***
Serum Corticosterone (ng/ml)	314.52 ± 16.70	167.81 ± 21.47***

Data presented as Mean ± SEM of n=4-6 for control and dexa group. * p value <0.05; *** p value <0.001 as compared to control.

DEXAMETHASONE TREATMENT DOES NOT ALTER BRAIN MORPHOLOGY IN RATS

The wet weight of whole brain as well as different regions of brain dissected under stereo-zoom microscope was taken. As shown in Table: 4.2 no significant difference was observed in weight of whole brain or regions in dexamethasone treated rats as compared to the control rats. However, a trend was observed towards reduction in brain weight in dexamethasone treated rats as compared to control. Also, no histological differences were noted in the brain of dexamethasone and control rats as evident by haematoxylin and eosin (H & E) staining as shown in Fig 4.3.

Table 4. 2 Absolute wet weight of different regions of rat brain.

Absolute wet organ weight (in gms)		
	CONTROL	**DEXA**
Hypothalamus	0.044 ± 0.011	0.038 ± 0.001 ^{ns}
Hippocampus	0.16 ± 0.017	0.15 ± 0.017 ^{ns}
Cortex	0.908 ± 0.02	0.91 ± 0.05 ^{ns}
Cerebellum	0.296 ± 0.03	0.275 ± 0.021 ^{ns}
Whole Brain	1.728 ± 0.04	1.67 ± 0.12 ^{ns}

Data presented as Mean ± SEM of n=6 for control and insulin resistant group. ns=p value >0.05 as compared to control.

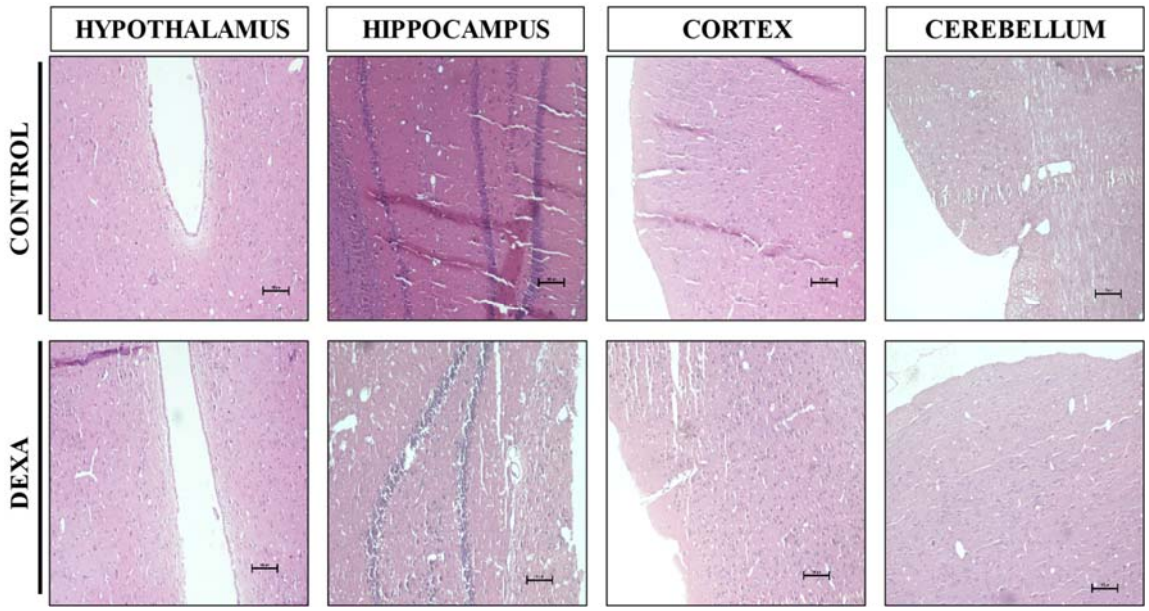
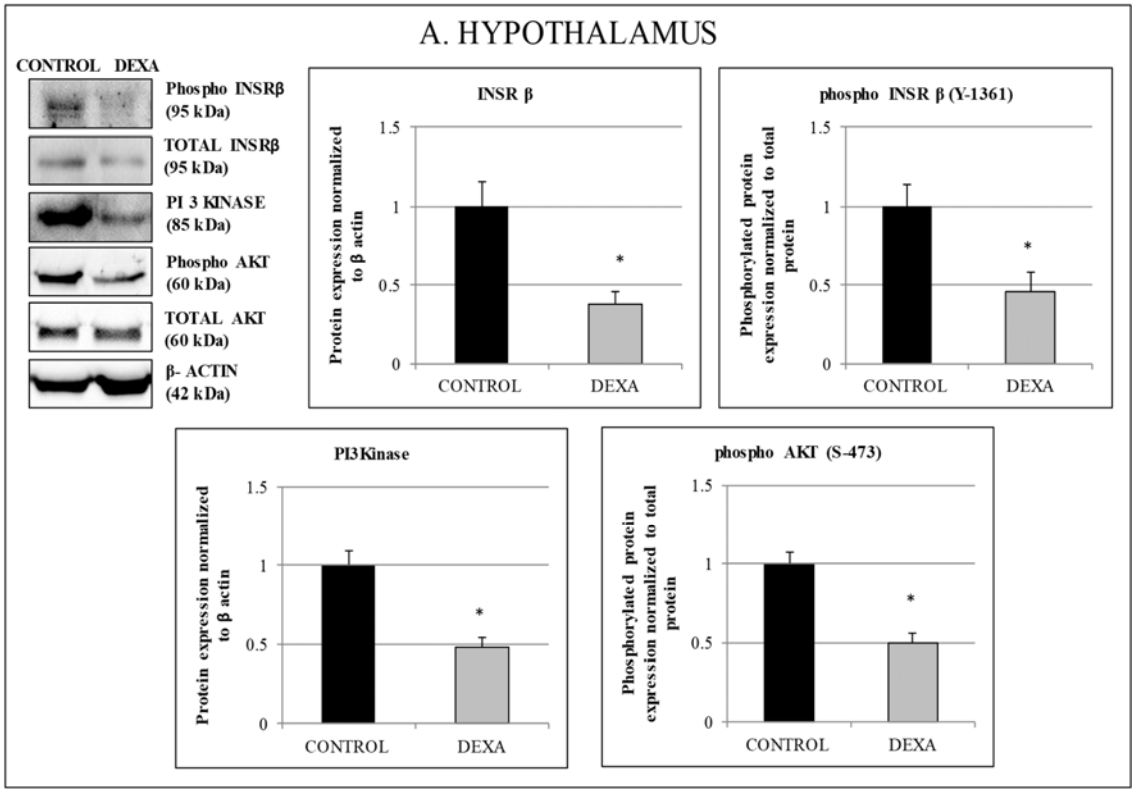
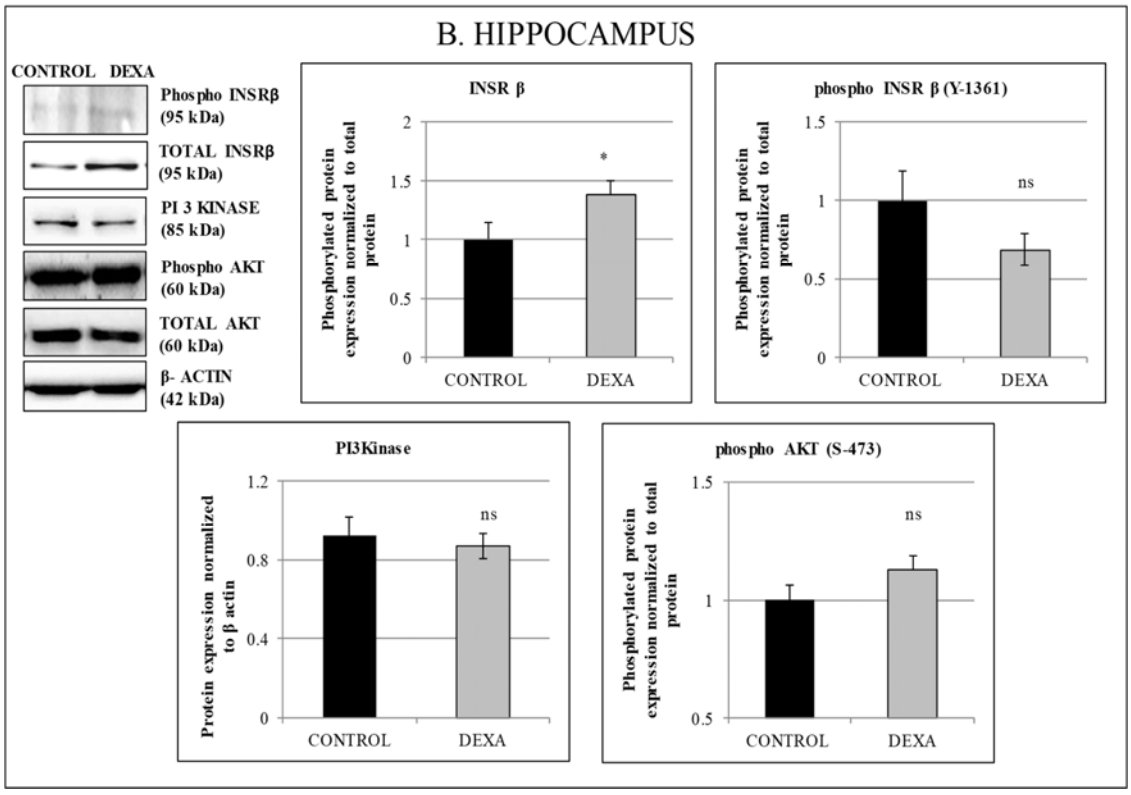


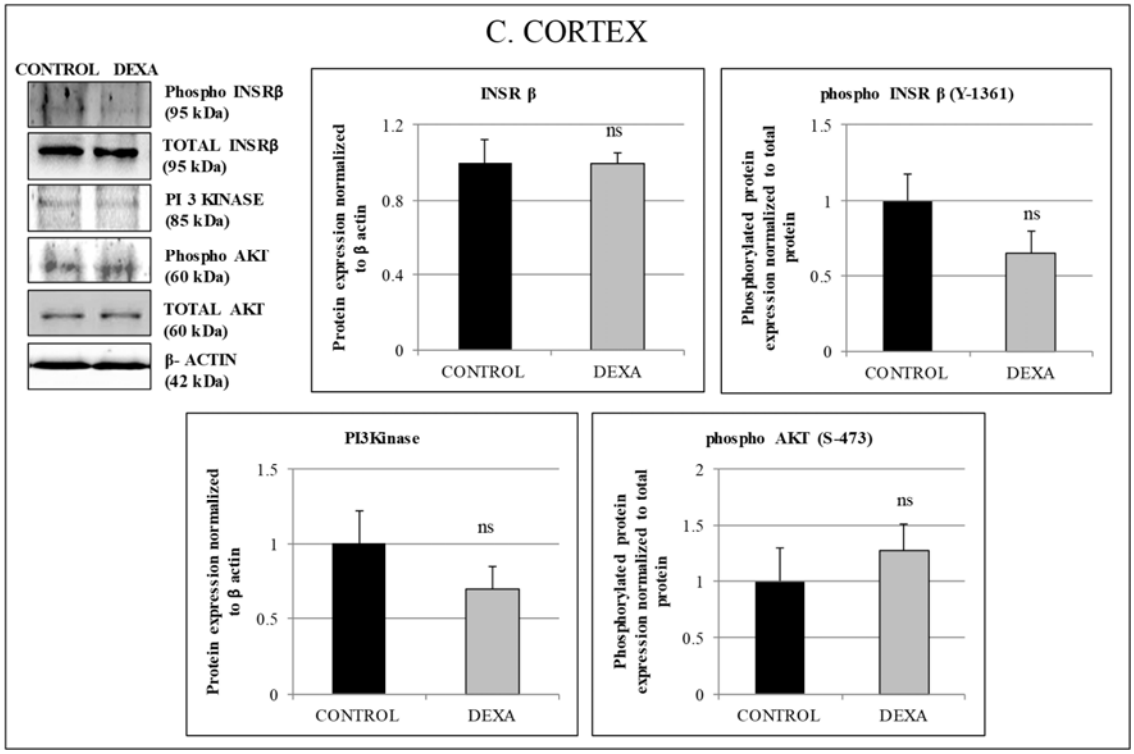
Figure 4. 3 Haematoxylin and eosin staining in different brain regions. Representative images of H & E staining in hypothalamus, hippocampus, cortex, and cerebellum of control and dexamethasone (dexa) treated rats. Scale bar represents 100 µm.

DEXAMETHASONE TREATMENT IMPAIRS INSULIN SIGNALING IN HYPOTHALAMUS WITH NO CHANGE IN HIPPOCAMPUS, CORTEX AND CEREBELLUM

It is known that binding of insulin to its plasma membrane receptor (INSR) elicits various intracellular signaling pathways, which mediate the effects of insulin. To determine whether dexamethasone treatment affects insulin signaling in brain, we examined the protein levels of phospho INSRβ (Y-1361), Total INSRβ, PI3Kinase, phospho AKT (S-473) and Total AKT in the hypothalamus (Fig. 4.4A), hippocampus (Fig. 4.4B), cerebral cortex (Fig. 4.4C) and cerebellum (Fig. 4.4D) of rats treated with saline or dexamethasone for 28 days. We found a regional difference in insulin signaling in brain following dexamethasone treatment. Specifically, dexamethasone treatment reduced insulin signaling in the hypothalamus (Fig. 4.4A) as shown by the INSRβ activity, PI3Kinase as well as AKT activity. However, no significant change was observed in hippocampus (Fig. 4.4B), cerebral cortex (Fig. 4.4C) and cerebellum (Fig. 4.4D) in dexamethasone treated rats as compared to control. Reduction in activated insulin receptor in hypothalamus of dexamethasone treated rats was also confirmed by the immunostaining of phospho-insulin receptor in brain sections as shown in Fig 4.5.


A. HYPOTHALAMUS
CONTROL DEXA
Phospho INSRβ (95 kDa)
TOTAL INSRβ (95 kDa)
PI 3 KINASE (85 kDa)
Phospho AKT (60 kDa)
TOTAL AKT (60 kDa)
β- ACTIN (42 kDa)
INSR β
Protein expression normalized to β actin
phospho INSR β (Y-1361)
Phosphorylated protein expression normalized to total protein
PI3Kinase
phospho AKT (S-473)
CONTROL
DEXA


B. HIPPOCAMPUS
CONTROL DEXA
Phospho INSRβ (95 kDa)
TOTAL INSRβ (95 kDa)
PI 3 KINASE (85 kDa)
Phospho AKT (60 kDa)
TOTAL AKT (60 kDa)
β- ACTIN (42 kDa)
INSR β
Phosphorylated protein expression normalized to total protein
phospho INSR β (Y-1361)
ns
PI3Kinase
Protein expression normalized to β actin
phospho AKT (S-473)
CONTROL
DEXA



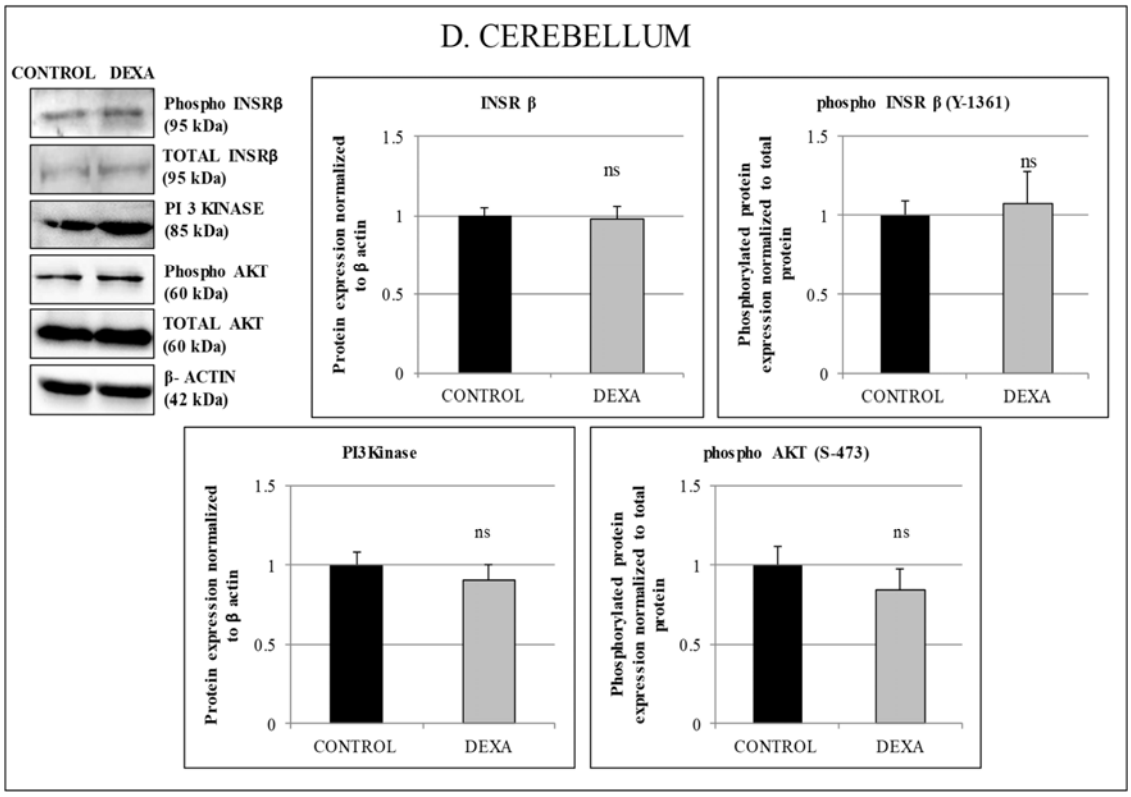
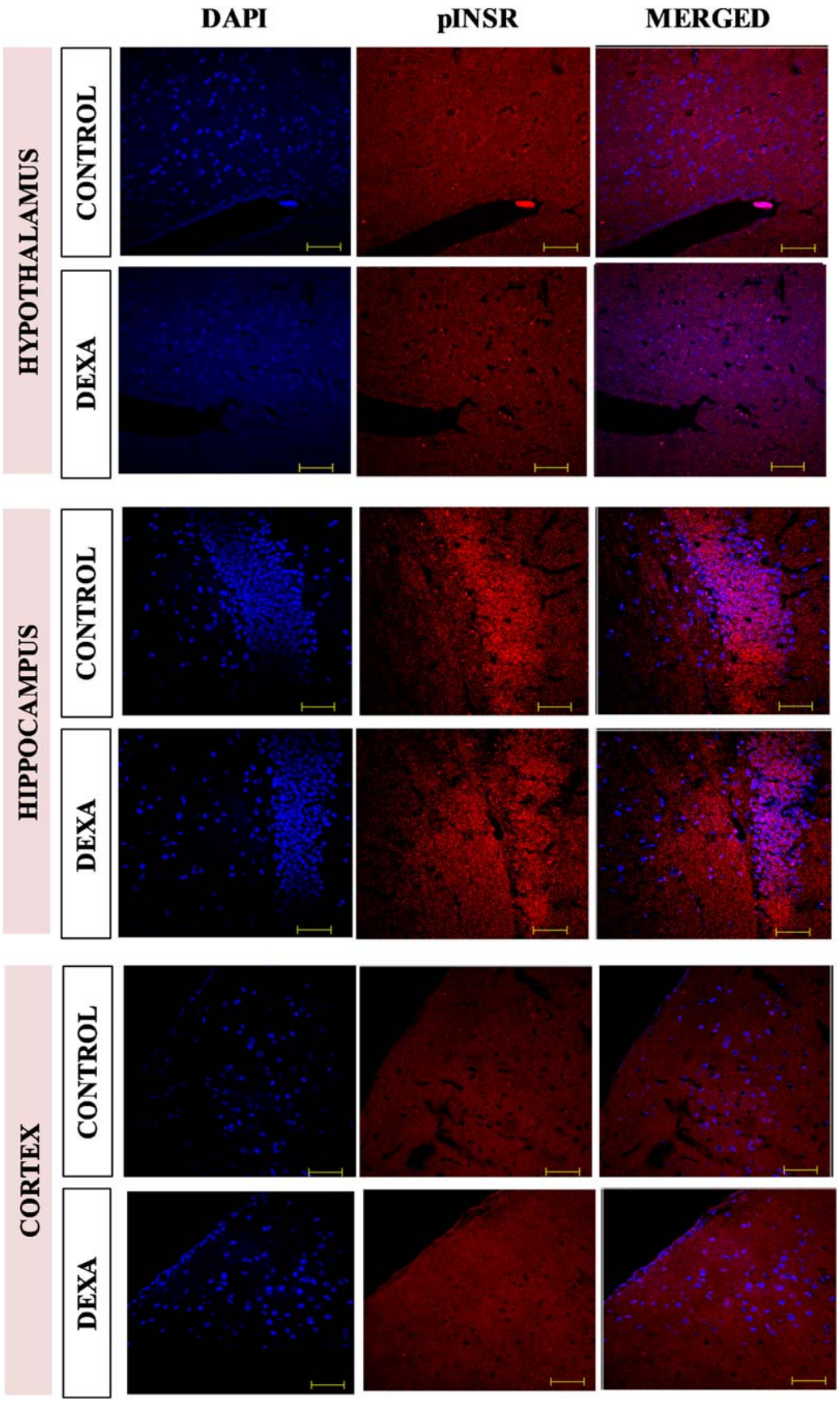


Figure 4. 4 Immunoblotting of insulin signaling proteins in brain :phospho INSRβ Y-1361, Total INSRβ, PI3Kinase, phospho AKT S-473, Total AKT keeping β-actin as endogenous control was done by western blotting for hypothalamus (A), hippocampus (B), cortex (C) and cerebellum (D) in control and dexamethasone (dexa) treated rats. Graph represents densitometric analysis done using Image J software. Data presented as Mean ± SEM of n=3-4 for control and dexa group. * p value <0.05, ** p value <0.01, and ns p value >0.05 as compared to control.


DAPI
pINSR
MERGED
HYPOTHALAMUS
CONTROL
DEXA
HIPPOCAMPUS
CONTROL
DEXA
CORTEX
CONTROL
DEXA

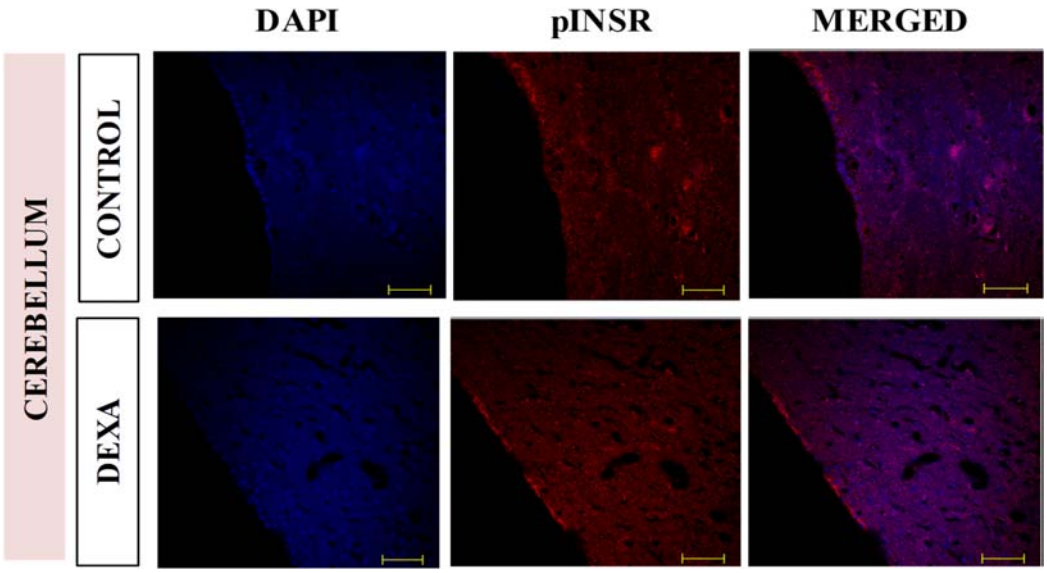


Figure 4. 5 Confocal photomicrographs showing immunostaining of brain sections. Different regions (hypothalamus, hippocampus, cortex and cerebellum) from control and dexamethasone (dexa) treated rat were stained with anti phospho-INSRβ (Y-1361) antibody (as shown in red) and Dapi (as shown in blue). Scale bar indicate 50µm.

DEXAMETHASONE LEAD TO NEURO-BEHAVIORAL ALTERATIONS IN RATS

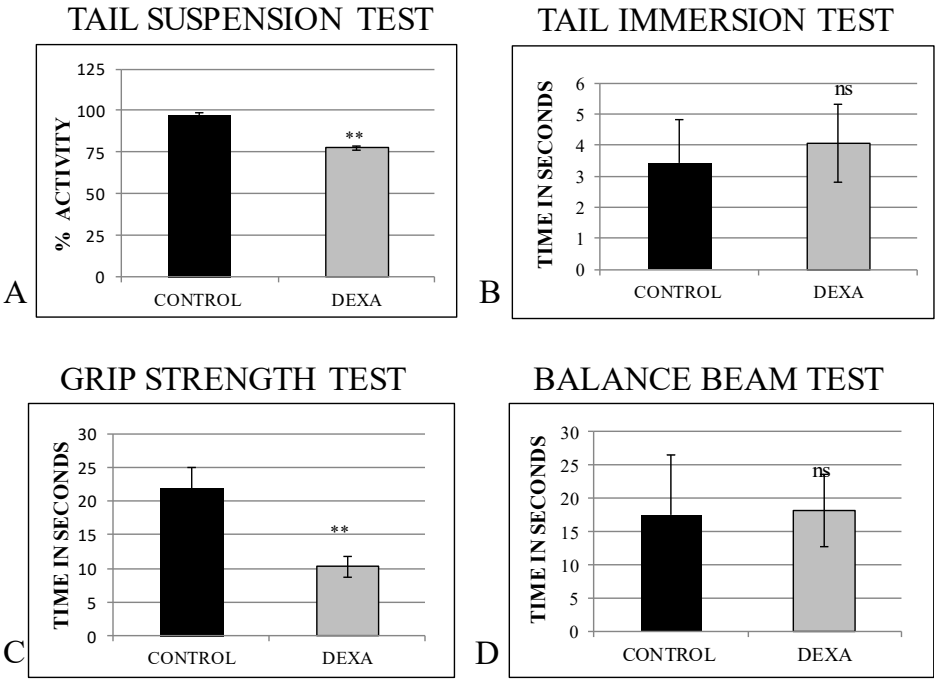
Different brain regions are involved in different functions. Hence, functional assessments in dexamethasone induced diabetes would link the physiological and psychological effects of dexamethasone and peripheral insulin resistance on brain. Thus, a battery of neuro-behavioral tests was performed on dexamethasone treated rats and were compared to that of control rats.

Downregulation of hypothalamic insulin signaling is associated with depressive like symptoms in rats (Grillo *et al.,* 2011). Thus, tail suspension test was used to assess the stress reactivity and depressive like symptoms in dexamethasone treated rats. As shown in Fig 4.6A, the dexamethasone treated rats had increased immobility as compared to the control rats. Pupil dilation test is one of the parameters to check for the retinopathic complication in type 2 diabetes. As shown in Table: 4.3, there was normal pupil dilation in dexamethasone treated rats. Since central and peripheral neuropathic symptoms are well recognized in the diabetic patients, reflex test based on brain stem reflex (or corneal reflex) (Table: 4.3) and spinal cord reflex (tail immersion test) (Fig 4.6B) were performed in both the groups. Corneal reflex test as well as the tail immersion test showed no marked change of function in dexa group as compared to control. Thus,

suggesting that there was no defect in reflex action in dexamethasone induced diabetic rats.

The motor function was assessed in experimental animals using grip strength test and gait analysis. As observed in grip strength test, (Fig 4.6C), the latency of dexamethasone treated rats to grip the wire decreased as compared to control. Sciatic function index (SFI) was calculated to check for the dysfunction of sciatic nerves (sciatic nerve pain) which is a common phenomenon in diabetic patient. As shown in Table: 4.4; the dexamethasone treated rats had SFI index of +11 indicating that they were on verge of motor impairment as compared to control. Cerebellum is the region of brain mainly associated with motor co-ordination and maintenance of body balance. The results of balance beam test as shown in Fig 4.6D clearly depicted that there was no defect in cerebellum function of dexamethasone treated insulin resistant rat.

In order to monitor the hippocampal cognitive function of spatial navigation, Morris water maze test was performed. Fig 4.6E represents acquisition phase where there was no difference in escape latency of dexamethasone treated and control. Also, no change was observed between the groups in probe test as shown in Fig 4.6F, suggesting that spatial navigation, an important function of hippocampus is not negatively affected after 28 days of severe insulin resistance. Dexamethasone treated rats had unchanged exploratory behavior as compared to control as evident in open field test (Table: 4.5).



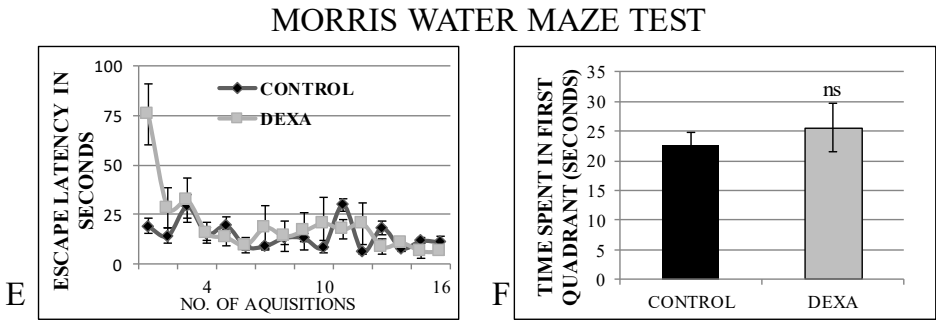


Figure 4. 6 Neurobehavior assessment performed in dexamethasone treated (Dexa) rats versus control rats. A: Tail Suspension Test; B: Tail Immersion Test; C: Grip strength test; D: Balance beam test; E: Morris Water Maze (MWM) test acquisitions; F: Probe test of MWM. Data presented as Mean ± SEM of n=6 for control and dexa group. ns p value >0.05 as compared to control, ** pvalue <0.01 as compared to control.

Table 4. 3 Pupil Dilation Test and Corneal Reflex Test performed in control and dexamethasone treated (Dexa) rats.

	A. PUPIL DILATION TEST	B. CORNEAL REFLEX TEST
CONTROL	√	√
DEXA	√	√

In pupil dilation and corneal reflex test, symbol √ depicted normal function. Gait Analysis Test results were calculated according to the SFI index by De Medicinalli and the value of +11 as observed in insulin resistant group represent the impairment in sciatic function.

Table 4. 4 Gait analysis in control and dexamethasone treated (Dexa) rats.

	PL	ITS	TS	TOF	SFI
CONTROL	2.53 ± 0.19	1.03 ± 0.05	1.85 ± 0.07	8.38 ± 0.13	0
DEXA	1.93 ± 0.02*	1.05 ± 0.05	1.63 ± 0.03*	8.3 ± 0.35	11.24

PL: print length; ITS: Intermediate toe spread; TS: Toe spread; TOF: distance to opposite foot; SFI: Sciatic function index. Data presented as Mean ± SEM of n=5 for control and dexa group. * p value <0.05 as compared to control.

Table 4. 5 Open field test in dexamethasone (Dexa) treated rats versus control.

	CONTROL	DEXA
CENTER	3.0 ± 1.7	3.5 ± 2.6 ^{ns}
OUTFIELD	18.0 ± 3.2	16.1 ± 1.1 ^{ns}
CORNER	165 ± 7.1	160.3 ± 13.4 ^{ns}

Data presented as Mean ± SEM of n=6 for control and dexa group. ns p value >0.05 as compared to control.

STEM CELL AND ASTROCYTIC MARKERS ARE ALTERED IN HIPPOCAMPUS OF DEXAMETHASONE TREATED RATS

Hippocampus is one of the major sites for neurogenesis in adult brain, hence we wanted to assess if hippocampal neural stem cells were affected in dexamethasone treated rats. Although hippocampal insulin resistance was not evident in dexamethasone treated rats, there was a significant decrease in protein expression of Nestin as shown in Fig 4.7A-B. Also, the gene expression markers responsible for stem cell maintenance i.e. *Notch1* (Fig 4.7C) and *Hes5* (Fig 4.7D) was decreased in hippocampus with an increase in Wnt signaling (Fig 4.7E) responsible for differentiation into neurons after treatment with dexamethasone.

Astrocytes aid in stem cell as well as brain micro environment maintenance. We observed that there was a modest decrease in the astrocytic marker GFAP in dexamethasone treated rats as compared to control (Fig 4.8A-B). Correspondingly, there was a two-fold reduction in the gene expression of astrocytic glutamate transporters viz. *Glast* (Fig 4.8D) along with that of lactate transporter *Slc16a1* (Fig 4.8E) and *Slc16a3* (Fig 4.8F). Glycogen levels were also reduced in hippocampus after dexamethasone treatment (Fig 4.8G). Also, a decline was observed in the levels of glutamate (Fig 4.9A) with no change in GABA (Fig 4.9B) in hippocampus of dexamethasone treated rats.

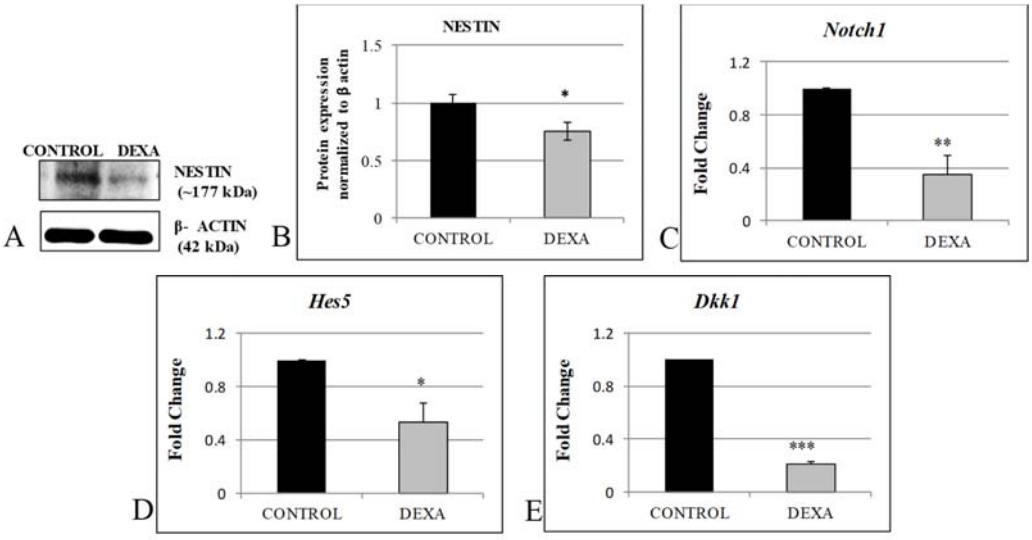


Figure 4. 7 Assessment of candidate players modulating hippocampal neural stem cell.
A: Representative image of immunoblotting of Nestin in hippocampus of control and dexamethasone treated (dexa) rats. **B:** Graph representing densitometric analysis of Nestin normalized against internal control β-actin. Quantitative real-time PCR analysis of *Notch1* **(C)**, *Hes5* **(D)**, and *Dkk1* **(E)** genes from hippocampus of control and dexamethasone (dexa) treated rats. Data presented as Mean ± SEM of n=3-4 for control and dexa group. * p value <0.05 as compared to control, ** p value <0.01 as compared to control, and *** p value <0.001 as compared to control.

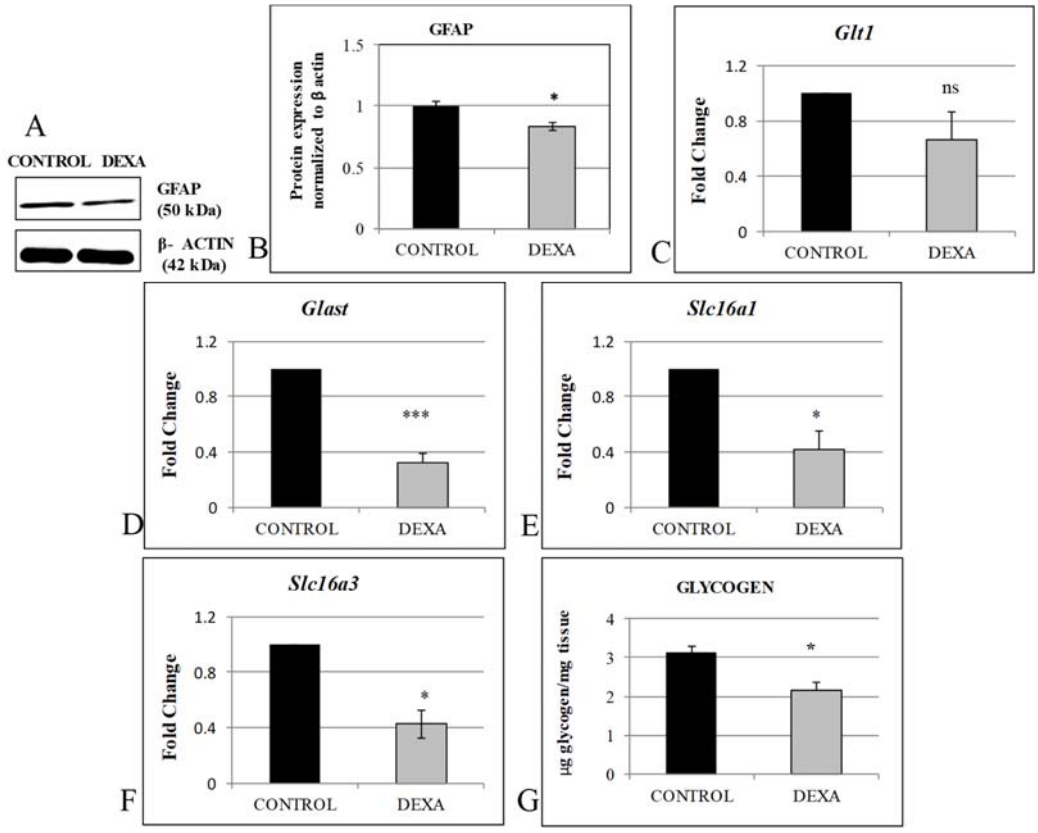


Figure 4. 8 Assessment of hippocampal astrocytic components.

A: Representative image of immunoblotting of GFAP in hippocampus of control and dexamethasone treated (dexa) rats. **B:** Graph representing densitometric analysis of GFAP normalized against internal control β-actin. Quantitative real-time PCR analysis of *Glast* **(C)**, *Glt1* **(D)**, *Slc16a1* **(E)**, and *Slc16a3* **(F)** genes from hippocampus of control and dexamethasone (dexa) treated rats. **G:** Glycogen content. Data presented as Mean ± SEM of n=3-4 for control and dexa group. * p value <0.05, ***p value <0.001 and ns p value >0.05 as compared to control.

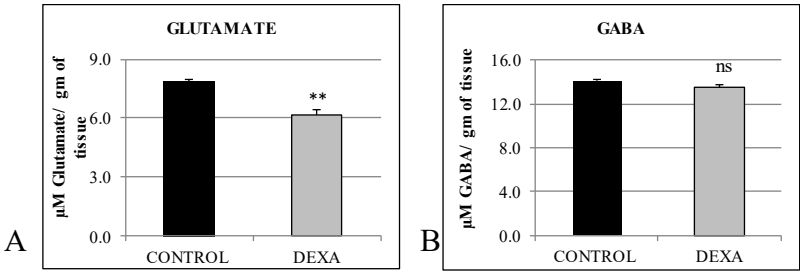


Figure 4. 9 Neurotransmitter estimation - Glutamate (A), and GABA (B) from the hippocampus of control and dexamethasone (dexa) treated rats. Data presented as Mean ± SEM of n=3-4 for control and dexa group. ** p value <0.01 and ns p value >0.05 as compared to control.

CANDIDATE MARKERS FOR APPETITE REGULATION IS DISTURBED IN HYPOTHALAMUS OF DEXAMETHASONE TREATED RATS

Since insulin signaling was remarkably reduced in dexamethasone treated rats along with alterations in appetite, we further dissected the molecular mechanisms involved in

the appetite regulation by hypothalamus in dexamethasone treated rats. Hypothalamic appetite regulation is mediated by aneroxic and orexic neuropeptides, which are modulated in response to peripheral signals like insulin, leptin, glucocorticoids etc. The gene expression of *Obrb* (Fig. 4.10A), which relays the signal of leptin to modulate the expression of neuropeptides remained unchanged because of dexamethasone treatment. However, there was a remarkable upregulation of *Agrp* (Fig.4.10B) and *Npy* (Fig. 4.10C) which acts as an orexic signal, whereas there was a downregulation of *Pomc* (Fig.4.10D) and *Mc4r* (Fig.4.10E), which are the anorexic signals. There was no significant change in the expression of *Cart* (Fig.4.10F) and *Crh* (Fig.4.10G) in dexamethasone treated group as compared to control.

Different lines of investigations suggested that disturbances of eating behavior are associated with hypothalamic neurotransmission. Hence, the level of neurotransmitters was analysed and results demonstrated that there was a significant decrease in the level of glutamate (Fig.4.11A), GABA (Fig. 4.11B) and dopamine (Fig. 4.11C) in hypothalamus of dexamethasone treated group as compared to control.

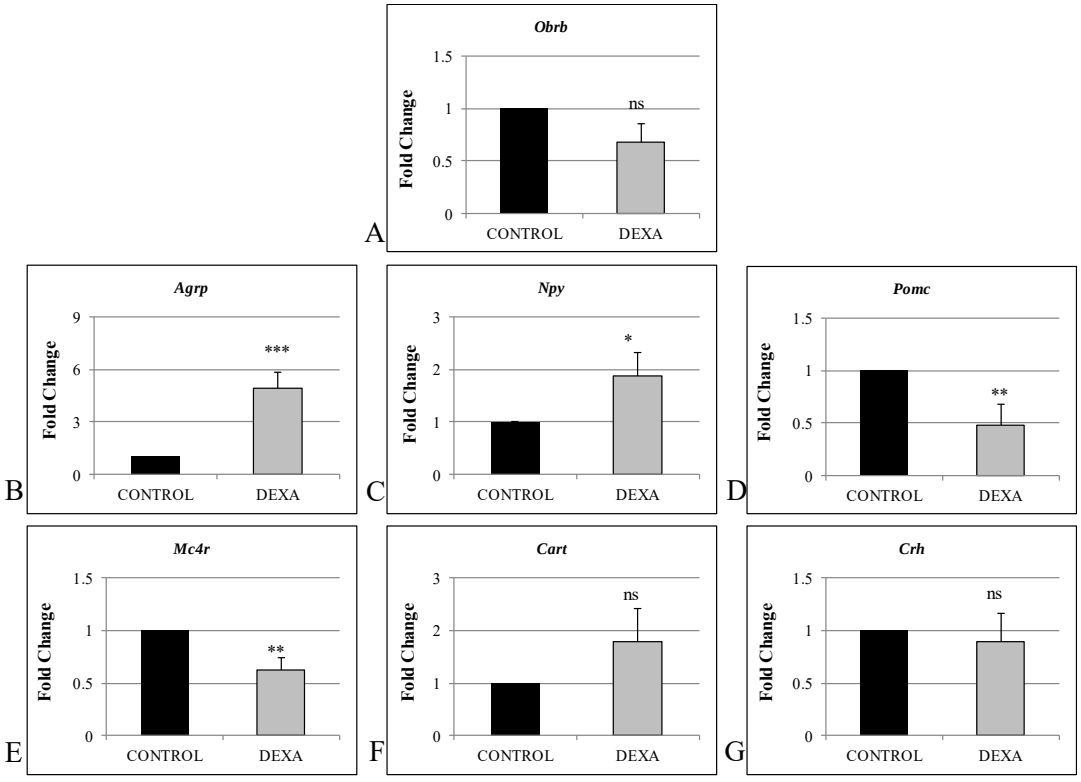


Figure 4. 10 Analysis of neuropeptides' gene expression levels. Quantitative real-time PCR analysis of *Obrb* (A), *Agrp* (B), *Npy* (C), *Pomc* (D), *Mc4r* (E), *Cart* (F), and *Crh* (G) genes from hypothalamus of control and dexamethasone (dexa) rats. Data presented as Mean ± SEM of n=3-4 for control and insulin resistant group. * p value <0.05; ** p value <0.01 and ns p value >0.05 as compared to control.

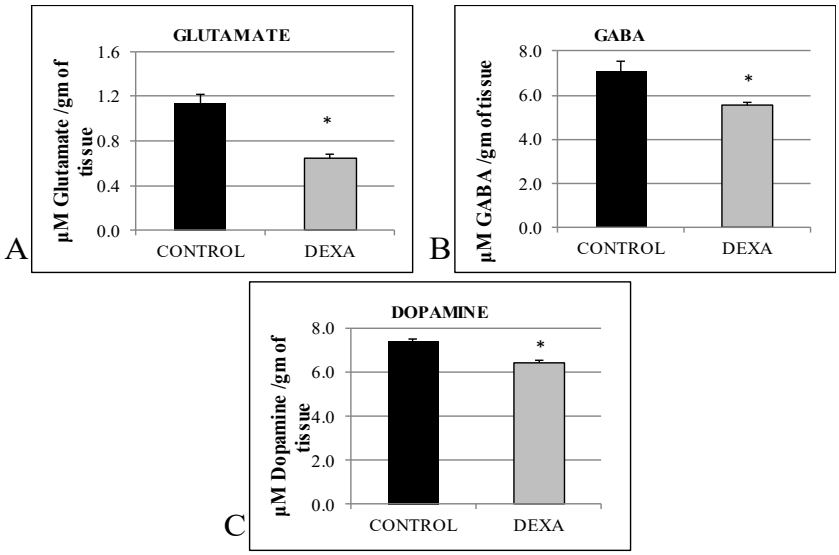


Figure 4. 11 Neurotransmitter estimation - Glutamate (A), GABA (B), and Dopamine (C) from the hypothalamus of control and dexamethasone (dexa) treated rats. Data presented as Mean ± SEM of n=3-4 for control and insulin resistant group. * p value <0.05 as compared to control.

Hypothalamic nutrient sensors play important role in maintaining whole body energy homeostasis by integrating information on energy status; hence we analysed the candidate nutrient sensors in hypothalamus. Glucose Transporter-1 (GLUT1) in the hypothalamic glial cells mediates glucose sensing while PPARs are widely known as metabolic sensors for lipids. We found that there was reduction in the protein expression of brain glucose transporter i.e. GLUT 1 (Fig.4.12B) and a key regulator of energy homeostasis i.e. PPARγ (Fig. 4.12D) with no change in PPARα (Fig.4.12C) in hypothalamus of dexamethasone treated rats as compared to control. Similarly, SirT1 and AMPK are also critical nutrient sensing pathways known to be associated with insulin signaling. The results demonstrated significant downregulation in the SirT 1 protein levels (Fig.4.12E) with no change in the activation of AMPK signaling (Fig.4.12F) in hypothalamus of dexamethasone treated rats as compared to control.

Astrocytic glycogen granules are the vital energy stores and are known to be mobilized during high energy requirement. Also, insulin and dexamethasone regulate the glycogen homeostasis i.e. its synthesis and breakdown. We found that there was drastic decrease in hypothalamic glycogen content in dexamethasone group as compared to control group (Fig.4.13A). The role of astrocyte specific glutamate transporter is very crucial in maintaining the glutamate homeostasis and are known to be altered in diseased condition. The gene expression study demonstrated that there was a significant decrease in the levels of *Glt1* (Fig.4.13C) with no change in *Glast* (Fig.4.13B), *Slc16a1*

(Fig.4.13D) and *Slc16a3* (Fig.4.13E) in dexamethasone treated group as compared to control.

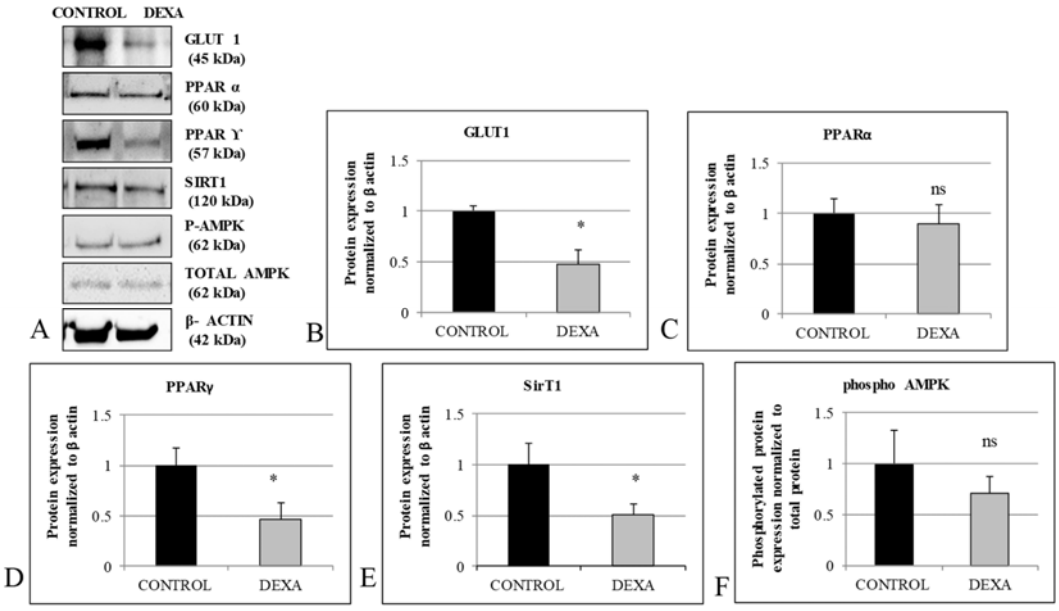


Figure 4. 12 Immunoblotting for hypothalamic nutrient sensors (A). The graphs represent densitometric analysis done using Image J software of target proteins normalized to β actin for GLUT1 (B), PPAR α (C), PPAR γ (D), and SIRT1 (E) and phospho AMPKα (Thr 172) / Total AMPKα (F). Data presented as Mean ± SEM of n=3-4 for control and dexa group. * p value <0.05 and ns p value >0.05 as compared to control.

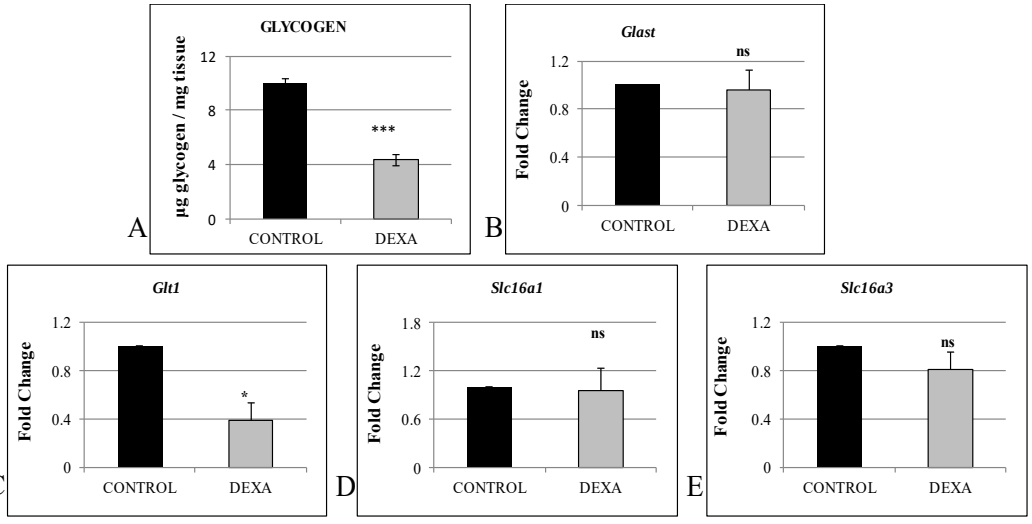


Figure 4. 13 Assessment of hypothalamus astrocytic components. A: Glycogen content in the hypothalamus of the dexa treated rats and control. Quantitative real-time PCR analysis of *Glast* (B), *Glt1* (C), *Slc16a1* (D), and *Slc16a3* (E) genes from hypothalamus of control and dexamethasone (dexa) treated rats. Data presented as Mean ± SEM of n=3-4 for control and dexa group. * p value <0.05, *** p value <0.001, and ns p value >0.05 as compared to control.

4.2 IMPACT OF GLUCOCORTICOID INDUCED MATERNAL INSULIN RESISTANCE ON HYPOTHALAMIC APPETITE REGULATING CIRCUITRY OF THE NEONATAL BRAIN.

4.2.1 PLAN OF WORK

3 mg of dexamethasone per kg of body weight was subcutaneously injected for 20 days in female young Charles Foster rats to develop diabetic rat model. One animal was kept per cage with ad libitum access to food and water. The body weight was monitored throughout the dosing period. After treatment period, confirmation of peripheral insulin resistance in dexamethasone (dexa) treated rats was done using serum insulin and glucose levels, and were compared to that of control rats (injected with equivalent dose of sterile normal saline). After 20 days of Dexamethasone treatment, Fasting Insulin resistance index (FIRI) of the rats were calculated and those females that were insulin resistant (FIRI>2.7) were kept for mating with male non-diabetic Charles Foster rats for 5 days. One female rat was kept with one male rat. Similarly, control females were also kept for mating with non-diabetic males for 5 days. At the end of pregnancy, the animals were allowed to deliver naturally, the day of birth was defined as postnatal day 0 (PND 0). The pups born to control and diabetic dams were separated based on their sex and sacrificed on PND 0 and their brains were dissected out. Hypothalamus was further processed for the assessment of the candidate appetite regulators. Please refer to Chapter 3 for detailed materials and methods. Since the dexamethasone administration was not there during gestation period, we affirm that the effect on hypothalamus of pups was only because of maternal insulin resistance and not because of direct effect of dexamethasone. The schematic plan of work for this objective is as shown in Fig 4.14.

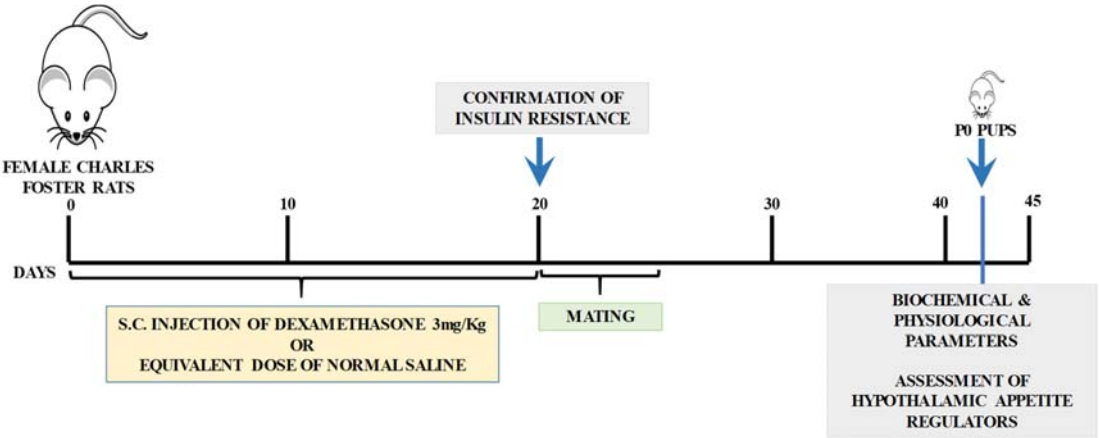


Figure 4. 14 Plan of work for assessment of maternal insulin resistance on fetal hypothalamic appetite regulatory circuit.

4.2.2 RESULTS

CONFIRMATION OF DEXAMETHASONE INDUCED MATERNAL INSULIN RESISTANT RAT MODEL

At the end of the dosing period, there was a significant rise in the fasting serum glucose and insulin levels in the dexamethasone treated rats as compared to control (injected with N. Saline). The Fasting Insulin Resistance Index was calculated from the values of fasting serum glucose and insulin, and the dexamethasone treated rats were found to positively correlate with the insulin-resistant phenotype with FIRI value substantially higher than control rats, as shown in Table 4.6. After confirmation of peripheral insulin resistance, the rats from both the groups were kept for mating with non-diabetic male Charles Foster rats for 5 days. The weight of the females was monitored throughout the gestation period as shown in Fig 4.15, no difference in the 'gain in body weight' was observed among both the groups.

Table 4. 6 FIRI values showing the development of insulin resistance in the dexamethasone treated (dexa) female rats after completion of dosing period.

	Fasting serum glucose (mMol/L)	Fasting Serum Insulin (µIU/L)	FIRI
CONTROL	2.55 ± 0.86	4.59 ± 1.71	0.4 ± 0.27
DEXA	4.7 ± 1.3*	85.50 ± 6.17***	16.4 ± 10.04***

Data presented as Mean ± SEM of n= 6-7 for control and dexa group. * p value <0.05 as compared to control; *** p value <0.001 as compared to control.

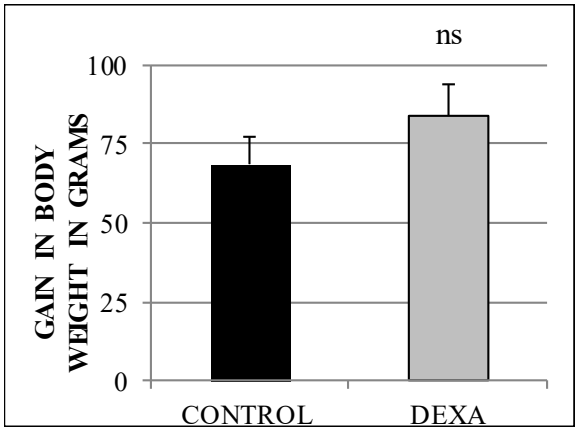


Figure 4. 15 Gain in body weight in Control and Dexamethasone (dexa) dams during gestation. Data presented as Mean ± SEM of n=6-7 for control and dexa group. ns p value > 0.05 as compared to control.

NO PHYSIOLOGICAL DIFFERENCES IN PUPS BORN TO DEXAMETHASONE TREATED DAMS AS COMPARED TO CONTROL DAMS

Maternal insulin resistance induced by dexamethasone did not result in changes in the litter size or survival rates of neonates as compared to control as shown in Table 4.7. Further, the physiological parameters were assessed in the PND 0 pups born to control and dexamethasone treated dams. No significant difference was seen in the body weight /crown to rump length between pups born to the dexamethasone treated and control dams (Table 4.8).

For all further studies, the male and the female pups were considered separately from both the groups to assess if there exist any sexual dimorphic changes. Blood was collected by cardiac puncturing and pooled from the pups born to same female for the estimation of serum glucose and insulin levels (Table 4.8). However, there were no rise in either glucose or insulin levels in male as well as female pups born to dexamethasone treated dams.

Table 4. 7 Litter size and mortality of delivered pups in control and dexamethasone (dexa) treated rats.

	LITTER SIZE	No. OF LIVE PUPS	No. OF DEAD PUPS
CONTROL DAMS	7 ± 3.56	6 ± 3.27	-
DEXA DAMS	7 ± 0^{ns}	6.7 ± 3^{ns}	1^{ns}

Data presented as Mean ± SEM for n= 6-7 for control and dexa treated dams. ns >0.05 as compared to control dams.

Table 4. 8 Estimation of ratio of bodyweight to crown to rump length, glucose, insulin and whole weight of brain from serum pooled from pups delivered to control and dexamethasone (dexa) treated rats at postnatal day 0 (PND0).

	MALE		FEMALE	
	CONTROL	DEXA	CONTROL	DEXA
Ratio of bodyweight to crown to rump length	1.21 ± 0.04	1.2 ± 0.14^{ns}	1.15 ± 0.11	1.16 ± 0.15^{ns}
GLUCOSE (mMol/L)	1.68 ± 0.38	2.84 ± 0.67^{ns}	1.78 ± 0.01	1.72 ± 0.48^{ns}
INSULIN (µIU/L)	4.32 ± 0.81	4.59 ± 0.72^{ns}	3.96 ± 0.77	5.01 ± 0.65^{ns}
Wet weight of whole brain	0.26 ± 0.03	$0.21 \pm 0.02^{**}$	0.24 ± 0.02	0.23 ± 0.04^{ns}

Data presented as Mean ± SEM of n= 10-20 for male and female pups born to control and dexamethasone (dexa) treated dams. ns >0.05; **p<0.01 as compared to control.

NO HISTOLOGICAL CHANGES IN BRAIN OF PUPS BORN TO DEXAMETHASONE TREATED DAMS AS COMPARED TO CONTROL DAMS

Wet weight of brain was measured and a significant reduction was seen in the whole brain weight of the male pups but not female pups born to the dexamethasone treated dams as compared to the control dams as shown in table 4.7. To check if the intra-uterine conditions had any effect on the histology of the hypothalamus, hematoxylin eosin staining was performed. Hypothalamic architecture analysis did not reflect any prominent changes in either male or female pups of the dexamethasone treated and control dams as shown in (Fig 4.16).

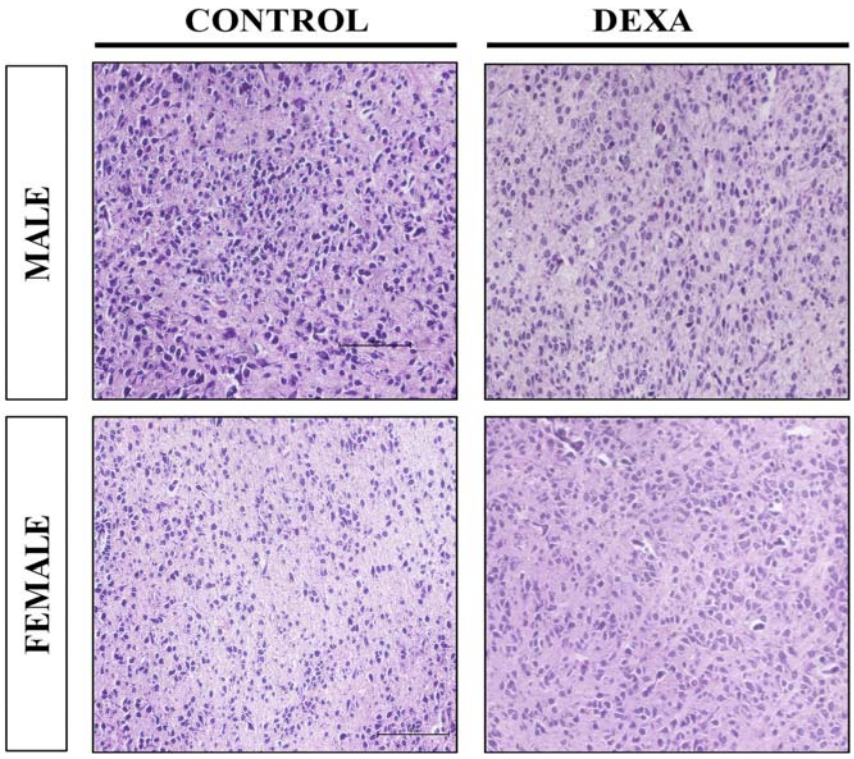


Figure 4. 16 Haematoxylin and eosin staining in hypothalamus of pups born to control and dexamethasone (dexa) treated dams at postnatal day 0 (Scale bar represents 100µm).

SEXUAL DIMORPHIC ALTERATIONS IN INSULIN SIGNALING IN PUPS BORN TO DEXAMETHASONE TREATED DAMS AS COMPARED TO CONTROL

The assessment of hypothalamic insulin signaling was directed by immunoblotting. Differential variation in the expression of the candidate insulin signaling proteins was observed in male and female pups' hypothalamus. As shown in Fig 4.17, in hypothalamus of the male pups born to the dexamethasone treated dams, there was a significant increase in the expression of PPAR-α (Fig 4.17F) as compared to pups born to the control dams.

However, no significant change was seen in the expression of activated INSR (Fig 4.17B), PI3K (Fig 4.17C), activated AKT (Fig 4.17D), and GLUT1(Fig 4.17E). To the contrary, in case of the hypothalamus of the female pups born to dexamethasone treated dams, there was a significant reduction in the expression of activated INSR (Fig 4.18B), PI3K (Fig 4.18C), GLUT1 (Fig 4.18E), and PPAR-α (Fig 4.18F), as compared to that of control.

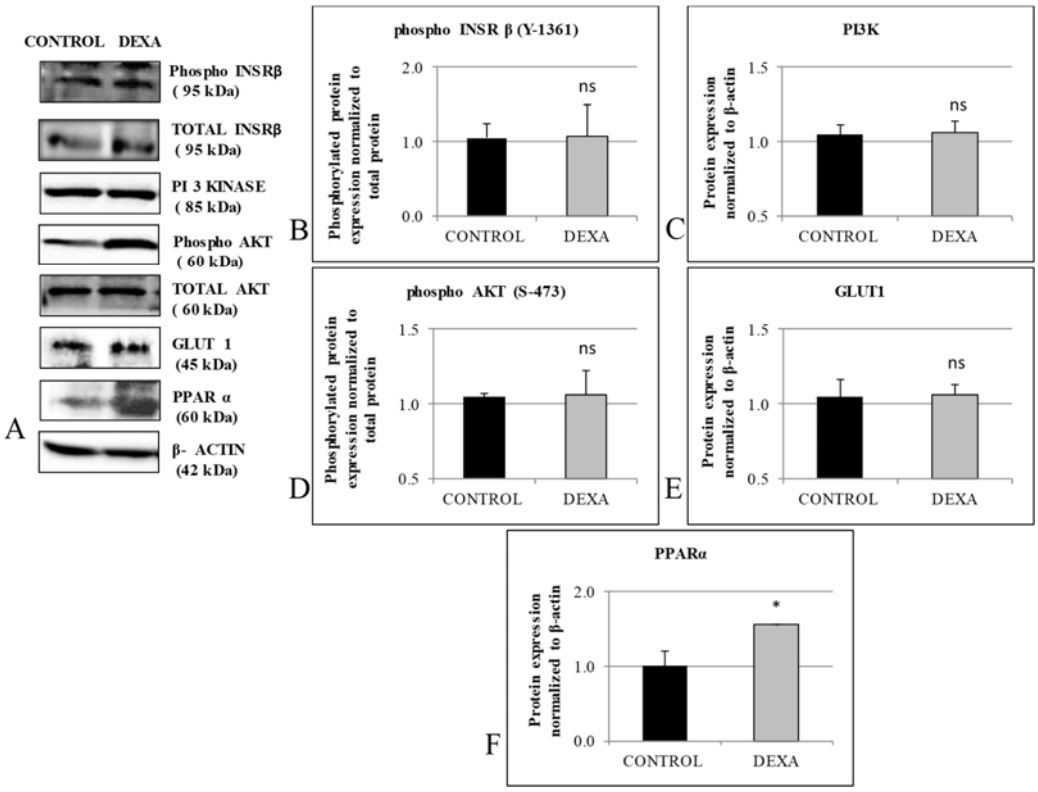


Figure 4. 17 Immunoblotting of candidate hypothalamic proteins in male pups born to control and dexamethasone (dexa) treated rats (A). Graph represents densitometric analysis done using Image J software for activated INSR **(B)**, PI3Kinase **(C)**, actvated AKT **(D)**, GLUT1 **(E),** and PPARα **(F)**. Data presented as Mean ± SEM of n=3 for control and dexa group. * p value <0.05 and ns p value >0.05 as compared to control.

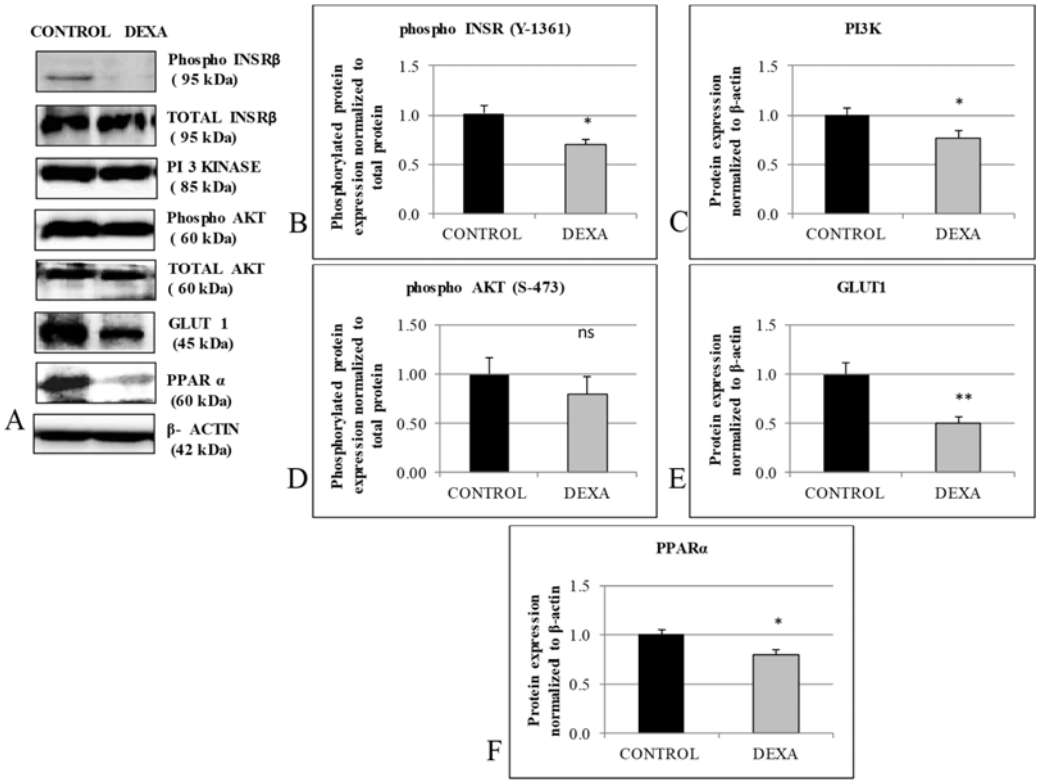


Figure 4. 18 Immunoblotting of candidate hypothalamic proteins in female pups born to control and dexamethasone (dexa) treated rats: (A) Graph represents densitometric analysis done using Image J software for activated INSR **(B)**, PI3Kinase **(C)**, actvated AKT **(D)**, GLUT1 **(E)**, and PPARα **(F)**. Data presented as Mean ± SEM of n=4 for control and dexa group. * p value <0.05, and ns p value >0.05 as compared to control.

SEXUAL DIMORPHIC ALTERATIONS IN HYPOTHALAMIC APPETITE REGULATING NEUROPEPTIDES IN PUPS BORN TO DEXAMETHASONE TREATED DAMS AS COMPARED TO CONTROL

The altered levels of insulin signalling proteins may affect the gene expression of appetite regulating neuropeptides. As observed from the results, the alterations in neuropeptides' gene expression in the hypothalamus of male and female pups were different. As shown in Fig 4.19, there was a significant increase in the gene expression of *Npy* (Fig 4.19B), *Pomc* (Fig 4.19C), and *Mc4r* (Fig 4.19D) in the hypothalamus of the male pups born to the dexamethasone treated dams as compared to the control dams. However, no significant change was observed in the gene expression of *Agrp* (Fig 4.19A), and *Cart* (Fig 4.19E). In similar fashion, when these neuropeptides were assessed in the hypothalamus of female pups born to dexa dams, we observed a marked reduction in the expression of *Agrp* (Fig 4.20A), *Npy* (Fig 4.20B), *Pomc* (Fig 4.20C),

and *Mc4r* (Fig 4.20D), while a significant increase was seen in the expression of *Cart* (Fig 4.20E).

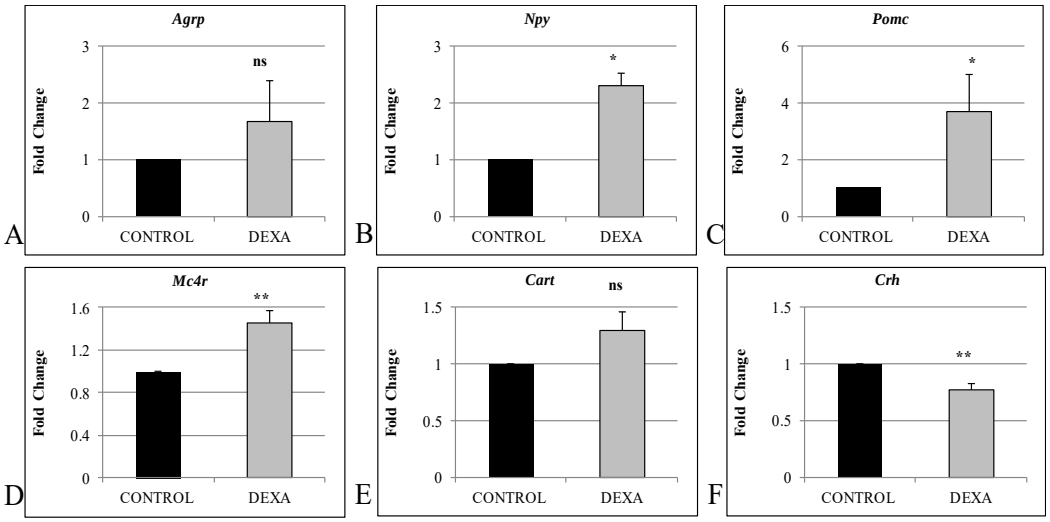


Figure 4. 19 Analysis of neuropeptides' gene expression - *Agrp* (A), *Npy* (B), *Pomc* (C), *Mc4r* (D), *Cart* (E), and *Crh* (F) in hypothalamus of male pups at postnatal day 0. Data presented as Mean ± SEM of n=3 for control and dexa group. * p value <0.05, ** p value <0.01, and ns p value >0.05 as compared to control.

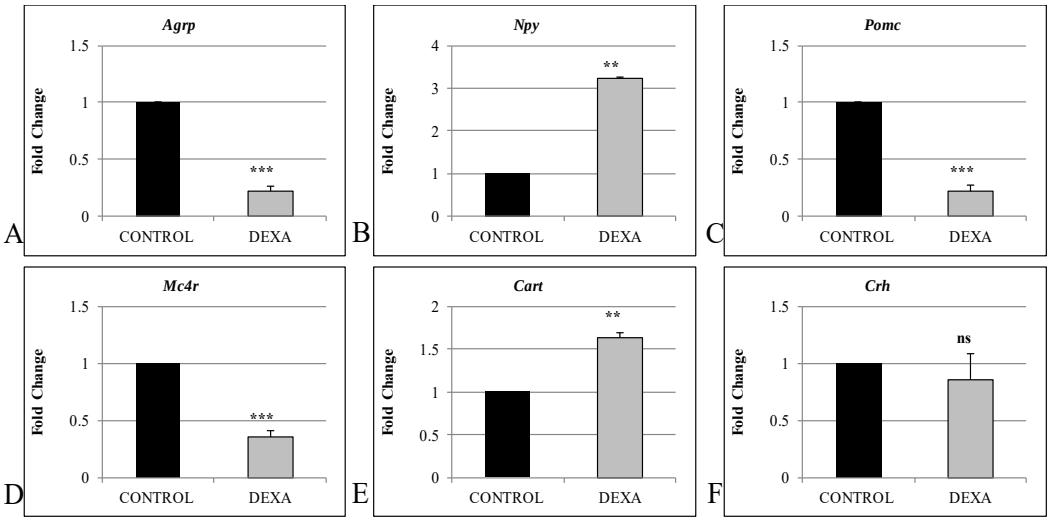


Figure 4. 20 Analysis of neuropeptides' gene expression - *Agrp* (A), *Npy* (B), *Pomc* (C), *Mc4r* (D), *Cart* (E), and *Crh* (F) **levels in hypothalamus of female pups at postnatal day 0.** Data presented as Mean ± SEM of n=3 for control and dexa group. * p value <0.05, ** p value <0.01, and ns p value >0.05 as compared to control.

The summary of outcome of appetite regulating hypothalamic factors in male and female pups born to dexamethasone treated rats with respect to control dams are as shown in Table: 4.9.

Table 4. 9 Summary of results of male and female pups as compared to respective control

	DEXA MALE PUPS	**DEXA FEMALE PUPS**
SERUM GLUCOSE	NO CHANGE	NO CHANGE
SERUM INSULIN	NO CHANGE	NO CHANGE
WHOLE BRAIN WEIGHT	DECREASED ↓	NO CHANGE
HYPOTHALAMIC INSULIN SIGNALING	NO CHANGE	DECREASED ↓
HYPOTHALAMIC GLUT1 LEVEL	NO CHANGE	DECREASED ↓↓
HYPOTHALAMIC PPARα LEVEL	INCREASED ↑	DECREASED ↓
OREXIC HYPOTHALAMIC NEUROPEPTIDES		
Agrp	NO CHANGE	DECREASED ↓↓↓
Npy	INCREASED ↑	INCREASED ↑↑
ANOREXIC HYPOTHALAMIC NEUROPEPTIDES		
Pomc	INCREASED ↑	DECREASED ↓↓↓
Npy	INCREASED ↑↑	DECREASED ↓↓↓
Cart	NO CHANGE	INCREASED ↑↑
Crh	DECREASED ↓↓	NO CHANGE

4.3 DISCUSSION

Brain was initially thought to be an insulin-independent organ, which now has begun to be considered as an insulin-responsive organ with the advent of the fact that insulin from blood can enter brain, where it can activate its cognate receptors to modulate several functions (Derakhshan and Toth, 2013). Dexamethasone-induced diabetic model is widely used *in vitro* and *in vivo* system to examine the pathophysiology of peripheral insulin resistance (Sakoda *et al.*, 2000; He *et al.*, 2015). But, very few reports emphasized that apart from hampering insulin sensitivity in insulin-dependent peripheral tissues, dexamethasone can also downregulate insulin signaling in brain (Park *et al.*, 2005). An attempt has been made in present study to understand the brain insulin resistance as well as to elucidate the links responsible for the appetite change after chronic dexamethasone treatment. In light of this, chronic high dose of dexamethasone was injected in rats to induce diabetes. These animals depicted glucose intolerance and higher FIRI index, thus confirming development of peripheral insulin resistance. Although brain appears to be protected against moderate amounts of synthetic glucocorticoids such as dexamethasone by a drug-exporting P-glycoprotein in the blood-brain barrier (Schinkel *et al.*, 1995), the chronic high dose of dexamethasone (3mg/kg body weight for 28 days) as used in the current study can access brain (Miller *et al.*, 1992).

Since insulin receptors are not evenly distributed throughout the brain, regional analysis of the brain insulin signaling was assessed in dexamethasone-treated and control rats. The foremost observation from objective 1 was that peripherally administered dexamethasone exerts region-specific effect on brain insulin signaling. Although there was no change in insulin signaling in cortex, cerebellum and hippocampus, there was a prominent decrease in hypothalamus. The probable reason being that hypothalamus has a leaky blood-brain barrier at several sites (Yin and Gore, 2010), making it more susceptible to peripheral hyperinsulinemia, and hyperglycemia, thus speculating that persistent hyperinsulinemia might have triggered hypothalamic insulin resistance as a compensatory mechanism. Also, there are glucose-sensing hypothalamic neurons which responds to glycemic status and results in counter regulatory imbalances in response to high glucose level or insulin resistance (Mastaitis *et al.*, 2005; Cotero and Routh, 2009). Thus, hypothalamic insulin resistance is one of the prime events in dexamethasone-treated rats.

Further, neurobehavioral assessment was conducted using a battery of test after the confirmation of peripheral insulin resistance in these animals. Diabetic neuropathy does develop in several diabetic rodent models but in a different manner, depending on the strain, the type of diabetes, the age of occurrence and the duration of diabetes (Dominique *et al.*, 2007). Insulin resistance is independently associated with peripheral and autonomic neuropathies as observed in diabetic patients along with hyperglycemia and hypocholesteremia (Lee *et al.*, 2012). Also, the brain stem reflex and the spinal cord reflex did not show any alteration in the dexamethasone treated group as observed by the corneal reflex test and tail immersion test respectively.

The observations in the grip strength test and gait analysis of the rats of diabetic group demonstrated that the animals were at the verge of nerve impairment and this might represent motor cortex defect. Dysregulation of central glucose and insulin in patients with poorly controlled diabetes may result in altered cerebral corticospinal motor function (Emerick *et al.*, 2005). However, in our model there was no change in insulin signaling in cortex probably because the analysis was done from whole rat cortex instead of only motor cortex. The cerebellum plays important function in the postural adjustments in order to maintain balance (Morton and Bastian, 2004), thus cerebellar damage often results in the balance disorders. Cerebellar function as well as its insulin signaling was not affected in dexamethasone treated rats as compared to control.

The exact effect of insulin on the hippocampal function of cognition is not very clear. The postmortem tissue from patients with neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease showed reduced mRNA and protein levels of INSR, implying a role for insulin signaling in neurodegenerative diseases (Aviles-Olmos *et al.*, 2013; Yarchoan *et al.*, 2014). However, in the current dexamethasone induced model, unaltered hippocampal insulin signaling and cognitive function was observed. Hippocampus is also the region comprising the major pool of NSCs with prominent adult neurogenesis (Anacker and Hen, 2017); hence, we assessed the level of Nestin (a marker for stem cells) as well as the candidate genes; Notch (*Notch1* and *Hes5*) and Wnt (*Dkk1)* responsible for deciding the fate of NSC differentiation. Notch mediated Hes5 signaling is necessary for neural stem cell maintenance by blocking neuronal differentiation and fate commitment (Zhang *et al.*, 2017). Decrease in Nestin, *Dkk1* and *Notch -Hes5* expression was evident in dexamethasone treated rat hippocampus. This reduced pool of NSCs might be because of marked inhibition of

proliferation induced by activation of the glucocorticoid receptor signaling by dexamethasone (Bielefeld *et al.*, 2017). Consequently, we predict that the same model after prolonged diabetic condition may depict cognitive dysfunction because of reduced NSC pool and neurogenesis, a similar phenomenon has been observed in diabetic patients with memory defects in later stage. Apart from intracellular signaling, NSC's behavior is likely to be affected by neighboring cells where we have limited our study on astrocytes (Wang *et al.*, 2011; Musaelyan *et al.*, 2014). There was decreased hippocampal expression of GFAP denoting the reduction in astrocyte number in dexamethasone treated rats as compared to control. Our analysis of key components of hippocampal astrocytes marked that these astrocytes were metabolically compromised and expressed remarkably reduced level of glutamate and lactate transporters as well as glycogen stores. Guo et al 2013 demonstrated that GLT-1 mediate the astrocytic regulation of glutamate and thus, modulates the cell fate of neural stem/progenitor cells (Guo *et al.*, 2013). Also, it is reported that glutamate regulates the proliferation of rat embryonic NSCs through regulation of the vascular endothelial growth factor (VEGF) expression in astrocyte, and cyclin D1 expression in NSCs (Liu *et al.*, 2015). Thus, perturbed glutamate homeostasis as observed in hippocampus because of decreased astrocytic expression of glutamate transporters as well as glutamate levels might lead to NSC exhaustion or aberrant proliferation. Although at tissue level, immunoblotting experiments demonstrated no change in insulin signaling in hippocampus of dexamethasone model, we conjecture that at cellular level there might be insulin signaling dysfunction specifically in astrocytes or neural stem cells. This hypothesis has been further addressed in our next objectives by *in vitro* experiments.

A work by Grillo CA *et al*., associated downregulation of hypothalamic *Insr* to depressive like behavior in rats (Grillo *et al.*, 2011). Our results were in accordance where dexamethasone treated group had increased immobility in Tail suspension test. The posture of immobility in context of tail suspension test was coined as 'behavior despair' largely based on the assumption that animals had given up hope of escaping (Porsolt *et al.*, 1987). Evidence suggests that there is greater psychiatric morbidity in diabetic population than in the general population, with depression being the most common psychiatric disturbance followed by anxiety (Knol *et al.*, 2006; Kogan *et al.*, 2007; Mezuk *et al.*, 2008; Golden and Mezuk, 2009). MRI studies have well documented the pathological alterations of limbic and cortical structures of brain in

depressive disorders (Lorenzetti *et al.*, 2009). Thus, we assert that there were adverse effects of stress induced peripheral insulin resistance in the depression centers of brain involving hypothalamic insulin signaling.

Hypothalamic insulin receptors are also crucial for the central regulation of appetite behavior. A frequent observation in this type of *in vivo* model of dexamethasone treatment has been reduction in food intake and body weight (Shimizu *et al.*, 2008); however, not much information is available on the effect of this treatment on hypothalamic regulation of feeding. Impaired insulin signaling with an emergence of insulin resistance-like condition was evident in the hypothalamus of dexamethasone-treated rats as compared to control rats. This desensitization of insulin signaling can be attributed to either direct dexamethasone treatment or dexamethasone-induced hyperinsulinemia. Contradicting the earlier reports, where hypothalamic insulin resistance can promote food intake leading to gain in body weight, dexamethasone-treated animals demonstrated decrease in body weight and were lean. These results clearly demonstrated that reduced insulin-mediated PI3K-AKT activation in hypothalamus is not always associated with obesity. It is in accordance with a report where impaired PI3K activation in ventromedial hypothalamus has been shown to resist the development of obesity (Klockener *et al.*, 2011).

Insulin signaling and the glycemic status are known to modulate the expression of GLUT1 (Barthel *et al.*, 1999), which is a major glucose transporter as well as a glucose sensor in brain (Chari *et al.*, 2011). There was a significant reduction observed in the expression of hypothalamic GLUT1 and also in SirT1, which is another metabolic sensor of glucose, thereby affecting not only glucose uptake but also glucose sensing by hypothalamus in dexamethasone-treated rats. Glucose also acts as a receptor stimulant for brain PPARγ (Qi *et al.*, 2012), and persistent hyperglycemia has been shown to decrease its expression in key hypothalamic regions involved in glucose homeostasis (Sarruf *et al.*, 2009). Studies with brain-specific PPARγ knockout mice demonstrates an increase in energy expenditure and a decrease in food consumption even when fed with high-fat diet (Lu *et al.*, 2011). Thus, in the present study, reduced PPARγ expression observed in hypothalamus of dexamethasone-treated rats justifies the reduction in food intake and weight loss.

Diabetes is strongly associated with changes in either turnover or activity of key enzymes involved in glycogen metabolism (Halse *et al.*, 2001; Krssak *et al.*, 2004), highlighting that brain glycogen content plays a critical role in diabetes. There are well-established effects of dexamethasone on glycogen metabolism in peripheral organs (Tavoni *et al.*, 2013), but only one report suggests that hypothalamic glycogen stores are depleted because of dexamethasone (Park *et al.*, 2005). Allaman *et al.,* demonstrated that dexamethasone inhibits glycogen synthesis in astrocytes induced by nor adrenaline (NA), and this inhibition does not result from a reduced rate of glucose transport or utilization (Allaman *et al.*, 2004). Thus, glucocorticoid-mediated hypothalamic insulin resistance causing reduced brain glycogen stores might be deleterious, since it may endanger neurons during subsequent periods of enhanced activity, as glycogen levels would be insufficient to ensure proper energy supply.

Hypothalamic insulin signaling in brain can upregulate anorexic neuropeptides such as POMC and downregulate orexigenic neuropeptides signals such as NPY and AgRP (Parker and Bloom, 2012). Hence, we checked status of these neuropeptide genes having noteworthy role in feeding behavior. Although our results were in line with the existing evidences where there was upregulation of gene expression of NPY and AgRP and downregulation of POMC and MC4R as a consequence of hypothalamic insulin resistance and dexamethasone treatment, the phenotype of these rats were totally paradoxical, thus suggesting that hypothalamic neuropeptides alone are not the sole factor decisive for the feeding behavior.

Tong *et al*. proved that while both NPY and AgRP stimulate food intake when infused into the brain, the detailed analysis established that there is weight loss when AgRP expressing cells are destroyed (Tong *et al.*, 2008). Also, genetic deletion of AgRP and NPY alone had little effect on feeding and body weight, and it is the GABAergic signaling that facilitates the feeding effect of NPY/AgRP at target sites in the hypothalamus (Wu *et al.*, 2009). These evidences indicate that the GABA is also required for the regulation of energy balance. Insulin resistance is known to decrease GAD65 mRNA expression and thus can reduce GABA level (Sato *et al.*, 2005). Thus, reduced GABA levels in dexamethasone-treated rats can contribute to the inability of AgRP/NPY to stimulate feeding. It is also supported by the fact that intracerebroventricular (i.c.v.) injection of GABA elicits an intense increase in food intake in rats and reciprocally i.c.v. injected GABA antagonist inhibits feeding (van den

Pol, 2003). Similar observation has been made, where central administration of glutamate (Stricker-Krongrad *et al.*, 1992) (Stanley *et al.*, 1993) and glutamate receptor agonists—kainic acid, AMPK, and NMDA —induced feeding, while glutamate receptor (mGlu5R) antagonist-6-methyl-2- (phenylethynyl) pyridine hydrochloride—decreased feeding (Fukumoto and Chaki, 2015) in rodents. Also, reduction in glycogen as seen in diabetic and stress condition, not only marks the depletion of the stored energy source but also disrupts glutamate and GABA homeostasis as proved in type 2 diabetic rodent models (Schousboe *et al.*, 2007; Sickmann *et al.*, 2012). Apart from GABA and glutamate, dopamine is other food intake-related neurotransmitters. In absence of neuronal insulin signaling, there is a rise in the dopamine-degrading enzymes such as MaoA and B, which results in increased dopamine clearance and hence reducing dopamine levels (Kleinridders *et al.*, 2015). Above facts further support the present observation of decreased glutamate, GABA, and dopamine levels negatively regulating the feeding behavior.

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 as summarized in Fig 4.21. Thus, dexamethasone treated model represents a promising rodent model to explore hypothalamic deregulation culminating into weight loss and dysregulated energy homeostasis. Also, it can be speculated that if prolonged, this model will also aggravate several other neurological complications and insulin signaling in other brain regions and thus can serve as an ideal model for stress and diabetes induced brain insulin resistance.

This hypothalamic network of appetite regulation is programmed prenatally in rodents and higher order mammals, where any intra uterine upsets are known to positively/negatively regulate this circuit (Nguyen *et al.*, 2017). It is well established that disconcerted maternal metabolic state such as insulin resistance (=diabetes), pre-disposes the foetuses to the risk of later age metabolic diseases (Hamilton *et al.*, 2010; Luo *et al.*, 2010; Jawerbaum and White, 2017). Hence, dexamethasone induced diabetic females with FIRI values above 2.67 were kept for mating with non-diabetic male rats to elucidate its impact on F-1 progeny.

Infants born to mothers with diabetes are at significantly greater risk for spontaneous abortion, stillbirth, congenital malformations and perinatal morbidity and mortality (Tennant *et al.*, 2014). However, there was no difference in the litter size, mortality as well as body weight in the pups born to the dexamethasone treated and control dams. Intrauterine environment affects the development of fetal organs, and infants born to diabetic mothers are known to have four-fold higher risks of neurologic structural abnormalities (Ornoy *et al.*, 2015). Our results demonstrated a remarkable reduction in the whole brain weight of the male pups born to the dexamethasone treated dams as compared to that of control dams. However, no such change was observed among the brain weight of female pups born to both groups.

As speculated fetal hypothalamic insulin signaling was differentially set in response to maternal insulin resistance induced by dexamethasone along with gender preferences in male and female hypothalamus. This sexual dimorphic effect can be attributed to the already existing differences in the brains of males and females, like the differential expression of insulin receptors at regions like hippocampus (Hami *et al.*, 2014). Also, there exists a difference in the roles of oestrogen and testosterone towards insulin sensitivity (Matsui *et al.*, 2013), which may be contributing to the onset of hypothalamic insulin resistance. Prominent reduction in insulin signaling was observed in the hypothalamus of the female pups and not the male pups born to the dexamethasone treated dams. There was also a steep reduction in the expression of GLUT 1 transporters in female pups of dexamethasone dams. Thus, confirming that an insulin resistance like condition evolved in the hypothalamus of the female pups born to dexamethasone dams, thus rendering them towards transformed appetite regulation in later stage.

As obvious from published reports and the observations in first part of our objective, insulin signaling works in concert with hypothalamic orexigenic and anorexigenic neuropeptides (Parker and Bloom, 2012). This perinatal disposition to hypothalamic insulin resistance as observed in female pups might also be associated with alterations in hypothalamic appetite regulating neuropeptides. In female pups of dexamethasone dams, there was a shift towards increased orexic signals (NPY) and reduced anorexic signals (Pomc and Mc4r). Although hypothalamic insulin signaling was not affected in male pups of dexamethasone dams, there was an increase in both orexic and anorexic neuropeptides. This can be attributed to the fact that there exists differences in the

expression level as well as response generated in these appetite regulating neurons in male and females (Urban *et al.*, 1993; Shi *et al.*, 2010). Although level of glutamate and GABA was unaltered in these pups, there was a decreased level of hypothalamic glycogen content in both gender of pups born to dexamethasone dams as compared to control.

PPAR-α is well expressed in different regions of the brain and its activation improves insulin sensitivity (M. M. Haluziki et al., 2006). Several studies exist that indicate an important role of brain PPAR α in physiological functions like neuroprotection and the control of whole-body glucose homeostasis during fasting. PPAR α target genes are upregulated during fasting. Since the melanocortin signaling is down regulated in the hypothalamus of females of dexamethasone treated dams, which would trigger the fed state resulting in downregulation of PPAR α. In case of males' hypothalamus there was opposite effect that was observed which resulted in upregulation of PPAR α.

Thus, the current neuroendocrine state of female pups of diabetic dams will plausibly push the feeding and energy homeostasis towards obese phenotype. The present study emphasizes the effect of maternal insulin resistance that may be caused due to diabetes, obesity, nutrition defects etc on the predisposition of infants to metabolic syndromes. Our study suggests that female hypothalamus is more prone to develop insulin resistance as compared to male brain as a consequence of maternal insulin resistance.

Thus overall, concluding that prolonged stress (= glucocorticoid) can not only lead to peripheral insulin resistance but can also perturb the insulin signaling in brain thereby negatively affecting functional aspects. The current study was delimited more towards hypothalamic aspects, and our results with accordance with other published researches asserted the decisive role of insulin signaling in appetite regulation in response to both prenatal as well as postnatal stress. Also, the changes in stem cell as well as astrocytic components lead to the formation of later objectives dealing with glucocorticoid exposure and insulin resistance in *in vitro* cellular models.

Thus, the outcome of this objective can be pictorially summarized as Fig 4.21.

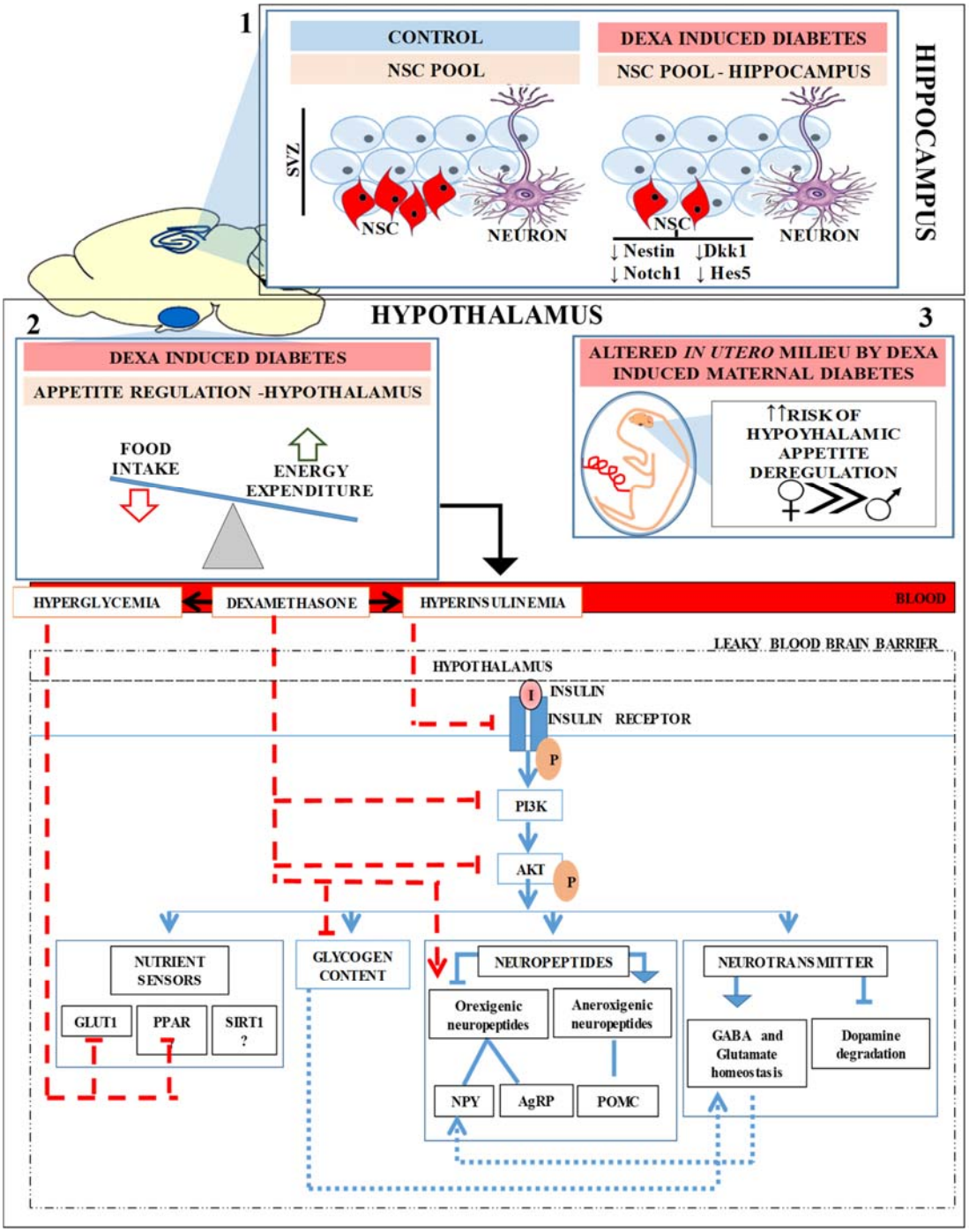


Figure 4. 21 Summary of "Regional and neurobehavioral study of brain insulin resistance in glucocorticoid induced diabetic rat model".

1. Reduced stem cell pool in hippocampus of dexamethasone (dexa) treated rats.
2. Hypothalamic appetite deregulation involving insulin resistance and dexa mediated changes in nutrient sensors, metabolites, neuropeptides and neurotransmitters, thus shifting dexa induced diabetic rats towards decreased food intake.
3. Dexa mediated maternal diabetes alters programming of appetite regulation in fetal hypothalamus. Gender differences were observed where female off springs were found to be more susceptible to hypothalamic changes than male off springs.