CHAPTER 3

MATERIALS & METHODS

3. MATERIALS AND METHODS

3.1 MATERIALS AND REAGENTS

Dexamethasone was procured from Zydus Alidac. Fine chemicals and consumables for western blotting was procured from Sigma-Aldrich and Biorad. Solvents were obtained from Sisco Research Lab (SRL). RNA isolation, cDNA synthesis, as well as real time PCR reagents were acquired from Invitrogen. Cell culture consumables were obtained from Eppendorf and Nunc. Cell culture media (DMEM F12), fetal bovine serum, growth factors (EGF, bFGF, B27 supplement minus insulin, Glutamine), as well as antibiotics (Penicillin-Streptomycin, Hygromycin) were procured from Sigma/ Invitrogen unless stated otherwise.

3.2 EXPERIMENTAL ANIMALS

Charles Foster rats are inbred strains of albino rats maintained in Animal House Facility, Department of Biochemistry. In the current study, virgin female rats weighing 180 – 220 g were used (Note that the only purpose of using female rats in the current study was that dose and duration of dexamethasone as well as phenotypic outcome in female rats were already well defined and published in the lab in context to ovarian insulin resistance). Rats were housed at Animal House Facility, Department of Biochemistry, with ad libitum access to water and chow (chow was procured from Pranav Agro Industries Ltd., Pune, India) in a well-ventilated animal unit (26–28 °C, humidity 60%, 12-h light-dark cycle). Care and procedures adopted for the present investigation were in accordance with the approval of the CPCSEA or Institutional Animal Ethics Committee (938/a/06/CPCSEA). For Objective 1A, the groups were control injected subcutaneously (s.c.) with normal saline, and dexamethasone treated group (dexa) with s.c. injection of 3 mg of dexamethasone per kg body weight daily for 28 days (Belani *et al.*, 2014). For objective 1B, control and dexamethasone treated group for 20 days were kept for mating with male non-diabetic Charles Foster rats 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. The detailed plan of work is discussed in Chapter 4.

3.3 CONFIRMATION OF INSULIN RESISTANCE AND ORAL GLUCOSE TOLERANCE TEST (OGTT)

Standard oral glucose tolerance test was performed after completion of dexamethasone treatment. After 12 h of overnight fasting, blood was collected from retro-orbital sinus for glucose and insulin measurement followed by oral administration of 2 g/kg body weight glucose. After glucose load, blood collection was performed at 0, 30, 60, 90, and 120 min for OGTT. The blood was allowed to clot at room temperature for 30 min followed by centrifugation at 2000g for 10 min for serum separation. Glucose was estimated from serum using glucose oxidase and peroxidase (GOD POD) method as per the manufacturers instructions (Reckon). Insulin levels were determined from fasting serum using Rat Insulin ELISA kit according to manufacturer's protocol (Mercodia, Germany). The fasting insulin resistance index (FIRI), a measure of the insulin sensitivity, was calculated according to the following formula: FIRI = [Fasting serum insulin (μIU/ml) x Fasting serum glucose (mmol/L)]/25 (Duncan *et al.,* 1995).

3.4 BEHAVIOUR STUDIES

After the treatment period and confirmation of insulin resistance in dexamethasone treated group, 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.

3.4.1 TAIL SUSPENSION TEST

A short piece of paper adhesive tape was attached along half the length of the tail. The free end of the adhesive tape was attached to a wooden plank such that the rat's head was about 20 cm above the floor as shown in Fig 3.1. Rats were observed for 3 min. Wriggling of the animal to avoid the aversive situation were recorded by an observer to calculate the % activity (Dunn and Swiergiel, 2005).

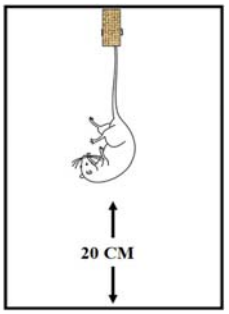

Figure 3. 1 Experimental setup for tail suspension test.

3.4.2 PUPIL DILATION TEST

The rat was held by hand and a 1.13W pen light was shined into the eye for 3 secs, followed by a 5-sec inter trial interval over three trials. The delay in dilation of the pupil was observed.

3.4.3 CORNEAL REFLEX TEST

The cornea of both the eyes of the rat was touched using cotton bud and the eye blink was observed. Animals with defects in corneal reflex would not show eye blink.

3.4.4 TAIL IMMERSION TEST

In the tail immersion tests, the noxious stimulus was given using hot water maintained at 52°C. Nociceptive reaction time i.e. the time taken for the rats to 'flick' their tail or to withdraw them from the hot water was recorded (Bhatt *et al.*, 2009).

3.4.5 GAIT ANALYSIS

The fore and hind paws of animals were stained with two different colours using non-toxic dye. The rats were trained to walk through an 8.2 x 42 cm walking track apparatus for two consecutive days, leaving their paw prints on blotting paper. On the third day several measurements were taken from the footprints, and used to calculate the sciatic function index (SFI) according to De Medinaceli (the normal value range between +11 and –11) from the equation described below (De Medinaceli L *et al.*, 1984). The temporal and spatial relationship of one footprint to another during walking were taken from the footprints. PL — distance from the heel to the third toe, the print length; TS — distance from the first to the fifth toe, the toe spread; IT — distance from the second to the fourth toe, the intermediate toe spread; and TOF — distance to opposite foot were measured as shown in Fig 3.2.

$$SFI = \left[\left(\frac{ETOF - NTOF}{NTOF}\right) + \left(\frac{NPL - EPL}{EPL}\right) + \left(\frac{ETS - NTS}{NTS}\right) + \left(\frac{EIT - NIT}{NIT}\right)\right]\frac{220}{4}$$

E stands for readings from experimental animals while **N** stands for that of normal (control) animals.

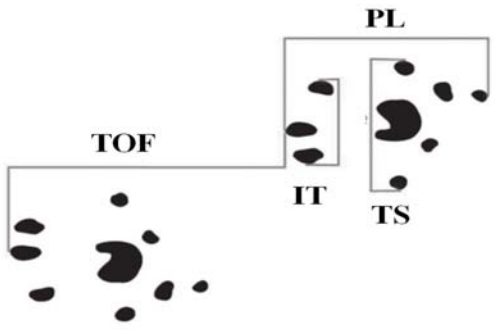

Figure 3. 2 Hindlimb prints of rats used to calculate Sciatic function index (SFI). PL - the print length; TS - the toe spread; IT - the intermediate toe spread; and TOF - distance to opposite foot.

3.4.6 GRIP ASSESSMENT TEST

Rats were placed on a metal wire 40 cm long, suspended 40 cm above a foam mat between 2 vertical bars as shown in Fig 3.3. They were introduced to the wire so that both front paws came in contact with the wire and there was an equal chance of grasping the wire. They were allowed to fall to the pad, remain hanging on the wire, or crawl along the wire to one of the supporting poles and the latency that a rat remained on the wire was measured (Sinz EH *et al.*, 1999).

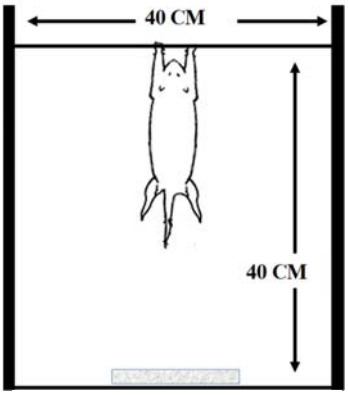

Figure 3. 3 Experimental setup for grip assessment test.

3.4.7 BALANCE BEAM TEST

The balance beam test was used to measure the ability of rats to traverse a horizontal narrow beam (1 cm×100 cm) suspended 50 cm above the ground as shown in Fig 3.4. During testing, animals were filmed crossing the beams and for successful performers, the latency to cross the beam was recorded. Training consisted of 3 trials, which served as baseline (M. Tariq et al, 2005).

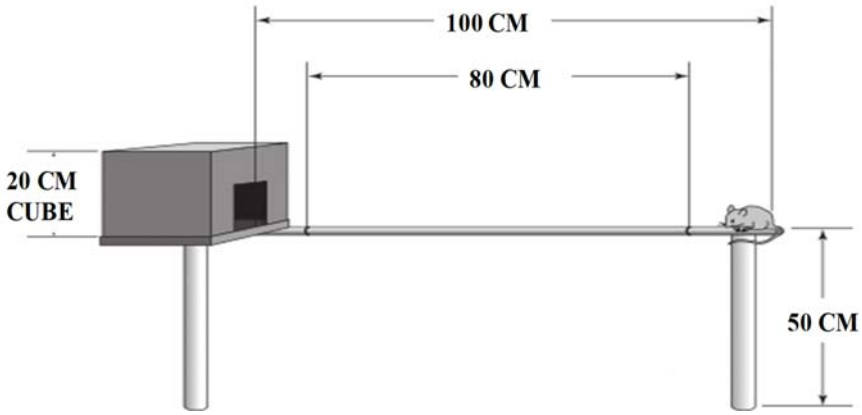

Figure 3. 4 Dimensions used in the set-up for balance beam test.

3.4.8 OPEN FIELD TEST

Rats were put in 100 X 100-cm arenas with 45-cm-high walls as shown in Fig 3.5. At the start of each trial, rat was grasped by the tail and placed in the centre of the arena. Trials were 3 min in duration. Relative time spent in the central area (infield, 20*20 cm), in the area close to the walls (outfield, 80 *80 cm) and in the corners, were recorded.

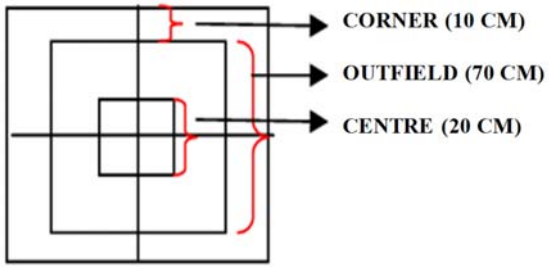

Figure 3. 5 Dimensions for open field test.

3.4.9 MORRIS WATER MAZE (MWM)

Cognition of each group was evaluated by the water maze Morris test for spatial reference task according to the protocol (Christie LA *et al.*, 2011). Testing occurred in a 180-cm diameter black pool centred within a rectangular room as shown in Fig 3.6. An overhead camera was connected to a video monitor to track the time (latency) spent to reach an invisible (black) platform placed 1 cm under the water surface. The MWM task was administered over 6 days and consists of 3 phases; a cued test with visible platform, an acquisition phase with hidden phase, and 24hr probe test with platform shifted to different quadrant.

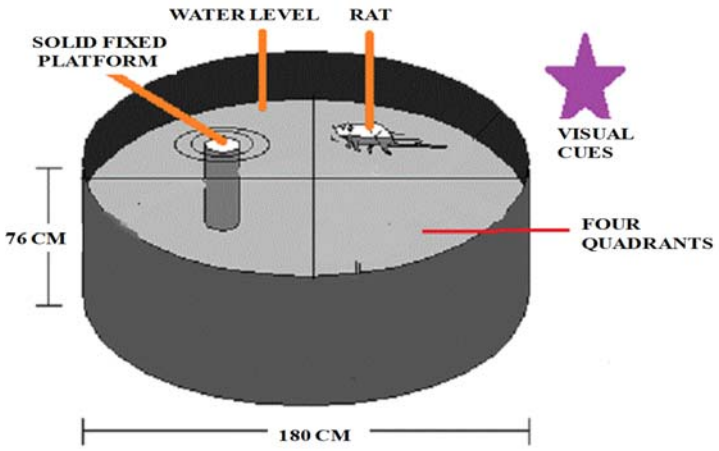

Figure 3. 6 Dimensions of pool used for Morris water maze test.

3.5 HISTOLOGICAL STUDIES

After treatment period, rats were anaesthetized with intravenous injection of xylazine (20 mg/kg bodyweight) and ketamine (150/kg body weight) and were transcardially perfused with heparinized sodium phosphate buffer (pH 7.4) followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Brain samples were dissected out, post-fixed by immersion in 4% paraformaldehyde (PFA) overnight at 4°C and then cryo-protected in 15% and 30% sucrose solutions in 0.1 M sodium

phosphate buffer (pH 7.4). These tissues were then embedded and frozen in tissue freezing medium (OCT, Leica). Sections were cut in the coronal plane on a cryostat (Leica CM1520) at 15 µm intervals and mounted onto Poly-L-lysine coated slides. These sections were then further processed for haematoxylin and eosin (HE) staining (using kit Biolab Diagnostics, Cat no. CY1577 as per manufacturer's instructions) and immunostaining. For immunostaining, sections were incubated in blocking buffer [2% fetal bovine serum serum, 2% bovine serum albumin, 0.1% Triton X-100 in Phosphate Buffer Saline (PBS) with pH 7.4] followed by incubation in primary antibody against phospho-INSRβ (Y-1361) at room temperature for 1 hour. After incubation, sections were rinsed in washing buffer (ten times diluted blocking buffer in PBS) and then incubated in goat anti-rabbit IgG CF 555 (Sigma Aldrich). Sections were then counterstained with the DNA stain- dapi and mounted with coverslip. Immuno-stained sections were viewed under confocal microscope (Zeiss LSM 710) and the fluorescence above the negative slides (only secondary antibody treated) were captured.

3.6 SERUM CORTICOSTERONE ESTIMATION

Corticosterone levels were estimated from serum separated from blood collected during 7–8 p.m. during pro-estrus stage. Briefly, corticosterone was extracted from serum using 1 mL dichloromethane (Sigma 270997). After centrifugation, aqueous phase was removed, washed with 1 mL 0.01 N NaOH and then centrifuged to remove the alkaline phase. Further, 30N sulfuric acid, 800 µl was added to the solvent phase, vortexed for 10–15 secs, followed by removal of the upper solvent layer. Acidic corticosterone containing phase was incubated at room temperature for 30 min (in dark), and the fluorescence of the sample was determined with an excitation wavelength of 472 nm and emission wavelength of 523 nm using Fluorescence Spectrophotometer (Hitachi F-7000). The fluorescence intensity was calculated from the standard curve prepared in charcoal-stripped serum, after correction for the low reading obtained with the blank carried through the same procedure (Silber *et al.*, 1958; Katyare and Pandya, 2005).

3.7 NEUROTRANSMITTER ESTIMATIONS

3.7.1 GLUTAMATE AND GABA ESTIMATION

The hypothalamus was dissected out, rinsed with ice cold PBS, weighed, and homogenized in 10% of 0.17M perchloric acid. The amino acid standards were

prepared by spiking known concentration of mixed standards (10, 20, 40, 80, and 160 ng/mL of each glutamate and GABA) in pooled brain homogenate. The homogenates were kept on ice for at least 30 min for complete protein precipitation. The homogenate was then centrifuged at 4 °C for 20 min at 12,000g. After centrifugation, supernatants were separated and either immediately analyzed or stored at −70 °C until assayed. The amount of glutamate and GABA was assessed by RP-HPLC coupled with electrochemical detector (model no. Waters 2465; Waters Corporation, Milford, USA). Estimation was performed according to the previously described method with minor modifications in the derivatization step (Bhattacharyya *et al.*, 2009). Briefly, a Sunfire® C18 column (4.6 × 150 mm, particle size 5 µm) was used, and separation was carried out using mobile phase containing a solution of 0.1M monosodium phosphate, and 0.5mM EDTA, 25% (v/v) methanol; pH was adjusted to 4.5, at a flow rate of 1.2 mL/min and an operating potential of 0.85 V. The solution was made in degassed deionized water. The derivatization reagent mixture consisted of 37mM orthopthaldehyde (OPA), 50mM sodium sulfite, 90 mM tetraborate buffer (which was set to pH 10.4 with sodium hydroxide, prior to addition of OPA), and 5% methanol (Reinhoud *et al.*, 2013). For preparation of standard calibration curve, 20 µl of derivatizing reagent was mixed with 1 ml of amino acid standard for 10 min. After incubation, 20 µl of sample was injected in HPLC. For sample analysis, 20 µl of the supernatant was mixed with 0.4 µl of the derivatizing reagent and incubated at room temperature for 10 min. Of the mixed supernatant, 20 µl was injected in HPLC. The standard curves were used to quantify the amount of glutamate and GABA in each sample by calculating the area under curve (AUC).

3.7.2 DOPAMINE ESTIMATION

For dopamine estimation, sample preparation was same as that for glutamate and GABA estimation. Of the deproteinized sample, 20 µL was injected in HPLC with Sunfire® C18 column (4.6 × 150 mm, particle size 5 µm), and separation was carried out using mobile phase containing methanol (15% v/v) in a solution (pH 4.2) of 32 mM citric acid, 12.5 mM disodium hydrogen orthophosphate, 0.5 mM octyl sodium sulfate, 0.5 mM EDTA, and 2 mM KCl, at a flow rate of 1.2 mL/min, an operating pressure of 3000 psi, and an operating potential of 0.61 V. The internal standard curves were prepared by spiking known amounts of mixed standard (10, 20, 40, 80, and 160 ng/mL

of dopamine (DA)) in 1 mL of pooled brain homogenates. The standard curves were used to quantify the amount of DA in each sample by calculating the AUC.

3.8 BRAIN CELL CULTURING

Post-natal day (PND) 0-2 neonatal pups of rats were procured from Animal house, Biochemistry Department. Care and procedures adopted for the present investigation were in accordance with the approval of the CPCSEA or Institutional Animal Ethics Committee. Primary cultures of astrocytes and neural stem cells (NSCs) were prepared from the PND 0–2 forebrains of neonatal rats as per the protocol by McCarthy and Pacey KK respectively. Animals were procured from Animal house facility, Department of Biochemistry. Care and procedures adopted for the present investigation were in accordance with the approval of the CPCSEA or Institutional Animal Ethics Committee (938/a/06/CPCSEA). Briefly 5-8 brains were extracted from PND 0-2 rat and placed in ice cold Phosphate buffered saline (PBS) with glucose. Meninges were removed and forebrain including hippocampus was dissected using stereo-zoom microscope (Leica). Tissue was triturated initially mechanically by passing through syringe (22 and 26 gauge), and later chemically by 0.05% trypsin-EDTA solution for 7-10 minutes at 37^0C. The trypsin action was inactivated using DMEM F12 (Gibco, Cat. no. 12500-062) with 10% Fetal Bovine Serum (FBS) (Gibco, Cat. no. 10270), tissue suspension was passed through 70µ strainer and centrifuged to pellet down the cells. These cells were then cultured in their respective media in an incubator at 37°C with 5% CO_2 and controlled humidity.

3.8.1 ASTROCYTE CULTURING (McCarthy and de Vellis, 1980)

For culturing astrocyte, media used was DMEM F12 with 10% FBS in cell culture treated non-vented flasks. When these cells reached confluency after 7-10 days of *in vitro* culturing, they were kept for overnight shaking in incubator shaker to remove other glial cells (microglia and oligodendrocytes). Cells were characterized after third to fourth passage using glial marker - Glial fibrillary acidic protein (GFAP) by immunocytochemistry and FACS analysis.

3.8.2 NEURAL STEM CELL CULTURING (Pacey *et al.*, 2006)

For culturing, NSCs, brain cells were seeded in non-adherent cell culture plates with DMEM F12 media containing B27 supplement (50X without insulin Gibco 05-

0129SA) with insulin, Epidermal growth factor (EGF) (Sigma, Cat. no. E4269), Fibroblast growth factor (FGF) (Sigma, Cat. no. F0291) and 2mM L-Glutamine (Sigma, Cat. no. G8540). Media was replenished at every third day and they were passaged when NSCs clusters (neurospheres) reached the size of 200µ. Cells were characterized at third passage using nestin and CD133 markers by immunostaining, and all the experiments were performed before 5th passage. For spontaneous differentiation, NSCs were plated on poly-l-lysine coated plates with differentiation media (DMEM with 1x B27supplement, 1% FBS, 2mM L-Glutamine and insulin) deprived of EGF and bFGF for 10 days. Media was replenished every third day.

3.9 SILENCING OF INSULIN RECEPTOR GENE

Plasmids for shRNA against rat insulin receptor (*Insr*) gene along with control plasmid were procured from Qiagen (Sure silencing shRNA plasmid Hygromycin KR44532H Cat. No. 336312) and the transfection was carried out using lipofectamine 2000 (Invitrogen). The pGeneClip™ Hygromycin Vector expressed a short hairpin RNA, or shRNA, under control of the U1 promoter and the hygromycin resistance gene as shown in Fig 3.7. Hygromycin resistance permitted the selection of transfected cells. Four pre-designed Sure silencing shRNA Plasmids were assessed for the knock down of *Insr* gene and the plasmid that gave maximum percentage of knockdown was used for all the experiments.

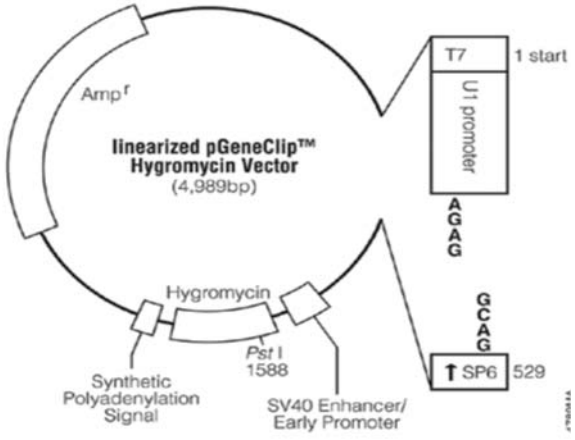

Figure 3. 7 pGeneClip™ Hygromycin Vector design

3.9.1 SILENCING OF INSULIN RECEPTOR GENE IN ASTROCYTES

The transfection in astrocytes 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.

3.9.2 SILENCING OF INSULIN RECEPTOR GENE IN NSCS

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

3.10 MTT ASSAY

Cells were plated in cell culture treated 96-well plate for respective treatments as mentioned in objective 2 (Chapter 5) and 3 (Chapter 6). After treatment period, 10 µl of 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution was added per 90 µl of the media followed by incubation for 3 hours at 37˚C in dark. After incubation, MTT containing media were gently removed, 100 µl acidic isopropanol (0.1N HCl in absolute isopropanol) was added, mixed well and incubated for 30 min at 37˚C in dark. These plates were then read at 570 nm.

3.11 CELL CYCLE ANALYSIS

Cells were harvested and the cell pellet was washed with 1XPBS. These cells were fixed using 70% ethanol at -20°C overnight followed by treatment of 50µg of RNase for 30 min at 37°C. The cells from each group were washed with staining buffer (1XPBS + 2%FBS +0.1% NaN_3) to stop RNase activity, and then incubated with 2.5µg of propidium iodide (PI; the excitation of PI was 536nm and the emission was 617nm) for 10 min at 37°C. After PI treatment, the cells' fluorescence was analysed by flow cytometry.

3.12 IMMUNO-PHENOTYPING USING FLOW CYTOMETER

Cells were harvested and then fixed with 2% PFA for 10 min at RT. After fixation, PFA was removed, and the cells were permeabilized with chilled absolute methanol for 5 min at 4°C. Further these cells were washed twice with staining media (PBS containing 1% BSA), and resuspended with 0.5 X 10^6 cells per 40 µl. Cells were then incubated with 2 µl of primary antibody for 45 minutes at RT followed by washes with staining media. Where ever primary antibody was not fluorescently tagged, the samples were further stained with its respective fluorescent labelled secondary antibody for 45 min at RT (Refer table no. 3.1 for specifications of antibody). The cells were resuspended in 200 µl staining media, and then kept at 4°C till cytometric analysis.

3.13 IMMUNOCYTOCHEMISTRY

Experiments were conducted on cells cultured on a cover slip. These cells were fixed using 4% paraformaldehyde, washed with 1X PBS, and permeabilized with 0.01% Triton X-100 for 2-4 min on ice. After washing with 1XPBS, these cells were proceeded for blocking with buffer containing 0.5% BSA+0.5% FBS in PBS for 1hour at room temperature followed by overnight incubation with primary antibody at 4°C. After incubation, washed with 1XPBS, and then incubated with secondary antibody (required only if primary antibody was not fluorochrome labelled) for 1 hour at room temperature (Refer table no. 3.1 for specifications of antibody). After washing steps, cells were counterstained with DAPI and then mounted on glass slides. The fluorescence images were captured using confocal microscopes.

3.14 GLYCOGEN CONTENT

Glycogen content was measured according to the Periodic Schiff Method (PAS) (Kilcoyne *et al.*, 2011) with slight modifications. In brief, 15mg of hippocampus / 1 x 10^6 cells was boiled in 30% KOH followed by the precipitation with absolute ethanol. The pellet was resuspended in appropriate amount of 0.2 M NaCl. 200ul of the sample was incubated at 37^0C for 2 hours with 20 µl of 50% of Periodic solution in 7% glacial acetic acid. After periodate oxidation, 20µl of Schiff's reagent was added and incubated for 30 min for the colour development. The absorbance was taken at A555 and the concentration was calculated as per the standard plot of glycogen (Range: 0-400 µg/ml) normalized to tissue weight / cellular protein content accordingly.

3.15 LACTIC ACID ESTIMATION

Astrocytes conditioned media was collected after the treatment period. Acetonitrile (HPLC grade) was added to conditioned media in 1:1 ratio and incubated at 4°C for 1 hour to precipitate out the protein. After incubation, cells were centrifuged at 12,000g for 10 min. The supernatants were collected and filtered through 0.22µm filter. These supernatants were dried completely under vacuum centrifugation, and re-suspended in 100µl of MilliQ water. Blank and spiked standards were prepared in DMEM F12 containing 0.5% charcoal stripped FBS to calculate lactic acid in unknown samples. Standard and samples were injected in HPLC with Reprosil™ gold C18 column and separation was carried out using mobile phase containing 0.05M phosphate buffer (pH 2.5) with 1% methanol at flow rate 0.8ml/min. The chromatographic run was for 12 minutes and detection was performed at 220nm (Biagi *et al.*, 2012).

3.16 WESTERN BLOT ANALYSIS

Tissue from *in vivo* experiments were dissected out, homogenized in buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, protease inhibitor cocktail) and stored in aliquots at –80°C. Tissue homogenates were sonicated at 45% amplitude for 2 min with 20 sec pulse on/off, centrifuged (14,000g) and aliquots of the clear extract were boiled in Laemmli SDS sample buffer. Cells from *invitro* experiments were directly harvested in Laemmli SDS sample buffer and sonicated at 45% amplitude for 20 secs with 20 sec pulse on/off. 40 µg (for tissue) or 15 µg (for

cells) of total protein as estimated by Bradford's method was resolved on 12% SDS-PAGE Tris-glycine gels and transferred to nitrocellulose membrane. Non-specific binding was blocked by incubating the membranes in 5% BSA and 0.1% Tween in Tris-buffered saline (TBS, pH 7.4) for 1 h at room temperature. The blots were subsequently incubated with primary antibodies against specific proteins (as mentioned in Table no. 3.1) overnight at 4 °C, with gentle agitation. Blots were washed with TBS containing 0.1% Tween (TBS-T) (4 × 15 min) and then incubated with respective secondary antibodies conjugated with HRP for 2 h at room temperature with gentle agitation. After four washes with TBS-T and one wash with TBS; specific bands of immune-reactive proteins were visualized using enhanced chemiluminescence (ECL) reagent (Millipore) in Chemidoc (Alliance Model 4.7). Densitometric analysis of the protein bands was calculated by Image J software. Intensity of target proteins were normalized with that of loading control i.e. β actin. The ratio of phosphorylated proteins to that of total protein was calculated after normalization to β actin.

3.17 GENE EXPRESSION STUDY

Total RNA was isolated from the samples (tissues/cells) using Trizol (Invitrogen). Total RNA was quantified and mRNA samples were reverse-transcribed into cDNAs using high capacity cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. Expression of target genes using specific primers (as listed in Table 3.2) was measured via real-time PCR technique using SYBR green chemistry (7500 Applied Biosystem Real Time PCR) or Taqman chemistry (Quant Studio Applied Biosystem). The Ct values of target genes were normalized to that of *Actb* levels to calculate ΔCt. The graph was plotted as relative fold change of expression of target genes which was quantified as $2^{-(\Delta\Delta Ct)}$, where ΔΔCt was ΔCt (target gene expression in test group) -ΔCt (target gene expression in control group).

3.18 STATISTICAL ANALYSIS

Results are presented as mean ± SEM of the indicated number of experiments. Statistical significance was determined using the unpaired students t-test or two-way analysis of variance using Bonferroni post-test using graph pad prism 3 software. P value less than 0.05 was considered to be statistically significant.

Table 3. 1 List of antibodies.

ANTIBODY TYPE	ANTIGEN	NAME PROVIDED BY SUPPLIER	SUPPLIER DETAILS	DILUTION
Primary Monoclonal	β actin	Anti β actin monoclonal antibody	Thermo scientific (Cat no.: MA1-91399	1:5000 (WB)
Primary Polyclonal	Insulin receptor β	Polyclonal rabbit anti-CD220 (Insulin receptor β)	BD transduction laboratories (Cat no.: 611277)	1:1000 (WB)
Primary Polyclonal	Phospho-Insulin receptor (Y-1361)	Phospho-IR pTyr1361 Antibody	ThermoFisher (Cat no.: PA5-35787)	1:1000 (WB) 1:100 (IHC)
Primary	GLUT 1	Anti-Glut 1, C-terminal antibody	Sigma Aldrich (Cat no.: SAB 4502803)	1:1000 (WB)
Primary Monoclonal	PPARγ	PPARγ (C26H12) Rabbit mAb	Cell signalling Technology (Cat no.: 2435)	1:1000 (WB)
Primary Monoclonal	PPARα	Monoclonal Anti Peroxisome Proliferator Activated Receptor α	Sigma (Cat no.: P 0869)	1:1000 (WB)
Primary Monoclonal	PI3 Kinase p85	PI3 Kinase p85 (19H8) Rabbit mAb	Cell signalling Technology (Cat no.: 4257)	1:1000 (WB)
Primary Monoclonal	Total Akt	Akt(pan) (C67E7) Rabbit mAb	Cell signalling Technology (Cat no.: 4691)	1:1000 (WB)
Primary Monoclonal	Phospho Akt	Phospho Akt (Ser473) (D9E) XP Rabbit mAb	Cell signalling Technology (Cat no.: 4060)	1:1000 (WB)
Primary Monoclonal	SirT1	SirT1 (D1D7) Rabbit mAb	Cell signalling Technology (Cat no.: 9475)	1:1000 (WB)
Primary Monoclonal	AMPKα	AMPKα (D63G4) Rabbit mAb	Cell signalling Technology (Cat no.: 5832)	1:1000 (WB)
Primary Monoclonal	Phospho-AMPKα (Thr172)	Phospho-AMPKα (Thr172) (40H9) Rabbit mAb	Cell signalling Technology (Cat no.: 2535)	1:1000 (WB)
Primary Monoclonal	Nestin	Anti-Nestin	Sigma (Cat no.: N5413)	1:1000 (WB)
Primary Monoclonal	CD133	PE anti mouse CD133	Biolegend (Cat no.: 141203)	1:100 (ICC)
Primary Monoclonal	GFAP	Alexa Fluor® 488 Mouse Anti-GFAP	BD Pharmigen™ (Cat no.: 561449)	1:1000 (WB) 1:100 (ICC)
Primary Monoclonal	MAP2	Purified Mouse Anti-MAP2	BD Pharmigen™ (Cat no.: 556320)	1:1000 (WB) 1:100 (ICC)
Primary Monoclonal	Nestin	PE Mouse Anti-Nestin	BD Pharmigen™ (Cat no.: 561230)	1:50 (IF)

Primary Monoclonal	O1	Monoclonal Anti Oligodendrocyte marker O1 Clone O1	Sigma (Cat no.: O7014)	1:50 (ICC)
Primary Monoclonal	Vimentin	Monoclonal Anti Vimentin Cy3 Conjugate Clone V9	Sigma (Cat no.: 9080)	1:100 (ICC)
Secondary	Anti-mouse IgG	Goat anti mouse Poly-HRP	Thermo scientific (Cat no. 32230)	1:2500 (WB)
Secondary	Anti-rabbit IgG	Goat anti rabbit Poly-HRP	Thermo scientific (Cat no. 32260)	1:2500 (WB)
Secondary	Anti-rabbit IgG	Antibody CF^{TM} 555 Conjugates	Sigma- Aldrich (Cat no. 32230)	1:250 (IHC)
Secondary	Anti-mouse IgG	Anti-mouse IgG FITC Conjugate	Sigma (Cat no. F8771)	1: 250 (ICC)

Western blotting (WB), Immunocytochemistry (ICC), Immunohistochemistry (IHC), Immuno flow cyometric analysis (IF).

Table 3. 2 List of primers used for gene expression studies.

A. PRIMERS FOR SYBR GREEN CHEMISTRY				
GENE		**FORWARD PRIMER 5'-3'**	**REVERSE PRIMER 5'-3'**	**ACCESSION**
Npy	Neuropeptide Y	AAT CAG TGT CTC AGG GCT GGA T	CCG CTC TGC GAC ACT ACA TC	NM_012614.2
Pomc	Proopiomelano -cortin	AAG AGC AGT GAC TAA GAGAGG CCA	ACG TCT ATG GAG GTC TGA AGC AGG	NM_139326.2
Agrp	Agouti-related protein	CGG AGG TGC TAG ATC CAC AGA	AGG ACT CGT GCA GCC TTA CAC	NM_033650.1
Crh	Corticotropin-releasing hormone	CCA GGG CAG AGC AGT TAG CT	CAA GCG CAA CAT TTC ATT TCC	NM_031019.1
Cart	Cocaine- and amphetamine regulated transcript	GCC AAG TCC CCA TGT GTG AC	CAC CCC TTC ACA AGC ACT TCA	NM_017110.1
Obrb	Leptin receptor long isoform	GCATGCAGAATCAGTG ATATTTGG	CAAGCTGTATCGAC ACTGATTTCTTC	NM_012596.1
Mc4r	Melanocortin 4 receptor	ACG CGC TCC AGT ACC ATA AC	AAA GAA CGC CCG ATA CTG TG	NM_013099.2
Glt1 or Slc1a2	solute carrier family 1 member 2 (SLC1A2) or Glutamate trasporter 1	CCGAGCTGGACACCAT TGA	CGGACTGCGTCTTGG TCAT	NM_017215.2
Glast or Slc1a3	solute carrier family 1 (glial high affinity glutamate transporter) or Glutamate Aspartate Transporter (GLAST)	CCATCCAGGCCAACGA AA	GCCGAAGCACATGG AGAA	NM_019225.2

Slc16a1	Solute carrier family 16 member 1 or Monocarboxylate transporter 1	TGGAATGTTGTCCTGT CCTCCTGG	TCCTCCGCTTTCTGT TCTTTGGC	NM_012716.2
Slc16a3	Solute carrier family 16 member 1 or Monocarboxylate transporter 4	TTCTCCAGTGCCATTG GTCTCGTG	CCCGCCAGGATGAA CACATACTTG	NM_030834.1
Actb	βactin	CTTCTGACCCATACCC ACCA	ATGGATGACGATAT CGCTGC	NM_031144.3
Notch1	Notch1	GAGGCTTGAGATGCTC CCAG	ATTCTTACATGGTGT GCTGAGG	NM_001105721.1
Dkk1	Dickkopf-related protein 1	GCTGCATGAGGCACGC TAT	AGGGCATGCATATTC CGTTT	NM_001106350.1
Hes5	Hairy and enhancer of split 5	GCACCAGCCCAACTCC AAAC	TGCAGGCACCACGA GTAGCC	NM_024383.1

B. PRIMERS WITH TAQMAN CHEMISTRY		
GENE		**Assay ID (Applied Biosystems)**
Actb	βactin	Rn00667869_m1
Nes	Nestin	Rn00564394_m1
Sox2	SRY (sex determining region Y)-box2	Rn01286286_g1
Pax6	Paired box 6	Rn00689608_m1
Neurod1	Neurogenic differentiation 1	Rn00824571_s1
Gfap	Glial fibrillary acidic protein	Rn00566603_m1
S100b	S100 calcium binding protein B	Rn04219408_m1
Dcx	Double cortin	Rn00584505_m1
Slc1a3	solute carrier family 1 (glial high affinity glutamate transporter) or Glutamate Aspartate Transporter (GLAST)	Rn00570130_m1
Fabp7	fatty acid binding protein 7	Rn01642066_m1
Olig1	oligodendrocyte transcription factor 1	Rn00572904_s1
Vim	Vimentin	Rn00579738_m1
Map2	Microtubule-associated protein 2	Rn00565046_m1
Dlg4	Discs	Rn00571479_m1
Mbp	Myelin basic protein	Rn01399619_m1
Alsh1l1	Aldehyde dehydrogenase 1 family	Rn00574839_m1
Insr	Insulin receptor	Rn00690703_m1
Slc2a3	Solute carrier family 2 (facilitated glucose transporter 3)	Rn00567331_m1
Slc2a1	Solute carrier family 2 (facilitated glucose transporter1)	Rn01417099_m1
Gsk3b	Glycogen synthase kinase 3 beta	Rn00583429_m1