CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Brain is the most complex of all the biological organs. It is composed of different cell types organized in signature patterns to make anatomically and functionally defined and interconnected areas which render distinctiveness to various behavior.

1.1 BRAIN PARCELLATION

For simplification, brain can be anatomically divided in major four divisions namely brain stem, cerebellum, diencephalon, and cerebral hemispheres as shown in Fig 1.1 and its function are as described in Table: 1.1 (Amaral, 2000; Tortora and Derrickson, 2009). There are also white matter connections that interconnect different regions of brain for coordinated fine-tuned functions (Nowinski, 2011) and cerebrospinal fluid (CSF) filled ventricular system (Mortazavi *et al.*, 2014).

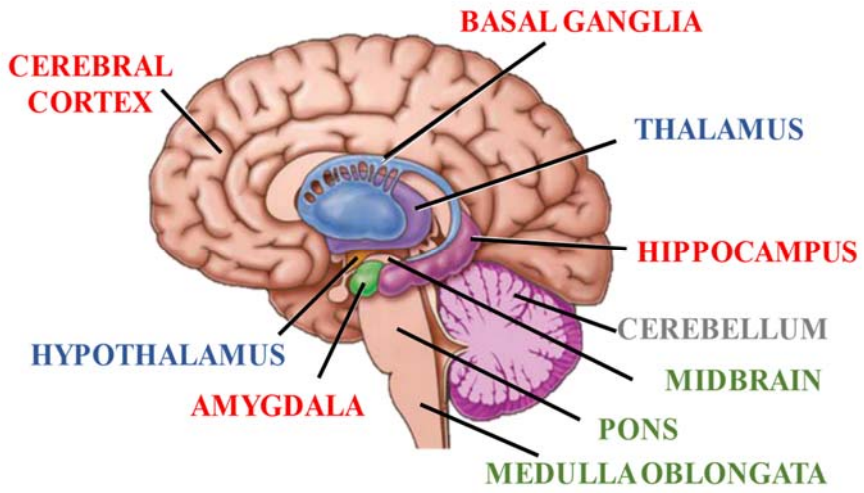

Figure 1. 1: Anatomical division of human brain in sagittal view: Brain stem (midbrain, pons, and medulla oblongata) as texted in green, cerebellum as texted in grey, diencephalon (thalamus, and hypothalamus) as texted in blue and cerebral hemisphere (cortex, basal ganglia, hippocampus, and amygdala) as texted in red (Tortora and Derrickson, 2009).

Table 1. 1: Function of different brain regions.

REGION	SUB-REGIONS	FUNCTION
BRAIN STEM	Medulla	Centre for respiration and circulation; sensory and motor control
	Pons	Relays sensory information between the cerebrum and cerebellum; sleep; control of autonomic function; arousal

	Midbrain	Response to sight and eye movement; hearing; body movement
CEREBELLUM		Receipt of somatosensory inputs and motor information; maintenance of balance
DIENCEPHALON	Thalamus	Transfer, gating and modulation of sensory information
	Hypothalamus	Energy homeostasis; thermogenesis; reproduction; regulation of pituitary hormones
CEREBRAL HEMISPHERE	Cerebral cortex	Perceptual and motor function; cognitive functions; emotion
	Basal ganglia	Convey information concerning movement, emotion, cognition
	Hippocampus	Cognition, long term memory, learning
	Amygdala	Analysing the emotional or motivational stimuli; co-ordination of variety of brain systems for appropriate response

Majority of the above-mentioned regions are analogous in mammalian brain. Hence, laying a strong foundation for the use of age-specific murine models to study different aspects of human brain. Though, there are some differences across these species as shown in Fig 1.2.

The generative capacity of the immature brain varies by brain region and cell type but in general, cell proliferative processes between rodents and humans are remarkably parallel, although the time scales are substantially different. They also exhibit some characteristic anatomical differences based on neural development as well as the size of the brain (Semple *et al.*, 2013). For instance, rodents have relatively more evolved olfactory bulbs (mediating their sense of smell) than humans. However, humans have relatively more developed language areas in their cerebral cortex. One very notable difference between the human and rodent developing brain is gyrification, which is essentially absent in the rodent brain. Also, both the species have evolved under different selection pressures that bias them to approach tasks with different strategies. Thus, the selection of an age-appropriate rodent model must be predicated on biochemical, developmental, behavioral and neuroanatomical differences to study specific pathological conditions before extrapolating to humans.

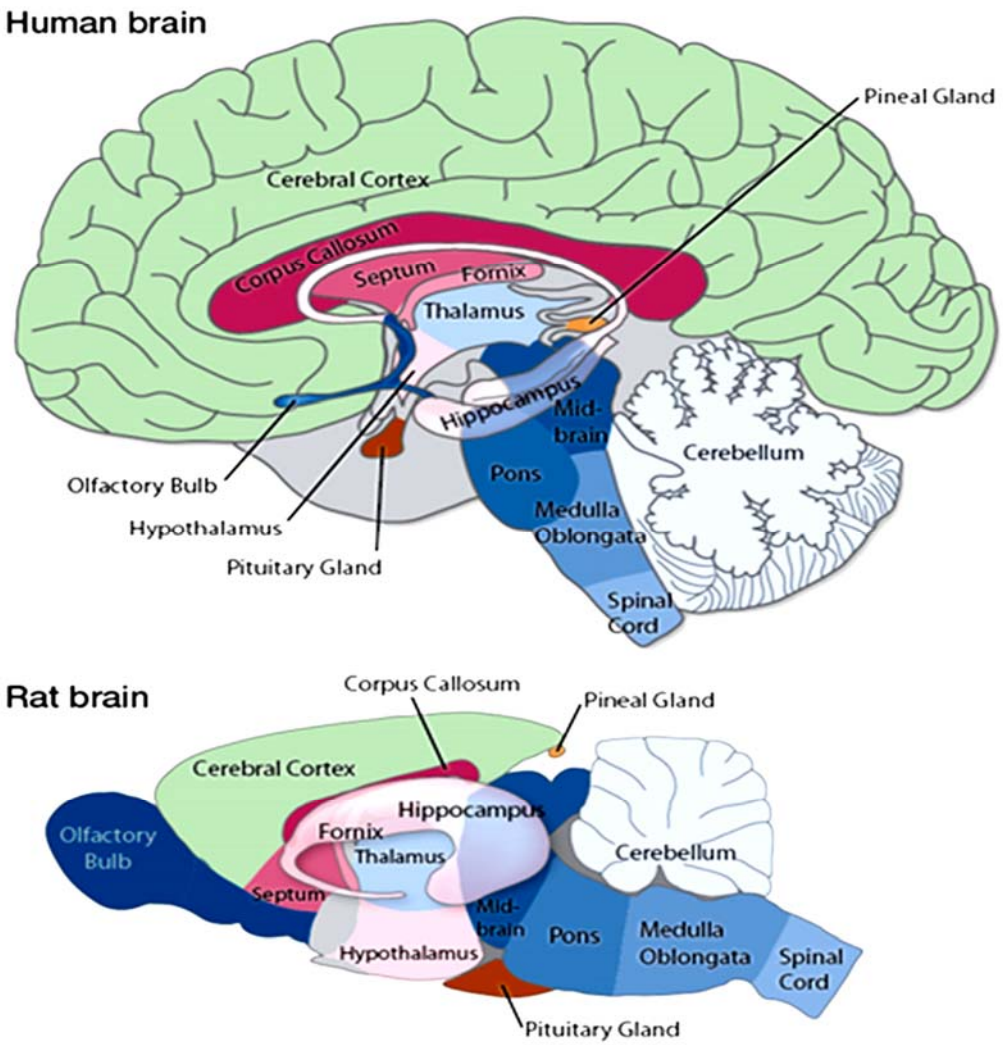

Figure 1. 2: Anatomical differences in brain: Human (top) and rat (bottom). (http://www.computescotland.com/extreme-reliability-of-design-and-the-agile-brain-5126.php)

1.2 BRAIN CELLS

Levelling down at cellular level, the brain comprises mesh of several types of specialized cells namely neural stem cells (NSCs), neurons, and non-neuronal cells or the glial cells [namely astrocytes, oligodendrocytes, microglia, ependymal cells] organized in a region configured manner. The major distinction between neurons and glia being that glial cells do not participate directly in synaptic interactions and electrical signaling, although their supportive functions help to define synaptic contacts and maintain the signaling abilities of neurons. The intercommunication and the interdependence among these different cells, are decisive in maintaining brain

homeostasis. The features and functions of these cells are as mentioned in the Table: 1.2.

Table 1. 2: Major types of brain cells, their specific markers, key features and functions.

BRAIN CELLS	SPECIFIC MARKERS	FEATURES	FUNCTION
NEURAL STEM CELL (NSC)	NESTIN, CD133, SOX2, PAX6, NEUROD1, NOTCH1, etc.	Self-renewing; asymmetric division; multipotent	Cellular regeneration during physiological cell turnover or injury
NEURONS	MAP2, DLG4, NeuN, NF, SY38, PSD95, etc.	Consists of the cell body, the dendrites, the axon, and the axon terminals; excitable cells; majority are post mitotic arrested in the G0-G1phase	Process and transmit information
ASTROCYTES	GFAP, GLAST, GLT-1, GS, S100-β, ALDH1L1, etc.	Abundant and heterogeneous type of glial cell	Provide trophic factors and nutrients; aid in the neurotransmission; maintenance of blood brain barrier; maintain metabolic homeostasis; maintenance of extracellular ion balance; repair
OLIGO-DENDROCYTES	OLIG 1, OLIG 2, OLIG 3, MBP, MOG, etc.	specialized cells synthesizing myelin sheath	Oligodendrocytic myelin sheath wrap around neuronal axons forming insulation barrier
MICROGLIA	CD11b, CD45, IBA1, F4/80, CD68, CD40, etc.	resident immune cells	Inflammatory response and removal of cellular debris
EPENDYMAL CELLS	CD133, CD 24, ARL13b, D2-TUBULIN, etc.	ciliated glial cells covering the ventricular surfaces	Secretion of trophic factors; uptake of glucose; dispersion of CSF components; modified ependymal cells in choroid plexus synthesize CSF

1.3 BLOOD BRAIN BARRIER

Brain is well separated from the blood by the tightly regulated barriers thus insulating brain from direct peripheral interference. Three barrier layers limit and regulate molecular exchange at the interfaces between the blood and the brain/neural tissue or its fluid spaces namely the blood brain barrier (BBB) formed by the cerebro-vascular endothelial cells between blood and brain interstitial fluid (ISF), the choroid plexus epithelium between blood and ventricular CSF, and the arachnoid epithelium between blood and subarachnoid CSF.

The BBB is a selective 'physical barrier' formed by the endothelial cells with tight junctions that line cerebral micro vessels associated with astrocytic end feet and pericytes as shown in Fig 1.3. Normally, the tight junctions only allow the penetration of small polar water-soluble compounds through paracellular aqueous pathway. Only small gaseous molecules (such as O2, CO2, etc.) and lyophilic agents (barbiturates, ethanol, etc.) can diffuse freely through the lipid membranes of BBB termed as transcellular lipophilic pathway.

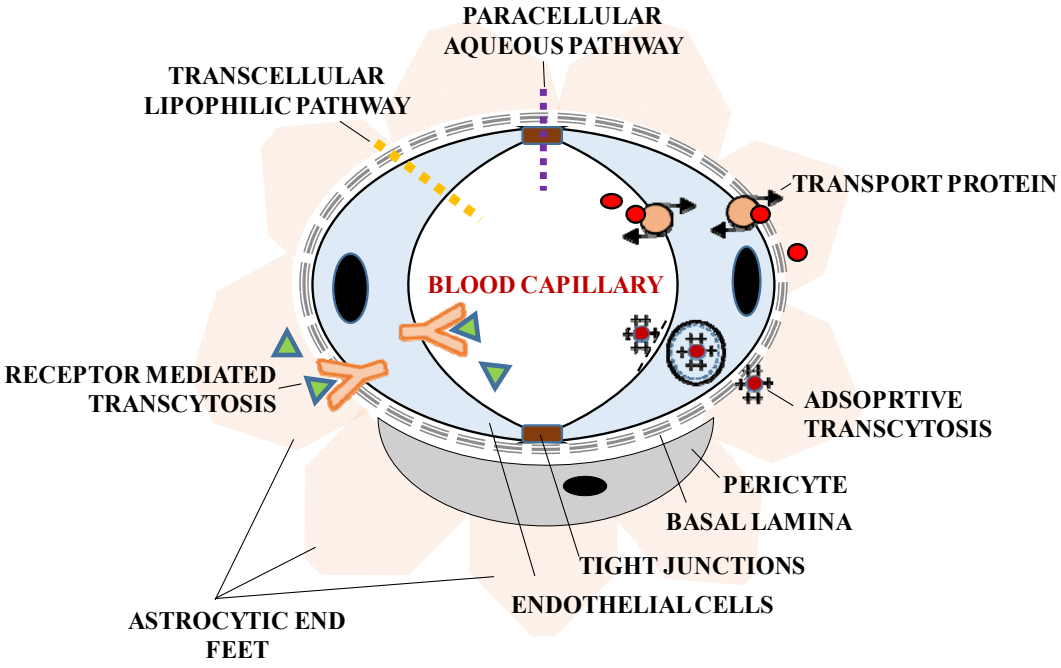

Figure 1. 3: Representation of blood brain barrier (BBB) and molecular trafficking: Endothelial cells, pericyte, and perivascular end feet of astrocytes forms the BBB. The main routes for molecular traffic across the BBB are paracellular aqueous pathway, transcellular lipophilic pathway, through transport proteins, receptor-mediated transcytosis and adsorptive transcytosis.

The endothelium contains transport proteins (carriers) for glucose, amino acids, purine bases, nucleosides, choline and other substances. Some transporters are energy-dependent (for example, P-glycoprotein) and act as efflux transporters for drugs such as azidothymidine. Large hydrophilic molecules such as peptides and proteins (insulin, transferrin, leptin, etc.) are transferred by specific receptor-mediated transcytosis. Native plasma proteins such as albumin are poorly transported, but cationization can increase their uptake by less specific adsorptive-mediated transcytosis. Further, there rests a combination of several intracellular (monoamine oxidase and cytochrome P450) and extracellular (peptidases and nucleotidases) enzymes which provides a 'metabolic barrier' to inactivate many neuroactive and toxic compounds (Abbott *et al.*, 2006).

The brain resides in an environment that is protected from humoral signals. However, the brain must assess key sensory information from the bloodstream, including levels of hormones, metabolites, and potential toxins to exert homeostatic control. Thus, there are "windows on the circulation" or circumventricular organs (CVOs) that serve as a conduit of peripheral cues into key neuronal cell groups that maintain homeostasis. CVOS, unlike BBB have fenestrated and rich supply of blood capillaries that allow relatively free passage of macromolecules to brain cells. They are located on the midline of the brain along the third and fourth ventricles. Therefore, the CVOs serve as a critical link between peripheral metabolic cues, hormones, potential toxins and cell groups within the brain that regulate coordinated endocrine, autonomic, and behavioral responses (Kaur and Ling, 2017).

1.4 PERIPHERAL SIGNALS CONVEYING INFORMATION TO BRAIN

Numerous peripheral signals contribute to regulation of brain functions. The best studied brain function in tune with peripheral signals is food intake and energy homeostasis. Short term energy regulators are nutrients such as glucose, amino acids, and gastro intestinal peptide hormones such as cholecystokinin whereas, and long-term energy regulators are insulin, leptin, ghrelin, glucagon-like peptide-1, etc. These are transported to brain where they modulate expression of hypothalamic neuropeptides which then regulate feeding behavior and body weight (Havel, 2001; Williams and Elmquist, 2012).

Similarly, there are several peripheral cues that interfere with psychological performance such as insulin, cytokines and glucocorticoids. Insulin receptor (INSR) has varied distribution in brain where it regulates learning, cognition, anxiety and emotions (Laron, 2009). Cytokines through their cognate receptors as well as by interaction with neurotransmitter systems, impact neurocircuits in the brain including the basal ganglia and anterior cingulate cortex, leading to significant changes in motor activity and motivation as well as anxiety, arousal, and alarm (Miller *et al.*, 2013). Glucocorticoids secreted from adrenal gland, have diverge effects on brain based on species, gender, age, hormone concentrations, timing, and duration of exposure. It entails on appetite, arousal, sleep, behaviour, cognition, memory, and mood. Two types of cytoplasmic receptors, namely mineralocorticoid and glucocorticoid receptors, having different brain distribution and functional pattern, mediate the hormonal activity through the stimulation or suppression of target gene transcription, depending on cell type (Fietta and Fietta, 2007).

Thus, transport of peripheral signals to brain and its integration is crucial for functional efficiency of an organism.

1.5 INSULIN TRANSPORT TO BRAIN

Among several peripheral cues, insulin is one of the very important peptide hormone secreted by pancreas that is required in brain for plethora of functions. Although insulin has a well described role in periphery, its exact role in alteration of brain functions ranging from cell survival to cognitive performance is yet not well defined. Thus, despite decades of extensive research, numerous unanswered questions still remain pertaining to the precise mechanism of insulin in brain during normal and diseased condition.

Insulin being a 51-amino acid peptide hormone, makes it impossible to diffuse across the BBB. Thus, for many decades it was deemed unlikely that insulin was relevant to CNS. However, with advent to the understanding of wide distribution of insulin receptor in various brain regions (Havrankova *et al.*, 1978; Marks *et al.*, 1990) as well as the biological outcomes of exogenous insulin administration in the CNS (Ono *et al.*, 1983; Danguir and Nicolaidis, 1984); there arose several hypotheses for the presence of insulin in brain. Due to lack of strong investigational proof for the endogenous

synthesis of insulin in brain cells, it was predicted and then also further verified that insulin is rather transported from peripheral circulation to brain. Experimental evidences suggested that there exists a dynamic insulin uptake via saturable receptor mediated transcytosis across blood brain barrier (Pardridge, 1986). Insulin produced in pancreatic β-cells is released into blood. This circulating insulin then binds to the insulin receptors on the luminal surface of the endothelial cells (ECs) of the BBB. ECs have insulin-binding sites that appear to have two distinct functions: as transporters of insulin across the BBB and as classic receptors. Insulin bound to the receptors for transport across BBB undergo receptor mediated transcytosis across the cytoplasm of the ECs to the abluminal surface where it is released into the brain interstitial space and acts upon brain cells to switch on the insulin signaling as shown in Fig 1.4.

Insulin signaling in ECs triggered by the binding of insulin to classical receptors affects the function of the barrier cell by activating intracellular machinery and mediating the effects, such as the increase in the transport of metabolites (tyrosine, tryptophan) from blood to brain, modification of the expression or activity of efflux transporters (P-glycoproteins), etc (Tagliamonte *et al.*, 1976; Liu *et al.*, 2009). The expression of the INSRs on ECs can be affected by the physiological as well as pathological conditions (Baura *et al.*, 1996; Kaiyala *et al.*, 2000; Gray *et al.*, 2017).

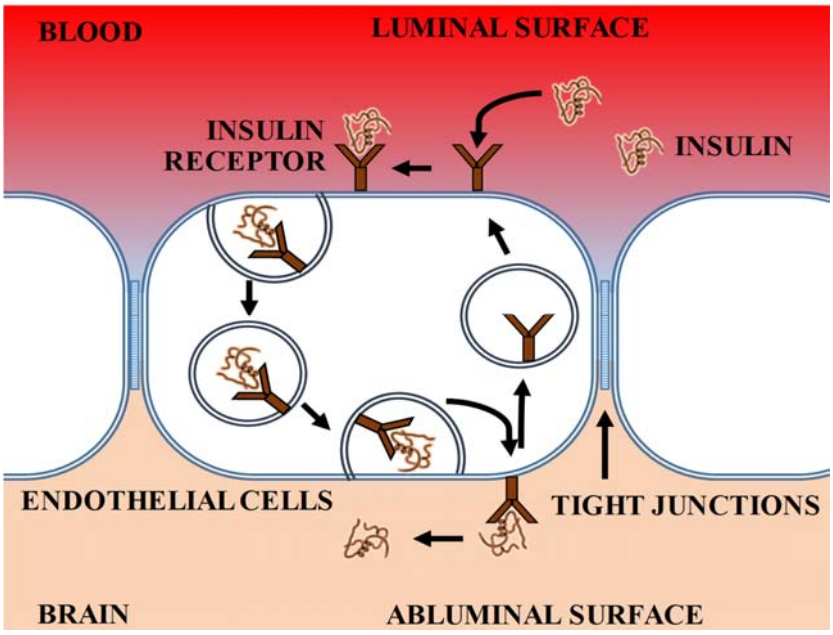

Figure 1. 4: Receptor mediated transcytosis of insulin across endothelial cells of blood brain barrier.

1.6 DISTRIBUTION OF BRAIN INSULIN RECEPTOR (INSR)

Two different isoforms of INSR are found in mammalian brain. One that is of *peripheral type*, detected in lower density on glial cells, and another one is a *neuron-specific type* that is widely distributed in the CNS. The major molecular skeleton as well as the biochemical properties of the neuronal INSR is indistinguishable from those found in the periphery. The major difference in these isoforms is the absence (neuron specific type) or presence (peripheral type) of 12 amino acids in the C-terminus of the alpha-subunit due to alternative splicing of exon 11 (Goldstein and Dudley, 1992). INSR without Exon 11 binds insulin with two-fold higher affinity than with Exon 11. The glycosylation pattern as well as the extent of negative cooperativity differs in these two types of insulin receptors (Heidenreich *et al.*, 1983).

INSRs are widely but unevenly distributed throughout the brain as shown in Fig 1.5. The differential density of INSR in different brain region might be one of the reason for the functional diversity of insulin signaling (Hill *et al.*, 1986). Also, regions that contain dendritic fields receiving rich synaptic input have high densities of INSR, suggesting its correlation with neuronal activity (Werther *et al.*, 1987). Neuronal cell INSR concentration is observed to be higher than glial cells with different downstream partners, thus generating different cellular response (Unger *et al.*, 1989). There also exists variation in INSR density between embryonic and mature brain, implying the developmental role of insulin (Kar *et al.*, 1993). The change in expression of brain INSR is also subject to physiological and pathological conditions where there are reports of downregulation of INSR with increase in age, diabetes, Alzheimer's disease, etc (Frolich *et al.*, 1998; Amessou *et al.*, 2010).

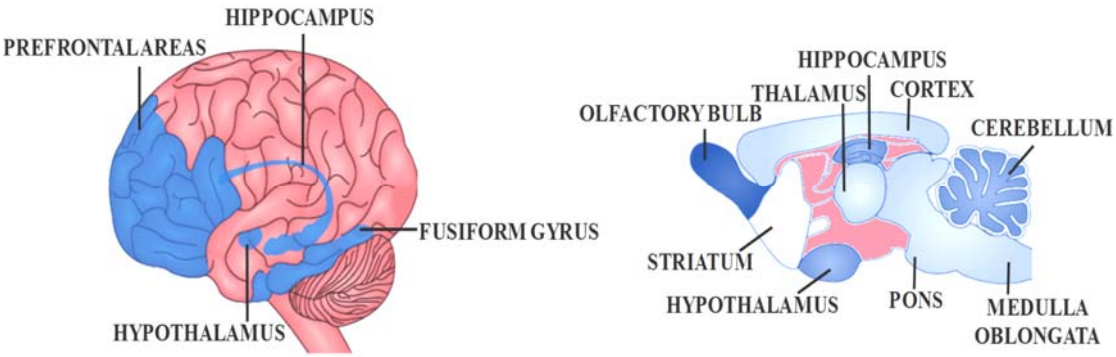

Figure 1. 5: Distribution of insulin receptor (INSR) in brain: Blue shading indicates relative abundance of INSR in different region receptor in human (left) and rat (right) brain Image Modified from:(Fernandez and Torres-Aleman, 2012; Heni *et al.*, 2015).

Thus, the diversity in the structure, distribution as well as function of the INSR defines the specificity and complexity of the role of insulin signaling in brain.

1.7 INSULIN SIGNAL TRANSDUCTION PATHWAY (Blazquez *et al.*, 2014)

The INSR protein is a hetero-tetramer consisting of two extracellular α-subunits and two transmembrane β-subunits held together by disulfide bonds. Binding of insulin to the α-subunit induces a conformational change in the receptor molecule, which brings the two β-subunits into close opposition resulting into subsequent auto-phosphorylation of β-subunit and the activation of INSR kinase activity. The activated INSR tyrosine kinase phosphorylates several intracellular substrates, including the most extensively characterized insulin receptor substrates (IRS-1, -2, -3, and -4), Shc, Gab1, Cbl, associate protein substrate (APS), and the signal regulatory protein family members. Each of these phosphorylated proteins provides specific docking sites for effectors containing Src homology 2 (SH2) domains that specifically recognize different phospho-tyrosine residues, including the regulatory subunit p85 of type 1A phosphoinositol-3-kinase (PI3K); the protein tyrosine phosphatase SHP2; the Src family of non-receptor-type tyrosine kinases, including FYN and CSK; the adaptor proteins GRB2; and the GTPase activating protein of Ras. In this complex cascade of biochemical signals, two major signaling pathways have been recognized, mediating either prevalent metabolic or mitogenic effects and originating by the activation of PI3K or RAS, respectively as shown in Fig 1. 6.

In the PI3K pathway, a regulatory p85 subunit and a catalytic p110 subunit phosphorylate phosphatidylinositol-(4, 5)-bisphosphate (PIP2), thereby generating phosphatidylinositol-(3, 4, 5)-trisphosphate (PIP3). PIP3 recruits, and activates pleckstrin homology (PH) domain containing proteins including enzymes, substrates, adaptors, and cytoskeletal molecules. Among these, PIP3 facilitates AKT activation by mediating its translocation to the membrane via the PH domain. AKT activation regulates metabolic enzymes, such as glycogen synthase kinase 3 (GSK3) and it is involved in glucose metabolism. Activated AKT also phosphorylates the Bcl-2 family member BAD, a proapoptotic protein and a family of forkhead box ‘Other’ (FOXO) protein, a signaling molecules that regulate various cell activities. Another pathway regulated by PI3K-AKT activation is the regulatory-associated protein of mTOR

(raptor) – mammalian target of rapamycin (mTOR) pathway, which regulates cell growth and metabolism and integrates signals coming from insulin, as well as other growth factors and nutrients.

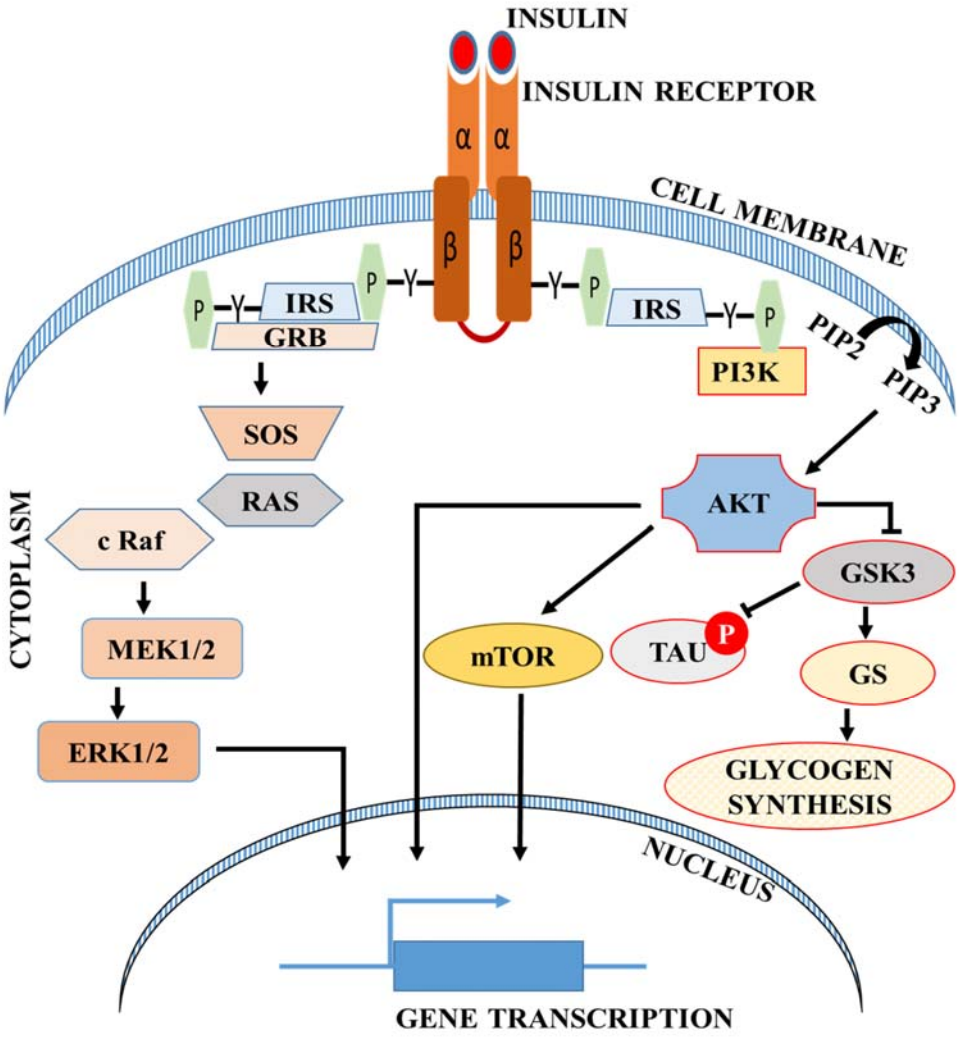

Figure 1. 6: Insulin signaling pathway: Insulin binding to its cognate receptor activates downstream cascade of biochemical signals mediating either metabolic or mitogenic effects originating by the activation of PI3K or Ras pathway.

In Ras pathway, GRB2 is an adaptor, that binds to phosphorylated IRS proteins through its SH2 domain. GRB2 further activates ras guanine nucleotide exchange factor mSOS (Son of Sevenless), which, in turn, activates p21RAS, a GTP-binding protein with GTPase activity toward active (GTP-bound) state. Phosphorylated INSR also activates p21ras through SHC proteins. Active p21RAS recruits and activates the serine/threonine kinase RAF, which, in turn, phosphorylates the dual specificity kinase Mitogen Activated Protein Kinase (MAPK) kinase (MEK1), which then phosphorylates ERK1/2, a kinase of the MAPK family. When phosphorylated, ERK1/2 trans-locates

to the nucleus, where it phosphorylates a number of substrates (SRC-1, PAX6, NF-AT, ELK-1, MEF2, C-FOS, C-MYC, and STAT3) involved in the activation of a complex transcriptional program.

1.8 ROLE OF INSULIN SIGNALING IN BRAIN CELLS

Brain insulin signaling exhibits plethora of functions both within brain as well as whole body. It plays a noteworthy role in the brain cells at various levels, may it be developmental, differentiation, survival or functional. This diversity in the cell types further complicates the insulin signaling at cellular level and its functional outcome. Insulin signaling is known to play various roles in different brain cells as shown in Fig 1.7.

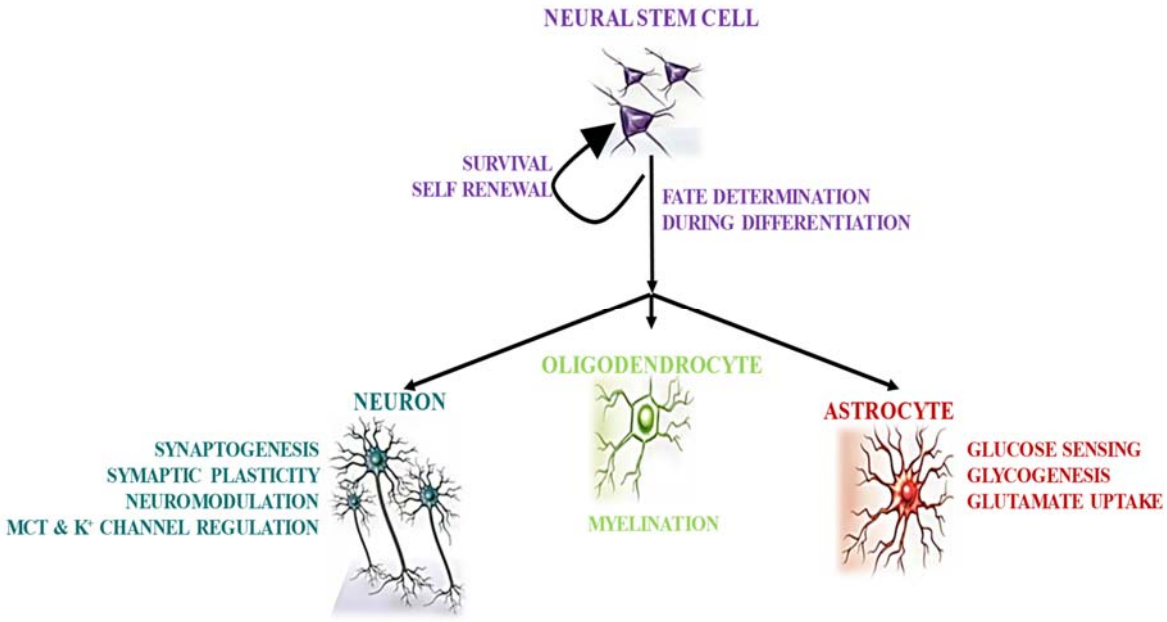

Figure 1. 7: Role of insulin in brain cells: Insulin regulates fate of neural stem cells (as shown by text in purple); synaptic activity and modulation in neurons (as shown by text in blue); myelination in oligodendrocytes (as shown by text in green) and glucose and glutamate metabolism in astrocytes (as shown by text in red).

1.8.1 INSULIN SIGNALING IN NEURAL STEM CELLS

Studies in the mammalian central nervous system supports the essential roles of insulin signaling in NSC self-renewal, survival as well as differentiation. Insulin acts as a crucial mitotic factor in conjunction with EGF receptor stimulation for cell-cycle progression of neural precursor cells, with insulin acting as a progression factor and EGF acting as a competence factor (Alagappan *et al.*, 2014). Also, following insulin

withdrawal, NSCs undergo a caspase-independent, autophagic cell death (Rhee *et al.*, 2013). Rodent studies have proved that altered fetal exposure to insulin level results in alteration in proliferation and differentiation of hypothalamic stem cells (Desai *et al.*, 2011). Thus, insulin in combination with various trophic factors are also reported to be instructive in deciding the fate of NSCs differentiation into neurons, astrocytes and oligodendrocytes (Rafalski and Brunet, 2011; Kuwabara and Asashima, 2012).

Apart from cell fate specification, insulin along with fibroblast growth factor (Fgf8) are decisive for the rostral-caudal specification of early neural development as demonstrated in mouse embryonic stem cells. They observed that low concentration of insulin combined with MAPK/ERK kinase (MEK) inhibitor induced the expression of Pax6 and Otx2, markers for the caudal forebrain, while suppressing the midbrain marker En2 (Shiraishi *et al.*, 2017).

1.8.2 INSULIN SIGNALING IN NEURONS

Several factors are responsible for the maintenance of neuronal survival, plasticity as well as signal transduction. There are reports that establish that insulin signaling via PI3K/ Akt signaling positively modulates the growth of neuronal cells. In cultured fetal neurons, insulin increases neurite outgrowth thus affecting synaptic plasticity (Gu *et al.*, 2014; Niyomchan *et al.*, 2015). Likewise, insulin increases the protein expression of the dendritic scaffolding protein post-synaptic density-95 (PSD-95) through the activation of the PI3K/mTOR pathway or by upregulation/stabilization of Tau protein, providing a molecular mechanism that could explain the effect of insulin on synaptogenesis, the modulation of the synaptic function as well as on the regulation of dendritic spine formation (Lee *et al.*, 2005; Nemoto *et al.*, 2011). Insulin signaling acts as a neuromodulator whereby it regulates the trafficking as well as activity of neurotransmitters (Rhoads *et al.*, 1984; Boyd *et al.*, 1985). Also, the expression of monocarboxylate transporters 2 (MCT2) which are involved in the uptake of lactate by the neurons during excess neuronal firing is increased under the influence of insulin mediated Akt signaling (Chenal *et al.*, 2008). Spanswick et al. demonstrated that insulin action in a few subsets of hypothalamic neurons is involved in regulation of ATP-sensitive K^+ channels (K^+ATP channels) (Spanswick *et al.*, 2000).

1.8.3 INSULIN SIGNALING IN ASTROCYTES

Insulin is reported to modulate the function of astrocytes. García-Cáceres et al. reported that insulin signaling in hypothalamic astrocytes co-controls CNS glucose sensing and systemic glucose metabolism via regulation of glucose uptake across the BBB. They showed a three-fold increase in the glycogen content when treated with insulin (Garcia-Caceres *et al.*, 2016). Likewise, insulin mediated Akt signaling leads to an increase in the expression levels of Glutamate Transporter 1 (GLT1) in astrocytes (Ji *et al.*, 2011).

1.8.4 INSULIN SIGNALING IN OLIGODENDROCYTES

There are *in vitro* reports which suggest that high concentration of insulin induces the differentiation of oligodendrocyte from the precursors. Insulin did not affect the rates of proliferation and/or differentiation of bipotential precursor cells but stimulated the differentiation into oligodendrocytes. Insulin also exerts anabolic effect on oligodendrocytes where it is known to stimulate the synthesis of sulpholipids which is an important component of myelin. The specific activities of the oligodendrocyte-specific enzymes 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) and Glyceraldehyde-3-Phosphate Dehydrogenase (G3PDH) were significantly diminished when the medium was deprived of insulin (van der Pal *et al.*, 1988).

1.8.5 INSULIN SIGNALING IN MICROGLIA

Neuroinflammation is reflected by microglial activation as observed in several neurodegenerative diseases. Insulin treatment lead to reduced virus replication and inflammatory gene expression in HIV infected microglia by restoration of peroxisome proliferator-activated receptor gamma (PPARγ) which is suppressed by HIV infection (Mamik *et al.*, 2016). Also, intranasal administration of insulin reduced the microglial activation in Alzheimer mice model (Chen *et al.*, 2014).

1.8.6 INSULIN SIGNALING IN EPENDYMAL CELLS

Ependymal cells have been reported to express the facilitative glucose carriers GLUT1, GLUT2, and GLUT4, as well as glucokinase (Maekawa *et al.*, 2000). They are therefore speculated to be part of the cerebral glucose sensing system and may also respond to insulin with alterations in their glucose uptake rate. The uptake of glucose by these cells is reported to be increased two-fold in presence of insulin (Verleysdonk *et al.*, 2004). Glucokinase (EC 2.7.1.1), which is responsive to insulin and insulin-like growth factor (IGF-1), is present in ependymal cells (Millan *et al.*, 2010).

1.9 PHYSIOLOGICAL OUTCOME OF BRAIN INSULIN SIGNALING

The brain insulin signaling is known to steer various functions of the peripheral tissues. Thus, any insult to brain insulin signaling can culminate into disturbed homeostasis in the whole body.

1.9.1 METABOLISM

In general, CNS insulin signaling regulates various aspects of metabolism via cross talk with peripheral tissues as evident by neuron-specific knockout of the insulin receptor in mice where whole-body insulin resistance and hypertriglyceridemia is observed (Bruning *et al.*, 2000).

In addition to its anorexigenic effect, insulin is a main player in CNS-dependent regulation of peripheral glucose fluxes. Central insulin infusion results in suppression of hepatic glucose production (HGP) as shown in Fig 1.8 (Obici *et al.*, 2002; Ramnanan *et al.*, 2011). The effect of insulin on hypothalamic glucose-sensitive neurons might be to induce an opening of the ATP-sensitive K+-channels, causing a cell-hyperpolarization that ameliorates the functional capacity to modify the glucose response of these glucose-sensitive cells. The signals generated in this process are transmitted to the vagal nerve that carries this information to the liver, which produces the appropriate response (Girard, 2006).

Hypothalamic insulin signaling regulates lipid metabolism in white adipose tissue (WAT). This has been demonstrated by Herzer *et al.,* where insulin infusion into the mediobasal hypothalamus (MBH) of Sprague Dawley rats lead to an increase in lipogenic protein expression, along with inactivation of hormone sensitive lipase (Hsl), thus suppressing lipolysis (Herzer *et al.*, 2015) as shown in Fig 1.8. Conversely, mice lacking the neuronal INSR exhibited unrestrained lipolysis and decreased *de novo* lipogenesis in WAT (Scherer *et al.*, 2011). Further, central administration of insulin modulated the brown adipose tissue (BAT) thermogenesis in a concentration dependent manner, where high dose of insulin increased while low dose decreased its sympathetic nerve activity (Rahmouni *et al.*, 2004).

In skeletal muscle, insulin signaling promotes glucose uptake and glycogen storage, thus is instrumental in reducing blood glucose levels as shown in Fig 1.8. Also,

hypothalamic insulin signaling inhibits endogenous glucose production by muscle (Coomans *et al.*, 2011).

Intracerebroventricular (i.c.v.) administration of insulin increases pancreatic insulin output, demonstrating that pancreatic β-cells are influenced by insulin-sensitive cells of the brain as shown in Fig 1.8 (Chen *et al.*, 1975). Moreover, insulin injection into the ventro-medial hypothalamus (VMH) inhibits glucagon secretion by pancreatic α-cells, indicating that insulin controls glucagon secretion via brain-mediated mechanisms (Paranjape *et al.*, 2010). Glucose-stimulated insulin secretion (GSIS) from pancreatic β cells is biphasic. Hypothalamic dysregulation of AMP-activated protein kinase (AMPK) during type 2 diabetes results into β cell dysfunction in fasting periods and controls first-phase GSIS via the sympathetic nervous system (SNS) (Koh and Lee, 2016).

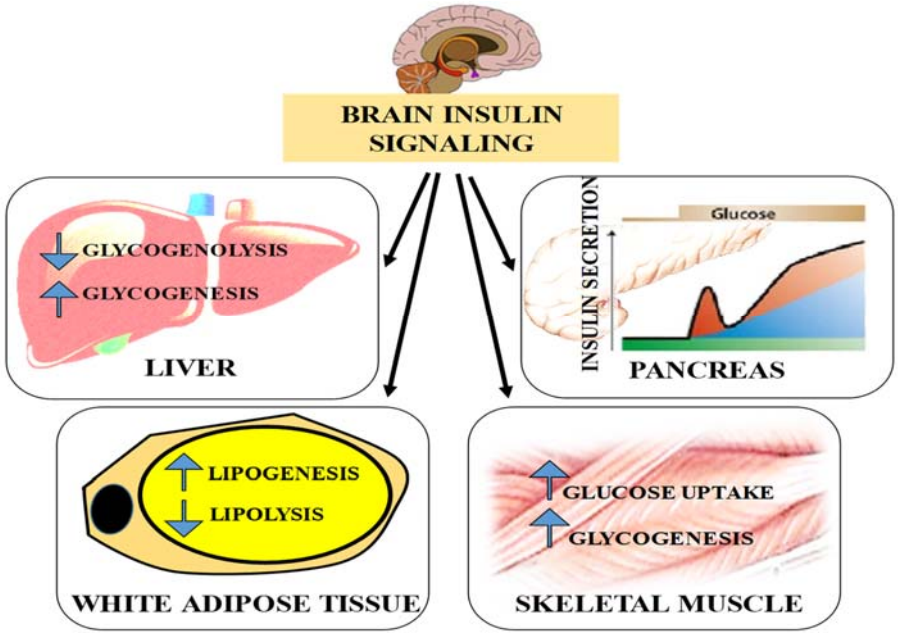

Figure 1. 8: Physiological effects of brain insulin signaling on liver, white adipose tissue, skeletal muscle and pancreas.

1.9.2 AUTONOMIC NERVOUS SYSTEM REGULATION

Epinephrine is released from the adrenal gland under the control of the autonomic nervous system and norepinephrine release reflects neurotransmission in the sympathetic branch of the autonomic nervous system. In humans, intranasal insulin influences concentrations of epinephrine (also known as adrenaline) and norepinephrine (also known as noradrenaline) (Stockhorst *et al.*, 2011; Scarlett and

Schwartz, 2015). Animal experiments have indicated that insulin signaling in brain can induce sympatho-excitation via central nervous autonomous centres. Immediate intranasal insulin administration in human beings is followed by an increase in blood pressure, as likewise seen after infusion of insulin to brain-perfusing vessels in animals (Benedict *et al.*, 2005).

1.9.3 APPETITE REGULATION

Injection of insulin into the cerebral ventricle or directly into the brain parenchyma profoundly inhibited food intake via activation of IRS-1 and -2 mediated PI3K -AKT signaling. Within the arcuate nucleus (ARC) in hypothalamus, insulin signaling act in parallel to that of other peripheral cues such as leptin to inhibit neuropeptide Y (NPY) and agouti-related peptide (AgRP) and to induce proopiomelanocortin (POMC) (precursor of α-melanocyte stimulating hormone; MSH) and cocaine and amphetamine related-transcript (CART) (Yu and Kim, 2012) as shown in Fig 1. 9.

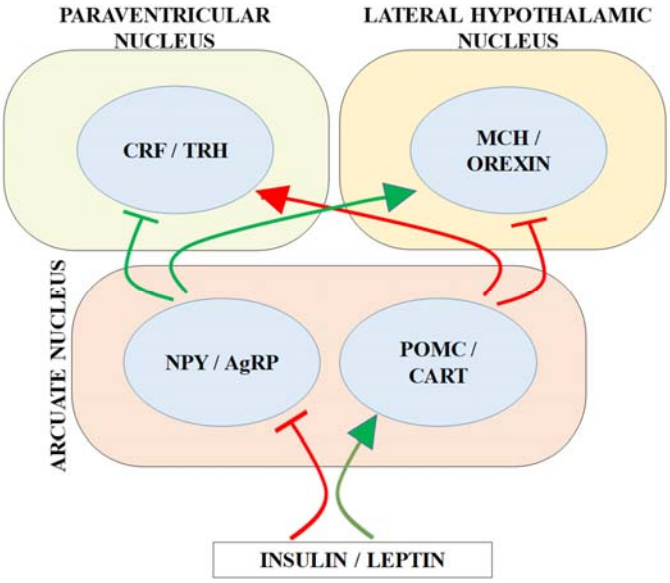

Figure 1. 9: Hypothalamic neural pathway regulated by insulin and leptin for stimulation of appetite (orexigenic pathways, green) or for suppression of appetite (anorectic pathways, red). NPY, neuropeptide Y; AgRP, agouti-related transcript; POMC, proopiomelanocortin; CART, cocaine- and amphetamine-regulated transcript; MCH, melanin-concentrating hormone; CRF, corticotrophin releasing factor.

1.9.4 REPRODUCTION

Insulin is involved in fine tuning reproductive function to that of metabolic activities via acting at hypothalamic, pituitary gland, and gonadal axis. Hypothalamic insulin signaling had a stimulatory effect on Luteinizing Hormone Releasing Hormone (LHRH) secretion with an increase in the luteinizing hormone pulse frequency (Dong

et al., 1991; Arias *et al.*, 1992; Miller *et al.*, 1995). Tanaka *et al.*, proposed that intracerebral insulin is a key regulator of pulsatile Gonadotropin-Releasing Hormone (GnRH) secretion in diabetic sheep (Tanaka *et al.*, 2000). However, there are still debates on whether its only insulin or insulin in conjugation with glucose concentration is mediating these effects (Bucholtz *et al.*, 2000).

1.9.5 BEHAVIOUR

Insulin action in the CNS has important effects on mood. The role of insulin in the modulation of cognition is a newly emerging field that is not yet fully understood. Insulin pathway modifies the topological characteristics of brain networks involved in cognition (Su *et al.*, 2017). Many studies have highlighted the effects of insulin on learning and memory, proposing that insulin regulates cognition by modulating synaptic plasticity, density, and neurotransmission, and also by regulating adult neurogenesis. High fat feeding of rats elevates neuronal corticosterone and impairs neuronal insulin signaling leading to long-term depression in the CA1 (Cornu Amonis 1) region of the hippocampus, probably through the change in the level of tyrosine phosphorylation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors and therefore affecting learning and memory process (Ahmadian *et al.*, 2004). Improper hippocampal insulin signaling reduces the levels of GluN2B subunit and phosphorylated GluA1 in the NMDA (N-Methyl-D-aspartate) receptor, thus hindering the synaptic transmission as shown in Fig 1.10.

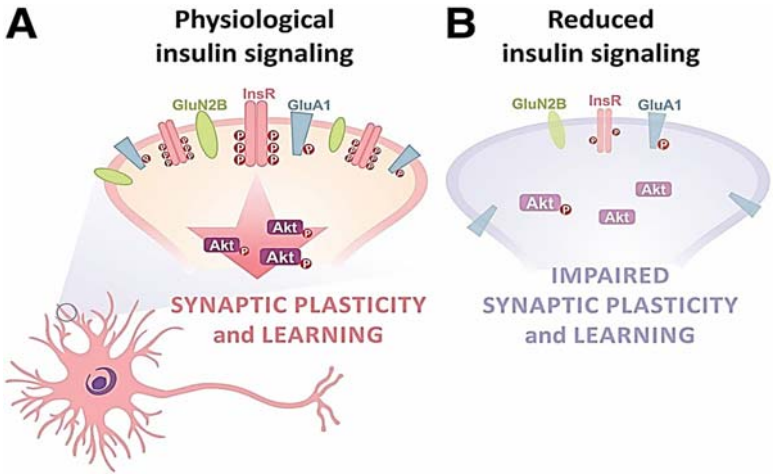

Figure 1. 10: Impact of insulin signaling at neuronal synapses: A: In physiological conditions, phosphorylation of insulin receptors (INSRs) and AKT reflects proper insulin signaling, which is linked to phosphorylation of GLUA1 and presence of GLUN2B at synapses, favouring synapse function learning. B: In pathological conditions, reduced insulin signaling decreases levels of GLUN2B and GLUA1 phosphorylation at synapses, leading to impaired synapse function (De Felice and Benedict, 2015).

The abundance of INSRs in the limbic system suggests that insulin participate in the regulation of synaptic activities deciding the mood. Neuronal insulin receptor knockout (NIRKO) mice exhibited depression like behaviour because of the increased brain monoamine oxidase (MAO) A and B in these brain regions, leading to an increase in dopamine turnover (Kleinridders *et al.*, 2015).

1.10 BRAIN INSULIN RESISTANCE

Brain insulin resistance is defined as a dysfunction of the insulin signaling cascade in the neural cells. It is manifested by reduced levels of insulin / INSR, reduced insulin binding or decreased responsiveness to insulin stimulation in brain /cerebrospinal fluid. The molecular mechanism for brain insulin resistance overlaps with that of diabetes affecting peripheral tissues, and thus to consolidate this concept, it is also referred as, 'Type 3 diabetes'. Since brain is central regulator of overall body function, any state leading to brain insulin resistance or Type 3 diabetes can manifest into mild to severe pathological condition at structural or functional level.

1.10.1 MOLECULAR MECHANISM OF BRAIN INSULIN RESISTANCE

Majority of insights into molecular mechanism of insulin resistance has been established in insulin dependent peripheral tissues. Since the signaling cascade is same as that of peripheral tissue, evidences support that these mechanisms stand true for even brain insulin resistance. At the molecular level, insulin resistance can be acquired through multiple mechanisms. One among these mechanisms being genetic abnormalities of one or more proteins of the action cascade can alter the signaling. For instance, there are reports of single-point mutations in INSR, such as F382V (delayed transport of INSR components to cell surface); R735S (insulin resistance due to the inhibition of precursor processing); L1018A (absence of tyrosine-kinase activity); and Y960F (multiple functional defects) resulting into insulin resistance or decreased insulin sensitivity (Taylor *et al.*, 1992) as shown in Fig 1.11A. There can also be defects at multiple levels of signaling as shown in Fig 1.11B. Aβs generated during Alzheimer's disease can reduce the binding of insulin to its receptor and receptor auto phosphorylation, triggering the dysfunction of brain insulin signal transduction (Ma *et al.*, 2009). Besides, if the regulatory subunits of PI 3-kinase are further increased, they can act as inhibitors of the normal dimeric form of the enzyme (Brachmann *et al.*, 2005).

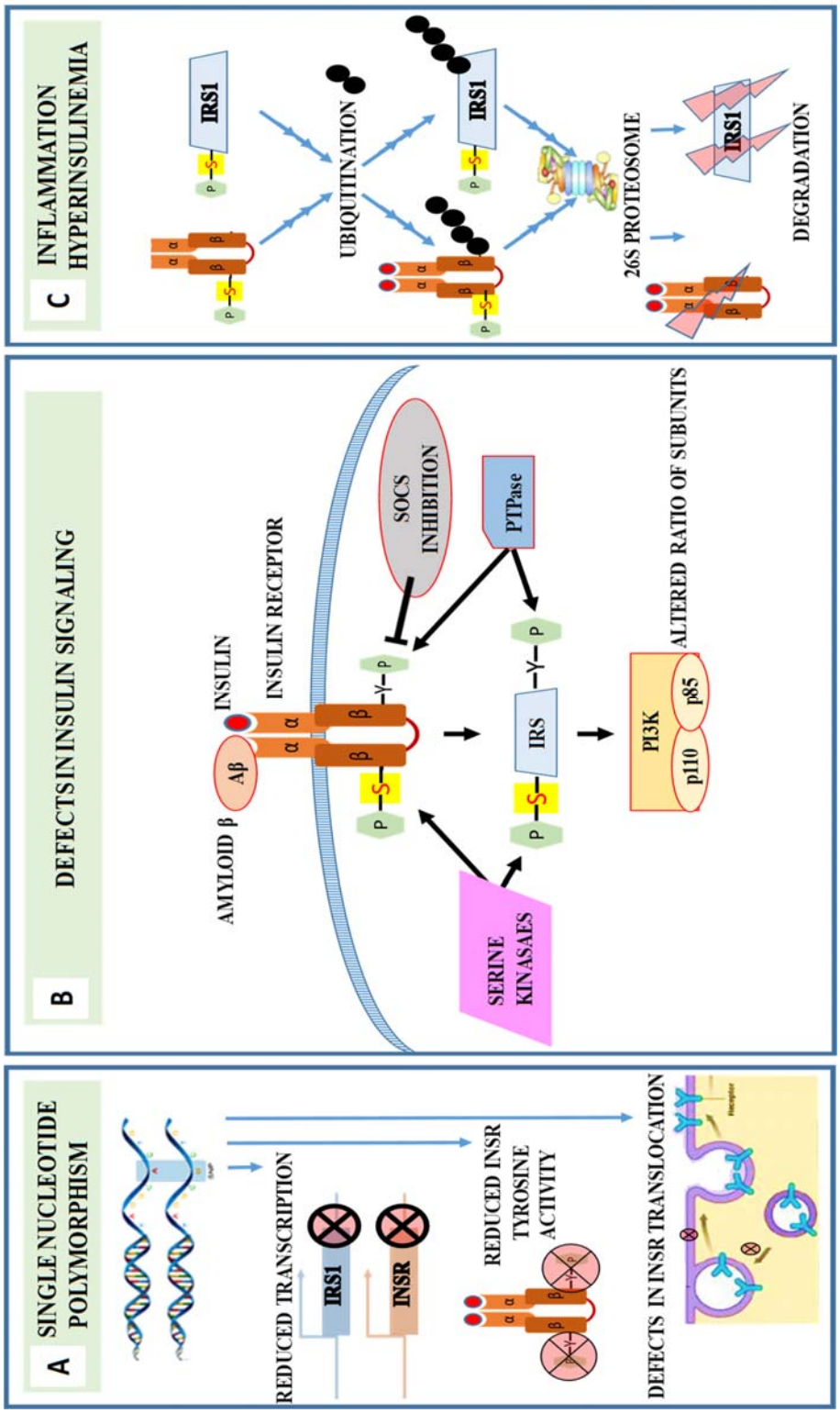

Figure 1. 12: Molecular mechanisms of Brain Insulin Resistance. A: Insulin signaling can be affected by genetic factors such as SNPs leading to decreased synthesis, activity or translocation of INSR or other components. B: Binding of amyloid βon INSR, post translational modifications such as serine phosphorylation of INSR or IRS; inhibition of tyrosine phosphorylation by rapid phosphatase activity or altered ratio of subunits of PI3K can hamper insulin signaling. C: Increased ubiquitination of components of insulin signaling can lead to insulin resistance.

There also exists negative feedback whereby any of the components can undergo posttranslational modifications affecting their activity. For example, the INSR and the IRS proteins can undergo serine phosphorylation by PKC, ERK, and the stress kinases, JNK and IKKβ (Liu *et al.*, 2001; Aguirre *et al.*, 2002). This phosphorylation blunts tyrosine kinase activity of INSR, and thus desensitizes the insulin signaling cascade. Further, serine phosphorylation can result into receptor down regulation by increasing its degradation.

There exist several inhibitors of the insulin signaling cascade that interact with the downstream signaling molecules. For example, the suppressors of cytokine signaling (SOCS) proteins, which are induced by inflammatory cytokines, tags IRS 1/2 for ubiquitin-mediated degradation and thus blocks insulin signaling (Rui *et al.*, 2002). Insulin resistance can also be due to increased activity or amount of the enzymes that normally reverse insulin action, including the phosphotyrosine phosphatases, e.g., PTP1b, and the PIP3 phosphatases, e.g., PTEN and SHIP (King *et al.*, 1991; Goldstein *et al.*, 1998). Conditionally, there can be downregulation of the important components of the insulin signaling cascade such as INSR, IRS-1, etc by either increased degradation (as shown in Fig 1.11C) or by decreased transcription (Kahn, 1980; Rui *et al.*, 2001).

Eventually, the phenotype of insulin resistance will depend on the exact components of cascade affected, the region of tissue, as well as the affected cell type.

1.10.2 CAUSES OF BRAIN INSULIN RESISTANCE

1.10.2A Genetic influences

Common genetic variants have been found to be linked to brain insulin sensitivity in humans. Apart from Single Nucleotide Polymorphisms (SNPs) in components of insulin signaling as described in earlier section of molecular mechanism of insulin resistance, there are several other SNPs influencing brain insulin sensitivity. Polymorphism in FTO (fat mass and obesity associated) locus is associated with brain's insulin responsiveness mediated by reduced insulin effect on beta activity (Tschritter *et al.*, 2007). Another common polymorphism associated with brain insulin action is located in the melanocortin 4 receptor (MC4R) gene which decreased insulin-stimulated cerebro-cortical theta activity (Tschritter *et al.*, 2011). Similar reports have

been obtained in context to SNP in the cannabinoid receptor 2 (CNR2) gene and apolipoprotein E (APOE) gene (Reger *et al.*, 2006; Ketterer *et al.*, 2014).

1.10.2B Intrauterine conditions

Fetuses of mothers with gestational diabetes mellitus are exposed to metabolic disturbances *in utero* which predispose them to metabolic diseases. Animal studies demonstrated an altered sensitivity to insulin in the brains (especially hypothalamus) of offspring of mothers with gestational diabetes is one of the cause for the predisposition to metabolic disturbances in later life (Vogt *et al.*, 2014). Also, diabetes and stress during pregnancy strongly influences the regulation of both IGF-1R and INSR in the developing hippocampus of the offspring (Glombik *et al.*, 2017). In rodents, maternal metabolic derangement influences the development of insulin-sensitive hypothalamic structures in the offspring which causes impaired brain insulin sensitivity (Gupta *et al.*, 2009; Linder *et al.*, 2014). Hence, speculating that brain insulin resistance might be programmed in humans during fetal development as shown in Fig 1.12. However, further research is required to extrapolate this mechanism in humans.

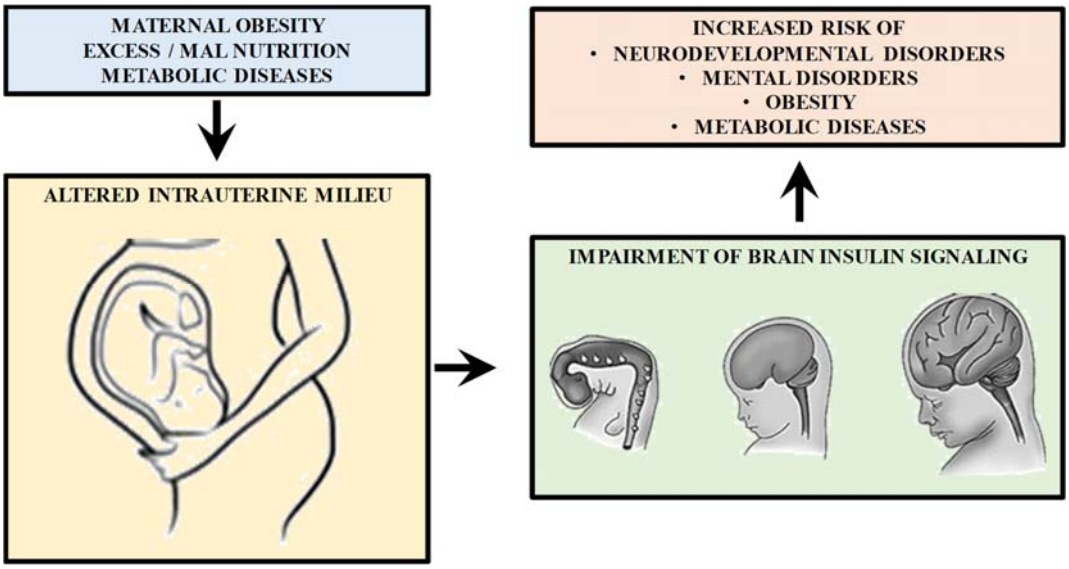

Figure 1. 12: Prenatal disposition to brain insulin resistance: Intrauterine disturbances can alter the insulin signaling in the brain of the offspring along with its predisposition to mental health problems as well as metabolic disorders.

1.10.2C High calorie diet and obesity

Enlarged visceral fat content along with increased levels of saturated free fatty acids during obesity are associated with brain insulin resistance especially affecting cerebral cortex and hypothalamus (Tschritter *et al.*, 2009). Furthermore, diet induced obesity

(DIO) mediated peripheral insulin resistance and steatohepatitis with elevated levels of ceramides are among the mediators for decreasing the brain insulin sensitivity (Lyn-Cook *et al.*, 2009; Tong *et al.*, 2009). Rodent models of high fat feeding exhibited decreased insulin signaling in hypothalamic regions responsible for appetite regulation and thus developing obesity (Melnyk, 1987; Klockener *et al.*, 2011). Decreased insulin sensitivity also contributes to cognitive impairment associated with the high fat-sucrose (HFS) diet in mice (Kothari *et al.*, 2017). Experimental obesity along with T2DM developed in rodents generated mild brain atrophy with brain insulin resistance, neuro-inflammation, oxidative stress, and deficits in cholinergic function (De Souza *et al.*, 2005; Rivera *et al.*, 2005). Even exposure to high calorie diet in early postnatal life can increase the susceptibility to hypothalamic insulin resistance. In this line, a study by Srinivasan M et al. demonstrated a remarkable decrease in the transcript levels of insulin receptor and leptin receptor in hypothalamus of the new-born rat pups artificially raised on high-carbohydrate milk formula along with adulthood obesity (Srinivasan *et al.*, 2008). Thus, suggesting that brain dynamically respond to dietary and hormonal alterations throughout the life.

1.10.2D Aging

The regulation of neuronal glucose metabolism during aging is diminished in the brain, as a result of complex age-associated alterations leading to decreased neuronal insulin signal transduction (Cholerton *et al.*, 2011). There is around 30-45% decrease in insulin / INSR level as well as INSR tyrosine kinase activity with aging in all brain regions in regionally specific manner, particularly in cortex [parietal, frontal and temporal] (Frolich *et al.*, 1998). Insulin signaling pathway in the brain interacts with signaling pathway of other factors, such as IGF-1 and brain-derived neurotrophic factor (BDNF) (Mattson *et al.*, 2004; van Dam and Aleman, 2004). Thus, reduction in BDNF and IGF signaling because of its overlap with the components of insulin intracellular signaling may contribute to insulin resistant brain state in aging. Also, age related diseases such as diabetes and Alzheimer's are underlying factors for the development of brain insulin resistance.

1.10.2E Diabetes

Decreased brain insulin content and decreased brain glucose metabolism accompanied with dysfunction of brain insulin signaling have been demonstrated in patients with Type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM) (Duarte, 2015).

These disturbances in signaling can be caused by inhibition of IRS or PI3K activity mediated by oxidative stress generated by hyperglycemia, advanced glycation end products (AGEs), increase in inflammatory cytokines, change in neurotransmitter levels etc (Lackovic *et al.*, 1990). Peripheral hyperinsulinemia associated with T2DM is known to decrease the transport of insulin as well as insulin signaling in several regions of brain. Thus, recent evidences supported the hypothesis that peripheral diabetes can increase the probability towards the progression of Type 3 diabetes as well as development of Alzheimer's disease as shown in Fig 1.13. Earlier, several independent studies have established that patients with T1DM and T2DM exhibited signs of premature aging, cognitive defects, as well as Alzheimer's disease (Munch *et al.*, 1998; Biessels and Kappelle, 2005; Biessels *et al.*, 2006; Shingo *et al.*, 2012; Ahmad, 2013; Bharadwaj *et al.*, 2017). Recent insights in this field have proposed that brain insulin resistance might be the main missing culprit.

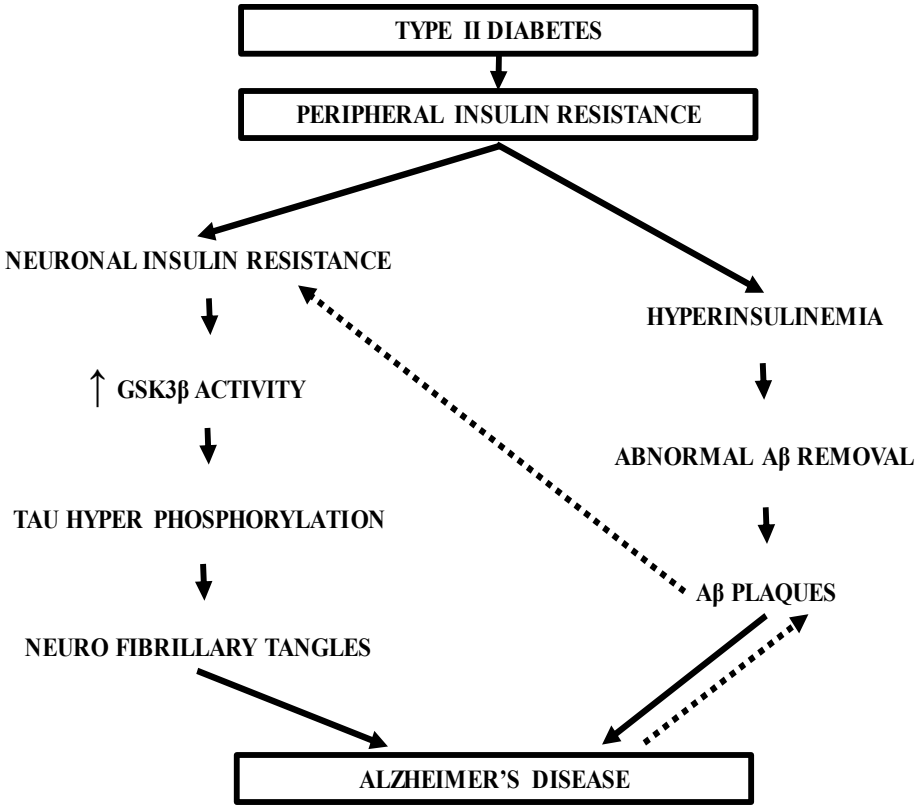

Figure 1. 13: Association between diabetes, hyperinsulinemia and Alzheimer's disease: Association between diabetes, hyperinsulinemia and Alzheimer's disease. Type 2 diabetes can lead to peripheral insulin resistance. This in turn can mediate tau hyper phosphorylation because of neuronal insulin resistance as well as incomplete removal of amyloid β leading to Alzheimer's disease. Otherwise amyloid β generated in Alzheimer's disease can interfere with neuronal insulin signaling and cause insulin resistance.

1.11.2F Alzheimer's disease

Alzheimer's disease is the most common form of dementia among older adults resulting from permanent generation of APP derivative Aβ which aggregates forming amyloid and plaques. Among several neurochemical alterations in the brain, growing evidence has identified a potential association of Alzheimer's disease to that of glucose metabolism and insulin activity (de la Monte and Wands, 2005). The reduced activity of INSR in the brain of people with sporadic Alzheimer's disease indicates a desensitization of insulin signaling along with an increase in cortisol and catecholamine levels. Insulin normally exerts a double-sided effect on Aβs, stimulating their neuronal release (mediated through GSK-3α kinase) and in the same time contributing to extra neuronal accumulation of Aβs by competing for insulin-degrading enzyme that degrades both insulin and Aβs. These Aβs in turn reduce the binding of insulin to its receptor and receptor auto phosphorylation, which in the early-onset type of Alzheimer's disease may be the cause of triggering the dysfunction of brain insulin signal transduction (Ma *et al.*, 2009). In physiological condition, insulin inhibits GSK-3β and consequently reduces tau phosphorylation, promoting binding of tau to microtubules. Following brain insulin dysfunction hyper phosphorylated form of tau protein builds neurofibrillary tangles, the other important pathological feature of the Alzheimer's disease (Blazquez *et al.*, 2014). Thus, Alzheimer's and brain insulin resistance shares a complex cause as well as consequence type of relation as shown in Fig 1.13. This also lead to the usage of the term 'Type-3-Diabetes' for Alzheimer's disease by the researchers because of the shared molecular and cellular features among T1DM, T2DM and insulin resistance associated with memory deficits and cognitive decline in elderly individuals (Kandimalla *et al.*, 2017).

1.10.2G Stress

The response to stress is mediated by the secretion of glucocorticoids from adrenal gland that signals different organs for appropriate outcome. Glucocorticoids have antagonistic effect as that of insulin and thus their elevated levels lead to increase in glucose, hence leading to peripheral insulin resistance and T2DM (Rafacho *et al.*, 2014) that can further precipitate into the development of insulin resistance in brain. The potential effects of glucocorticoids on brain insulin could be a result of both peripheral and central action, where local regulation has been suggested to involve effects via glucose and serotonin. In rodent models, chronic corticosterone or dexamethasone

administration peripherally as well as centrally can lead to region specific down regulation of insulin signaling or insulin transport through BBB (Baura *et al.*, 1996; Buren *et al.*, 2002). Apart from being released during stress, glucocorticoid level also increases in conditions such as obesity, Cushing's syndrome, aging etc., thus again being a causative factor for the development of brain insulin resistance (Landfield *et al.*, 2007).

1.11 THERAPEUTIC IMPLICATIONS OF BRAIN INSULIN SIGNALING

It has been now well established that brain insulin signaling is involved in cognition as well as energy metabolism thus, opening the ways for its therapeutic implications in brain defects. However, the systemic injection of insulin for treatment of brain diseases will have side effects on peripheral tissues as well as will limit the insulin availability and transport to brain. To overcome this, direct brain injections as well as intranasal inulin delivery are the suggested modes of insulin delivery to the brain.

In several rodent models of dementia, Alzheimer's disease as well as Parkinson's disease, ICV injection of insulin has been demonstrated to have a promising ameliorative effect (Bhattacharya and Saraswati, 1991). Also, obesity induced by high fat feeding, diabetes as well as hyperphagia can be reverted by brain insulin treatment (Sipols AJ *et al.*, 1995).

Direct administration in brain stereotaxically being an invasive technique, scientists have now come up with intranasal drug delivery. The nasal mucosa in the upper third of the nasal cavity provides a direct pathway from the external environment to the brain through olfactory nerves and thus can be used to noninvasively deliver therapeutics into the brain as shown in Fig 1.14. This pathway effectively bypasses the BBB and avoids the systemic exposure and side effects associated with insulin entering the bloodstream.

In rodents, intranasal insulin restores insulin signaling, increases synaptic proteins, reduces amyloid beta level, neurodegeneration and microglial activation in Alzheimer's brain (Chen *et al.*, 2014; Rhea *et al.*, 2017). Extrapolating this in human subjects, intranasal insulin improved memory (Benedict *et al.*, 2004; Benedict *et al.*, 2007). Also, a single intranasal insulin administration effectively lowers stress-induced HPA axis responsiveness and, thus offers a therapeutic potential to prevent hyperactivity of the

HPA system (Bohringer *et al.*, 2008). Also, pre-treatment of mice with the insulin sensitizer dicholine succinate prior to social defeat stress diminished anhedonia and behavioural despair (Cline *et al.*, 2015). Intranasal insulin can also be used as a viable therapy for traumatic brain injury as it ameliorated memory, increased cerebral glucose uptake, decreased neuroinflammation and hippocampal lesion volume in murine model of controlled cortical impact injury (Brabazon *et al.*, 2017). In healthy humans, intranasal insulin administration led to decrease in body weight and fat in male but not in female subjects, thus acting as a negative feedback regulation of adiposity in a gender specific manner (Hallschmid *et al.*, 2004).

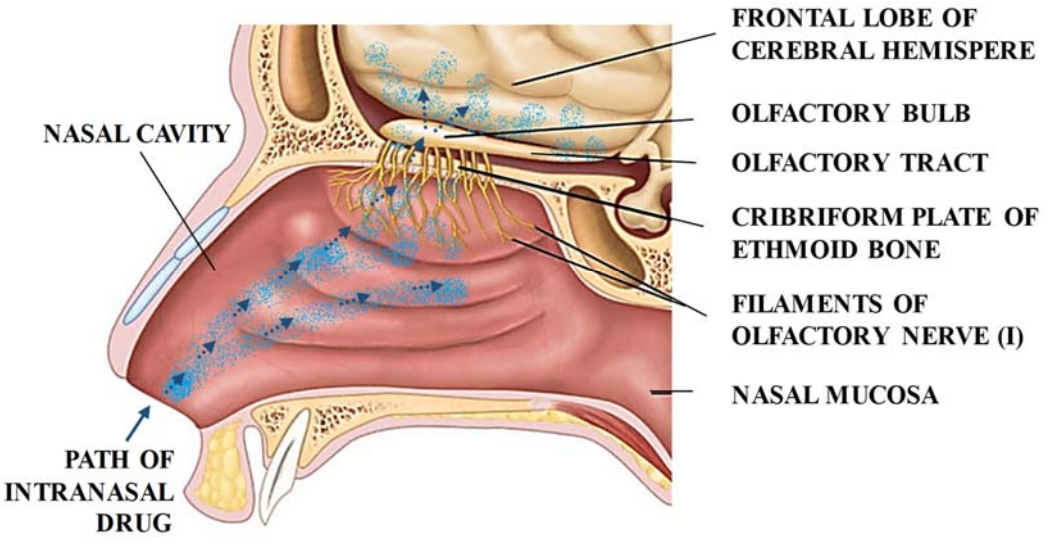

Figure 1. 14: Intranasal delivery routes of drugs: Drugs (as shown by blue dots) can travel through olfactory nerves and can enter brain, thus bypassing blood brain barrier and peripheral blood circulation. Modified from (Tortora and Derrickson, 2009)

Thus, central insulin signaling has a much broader significance in maintenance of whole body homeostasis than predicted when first discovered in brain. Also, modulation of brain insulin signaling holds a promising therapeutic potential in psychotic, metabolic as well as endocrine disorders. However, there are still several mislaid links in the brain insulin signaling at cellular, regional, and functional level which limits its unabridged exploitation for clinical applications.