

Deciphering Brain Insulin Receptor and Insulin-Like Growth Factor 1 Receptor Signalling

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Journal of Neuroendocrinology

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Insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) are highly conserved receptor tyrosine kinases that share signalling proteins and are ubiquitously expressed in the brain. Central application of insulin or IGF1 exerts several similar physiological outcomes, varying in strength, whereas disruption of the corresponding receptors in the brain leads to remarkably different effects on brain size and physiology, thus highlighting the unique effects of the corresponding hormone receptors. Central insulin/IGF1 resistance impacts upon various levels of the IR/IGF1R signalling pathways and is a feature of the metabolic syndrome and neurodegenerative diseases such as Alzheimer's disease. The intricacy of brain insulin and IGF1 signalling represents a challenge for the identification of specific IR and IGF1R signalling differences in pathophysiological conditions. The present perspective sheds light on signalling differences and methodologies for specifically deciphering brain IR and IGF1R signalling.

Key words: insulin and IGF1 receptors, brain, diabetes

doi: 10.1111/jne.12433

In the last 20 years, substantial progress has been made in understanding insulin/insulin-like growth factor (IGF) 1 signalling in the central nervous system (CNS), a former nonclassical insulin responsive tissue, which is now considered as an insulin sensitive organ (1). This perspective approaches the longstanding challenge of identifying differences of insulin and IGF signalling in the brain and deals with phenotypic discrepancies of perturbations of the insulin receptor (IR) and IGF1 receptor (IGF1R) signalling cascade in the brain. Because diabetes is still on the rise worldwide, the precise understanding of these crucial signalling pathways, which are altered in metabolic diseases, is of considerable importance. The presence of brain insulin and IGF resistance in neurodegenerative disease, as exemplified by a reduction in insulin and IGF-1 sensitivity in postmortem brain tissues from Alzheimer's disease (AD) patients (2), highlights the importance of these pathways for brain physiology. Thus, understanding and discriminating these closely related signalling pathways is central to current research and crucial for potential therapeutic interventions for metabolic, neurological and neurodegenerative diseases.

The insulin and IGF signalling pathway

The pancreas-derived hormone insulin and the liver-secreted hormones IGF1 and 2 exert signalling effects by binding and activating their congeneric receptors: IR and IGF1R. Although IGF1R exists as a single isoform, IR is found in two distinct isoforms: IR-A (exclusion of exon 11) and IR-B isoform (inclusion of exon 11). The IR-A isoform exhibits a two-fold increased affinity to insulin and an increased affinity for IGF2 compared to IR-B isoform (3,4). The IR-A isoform appears to exert enhanced mitogenic effects, whereas the IR-B isoform strengthens metabolic effects upon insulin stimulation. Interestingly, IR-A is the major isoform in the brain and in neurones, whereas glia cells exhibit predominantly IR-B isoform expression (5). This expression pattern is conserved throughout different species, indicating that the mitogenic effects of central insulin signalling are important for brain development and function (6). The occurrence of IR and IGF1R homo- and heterodimers further increases the complexity of these hormone receptor interactions. Homo- and heterodimers display altered affinities for insulin and IGF, allowing this system to react accurately to different insulin/

IGF1 concentrations (7). Homodimers of IR and IGF1R exhibit higher ligand affinity towards their endogenous ligands. Heterodimers exhibit almost similar affinity to IGF1 compared to IGF1R homodimers but show substantially decreased insulin binding affinity (7). Although heterodimers consisting of IR-A or IR-B and IGF-1 receptor bind IGF1, IGF2 and insulin with similar affinity, they exhibit higher affinity towards IGF1 compared to insulin (8,9), indicating that the action of IGF1 is crucial for brain physiology. In the rabbit brain, IRs exist predominantly as heterodimers and approximately 50% of all IGF1Rs form heterodimers (10). The abundance of heterodimers in distinct brain regions has not been investigated in detail, although receptor heterodimerisation appears to be dependent on the ratio of expressed receptor levels in a certain region (10). Ligand binding (insulin, IGF1 and 2) to IR and IGF1R causes autophosphorylation and activation of their tyrosine kinase domain with subsequent binding and phosphorylation of insulin receptor substrate (IRS) proteins. IRS proteins, which, in the brain, comprise IRS1, IRS2 and IRS4 (IRS4 is mainly restricted to the hypothalamus), are central to these hormone signalling pathways. They act as a hub and enable two distinct signalling pathways: the phosphoinositide 3-kinase (PI3K)-AKT [also known as protein kinase B (PKB)] pathway and the mitogen-activated protein kinase-extracellular

signal regulated kinase (MAPK-ERK) pathway (11,12). Binding of PI3K to IRS proteins activates the PI3K catalytic subunit, converting phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol (3,4,5)-triphosphate (PIP₃). Subsequently, phosphoinositide-dependent protein kinase 1 binds PIP₃ and activates AKT, enabling downstream signalling pathways such as mammalian target of rapamycin complex 1, glycogen synthase kinase 3 β and forkhead box O (FoxO) signalling, thereby regulating neuronal protein content, autophagy, synaptic function, plasticity and proliferation (13–15) (Fig. 1). Thus, dysregulation in any of the aforementioned insulin/IGF-activated pathways causes deterioration of neuronal function and viability. Insulin/IGF1 stimulation of the second major pathway downstream of IRS proteins (the MAPK-ERK pathway) impacts upon brain cell proliferation and differentiation. Here, the SH2 domain containing adapter molecules growth factor receptor-bound protein 2 (Grb2) and Shc bind to the phosphorylated receptors and IRS proteins. Grb2 binds then to, for example, son-of-sevenless (i.e. SOS), which activates the guanine nucleotide-binding protein Ras by catalysing the release of GDP (inactive Ras) and the binding of GTP (active Ras). Subsequently, active Ras stimulates the downstream kinase cascade consisting of Ser/Thr kinase Raf, which further phosphorylates mitogen-activated protein kinase kinase

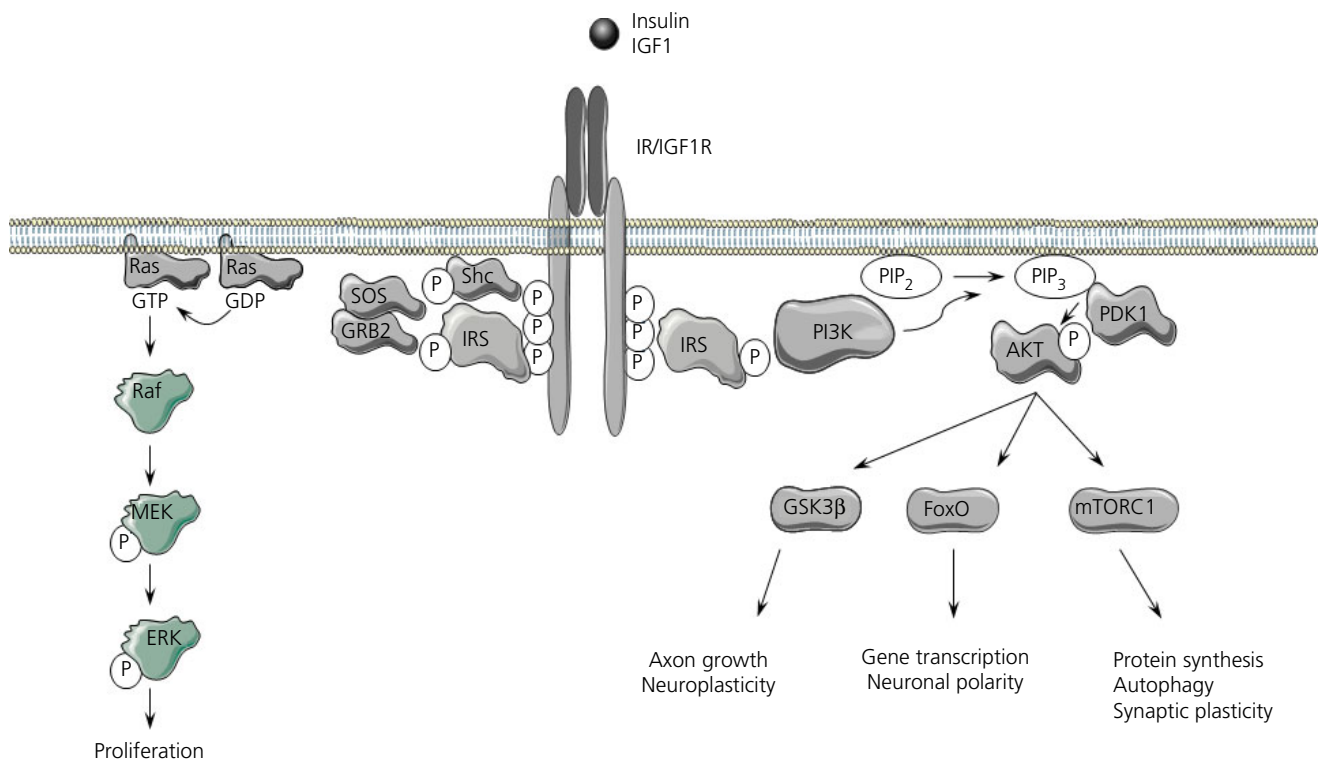


Fig. 1. Insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) signalling pathway. Insulin and IGF1 bind to their receptors, inducing a conformational change and autophosphorylation of the IR and IGF1R beta subunit. Subsequently, insulin receptor substrate (IRS) proteins or Shc are recruited and phosphorylated. Shc activates the mitogen-activated protein kinase-extracellular signal regulated kinase (MAPK-ERK) pathway and IRS proteins predominantly induce activation of the phosphoinositide 3-kinase (PI3K)-AKT pathway. Here, activation of PI3K causes phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol (3,4,5)-triphosphate (PIP₃) conversion and activation and phosphorylation of AKT by phosphoinositide-dependent protein kinase 1. AKT-dependent regulation of forkhead box O (FoxO), mammalian target of rapamycin complex 1 (mTORC1) and glycogen synthase kinase 3 β (GSK3 β) signalling regulates axon growth, gene transcription, protein synthesis and neuronal plasticity. MEK, MAPK/ERK kinase; PDK1, phosphoinositide-dependent protein kinase 1; SOS, son-of-sevenless. [Adapted from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License].

(MEK), followed by phosphorylation of ERK1 and 2, thereby regulating proliferation and cell survival (Fig. 1). Consistently, a deficiency of ERK signalling in the brain affects neuronal survival and causes neuronal death (16,17), demonstrating that this pathway is crucial for proper brain development.

Although all components of the IR and IGF1R signalling pathway are ubiquitously expressed throughout the brain, they exhibit different expression rates in certain brain regions (1,5,18,19), which may partially account for different magnitudes of central insulin and IGF1 signalling. IR gene expression is higher in the arcuate nucleus of the hypothalamus (ARH), piriform cortex and amygdala compared to IGF1R, which are regions important for controlling food intake, energy expenditure, olfaction and anxiety (18). IGF1R exhibits higher expression levels in circumventricular organs, as well as the pituitary and cerebellum, thus indicating a more prominent role for brain development and sensory function (18,20). Expression levels of IR and IGF1R in brain change with age. Central IR expression is higher in younger animals and decreases with age (21). Spatial memory training increases IR expression in hippocampal regions, a region already displaying high IR and IGF1R expression (18), indicating that the age-associated reduction of IR expression may be attenuated in certain brain areas by training (22). In the brain, IGF1R is also highly expressed during development and decreases soon after birth to adult levels (23). Moreover, IGF1R expression in brain negatively correlates with longevity in 16 different rodent species, suggesting that modulation of brain IGF1 signalling may affect lifespan (24,25). On average, *Igf1r* and *Irs2* mRNA expression is approximately two-fold higher than *Ir* and *Irs1* gene expression in the brain of adult mice, suggesting a dominant role of IGF1R signalling for brain development (1). The importance and impact of regional expression differences of insulin/IGF1 signalling proteins, such as AKT and ERK and its influence on exerting insulin/IGF1 action on brain physiology needs further investigation.

The impact of central IR and IGF1R signalling

Although the existence of insulin in the brain was revealed in the late 1960s (19,26,27), the importance of these signalling pathways was only first appreciated 10 years later. Woods *et al.* (28) demonstrated that central application of insulin reduced food intake in baboons. The discovery that the pancreas-derived hormone insulin can act in the brain as an anorectic hormone changed the old view of the brain as a non-insulin responsive organ to an insulin-sensitive organ. The importance of IGF1 signalling on brain physiology was unveiled starting in the 1980s, initially describing IGF1 signalling as a major growth promoting factor for the brain, affecting neurogenesis, neuronal survival and myelination, which was later also confirmed for IGF2 (25,29–32). Consistently, studies using genetically engineered animal models clearly support the growth promoting effect of IGF signalling on brain, whereas insulin signalling has a modulatory role for neuronal proliferation. In cultured rat brain cells, insulin stimulation induces DNA synthesis and deficiency of insulin 1 and 2 causes reduced brain growth (33–36), although the loss of IR in the brain does not affect neuronal survival (37).

IGF1 signalling is complex as a result of the existence of several IGF binding proteins (IGFBP) which serve as carrier proteins for IGF and enhance or attenuate IGF signalling (38). Loss of IGF1, IGF2, IGF1R or overexpression of IGFBP1 and 3 (antagonising IGF1R signalling) reduces brain size (25,29,31,39–44), whereas deficiency of IR does not influence brain development (37,45). Moreover, overexpression of IGF1 increases brain size, whereas mice with elevated IGF2 levels display neonatal overgrowth (32,46) and IGFBP2 overexpression does not affect brain size (47). Although both receptors use IRS1 and 2 as downstream targets to exert their effects, IRS2 may occupy a dominant role for central IGF1R signalling. This hypothesis emerges from the observation that IGF1R and IRS2 deficiency (25,48,49) severely impacts upon brain development with decreased brain size, whereas mice with a neuronal knockout of insulin receptor or mice deficient for IRS4 do not exhibit any signs of altered brain size (37,50). IRS1 knockout animals not only exhibit a strong reduction in body size, but also display an increased brain/body weight ratio (48), and IRS1 deficiency does not prevent IGF-I stimulated brain growth (48,51). Although IRS2 deficient animals possess smaller brains, neuronal IRS2 overexpression does not impact brain growth (52), highlighting a modulatory role of IRS2 on IGF1R-dependent brain growth (53), as well as compensatory regulation by other IRS proteins or a yet unidentified role of IRS2 for brain growth through non-neuronal cells (Table 1). In addition, in postmortem brain tissue from humans, 1 nM insulin activates IR binding to IRS1 without activating IR–IRS2 interaction in the hippocampus and cerebellar cortex. Conversely, 1 nM IGF1 induces IGF1R binding to IRS2 without affecting IRS1 binding (2). Interestingly the IGF1R–IRS2 axis occupies a crucial role for neuronal proliferation and brain development. It is currently unknown whether IGF1 signalling through IRS2 observed in the hippocampus and cortex has relevance for IR/IGF1R signalling to the brain more widely. Exclusive interaction partners for IRS1 and IRS2 such as Csk (for IRS1) or DOCK-6 (for IRS2) have been identified in muscle cells and Csk interacts specifically with the IR (54). Whether these specific interactions are also present in brain remains to be investigated.

Over recent decades, immense progress has been made in unravelling additional effects of insulin and IGF signalling on brain function and metabolism.

Insulin can act as an anorexigenic hormone in the arcuate nucleus by modulating pro-opiomelanocortin (POMC; anorexigenic), agouti-related protein (AgRP; orexigenic) and neuropeptide Y (NPY; orexigenic) neurones to promote satiety (28,55–57), whereas IGF2, but not IGF1, reduces NPY release to a similar extent as insulin (58). In addition, the injection of an enriched preparation of IGFs in the lateral ventricle decreases food intake in rats compared to saline injection (59). Yet, central inhibition of IGF1R signalling reduces short-term food intake (60), although adult brain-specific IGF1R heterozygous mice exhibit slightly greater body weight (25). Thus, the precise effect of central IGF1 and 2 signalling on food intake remains unclear. Insulin and IGF1 act in the brain and promote fertility by regulating luteinising hormone, spermatogenesis and ovarian follicle maturation (55,61) and increase thermogenesis via brown adipose tissue activity (62). IR signalling suppresses hepatic

Table 1. Effect of Genetic Deletion of Proteins of the Insulin Receptor (IR) and Insulin-Like Growth Factor 1 Receptor (IGF1R) Signalling Cascade on Brain Size and Metabolism.

Mouse model	Brain size	Metabolic phenotype	References
IR Nes-Cre	Normal	Brain KO: obesity, increased food intake (♀), mild insulin resistance	(37,55)
IGF1-R Nes-Cre	Decreased	Heterozygous brain KO: reduced body weight, increased adiposity, hyperleptinemia, glucose intolerant	(25)
IRS-1 KO	Slightly decreased, but increased brain/body weight ratio	Whole body KO: decreased body size and weight, glucose intolerant, insulin resistant	(33,36,48)
IRS-2 KO	Decreased	Whole body KO: decreased body weight, glucose intolerant, insulin resistant, diabetic	(48,49)
IRS-4 KO	Normal	Whole body KO: slightly decreased growth in males, glucose intolerant	(50)
Ins 1/2 KO	Decreased	Whole body KO: growth retardation, diabetes and ketoacidosis, glucosuria, liver steatosis	(34)
IGF-1 KO	Decreased	Whole body KO: severe reduction in growth	(29,31,39)
IGF-2 KO	Decreased	Whole body KO: severe reduction in growth	(39,40)
IGFBP-1 OE	Decreased	Whole body OE: decreased body weight, hyperglycaemia	(42–44)
IGFBP-2 OE	Normal	Whole body OE: decreased body weight, reduction in fasted serum glucose levels	(47)
IGFBP-3 OE	Decreased	Whole body OE: Increased IGF-1 levels; reduced birth size, increased adiposity when overexpressed under the control of a CMV promoter	(41)
IGF-1 OE	Increased	Brain OE: normal body weight,	(46)
IGF-2 OE	Normal, but decreased brain/body weight ratio	Whole body OE: overgrowth, increased body weight	(32)
IRS-2 OE	Normal	Neuronal OE: decreased activity and energy expenditure, increased fat mass, age-dependent glucose intolerance and insulin resistance	(52)

OE, overexpression; KO, knockout.

gluconeogenesis and recent data indicate that this might be also true for IGF signalling (63,64). In addition, the central action of IR signalling also regulates the counter regulatory response to hypoglycaemia via epinephrine regulation (65) and insulin/IGF1 reduces mean arterial blood pressure in part by activating endothelial nitric oxide synthase, which increases levels of the vasodilator nitric oxide (66–68). Strikingly, both hormones exhibit pro-survival effects on neurones and increase mood and cognitive function, showing that central effects of these hormones impact whole body physiology, even beyond metabolism (1,45) (Fig. 2). Because intranasal insulin application has been shown to attenuate the cognitive decline in a small patient cohort suffering from AD (69) and IGF1 application reduces neuronal injury and improves neurologic function in rodent stroke models (70,71), the central regulation of these receptor signalling pathways represents a therapeutic target for the treatment of metabolic and neurodegenerative diseases. However, it has been suggested that neuronal IGF resistance may represent an endogenous, protective mechanism for the prevention of A β accumulation because neuronal IGF1R deficiency in a mouse model of AD resulted in decreased A β accumulation and amyloid plaques (72). Thus, further research is needed to clarify whether enhancing IGF signalling affects AD progression. In addition, the use of insulin sensitiser glucagon-like peptide 1 agonist exenatide is currently being studied in a clinical trial for the treatment of Parkinson's disease (PD), another neurodegenerative disease associated with diabetes mellitus (73,74). Whether the use of insulin sensitisers in PD patients affect brain insulin signalling remains unknown.

Brain insulin and IGF concentration

A central feature of the metabolic syndrome is peripheral and central insulin/IGF resistance, which is also present in brains of AD patients, indicating a link between altered metabolism and neurodegenerative diseases. The term 'insulin/IGF resistance' describes a phenomenon where the body exhibits a blunted activation of the IR and IGF1R signalling cascades. To counteract this resistance, beta cells increase the production and secretion of insulin to propagate sufficient insulin signalling, which can lead to hyperinsulinaemia. One difference between central and peripheral insulin resistance is the occurrence of high insulin concentrations only in the periphery. Although obese, insulin-resistant patients display elevated blood insulin levels, to counteract reduced insulin sensitivity, the cerebrospinal fluid (CSF), which is produced from arterial blood by the choroid plexuses of the lateral and fourth ventricles, exhibits decreased, rather than increased, insulin levels (75–77). In addition, there is no difference in brain insulin content between nondiabetic and diabetic animals (76). This observation is of particular interest because: (i) peripheral insulin is able to enter the CSF (78); (ii) basal plasma insulin levels correlate with insulin levels in the CSF in large animals (79); and (iii) type 1 diabetic rodent models exhibit increased insulin uptake (80). Yet, impaired insulin transport across the blood–brain barrier has been demonstrated in obese and insulin-resistant humans (77,81) and may exclude brain hyperinsulinaemia as a phenomenon of central insulin resistance. IGF1 and IGF2 levels are also reduced in brain samples of diabetic rodents (82). Because insulin and IGF1 up-regulate IGF2 levels, the decrease

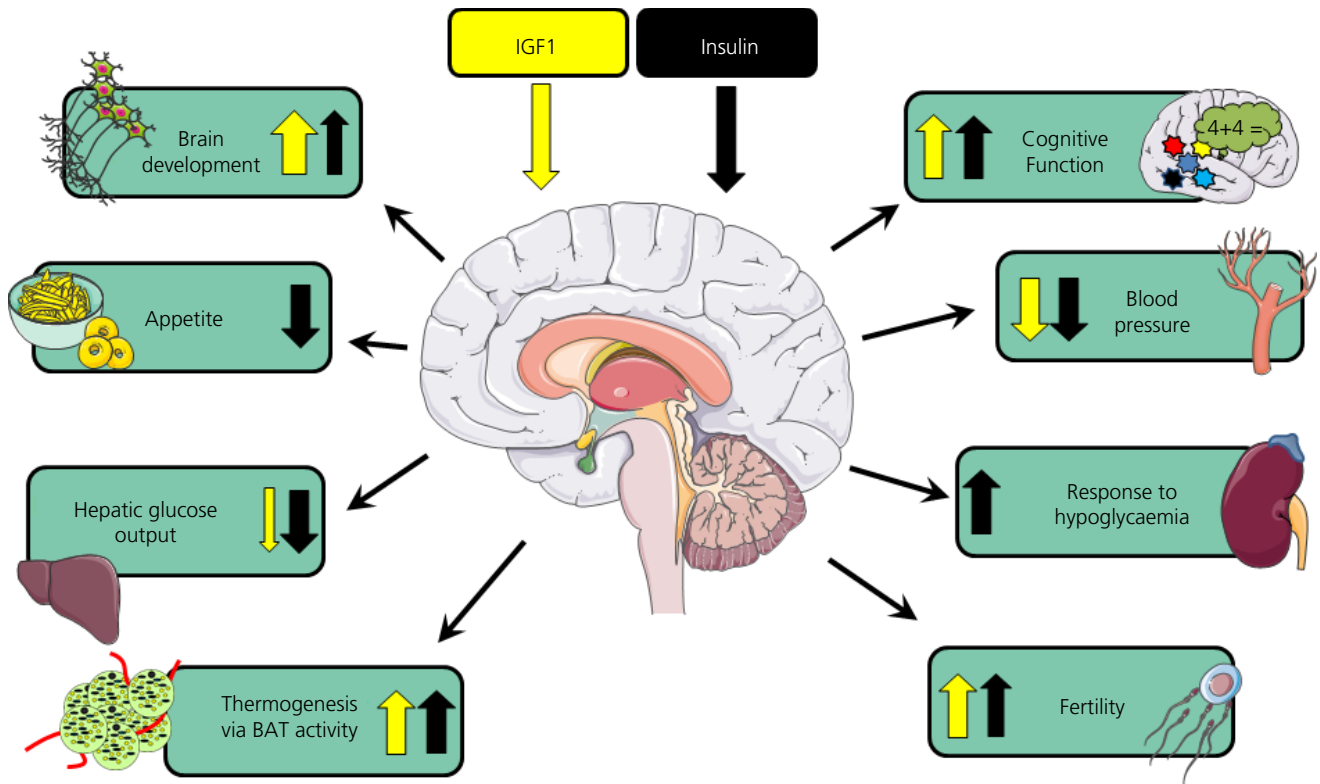


Fig. 2. Central insulin and insulin-like growth factor 1 (IGF1) signalling impacts on metabolism and cognition. Brain insulin receptor (IR) and IGF1R signalling increase neuronal proliferation and synaptic plasticity and affect brain development and cognitive function. In addition, central insulin signalling reduces appetite via regulation of hypothalamic neurones such as pro-opiomelanocortin (POMC) and agouti-related protein (AgRP) neurones, reduces hepatic glucose output and increases thermogenesis via brown adipose tissue (BAT) activity. Both hormones increase fertility by regulating luteinising hormone and thus spermatogenesis and ovarian follicle maturation and reduce mean blood pressure by neural endothelial nitric oxide synthase activation and increased blood flow to skeletal muscle. Furthermore, central insulin signalling regulates the counter-regulatory response to hypoglycaemia (the arrow size resembles the strength of impact). [Adapted from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License].

in IGF2 levels in diabetic brains may be part of decreased central insulin and IGF1 function in diabetic conditions (83,84), thus highlighting the complex interplay of these related hormone cascades. In summary, insulin and IGF1 signalling is reduced in brains of patients suffering from the metabolic syndrome and AD, which does not result in apparent hyperinsulinaemia in the CSF. Why this should be different between the brain and periphery remains unknown.

In addition to controversies about the relative insulin concentrations measured in the brain compared to serum (76,85), insulin mRNA has been detected locally in the brain of rodents and rabbits (86–88). Multiple mouse lines expressing Cre recombinase under the control of the *Ins2* promoter (RIP Cre) display recombination events in various regions of the brain (89,90), indicating that insulin mRNA may be expressed in neurones. These particular neurones have been shown to regulate energy expenditure and adiposity, highlighting the importance of *ins2* positive neurones for metabolism (91). Nevertheless, the majority of brain insulin will very likely originate from the periphery and the possible effects of locally produced insulin mRNA in the brain on neuronal functions are unknown.

The median eminence is a circumventricular organ, close to the ARH, and is characterised by a fenestrated blood–brain barrier and

hence has almost direct contact with blood metabolite and hormone concentrations. Thus, insulin concentrations in specific brain regions are different, with the highest levels in close proximity to a fenestrated blood–brain barrier (92). Circumventricular organs are characterised by a high density of IGF1R, indicative of increased IGF1R action, and this might have a modulatory role for hypothalamic insulin action. An unanswered question is whether different hormone threshold levels are required to fully activate IR and IGF1R signalling in certain brain regions. The ARH exhibits high insulin sensitivity as a result of locally elevated insulin concentrations, as well as IR protein levels, and hypothalamic insulin signalling is important for regulating food intake and hepatic gluconeogenesis, as well as modulating energy expenditure (93). Consequently, diabetic animals exhibit hypothalamic insulin resistance (94). Yet, although AgRP neurones in the ARH become insulin-resistant in diet-induced obesity, SF1 neurones, which reside in the ventromedial hypothalamus (VMH) and in animals fed a high-fat diet, do not (64,95,96). This difference in insulin sensitivity may reflect different insulin concentrations in sub-regions of the hypothalamus or indicate that certain neuronal populations possess different insulin sensitivities to enable proper insulin signalling. Whether insulin concentrations differ between brain regions in diabetic patients is

unknown, although selective insulin resistance in distinct brain compartments has been observed in humans (97), indicating that brain insulin responsiveness also varies in human metabolic disorders.

Causes of central insulin/IGF1 resistance

Despite the aforementioned differences and difficulties in analysing the insulin concentration and sensitivity in the brain, the peripheral tissues and the brain both exhibit clear signs of insulin resistance in metabolic disorders (98,99). Causes of central insulin/IGF resistance are multifactorial and can impact on multiple levels of their signalling cascade (11). Pathophysiological concentrations of nutrients in blood or tissues, hyperglycaemia (high blood sugar), lipotoxicity (a syndrome of excessive accumulation of lipid intermediates in non-adipose tissue, which can cause cellular dysfunction and cell death) or cellular perturbations of organelles, such as endoplasmic reticulum (ER) and mitochondria, can alter insulin/IGF signalling. Common to these causes is the downstream activation of serine/threonine kinases (e.g. c-Jun kinase and I κ B kinase), which induce serine and threonine phosphorylation of IR, IGF1R, IRS proteins or AKT. These phosphorylation events have been shown to be either associated with or of being a causal factor of insulin/IGF resistance (100).

Substantial progress has been made in understanding how inflammatory pathways can modulate insulin/IGF signalling (101) and it emerges that perturbations in cellular organelles are important triggers in this process. A well described perturbation is the unfolded protein response of the endoplasmic reticulum (UPRer). Proper UPRer restores cellular homeostasis, induces a stop of protein translation, and up-regulates chaperones and proteases to cope with the burden of misfolded proteins by refolding and/or degradation of misfolded proteins. A reduction of obesity-induced ER chaperone activity improves insulin signalling and excessive activation of UPRer induces central insulin resistance (102,103). Consistently, expression of ER chaperones is increased in obese and diabetic conditions, as well as in neurodegenerative diseases (102,104). Mitochondrial dysfunction has also been shown to cause central insulin/IGF1 resistance. The unspecific term 'mitochondrial dysfunction' describes various forms of altered mitochondrial function (e.g. dysregulated mitochondrial dynamics, alterations in the membrane structure, dysregulated calcium homeostasis or perturbations of mitochondrial proteins) (105–109). Common to these alterations is often an increased production of reactive oxygen species, activation of serine/threonine kinases, which induce inhibitory phosphorylation of insulin/IGF signalling proteins. These events are also associated with type 2 diabetes, ageing and neurodegenerative diseases, highlighting the importance of proper mitochondrial function for whole body physiology (110,111). Dysregulation of mitochondrial dynamics in the hypothalamus results further in a disrupted mitochondrial-ER cross-talk in POMC neurones, underlining close interaction of these organelles in the hypothalamus and the complex modulation of insulin/IGF signalling by these organelles (105,107). Interestingly, treating mice with the insulin sensitiser rosiglitazone can reverse insulin resistance and ameliorate mitochondrial dysfunction in the

CNS (112), underlining the interplay of insulin signalling and mitochondrial function in the brain.

Discrimination between central IR and IGF1R signalling

Although insulin and IGF1 signalling have been extensively studied over recent decades, the molecular discrimination between IR and IGF1R signalling events is far from trivial (113). Because both receptors use the same intracellular downstream signalling cascade (114), unique activation markers do not exist. The classical approach for investigating and discriminating central insulin and IGF1 signalling comprises *in vitro* stimulation using low concentrations of both hormones (1–10 nM), followed by immunoblotting for phosphorylated receptors, IRS proteins, AKT and ERK (2,115). For *in vivo* approaches, insulin/IGF can be applied intranasally in the form of a nasal spray to bypass the blood–brain barrier, which allows the delivery of insulin/IGF into the brain with no or only marginal effects in peripheral tissues (116–118). This is followed by magnetic resonance imaging to measure cerebral blood flow as an indicator of increased brain activity. In addition, insulin/IGF can be injected (i) into the inferior vena cava, thereby reaching the brain via the circulation (37,94,119) or (ii) directly into the brain using stereotaxic injection in fasted animals (37,120,121), followed by brain dissection and immunoblotting of phosphorylated signalling pathway molecules. Although several phospho-tyrosine and serine IRS1 antibodies are available to decipher altered insulin/IGF action, the detection of specific IRS2 phosphorylation is more difficult because there is a lack of site-specific phospho antibodies. Because novel IRS2 serine and tyrosine phosphorylation sites have been detected in normal and resistant states (12,122), there is a need for new, specific phospho-IRS2 antibodies to improve the understanding and analysis of selective insulin signalling. In the brain, insulin/IGF signalling can also be visualised using immunohistochemical detection of, for example, PIP₃ (123), or the general marker for neuronal activation c-fos (124). However, these markers are not specific for IR or IGF1R activation.

Another possibility for differentiating between IR and IGF1R signalling is the use of specific inhibitors and agonists or the genetic modulation of proteins in these signalling pathways (Table 2). The use of specific agonists or antagonists, which modulate receptor activation on different levels, facilitates the discrimination of specific signalling. One specific IR antagonist comprises the covalently dimerised insulin derivative B29-B29', which inhibits insulin binding and downstream signalling (125). The monoclonal antibody XMetA is a partial agonist, acting in an allosteric manner by enhancing insulin binding to its receptor but selectively activating the PI3K-AKT node, whereas the ERK pathway does not appear to be affected (126). Because selective insulin resistance exists in diabetic conditions by exclusive alteration either of the AKT pathway or the ERK pathway (127), the use of XMetA is of particular interest for investigating selective brain insulin signalling. The peptide S961 possesses agonistic and antagonistic properties on IR signalling. When used at a high concentration, S961 exhibits antagonistic characteristics (128) and induces systemic insulin resistance. However, when used at a concentration in the range 1–10 nM, S961

Table 2. Different Methodologies to Detect and Investigate Altered Insulin and Insulin-Like Growth Factor 1 (IGF1) Signalling in the Brain.

Methods	Insulin signalling analysis	IGF-1 signalling analysis	References
Analysis of inhibitory phosphorylation Intranasal application	IRS-1, IRS-2 and IR phosphorylation Insulin	IRS1, IRS-2 and IGF-1R phosphorylation IGF-1	(2,115) (116–118)
Analysis of hormone stimulation via vena cava or stereotaxic injection	pAKT, pERK, PIP ₃ staining, c-fos	pAKT, pERK, PIP ₃ staining, c-fos	(37,120,121,123,124)
Use of agonist/antagonist	B29-B'29, S961 peptide, XMetA	Jb1, picropodophyllin, AG1024, α -IR3	(125,126,128–130,132–135)
Genetic modification	IR KO, IR-AS, AAV, CRISPR/Cas9	IGF-1 R KO, siRNA, AAV, CRISPR/Cas9	(138,141–143) (see also references in Table 1)

AAV, adenoviral-associated virus; IR, insulin receptor; IRS, insulin receptor substrate; KO, knockout; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol (3,4,5)-triphosphate.

acts as a partial agonist *in vitro*, increases IR and AKT phosphorylation and promotes mitogenic effects without affecting metabolic endpoints, inducing selective insulin signalling (129).

For IGF1R signalling, the peptide analogue JB1 specifically binds to the N-terminus of IGF1R, prevents IGF1 binding and blocks IGF1R signalling via both the downstream AKT and ERK pathways (130). The cyclolignan picropodophyllin (ppp) not only inhibits IGF1R activity by inhibiting its tyrosine phosphorylation, but also causes a down-regulation of IGF1R protein levels, mimicking closely changes to central IGF1R signalling in diabetic conditions (131–133). Importantly ppp also induces apoptosis in IGF1R deficient cells, indicating the nonspecific IGF1R effects of this inhibitor. Thus, good controls are needed when using this antagonist to specifically address IGF1R signalling. When used at a low concentration, the synthetic protein tyrosine kinase inhibitor tyrphostin AG1024 exhibits a preference for inhibiting IGF1R, as indicated by almost eight-fold lower IC₅₀ values for tyrosine kinase activity and four-fold lower IC₅₀ values towards exogenous substrates compared to IR inhibition (134). The use of monoclonal antibody alpha IR-3 specifically inhibits IGF1 binding to its receptor and suppresses IGF1R-mediated ERK and AKT activation, and can also be used to address IGF1R signalling *in vitro* (135). Moreover, ligand specific markers have been described for IR and IGF1R signalling, such as 14-3-3 proteins and GIPC1, which have been shown to solely interact with IGF1R in yeast systems (136). In addition, glypican 4 has been shown to interact with unoccupied IR but not with IGF1R, whereas binding of the ligand causes dissociation of glypican 4 from IR but binding to IGF1R (137). This is of specific interest because the interaction might be a marker for non-activated and activated IR compared to IGF1R signalling. However, whether all these interactions hold true in the brain is currently unknown. Finally, stimulation of insulin or IGF1 in the context of a knockout of the IR or IGF1R, or the use of antisense oligodeoxynucleotides directed against the receptors (138), results unequivocally in specific outcomes for their signalling pathway, whereas the use of genetic ablation of downstream signalling proteins, such as IRS proteins in the brain, will influence both pathways. The use of specific Cre-lines for a variety of brain cell populations helps to delineate the effect of insulin/IGF signalling in distinct brain subpopulations (139). Mice with a knockout of IR in POMC neurones exhibit unaltered energy homeostasis, whereas

mice deficient for IR on AgRO neurones display increased hepatic glucose output, demonstrating that insulin receptor signalling in distinct neurones differentially influence metabolism (64). The novel site-specific recombinase (SSR) Dre, which recognises palindromic sequences termed 'rox' sites, extends the portfolio of available SSRs and may be used to investigate multiple gene splice variants within one mouse model using Cre and Dre technology (140). The use of adenoviral-associated virus (AAV) expressing Cre recombinase will further accelerate the understanding of these crucial pathways allowing for fast and precise genetic modulation/ablation of insulin/IGF signalling molecules in discrete brain subpopulations or regions (141,142). The CRISPR/Cas9 technique can be used both to generate fast genetic knockout models and to introduce disease-relevant point mutations in, for example, the IR or IGF1R, allowing the rapid analysis of their effects (143,144). These results and opportunities highlight the power of genetic modifying techniques for revealing novel insights into the complexity of brain insulin/IGF signalling.

Although the use of genetic knockout models of brain IR and IGF1R reveals distinct phenotypes, indicating different signalling events, it often does not accurately reflect observed signalling alterations in metabolic disease models, which can occur later in life. Examples include a reduction of brain IR expression during ageing or the presence of neuroinflammation-associated brain insulin resistance, which is absent in brain insulin receptor knockout animals (22,37,45,145). Conventional knockout strategies for IR and IGF1R signalling molecules can cause developmental dysregulations, as observed for neuronal IGF1R deficiency. Thus, use of inducible Cre lines or AAV injection in older animals can help to improve our understanding of the effects of brain insulin/IGF resistance on physiology during ageing.

In summary, the toolbox of different agonists, antagonists and genetic modifications of the IR and IGF1R signalling cascade will help to decipher selective insulin/IGF1 signalling and resistance in the brain, as well as its effect on metabolism, as reflected in human pathophysiological conditions.

Human brain insulin signalling: where are we?

The human brain is an insulin-sensitive organ. As in rodents, human brain insulin signalling modulates food intake and body

weight and impacts upon cognitive function. Recent data even indicate a role for human brain insulin signalling in regulating peripheral glucose and fatty acid metabolism, highlighting the importance of central insulin signalling for human physiology (98). Importantly, brain insulin resistance has also been demonstrated in humans. Obesity, ageing, and increased levels of saturated fatty acids are linked to brain insulin resistance. Causes for human brain insulin resistance are not well understood and are the subject of current research worldwide. Recently, it has been shown that gestational diabetes impairs foetal postprandial brain activity (146), which may be a result of brain insulin resistance, as demonstrated in rodents (147). Intranasal insulin appears to be less effective in obese compared to lean individuals (97). In addition, selective brain insulin resistance has been characterised in humans. Here, intranasal insulin fails to alter neuronal networks, which influence body weight control but improve mood and declarative memory (148). Whether this is a result of differential uptake and/or the enrichment of insulin in specific brain parts has not been fully clarified.

Whether intranasal application of insulin is able to attenuate or even overcome brain insulin resistance in humans, a condition not characterised by brain hyperinsulinaemia, is still unknown. However, systemic application of the long acting insulin analogue insulin detemir, which is able to cross the blood–brain barrier, causes a marked reduction in food intake compared to regular human insulin in healthy volunteers, indicating that enhanced insulin action, even in healthy people, modulates food intake (149,150). In addition, peripheral insulin detemir application improves the action of brain insulin in overweight, nondiabetic humans (151), showing that central insulin application via nasal sniffing may improve brain insulin resistance. Insulin resistance can be also attenuated by insulin sensitisers, such as metformin, which is widely used in clinics. Interestingly, metformin has been shown to penetrate the brain, which makes it an attractive drug for counteracting central insulin resistance (152). The effect of other insulin sensitising agents on brain insulin signalling in humans, such as thiazolidinediones (TZD) or glucagon-like peptide 1 receptor agonist, has not been extensively tested. Although TZDs only modestly penetrate the brain, their peripheral administration has been shown to reduce brain insulin resistance in animal models (112), suggesting that TZDs can influence central insulin signalling in humans.

A crucial milestone for the diagnosis and treatment of brain insulin resistance is the identification of a specific marker that is reliable and simple to measure. The identification of such marker(s) might not only facilitate the diagnosis of central insulin resistance but also improve the understanding of its influence on diabetes-related neurological alterations.

Conclusions

Overall, IGF1R signalling is important for brain development and neuronal proliferation, whereas brain IR signalling is a crucial homeostatic factor modulating various aspects of brain physiology.

Although insulin and IGF signalling share signalling molecules and exert several similar effects on brain physiology, varying in strength, they also exhibit specific physiological differences (e.g. the effect of central insulin receptor signalling on neuroinflammation and brain development compared to brain IGF1R signalling). Still, it is very difficult to specifically dissect and differentiate between IR and IGF1R signalling. The use of specific receptor agonists and antagonists will help shed light on this scenario and decipher selective insulin resistance as observed in obese patients. Because human brain insulin/IGF resistance is present in metabolic disorders and neurodegenerative diseases, its accurate diagnosis, understanding and potential reversal is of utmost importance, especially in the wake of an obesity pandemic and the worldwide increase of patients suffering from neurodegenerative diseases.

Acknowledgements

The work described in the present study was supported by the Deutsche Forschungsgemeinschaft (DFG) grant project KL 2399/4-1 to AK and by the Federal Ministry of Education and Research (German Center for Diabetes Research, Grant No. 01GI092).

Declaration of interest

The author declares that there are no conflicts of interest.

Received 1 May 2016,
revised 12 September 2016,
accepted 12 September 2016

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