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Intranasal insulin and orexins to treat age-related cognitive decline

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Abstract

The intranasal (IN) administration of neuropeptides, such as insulin and orexins, has been suggested as a treatment strategy for age-related cognitive decline (ARCD). Because dysfunctional neuropeptide signaling is an observed characteristic of ARCD, it has been suggested that IN delivery of insulin and/or orexins may restore endogenous peptide signaling and thereby preserve cognition. IN administration is particularly alluring as it is a relatively non-invasive method that directly targets peptides to the brain. Several laboratories have examined the behavioral effects of IN insulin in young, aged, and cognitively impaired rodents and humans. These studies demonstrated improved performance on various cognitive tasks following IN insulin administration. Fewer laboratories have assessed the effects of IN orexins; however, this peptide also holds promise as an effective treatment for ARCD through the activation of the cholinergic system and/or the reduction of neuroinflammation. Here, we provide a brief overview of the advantages of IN administration and the delivery pathway, then summarize the current literature on IN insulin and orexins. Additional preclinical studies will be useful to ultimately uncover the mechanisms underlying the pro-cognitive effects of IN insulin and orexins, whereas future clinical studies will aid in the determination of the most efficacious dose and dosing paradigm. Eventually, IN insulin and/or orexin administration may be a widely used treatment strategy in the clinic for ARCD.

Keywords

Insulin; Orexins; Intranasal administration; Aging; Cognition

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1. Introduction

1.1 The aging population and cognitive decline

The brain exhibits numerous changes throughout the aging process [1]. Some of these changes may be neuroprotective or irrelevant to cognitive function, as some older adults retain exceptional cognitive abilities well into their later years [2]. Other brain changes, however, may contribute to age-related cognitive decline (ARCD). ARCD encompasses a spectrum of deteriorating cognition, from a subtle loss of cognitive abilities in healthy aging to neuropathologies such as Alzheimer's disease (AD) and other forms of dementia [3, 4]. While many aging individuals exhibit slight cognitive changes that may be a natural part of the aging process, others undergo pathological brain aging. This patient population, due to underlying neurodegeneration that causes structural changes in the brain, demonstrates accelerated cognitive impairment that leads to the loss of ability to complete daily activities and therefore a loss of independence. ARCD is becoming a growing public health concern as the global population over age 60 is increasing to a projected 2 billion by 2050, up from 900 million in 2015 [5]. A contributing factor to this surge is the aging "baby boomer" generation, who began turning 60 in 2006, as well as medical advances that have significantly increased life expectancy [6, 7]. Unfortunately, there is no treatment that effectively ameliorates ARCD. Current therapeutic strategies include diet and exercise interventions, nutraceuticals, vitamins, hormone therapy, cognitive training, non-steroidal anti-inflammatories, anti-hypertensives, lipid-lowering and diabetes treatments, and anti-dementia drugs. However, a recent review of 263 studies found little evidence for the success of any of these interventions in the delay or prevention of ARCD [8]. The lack of efficacious treatments, especially with regard to pathological brain aging, poses a significant societal problem as ARCD imposes substantial emotional, social, and financial burdens on not only the affected individual but also the public healthcare system, their caregivers, and most importantly their loved ones [4, 9]. Accordingly, identification of new treatment strategies that can maximize cognitive function and improve quality of life in these individuals is a necessity [10]. Intranasal (IN) delivery of neuropeptides to the central nervous system (CNS) has been proposed as a potential strategy to treat cognitive dysfunction, AD, and other neurodegenerative disorders [11, 12]. In support of this approach, AD has been shown to involve dysfunctional neuropeptide signaling in the brain [13, 14]. Thus, IN delivery of specific neuropeptides may directly target the brain and thereby restore signaling, and ultimately, cognition.

1.2 IN therapeutics

Initially, drug administration via the IN route was used clinically to treat local disorders such as rhinorrhea, sinusitis, allergies, and nasal congestion [15, 16]. However, by 1986 it was demonstrated that the large tracer molecule horseradish peroxidase reaches the CNS of rodents and primates within 45–90 minutes after IN administration [17]. Years later in the first quantitative analysis of the intraneuronal transport of a protein to the brain following IN administration, Thorne et al. reported a biologically-relevant mean concentration of 140 nM wheat germ agglutinin-horseradish peroxidase in the olfactory bulb [18, 19]. These findings led to IN studies of various tracer materials, heavy metals, and peptides being transported across the olfactory epithelium into the CNS [20]. In 1989, William Frey suggested the use

of IN delivery as a way to bypass the blood-brain barrier and target therapeutic agents to the CNS [21, 22]. IN administration of a number of peptides has since been explored for the treatment of AD and cognitive decline, including vasoactive intestinal peptide [23], vasopressin [24], exendin [25], leptin [26], an eight amino acid peptide fragment of activity-dependent neuroprotective protein [27], pituitary adenylate cyclase-activating polypeptide [28], insulin [29], and orexins [30]. This review will focus exclusively on the use of IN insulin and orexins as potential treatments for ARCD. Both insulin and orexin receptors are widely distributed in the hypothalamus, a brain region touted for its critical role in the integration of endocrine and behavioral factors to regulate body weight and metabolism [31, 32]. Insulin and orexins are also known to play a key role in glucose homeostasis [33, 34] and have demonstrated anti-inflammatory effects [35, 36]. Additionally, orexin neurons have not only been suggested as essential for the prevention of peripheral insulin resistance with aging [37], but also serve as a critical link between energy balance and the sleep/wake state [38, 39]. This link may be mediated, at least in part, by the sensitivity of orexin neurons to levels of extracellular glucose, which gates a tandem-pore potassium channel on these neurons [40]. Indeed, this relationship may have particular significance for cognitive correlates of diseases of glucose dysregulation, such as diabetes [41]. Importantly, alterations in physiological homeostasis (i.e., energy balance, fuel metabolism, sleep-wake cycle) are early predictors of subsequent cognitive decline in the elderly [42–44]. Thus, insulin and orexins, peptides that are known to play significant integrative roles in central correlates of homeostasis while also interacting with neural circuits underlying cognition, may hold particular promise as targets for ARCD.

1.3 Advantages of IN administration

The IN-delivery method has several benefits over other forms of therapeutic administration. First, the IN technique allows substances to be conveyed rapidly and directly to the CNS [45–48]. This minimizes exposure of the drug to peripheral organs and tissues, thereby reducing adverse systemic side effects [45–47, 49]. For example, peripheral insulin administration triggers hypoglycemia which can subsequently activate endocrine stress axes and impair cognition [49, 50], while peripheral orexins may have effects on the male reproductive axis, endocrine functions, and gastric emptying [51–53]. Elimination of contact with the bloodstream not only permits physiologically effective drug concentrations to reach the brain without eliciting systemic side effects, but also allows peptides that are typically degraded rapidly in the blood to avoid first-pass elimination by the liver and gastrointestinal system [15, 45, 50, 54].

Additionally, systemic administration forces drugs to contend with the blood-brain barrier, which impedes 98% of systemically-administered small molecules and almost 100% of large molecules from reaching the brain [45, 46, 55]. IN delivery is an administration method that allows drugs to bypass this barrier. While intracerebroventricular (ICV), intraparenchymal, and intrathecal administration also target the CNS, these techniques require invasive surgery and are expensive, and therefore are generally not used in humans [46, 49, 54, 56]. Also, the amount of drug reaching the CNS with these methods can be limited due to slow diffusion from the injection site [46]. IN administration, on the other hand, is regarded as a safe and relatively non-invasive method of targeting peptides to the brain [45–47, 55]. This could be

especially important for the treatment of chronic diseases, where frequent dosing may be required to yield a therapeutic effect [54].

Today, most of the IN-administered drugs used clinically are in the form of nasal sprays [57]. These allow for self-administration and quick dispersion and absorption through the nasal mucosal surfaces [57]. A few studies have observed limited absorption across the nasal epithelium, which is regarded as the main disadvantage of IN administration [45, 56]. This may be due to a low surface area available for delivery, the presence of peptidases in the nasal mucosa, and/or rapid mucociliary clearance [16, 56–59]. This can be overcome, however, by the use of especially potent drugs or in combination with absorption enhancers including cell-penetrating substrates (cyclodextrins, surfactants, bile salts, fatty acids, phospholipids, chitosan), focused ultrasound, and PEGylation [16, 45, 56, 59, 60]. Mucolytic agents that decrease the viscosity of mucus can also be used to improve nasal absorption [61]. Mild irritation of the nasal cavity has been cited clinically [45] as another disadvantage of this technique, although this side effect should be weighed against the potential therapeutic benefits of the IN delivery method.

1.4 IN delivery pathway

The pathway a drug takes from the nose to the brain varies according to several factors including the lipophilicity, charge, molecular weight, pH, osmolarity, viscosity, solubility, stability, concentration, volume, and formulation of the drug as well as anatomical and physiological factors such as age, membrane transport, area of deposition, dissociation, degradation, and clearance mechanisms [16, 20, 48, 60, 62]. The clearance of a drug from the nasal cavity depends on the location of its deposition; anteriorly-deposited drugs (e.g. nasal sprays) tend to have slower mucociliary clearance compared to posteriorly-deposited (nasal drops) drugs [59]. Nasal drops appear to be the simplest and most convenient form, but can lead to rapid nasal drainage and it can be difficult to quantify the amount of drug delivered [59]. Gel-devices can be used instead to reduce postnasal drip, improve the accuracy of delivery, and potentially increase nasal absorption by fixing the drug in the nasal mucosa and reducing mucociliary clearance [59].

The mechanism of IN neuropeptide absorption is still not completely understood, particularly for the orexins. There has been further investigation of the functional effects and several more proof-of-concept studies of IN insulin to date. However, it is thought that, due to the properties and molecular weights of insulin and orexins, these drugs pass through the mucous layer and are then transported by transcellular processes across the olfactory or respiratory epithelia in the nasal passages [48, 56, 59, 60]. Next, they move from the nasal mucosa to other areas of the CNS via rapid, extracellular bulk flow along the olfactory and trigeminal nerves [56, 63]. As part of the olfactory pathway, insulin and orexins travel from the externally-exposed dendrites of the olfactory sensory neurons and follow the olfactory axon nerve bundles through the foramina on the cribriform plate of the ethmoid bone to the surface of the olfactory bulbs [64]. In contrast, these drugs can move through the perineural spaces along the trigeminal nerve to reach the more caudal regions of the brain. The maxillary (V_2) branches of the trigeminal nerve provide somatosensory innervation of the olfactory and respiratory epithelia and then merge at the trigeminal ganglion to enter the

CNS at the level of the pons and terminate at the spinal trigeminal nuclei in the brainstem [15], although a small portion of the trigeminal nerve also terminates in the olfactory bulbs [65]. After transport along the olfactory or trigeminal nerves, insulin and orexins can diffuse into the subarachnoid space and move into the perivascular spaces of the cerebrovasculature where they are then rapidly transported via a “perivascular pump” [66] through the brain to their therapeutic targets [46, 56]. Although the olfactory and trigeminal pathways are more frequently discussed, more recently the rostral migratory stream has also been suggested to play a role in the delivery of IN-administered drugs to the CNS, as its surgical transection was shown to block uptake of IN-administered radioligands [67]. The rostral migratory stream connects the olfactory bulb to the periventricular regions. In addition to these direct pathways, neuropeptides can indirectly enter the CNS from the nasal passages by the cerebrospinal fluid (CSF), blood vasculature, or the lymphatic system [15, 46]. This is helped, in part, by the highly vascularized nature of the nasal mucosa. Once in the systemic circulation, however, the drug would then have to cross the blood-brain barrier in order to reach the CNS [15].

2. Insulin to treat age-related cognitive decline

2.1 Brain insulin signaling

Insulin is a 5.8 kDa hormone composed of 51 amino acids in two peptide chains [68]. Its role in the periphery is well-known; insulin is released from the pancreas in response to increased plasma glucose levels, where it binds to and activates insulin receptors to promote glucose uptake and utilization in liver, muscle, and adipose tissue [69, 70]. The identification of insulin [71] and insulin receptors [72] in the brain later suggested that insulin can also act on the CNS [73].

The insulin receptor is a heterotetrameric transmembrane glycoprotein comprised of two α and two β subunits linked by disulfide bonds [74, 75]. Insulin binds to the 135 kDa extracellular α subunit at the ligand binding site, activating the intrinsic tyrosine kinase activity of the 95 kDa intracellular β subunit which initiates a phosphorylation cascade [76, 77]. The activated insulin receptor first phosphorylates insulin receptor substrate (IRS) proteins at multiple tyrosine residues, which bind to Src homology 2 (SH2) domains on various signal transduction proteins to trigger one of two major downstream signaling pathways: the phosphoinositide-3 kinase (PI3K)/Akt and the Ras/mitogen-activated protein kinase (MAPK) pathways [75, 77]. We will focus here on the PI3K pathway, which is known to mediate the metabolic functions of insulin [78]. PI3K is composed of two subunits, a 110 kDa catalytic (p110) subunit and an 85 kDa regulatory (p85α) subunit, which houses two SH2 domains [75]. The interaction of phosphorylated IRS-1 (the principal substrate, although there are several others including IRS-2/3/4) with p85α initiates PI3K activity, catalyzing the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [75, 79]. This then stimulates the serine/threonine kinase phosphoinositide-dependent kinase (PDK1), which phosphorylates and activates a number of downstream effectors such as Akt (also known as protein kinase B [PKB]), mammalian target of rapamycin (mTOR), and p70 S6 kinase (S6K1) [78]. Akt phosphorylates, and thus inhibits, glycogen synthase kinase 3β (GSK3β) at Serine 9 [80]. As GSK3β is a constitutively active enzyme that represses several

proteins, inhibition of GSK3β activates pathways that are normally repressed [81]. Specifically, the inhibition of GSK3β is known to phosphorylate glycogen synthase [82], which stimulates glycogen synthesis, and tau protein [83]. Tau is a microtubule-associated protein, essential for assembling and stabilizing microtubules, that plays a key role in axonal elongation when phosphorylated by GSK3β [84, 85].

In addition to the activation of Akt, PDK1 is necessary for the translocation of the insulin-sensitive glucose transporter GLUT4 from intracellular stores to the plasma membrane [86]. The aforementioned insulin-stimulated glucose uptake in the periphery occurs mainly through GLUT4 [70]. However, this also occurs in the brain, as we have previously demonstrated a PI3K-dependent stimulation of GLUT4 translocation to the plasma membrane in the rat hippocampus in response to plasma [87, 88] and brain [89] insulin. This is important as the concentration of GLUT4 in the plasma membrane is known to regulate the transport of glucose [69, 70], which is regarded as the major energy source used by the brain [90, 91]. Interestingly, it has also been reported, using ^{31}P-magnetic resonance spectroscopy, that IN insulin increases brain cell energy in the form of adenine triphosphate (ATP) and phosphocreatine in healthy young adult men [92]. A key difference between peripheral and central insulin-stimulated glucose utilization is that, within the CNS, glucose functions not only as an energy source but also in the enhancement of cognition. In this regard, improvements in memory have been observed following glucose administration [93–95]. Thus, proper insulin signaling and the subsequent activation of downstream targets of the insulin receptor, including the translocation of GLUT4, is thought to play an integral role in cognitive functioning [87]. Accordingly, it is not surprising that a deficit in insulin activity, i.e., insulin resistance, is a pathological state characteristic of aging and AD-type processes [96, 97].

2.2 Insulin signaling in the cognitively impaired brain

The literature supports the concept that ARCD is associated with a disruption of insulin receptor signaling, which was first suggested by Siegfried Hoyer over 30 years ago [98]. Several studies have demonstrated reductions or aberrations in various insulin signaling proteins with cognitive impairment. In this regard, tyrosine kinase activity was shown to be significantly decreased in several brain regions in AD brains [99, 100]. Significantly lower levels of IRS-1 and IRS-2 were also found in post-mortem human AD brains, and the reductions related strongly to the severity of the disease [101, 102]. Additionally, a reduction in IRS-1 was observed in the hippocampal membrane extract of a mouse model of AD (3xTg-AD) compared with wild-type mice [103]. As discussed above, tyrosine phosphorylation of IRS-1 leads to the downstream activation of PI3K, Akt, mTOR, and the inhibition of GSK3β. However, the phosphorylation of IRS-1 on multiple serine residues can inhibit IRS-1 activity, leading to insulin resistance and the acceleration of disease pathogenesis [102, 104]. It was unsurprising, then, that post-mortem analyses revealed elevated levels of IRS-1 phosphorylated at Serine 312 (IRS-1 pSer312), IRS-1 pSer616, and IRS-1 pSer636 in AD brains relative to controls [102, 104–107], with a progressive increase in the frequency of phosphorylated IRS-1-filled neurons from non-cognitively impaired to mild cognitive impairment (MCI) to AD patients [105]. The staining pattern of IRS-1 pSer312 was also shown to be localized predominately to nuclei in aged cognitively-normal

patients but altered in the hippocampus of AD brains, where it may be found in granules of the cytosol [103]. AD mouse models also demonstrated increased IRS-1 pSer312 and IRS-1 pSer636 levels with a more cytosolic localization, compared to wild-type mice with lower levels and a more nuclear localization [103, 107]. Lower levels of the PI3K subunit p85α were also observed in the hippocampus and hypothalamus of AD relative to control tissues [101], and an ex vivo stimulation protocol revealed marked reductions in IRS-1 binding of p85α in response to 1 nM insulin in the cerebellar cortex and hippocampal formation of AD brains [106]. These results indicate that proteins at various points along the insulin signaling pathway show decreased expression or disrupted activation with cognitive impairment.

These disruptions in insulin signaling are thought to be involved in several other defects observed in the aged cognitively impaired brain. While proper insulin receptor activation can stimulate translocation of GLUT4 to the plasma membrane (as described above), a disruption in signaling can dysregulate glucose transport and utilization. Thus, it is unsurprising that several studies with MCI and AD patients have revealed reduced cerebral glucose metabolism on positron emission tomography scans with 2-[^{18}F]fluoro-2-deoxy-D-glucose (FDG–PET) [108–114]. Additionally, while insulin is known to downregulate and inhibit GSK3β to ultimately phosphorylate tau protein, dysregulation of insulin signaling can lead to the overactivation of GSK3β [115]. This has been shown to promote abnormal hyperphosphorylation of tau [116], which can aggregate into neurofibrillary tangles, a known pathophysiological hallmark of AD. It has also been suggested that the changes in expression of the insulin receptor and downstream signaling proteins may precede an increase in accumulation of amyloid β in the brain, another widely accepted feature of AD [117]. Interestingly, insulin administration has been shown to reduce levels of tau and completely reverse the increase in amyloid β that was induced with a high-fat diet in a mouse model of AD (3xTg-AD) [118]. Insulin also significantly increased levels of insulin degrading enzyme (IDE), an intracellular protease responsible for catabolizing insulin and other peptide hormones [119], in primary hippocampal neurons [120], an effect that was blocked with PI3K inhibition. In addition to its role in insulin degradation, IDE has also been shown to break down amyloid β [119, 121]. Thus, it is unsurprising that activity and levels of IDE were decreased in an AD mouse model (Tg2576) subjected to a high-fat diet [122] and in the hippocampus and temporal cortex of AD brains [120], while amyloid β burden increased. Amyloid β, often present at high levels in AD brains, has been shown to decrease PI3K activity [123], which would inhibit IDE activity and ultimately increase levels of amyloid β, subsequently creating a vicious cycle [120]. It is plausible that insulin administration would break this cycle by stimulating the insulin signaling cascade and upregulating PI3K activity [120].

Accordingly, CNS insulin resistance, defined as decreased cellular responsiveness to a normal level of insulin [124], or more simply, defective signal transduction [76], has been proposed as a mechanistic mediator of the cognitive dysfunction seen with AD [14]. It is plausible then, that the restoration of CNS insulin signaling may be necessary to slow the development of AD and ARCD [14, 106, 125–129]. Specifically, there has been a call for treatments to attenuate CNS insulin resistance by raising insulin receptor and IRS-1 activation to enhance signaling in the PI3K/Akt pathway [106, 129]. CNS administration of

insulin may represent a potential strategy to restore insulin signaling in the brain and thus overcome insulin resistance and the often-related cognitive deficits (Figure 1).

2.3 Central administration of insulin

In support of the hypothesis that restoration of brain insulin signaling could be an effective approach for the treatment of ARCD, several preclinical studies have investigated the effects of direct administration of insulin into the CNS. ICV insulin in rats has yielded both impaired [130] and enhanced performance [131] on a passive avoidance task. These contrasting findings could be dose-dependent, as another study [132] revealed a significant improvement in spatial memory with higher (16 and 32 mU), but not lower, doses. In addition, chronic ICV insulin (5 mU/day for 4 weeks) was also found to enhance spatial memory in untreated rats and restore spatial memory impaired in a model of chronic neuroinflammation [133]. However, this improvement in memory performance was only seen in young, but not aged, rats. Several studies have also demonstrated that intrahippocampal insulin administration dose-dependently enhances spatial working memory rats [134–136]. Additionally, it was found that intrahippocampal insulin reversed the spatial memory deficit induced by a high-fat diet in C57BL/6J mice [137], however, it was not sufficient to restore spatial learning and memory deficits induced by scopolamine in rats [138]. Intrahippocampal insulin also improved passive avoidance learning in rats [139]. Collectively, most studies support the concept that direct administration of insulin into the brain has cognitive-enhancing effects.

2.4 IN insulin in young healthy human volunteers

As previously mentioned, ICV and intrahippocampal administration rapidly target insulin to the brain but obviously are not translatable to the clinical setting. Thus, human studies have utilized the non-invasive IN approach to deliver insulin to the brain, with the intent of exploring the ability of this method to restore insulin signaling and, ultimately, cognition. These studies were prompted by the U.S. patent, issued to William Frey in 2001, for the use of IN insulin to bypass the blood-brain barrier, target the brain, and treat neurodegenerative disorders including AD and Parkinson’s disease [140]. In 2002, Jan Born and colleagues then published the first clinical trial demonstrating that IN insulin reached the CSF without uptake into the circulation; thus, no change to blood levels of insulin or glucose [13]. This was followed by several studies led by Christian Benedict that examined the effects of IN insulin in healthy human volunteers. They demonstrated, in the first clinical trial for efficacy of IN insulin, that 8 weeks of IN insulin (4 × 40 IU/day) improved delayed recall of word lists and enhanced mood in young men and women [141]. Subsequent studies using a similar dosing regimen showed that IN delivery of a fast-acting insulin aspart (NovoLog) to young men improved word list recall to an even greater extent [142]. The authors suggested this could be due to the different pharmacokinetic properties of insulin aspart: while regular insulin consists of hexamers, insulin aspart forms monomers and therefore more aspart molecules may travel from the nose to the brain [142]. Although 8 weeks of IN insulin or insulin aspart successfully improved memory, an acute dose of either formulation did not improve word recall in these studies [141, 142].

Ensuing studies continued to explore the effects of acute IN insulin in young healthy volunteers. A single dose (160 IU) of IN insulin decreased food intake from an *ad libitum* breakfast buffet in young men but improved performance on spatial learning and working memory tasks in young women, indicating sex-dependent effects of acute IN insulin [143]. As a follow up, the effects of an acute dose (160 IU) of IN insulin on food intake and memory in healthy postmenopausal women (57.6 ± 1.1 years) were examined in order to determine if estrogen level mediates the response to insulin [144]. However, the results were similar to those seen in young women; specifically, IN insulin did not affect food intake but enhanced working memory. This suggested that the contrasting responses of men and women to IN insulin may be unrelated to estrogen concentration, and instead regulated by another factor. Additionally, evidence began to emerge that indicated that IN insulin may only have effects on certain memory tasks. In young men, IN insulin (40 IU) did not affect declarative or spatial memory performance on olfactory or visual mazes [145] or immediate odor-cued recall of spatial memory, place, or odor recognition [146]. However, this dose did improve delayed odor-cued recall of spatial memory [146]. The authors suggested that IN insulin increased learning success during the encoding tasks, as the subjects' accuracy improved significantly during the delayed task [145]. Interestingly, a few other studies have examined the link between insulin and olfactory effects. IN insulin was found to improve olfactory sensitivity in anosmic [147], hyposmic [148], and normosmic [149] volunteers. However, there have been some contrasting results, as other studies demonstrated reduced olfactory sensitivity in normosmic subjects [150, 151]. It is possible that the results may be mediated by sex, smelling ability, dose of insulin, and odor (i.e., food-related vs. neutral). Regardless, investigation of the effects of IN insulin on olfactory performance is important because olfactory impairments are known to be associated with and a predictor of MCI and/or AD [152].

Finally, IN insulin (160 IU) was also administered to young men and women just prior to 8 hours of nocturnal sleep, in order to investigate its effects on memory consolidation [153]. IN insulin impaired the acquisition of new declarative and procedural information but did not affect retrieval of original memories learned before sleep, implying that insulin may contribute to memory formation by reducing the interfering influence of new information. These results can be connected to a follow-up study in which young healthy men self-administered placebo or IN insulin (160 IU/day; immediately after awakening or before bed) for 8 weeks [154]. Evening insulin administration elicited a modest but significant improvement on delayed word recall after 5 weeks of treatment, and lessened memory decay (a measure of forgetting) between immediate and delayed word recall within one week of treatment. This suggests that it is not only time of administration that may exert differential effects of IN insulin, but also how often the dosage is delivered, as the memory enhancement seen with 8 weeks of 4 × 40 IU/day [141, 142] appeared greater, albeit later, than that observed with a single 160 IU/day dose for 8 weeks.

Thus, the results from these studies (Table 1) suggest that the effects of IN insulin in young healthy volunteers are influenced by sex, dose, length of dosing regimen, time of dose, drug formulation, and type of memory task. It is probable that the most effective combination of factors has not yet been determined; therefore, further investigation is needed. In this regard,

it is particularly important to assess the effects of IN insulin in aged memory-impaired individuals, as this has been suggested as a treatment for ARCD.

2.5 IN insulin in patients with MCI or AD

Several studies and pilot clinical trials led by Suzanne Craft have demonstrated the memory-enhancing effects of IN insulin in cognitively impaired patients. Acute IN insulin (both 20 and 40 IU) was found to improve verbal memory in MCI and AD patients in their 70s who lacked the APOE ε4 allele [155]. This allele is a strong genetic predictor of AD; specifically, the presence of one ε4 allele increases the risk of developing AD two- to three-fold, and having two ε4 alleles increases the risk by about 15-fold [156]. A dose-response curve was then completed with this same population and revealed that verbal memory was facilitated with acute 10, 20, and 40 IU doses of IN insulin, but not 60 IU; the 20 IU dose led to the greatest improvement in memory [157]. Individuals with the APOE ε4 allele did not have memory enhancement following IN insulin, providing more evidence that APOE genotype may play a role in AD prognosis and response to treatment. In a follow-up study, the efficacy of a 20 IU dose of IN insulin was confirmed by observed improvements in verbal memory, selective attention, and functional status in MCI and AD patients [158].

These investigators then transitioned from the use of an acute IN insulin dose to a chronic dosing regimen in this patient population. They found that 4 months of IN insulin (20 or 40 IU/day) preserved general cognition and functional ability, and 20 IU/day also improved delayed memory [159, 160]. A follow-up study then showed that sex and APOE genotype may differentially affect response to 4 months of treatment with IN insulin [161]. In individuals lacking the APOE ε4 allele, the 20 IU/day dose improved delayed memory for both men and women, but only men showed improved cognitive performance with the 40 IU/day dose. Those with the APOE ε4 allele remained cognitively stable. Functional abilities appeared preserved for women with either dose compared to men. Unfortunately, a recent large multisite randomized double-blind clinical trial assessing the efficacy of 12 months of IN insulin (40 IU/day) in aged MCI or AD patients yielded disappointing results [162] with regard to the primary intention-to-treat cohort. No cognitive or functional benefits were observed with 12 months of IN insulin, as measured by changes in scores on the AD Assessment Scale-cognitive subscale 12 (ADAS-cog-12), AD Cooperative Study Activities of Daily Living Scale for MCI (ADL-MCI, the Clinical Dementia Rating Scale Sum of Boxes, and a memory composite evaluation. However, malfunction of the IN-delivery device partway through the trial necessitated the switch to another device that utilized a different delivery strategy. Thus, the investigators warn that interpretation of the results are complicated by the device malfunction. Further analysis of a secondary cohort revealed that IN insulin delivered via device 1 significantly improved ADAS-cog-12 and ADL-MCI scores beginning 12 months after treatment. These results warrant cautious interpretation but also demonstrate the importance of examining the long-term effects of IN insulin in a cognitively-impaired patient population, as the number of studies to date is relatively limited.

The ability of IN-delivered insulin analogues to restore cognitive function in ARCD has also been examined. Rosenbloom et al. found that acute IN glulisine (20 IU; a rapid-acting

insulin analogue lacking zinc-containing compounds) administration did not improve performance on learning, memory, executive function, language, or visuospatial function tasks in mild to moderate AD patients with the APOE ε4 allele [163, 164]. Additional studies determined that 3-week IN administration of insulin detemir (a long-lasting insulin analogue) in MCI and AD patients with the APOE ε4 allele improved visuospatial (20 or 40 IU/day) and verbal working memory (40 IU/day), but did not affect daily or executive functioning [165, 166]. Subsequent studies by these same investigators compared the effects of 4 months of IN insulin detemir (40 IU/day) administration with placebo and regular human insulin (40 IU/day) on older adults diagnosed with MCI or mild to moderate AD [167, 168]. Regular IN insulin treatment was associated with better delayed memory composite scores (measured by delayed story recall and delayed Selective Reminding Test recall) after 2 and 4 months, preserved volume of four brain regions of interest (left superior parietal cortex, right middle cingulum, left cuneus, and right parahippocampal gyrus), and reduction in the tau-P181/Aβ42 ratio compared with placebo-treated subjects. Interestingly, no significant effects were seen with IN insulin detemir compared with placebo.

Additionally, an acute dose of IN insulin (40 IU) was shown to improve visuospatial memory in a group of older individuals (62.0 ± 7.9 years) with type 2 diabetes mellitus [169]. A follow-up study investigating the effects of 24 weeks of daily IN insulin administration is currently being conducted [170]. This is important as diabetes has been shown to accelerate brain aging and increase the risk of AD [169]. Furthermore, both cognitive impairment and diabetes are hypothesized to share the pathogenic feature of insulin resistance [171].

These findings indicate the potential for IN insulin administration to preserve brain structure and improve various aspects of memory in patients with MCI or AD. However, it is clear that the response to treatment may vary by APOE genotype, sex, dose, length of dosing regimen, and insulin formulation. More studies are needed to fully explore these avenues. Currently, it appears that individuals lacking the APOE ε4 allele respond more favorably to IN insulin, although APOE ε4 carriers may see memory improvements with longer-lasting insulin detemir [172]. Additionally, 20 IU/day appears to be the most efficacious dose of IN insulin. This is lower than the 40 IU/day dose that elicited the most favorable response with IN insulin detemir, although as noted in [165], this discrepancy could be a result of the different pharmacodynamic profiles of these insulin formulations. Insulin detemir has a longer half-life, is more lipophilic, and binds to albumin, thus resulting in slower absorption and a greater overall exposure [165, 173]. On the other hand, regular insulin mimics postprandial insulin release and reaches higher peak levels, potentially activating mechanisms essential to memory at lower doses [165]. As noted above, there are also differential outcomes depending on the duration of treatment (acute vs. subchronic vs chronic). Both an acute dose and 4 months of IN insulin appeared to elicit pro-cognitive effects, while 12 months of IN insulin did not. It is interesting to speculate that chronic daily administration of IN insulin may elicit insulin receptor downregulation and/or desensitization. Under such conditions, it is possible that a dosing paradigm with IN insulin administered every other day or a few times a week would allow for pro-cognitive effects but would eliminate potential pharmacodynamic deficits. Similarly, it is possible that the IN insulin builds up to a high level with a chronic administration. Studies [135, 157] have

indicated that IN insulin may elicit cognitive-enhancing effects according to an inverted U-shaped distribution; thus, too high of a dose will be unsuccessful in treating ARCD. Alternatively, it is possible that, similar to the currently available drugs used to treat cognitive decline, the pro-cognitive effects are only temporary as they do not target the underlying neurodegeneration that occurs with cognitive decline. Clearly the total dosing length, as well as number and timing of doses each day, warrants further investigation.

The memory enhancements observed in young healthy, as well as aged memory-impaired individuals (Table 2), indicate that IN insulin may be a clinically-relevant therapeutic for the improvement of memory, and ultimately quality of life, in individuals during both pathological and non-pathological aging. Additionally, this may be an intervention strategy that can be used during the prodromal phase of AD (i.e., MCI), when changes are already occurring in the brain but before the manifestation of symptoms. While human studies are important in the understanding of IN insulin efficacy in a clinical population, preclinical studies are essential for the identification of the mechanism(s) through which IN insulin promotes cognitive function.

2.6 IN insulin in young and aged rodents

Within the last decade, several preclinical studies have revealed improved performance on memory tasks, in support of the aforementioned clinical observations. For example, Woo and colleagues observed downregulated insulin signaling and impaired spatial working memory (Y-maze test) in young (2 months) C57BL/6 mice subjected to chronic restraint stress [174]. However, these deficits were restored to that of a control group after one week of administration of IN insulin (100 μg) 30 min before restraint stress. In addition, Mao et al. found that 6 weeks of IN insulin in young (4.5 month) APP/PS1 mice (a model of AD) decreased anxiety-related behaviors and improved spatial memory plasticity, as shown by a reversal Morris Water Maze task [175]. In rats, 2 weeks of IN insulin restored spatial memory that was impaired by streptozotocin [176, 177] and amyloid β [178]. Similarly, Guo et al. demonstrated that 6 weeks of IN insulin also restored memory on the Morris Water Maze in streptozotocin-treated rats [179]. A study by Fadool and colleagues also showed positive effects of 1 week of IN insulin administration in young mice [180]. They demonstrated that one week of human recombinant IN insulin increased short- and long-term object memory recognition, anxiolytic behavior, and odor discrimination in C57BL/6 mice (postnatal days 41–60). However, in a follow-up study, they treated male C57BL6/J mice (beginning at 4 months) with IN insulin (450 μg twice daily) for 4 and 8 weeks, and found that a more chronic administration of IN insulin did not enhance olfactory ability, object memory recognition, or a majority of systems physiology metabolic factors in young mice [181]. This is very interesting in that it, in some ways, mimics the results seen with chronic IN administration in the human clinical trials, where 4 months of IN insulin preserved cognition in MCI and AD patients [159], but 12 months did not [162].

While the above preclinical studies were performed in young rodents, IN insulin-induced memory enhancement has also been explored in aged mice. One week of IN insulin (1.75 IU/day) prevented an anesthesia-induced deficit in spatial learning and memory, as measured by the Morris Water Maze, in aged (17–18 months) C57BL6/129 mice [182]. Li et al. also

found that 26 days of IN insulin (1.75 IU/day) administered to aged C57BL/6 (17–18 months) mice prior to anesthesia prevented long-term anesthesia-induced impairments on the novel object recognition and contextual-dependent fear conditioning tasks [183]. In addition, 4 weeks of IN insulin (0.024 IU) administered to aged (18 months) C57BL/6 mice significantly improved performance on the radial arm water maze [184]. However, there have been some contrasting results. When Marks et al. observed aged (15 months) male mice following 12 months of a high-fat or regular control diet, they found that one week of IN insulin elicited anxiety-like behaviors in the aged animals on the control diet [180]. Additionally, in the diet-induced obese animals, IN insulin did not improve object memory performance and elicited an anxiogenic response in both the light-dark box and elevated plus maze tests. The group led by Olivier Thibault has also explored several formulations of IN insulin in aged male Fischer 344 rats, because, as noted above, different formulations of insulin may produce different outcomes. They first demonstrated that 8–11 days of low-dose (0.0715 U) IN insulin (both Levemir and Humalog formulations) restored memory recall on the Morris Water Maze in aged (21 months) male Fischer 344 rats to that of a young cohort (3 months) [185]. This was not seen with higher Levemir doses (0.143 and 0.286 U/day for 5 days). They next explored Apidra, a zinc-free formulation with slightly faster pharmacokinetics than regular human insulin but similar affinity for the insulin receptor [186]. Neither acute nor chronic (9 days of 0.0715 IU/day) IN Apidra improved memory or recall of the platform on the Morris Water Maze in young (3 months) or aged (21 months) male Fischer 344 rats [186]. In fact, chronic IN Apidra appeared to have a negative impact on learning in the aged animals. They then examined a fast-acting insulin aspart, a higher-penetrance insulin analogue with molecular modifications that increase absorption and peak plasma concentrations to almost twice that of human insulin [187]. Three months (0.0715 IU 5x per week) of IN insulin aspart in young (2 months) and aged (18 months) male Fischer 344 rats did not significantly enhance learning on the Morris Water Maze, but the aged animals showed a 30% increase (although non-significant) in time spent in the correct quadrant [187].

Overall, the results from these rodent studies illustrate that IN insulin improves performance on various memory tasks (Table 3). However, similar to observations in humans, the beneficial effects on IN insulin may vary according to treatment conditions (dose, length of dosing regimen, insulin formulation, age and strain of animal, etc.). More studies (both preclinical and clinical) are needed to optimize these conditions and to further explore the activation of the insulin signaling pathway following IN insulin.

2.7 Potential mechanisms underlying the pro-cognitive effects of insulin

While basic science studies such as the ones mentioned above can be important for exploring behavioral outcomes, they are also essential in providing mechanistic insights into how IN insulin effects cognition. Currently, the underlying mechanism is unknown, although several investigators have offered suggestions, as to potential cellular and molecular targets affected upon insulin administration. Insulin may play a role in synaptic transmission and plasticity [188], particularly as it has been shown to enhance *N*-methyl-*D*-aspartate (NMDA) receptor trafficking [189] and activity [190, 191]. It has also been suggested that insulin enhances neuronal communication and thus improves synaptic plasticity and membrane

excitability by reducing the calcium-dependent slow afterhyperpolarization amplitude and duration [185]. Insulin has also been shown to increase GABA receptor expression and activation [192], and presynaptically, to modulate catecholamine neurotransmission [193]. Furthermore, insulin may support memory processes by increasing local glucose metabolism [135] and, as aforementioned, by stimulating the transport of GLUT4 to the plasma membrane [87–89]. Finally, insulin has also been regarded as anti-inflammatory [35], as it has been shown to suppress pro-inflammatory cytokines [194–196] and induce anti-inflammatory mediators [197]. These are only a few of the several hypotheses regarding how insulin may exert pro-cognitive effects; unfortunately, the mechanism has yet to be identified and the above suggestions are purely speculative. Future preclinical studies will be essential to determine this underlying mechanism, which will be important to understand if IN insulin will one day be used in the clinical setting to treat ARCD. However, as previously mentioned, insulin is not the only neuropeptide that demonstrates potential to treat ARCD. IN orexin represents another plausible treatment strategy.

3. Orexin to treat age-related cognitive decline

3.1 Brain orexin signaling

Orexin A (hypocretin 1; 3.6 kDa, 33 amino acids) and Orexin B (hypocretin 2; 2.9 kDa, 28 amino acids) are neuropeptides synthesized by a population of neurons restricted to the lateral and dorsomedial hypothalamus and the perifornical area [198]. These neurons were simultaneously discovered in 1998 by two separate research teams [198, 199]. The orexin/hypocretin peptides, derived from the prepro-orexin gene, bind to their G protein-coupled receptors: orexin/hypocretin receptor type 1 (Ox1R/HcrtR1) and type 2 (Ox2R/HcrtR2) [198]. Orexin A/hypocretin 1 (herein referred to as orexin A) preferentially binds Ox1R, with a 10- to 100-fold greater affinity then orexin B/hypocretin 2 (herein referred to as orexin B) [198, 200]. However, both orexin A and B have similar affinities for the less-selective Ox2R [198, 200]. Unlike the widely characterized insulin signaling pathway, the orexin signaling cascade is still not completely understood. Briefly, both orexin receptors can couple to members of the G protein families ($G_{q/11}$, $G_{i/o}$, and G_s) or other proteins such as β-arrestin to modulate non-selective cation channels, phospholipases, adenylyl cyclase, and protein and lipid kinases (i.e., PI3K, MAPK) [201]. These enzymes mediate postsynaptic depolarization through the elevation of calcium levels (through voltage-gated calcium channels or from intracellular stores) [198, 201, 202], inhibition of potassium channels [203], and/or activation of the sodium/calcium exchanger [201, 204, 205], all of which results in neuronal excitation [206]. Orexin receptors can also regulate presynaptic glutamate and γ-aminobutyric acid (GABA) neurotransmitter release to elicit downstream neuronal effects and increase the number of NMDA receptors in the cell membrane to produce long-lasting increases in neuronal excitability [206–209].

Orexin receptor signaling influences multiple CNS functions, particularly those involved in homeostasis. However, this system has more recently been suggested to not only mediate homeostasis and integrate physiological functions [210, 211], but also modulate cognitive functions including attention, wakefulness, and learning and memory [212]. For one, feeding is a homeostatic function regulated by orexins that also includes cognitive components

(interoceptive awareness of hunger, memories of where to obtain food, attention to food-related cues, etc.) [213]. Additionally, orexins are known to promote arousal and wakefulness [214] and may stabilize sleep/wake transitions [215–218]. This is important as sleep is essential for memory consolidation [219]. It is not surprising, then, that a selective loss of orexin neurons is a classic feature of narcolepsy, a sleep disorder characterized by excessive daytime sleepiness [220–224]. While the most widely-known symptom of this disorder is a disruption in the sleep/wake cycle, patients also exhibit subtle cognitive deficits (i.e., attentional and olfactory discrimination) even during periods of apparently normal wakefulness [225, 226]. Interestingly, these cognitive deficits in narcolepsy show subtle similarities to those in ARCD, further supporting the concept that orexin promotes cognitive function [227, 228]. Moreover, orexin neurons are known to project to and activate brain regions implicated in cognition and memory, including the medial prefrontal cortex (PFC), basal forebrain, amygdala, nucleus accumbens, and hippocampus [213, 229–232], and have been found to enhance long-term potentiation, a cellular correlate of learning and memory [233–235]. While more widely known as physiological integrators [211], these results support the concept that orexins also play a key role in memory and cognition. Indeed, physiological disturbances in the elderly, including unexplained weight loss and disruptions in the sleep-wake cycle, are thought to be early phenotypic manifestations of brain changes that subsequently predict, and may contribute to, cognitive decline [43, 236–238]. Thus, it is plausible that IN orexin administration could serve as a treatment strategy for cognitive dysfunction [213, 227].

3.2 Orexins in the aging brain

Many of the physiological processes moderated by orexins, including sleep, food intake, metabolism, and cognition, undergo adverse changes with aging [210, 239]. It has therefore been suggested that aging coincides with a decline in orexin neuron number and sensitivity [210]. In this regard, recent work in humans showed a 23% decrease in orexin neurons from infancy to late adulthood, with a 10% decrease occurring between early and late adulthood [240]. The loss of orexin neurons with aging has been studied more extensively in animals. For example, the number of orexin-A immunopositive neurons in the lateral hypothalamus was shown to begin to decrease between 400–800 days in C57BL/6 mice [241] and after 8 months in male Wistar rats [242], with greater than 40% reduction in male Fischer 344/Brown Norway F1 hybrid rats by 26 months [231]. Additionally, the number of cells expressing the prepro-orexin gene, as well as levels of prepro-orexin gene expression, were significantly decreased in aged (24 months) male Hannover-Wistar rats in comparison to a young adult (3 months) cohort [243]. Enlarged axon terminals were also found in orexin neurons of aged cats, indicating morphological changes may occur in these neurons with age [244]. Finally, a decrease in orexin receptor mRNA expression in various brain regions was also found in aging C57BL/6 mice [245], indicating that it is not just the number and morphology of orexin neurons that changes with age, but also the expression of orexin receptors.

Orexin loss has been observed not only with non-pathological aging, but also in patients with neurodegenerative diseases such as AD. Post-mortem analysis identified a significant decrease in orexin A-immunoreactivity in the hypothalamus and ventricular CSF of AD

patients when compared to controls [246]. Downregulation of orexin receptors in subregions of the hippocampus has also been seen in patients with early- and late-onset familial AD [247]. Such observations support the hypothesis that reductions in orexin neurons and receptors may be a contributing factor to the severe cognitive impairment observed in AD patients. In fact, a recent study found a significant positive correlation between orexin A levels in the CSF and cognitive function (as measured by scores on the Mini-Mental State Examination and Montreal Cognitive Assessment) in patients with AD [248]. Interestingly, a decline in CSF orexin A has also been observed in patients with other clinical parameters that influence cognitive function. Orexin A concentrations in the CSF were inversely correlated with body weight and water content [249], and in narcoleptic patients, lower CSF orexin levels seemed to be associated with the occurrence of obesity [250].

3.3 Potential mechanisms underlying the pro-cognitive effects of orexins

A key characteristic of age-related cognitive changes is a decline in attentional capacity [251], which may also lead to other symptoms such as disruption of working memory [239]. These attentional and cognitive deficits may stem from alterations in several interacting neurotransmitter systems, particularly the cholinergic system [239]. In fact, evidence indicates that it is not a loss of cholinergic number, but instead a reduction in or dysregulation of cholinergic activity that leads to impaired cognition [252–254]. Previous studies from our laboratory suggest that it is the failure of normal afferent regulation of the basal forebrain cholinergic system, including alterations in orexin modulation of this system, that plays a key role in ARCD [239]. We have previously demonstrated a widespread distribution of orexin-immunoreactive fibers in cholinergic regions of the basal forebrain [255] and a significant decrease in orexin fiber innervation to cholinergic cells in aged rats [232]. Additionally, orexin A administration to the basal forebrain of young adult male rats has been shown to increase acetylcholine efflux in select brain regions: orexin A administration to the ventral pallidum/substantia innominata increased acetylcholine release in the PFC [255] and administration to the medial septum increased hippocampal acetylcholine release [232]. These high levels of acetylcholine set the appropriate dynamics for increased attentional capacity [256]. In addition, elimination of orexin neurons with the orexin B-saporin toxin, blockage of the Ox1R with the selective antagonist SB-334867, or downregulation of orexin expression by virus-mediated gene transfer all significantly blunted the cholinergic and behavioral response to presentation of a food stimulus [257, 258]. Collectively, these data indicate that the failure of the orexin system to activate the basal forebrain cholinergic system may play a role in the attentional deficits and inappropriate behavioral and cognitive responses to homeostatic challenges often seen in neurodegenerative conditions such as ARCD [239, 259]. Administration of orexins to the brain, however, could improve cognition in aging individuals via activation of the cholinergic system (Figure 2).

Interestingly, it has also been suggested that orexins confer pro-cognitive effects by reducing inflammation and apoptosis [36, 260], possibly through the modulation of microglia [261, 262] and/or antioxidant effects [263, 264]. Several studies have revealed data in support of this hypothesis. Treatment with orexin A inhibited the production of pro-inflammatory markers in murine BV2 microglial cells following exposure to palmitic acid [262] and

lipopolysaccharide (LPS) [261], and in human hepatocytes treated with Hepatitis B virus-encoded X protein (HBx) [265]. Orexin A was also found to mitigate HBx-induced oxidative stress, as shown by decreased production of reactive oxygen species (ROS), 4-hydroxynonenal (4-HNE; an indicator of ROS production) and the NADPH subunit NADPH oxidase 4 (NOX-4) [265]. Similarly, orexin A treatment attenuated the increased production of mitochondrial ROS, expression of NOX-4, activation of the NLRP3 inflammasome, and levels of pro-inflammatory cytokines in human coronary endothelial cells stimulated with high glucose [263]. These results have been echoed in *in vivo* rodent studies. In a mouse model of multiple sclerosis, peripheral administration of orexin A was found to significantly reduce CNS inflammation, as shown by decreased immune cell infiltration, expression of chemokines and pro-inflammatory cytokines, demyelination, astrogliosis, and microglial activation [266]. Subcutaneous and ICV orexin A administration in mice was also shown to attenuate the amount of pro-inflammatory cytokines activated in response to LPS [267], and in a rat gastric ischemia-reperfusion model, orexin A infusion decreased neutrophil activation and lipid peroxidation [264]. In rats subjected to transient middle cerebral artery occlusion (MCAO; a model of ischemic stroke), orexin A significantly decreased infarct size [268] and number of apoptotic neurons, attenuated neurologic deficits, and increased expression of hypoxia-inducible factor-1∝-positive neurons [269]. Similarly, Xiong et al. found that ICV Orexin A pretreatment significantly decreased infarct volume in response to MCAO in both wild-type and orexin/ataxin-3 (O/A3) mice, a genetic model of selective orexigenic neurodegeneration [261]. Findings from other studies utilizing these transgenic mice indicate that lack of orexins may be associated with impaired cognition and increased inflammation, as O/A3 mice have demonstrated increased levels of pro-inflammatory cytokines compared to wild-type mice [270]. Additionally, in comparison to their wild-type counterparts, O/A3 mice had significantly larger infarcts, increased numbers of activation macrophages and microglia, worse neurological scores, and decreased spontaneous locomotor activity in response to MCAO [261] and significantly impaired long-term memory and increased Iba1 expression (a marker of microglial activation) in response to a high-fat diet [270].

These studies indicate that, while a lack of proper orexin signaling may be associated with neuroinflammation and impaired neurological function, administration of exogenous orexins can ameliorate these effects. Thus, orexin-induced neuroprotection may be mediated not only by activation of the basal forebrain cholinergic system, but also through anti-inflammatory effects. More studies are needed to fully examine the mechanistic basis of the pro-cognitive effects of orexin.

3.4 Central administration of orexins

As aforementioned, orexin administration directly into the CNS has been shown to increase acetylcholine efflux in select brain regions [232, 255] and reduce inflammation [261, 267, 268]. However, central administration of orexins has also been shown to improve performance on cognitive tasks, providing evidence that it could be a successful treatment for ARCD. ICV administration of orexin A [271] or orexin B [272] has been shown to improve learning, consolidation, and retrieval of memory in a one-way passive avoidance task in rats, while the selective Ox2R antagonist *N*-ethyl-2-[(6-methoxy-pyridin-3-yl)-

(toluene-2-sulphonyl)-amino]-*N*-pyridin-3-ylmethyl-acetamide (EMPA) reversed the effects of orexin B on memory consolidation [273]. Similarly, ICV administration of orexin A ameliorated the spatial learning and memory deficits seen in adult male epileptic rats (treated with pentylenetetrazol), an effect that was attenuated by ICV administration of the selective Ox1R antagonist SB-334867 [274]. ICV orexin A administration also improved the poor attentional task performance in seen rats with 192 IgG-saporin-induced lesions of cholinergic projections to the medial PFC [275] and improved memory retention in both T-maze footshock avoidance and one trial step-down passive avoidance in both CD-1 and SAMP8 mice [276]. Finally, ICV administration of the selective Ox1R antagonist SB-334867 impaired performance on spatial and simultaneous visual discrimination tasks in male rats [277, 278]. Interestingly, Aou et al. found contrasting results; this study demonstrated impaired spatial learning on the Morris Water Maze in male rats after ICV orexin A administration [279]. However, it should be noted that this study was done in young adult rats. Therefore, it may be that simply adding more orexin into an already healthy, young brain may not be beneficial and may actually impair cognition.

In contrast to the ICV method that delivers these peptides to the CSF in the cerebral ventricles [280], orexins can also be targeted to specific brain regions to allow for observation of selected behaviors. Basal forebrain administration of orexin A improved performance on tests of executive function [281] and attention [275] in male rats, while administration of the Ox1R antagonist SB-334867 decreased attentional performance [259]. Medial PFC administration of orexin B also improved accuracy on a task of high attentional demand in male rats [229]. Similarly, administration of orexin A into the CA1 region of the hippocampus improved performance of female O/A3 mice on the two-way active avoidance task [282], while administration of Ox1R antagonist SB-334867 into the CA1 or DG regions of the hippocampus impaired various aspects of memory on the Morris Water Maze [283, 284] and passive avoidance [285] tasks in male rats. Clearly, the results from these studies indicate that central orexin administration can lead to improvements on a variety of cognitive tasks, an effect that is subsequently impaired with blockade of the orexin receptors. However, it is particularly important to focus on the effects of the clinically relevant IN administration of orexins.

3.5 IN administration of orexins

As mentioned above, IN administration has several advantages over other central delivery methods. IN delivery has been shown to target orexin A to the CNS, with the highest concentrations observed in the trigeminal nerve, olfactory bulbs, and anterior olfactory nucleus [30, 286]. This method also resulted in significantly less delivery to the blood and peripheral tissues in comparison with intravenous (IV) delivery [286]. Thus, IN orexin administration could be a promising non-invasive treatment for ARCD. Unfortunately, there are only a few studies that have assessed the effects of IN orexin administration on aspects of cognition. However, this appears to be a growing area of interest, especially with mounting evidence suggesting the role of orexins in cognition. For example, IN orexin A (1 μg/kg) was found to significantly improve performance on a multi-image delayed match-to-sample short-term memory task in sleep deprived adult male rhesus monkeys [287]. This effect was greatest on high-cognitive load trials (long duration trials with more distracter

images) and larger in comparison to IV-administered orexin A (10.0 μg/kg); large enough, in fact, to return mean performance to non-sleep-deprived levels. IN orexin A also restored local cerebral glucose metabolism in the dorsolateral PFC, striatum, and thalamus to non-sleep deprived levels, which was also a greater effect than that seen with IV administration. Another study then examined the effect of IN orexin A on hippocampus-dependent social memory in mice [288]. IN orexin A (0.8 nmol) partially restored social memory, as measured by the two-enclosure homecage test, in adult (3–6 months) male and female O/A3 mice to that of their wild-type counterparts [288]. A few studies have also demonstrated the ability of IN orexin A to ameliorate neuroinflammation. IN orexin A significantly decreased levels of pro-inflammatory cytokines, increased production of anti-inflammatory cytokines and orexin receptors, as well and improved neurological behavioral deficits in a rat model of cardiac arrest [289] and a mouse model of intracerebral hemorrhage [290].

Recent studies from our laboratory have begun to assess the anatomical, neurochemical, and behavioral effects of IN orexin administration. In young (3–4 months) and aged (26–28 months) male Fischer 344/Brown Norway F1 rats, IN orexin A (100 μM) increased cFos expression (a marker of neuronal activation) in the prelimbic and agranular insular cortices, as well as the ventral orbital cortex, piriform cortex, and pedunculopontine nucleus in young and the claustrum and dentate gyrus in aged animals [227, 228]. In another cohort of young male Fischer 344/Brown Norway F1 rats, IN administration of the modified orexin B peptide [Ala_{11},D-Leu_{15}]-OxB, which has been reported to have a 400-fold higher affinity for the Ox2R vs. the OxR1 [291], significantly increased cFos expression in the agranular insular and piriform cortices and the nucleus basalis/substantia innominata [212]. IN [Ala_{11},D-Leu_{15}]-OxB in young rats and IN orexin A in both young and aged rats also increased cFos expression in cholinergic (choline acetyltransferase-positive) neurons of several regions of the basal forebrain [212, 227, 228]. Additionally, IN orexin A decreased cFos expression in GABAergic (parvalbumin-positive) neurons of the prelimbic cortex in both young and aged rats, suggesting that rather than globally increasing neuronal activation, IN orexin A targets specific brain regions [227, 228]. These studies also examined cholinergic and glutamatergic efflux in the PFC, a brain region important for multiple aspects of executive and cognitive function [227, 228]. IN orexin A significantly increased acetylcholine efflux in the PFC in both young and aged rats and glutamate efflux in young rats beginning 15 minutes after administration [227, 228]. Finally, performance on the attentional set-shifting task, a PFC-mediated test used to measure attention and cognitive flexibility in rats, was assessed in young and aged rats following IN orexin A administration. Aged saline-treated animals performed worse on the extra-dimensional set-shifting stage of the task than the young saline-treated group [228]. While IN orexin A did not significantly improve performance of the aged animals on this stage when compared to aged saline-treated animals, it restored performance to levels not significantly different from young animals. Interestingly, IN orexin A impaired the performance of young animals on the reversal stage of the task [228]. This indicates that, similar to the results of Aou et al. [279] (discussed above), orexin A administration to cognitively intact younger subjects may actually impair cognition, possibly due to excess ACh release in the brain. Collectively, these studies indicate that IN- administered orexin A rapidly targets the brain and increases neuronal activation and neurotransmitter efflux in several brain regions that mediate

cognitive functions (Table 4). This could be an underlying mechanism as to how IN orexin A alters cognitive behaviors.

While there have been no studies to date that have investigated the pro-cognitive effects of IN orexins in human subjects to treat ARCD, IN orexin A administration has been explored in patients with narcolepsy with cataplexies. IN orexin A improved olfactory threshold scores [292], had a stabilizing effect on REM sleep [293], and reduced REM sleep duration and wake-to-REM transitions [294]. These results are particularly interesting, as they provide evidence that IN orexins may be efficacious not only in rodents and non-human primates, but also in the clinical setting. More importantly, though, the findings of these studies indicate that IN administration of orexins can be used to induce effects on olfaction [295], alertness, and sleep [296], functions that are often dysregulated with aging and AD. Thus, these studies provide evidence that orexin signaling can lead to cognitive improvement. However, it is clear that far fewer studies of IN orexins have been conducted in comparison to IN insulin. There is still considerable work that needs to be done, including investigating the effects of chronic IN orexin administration in aged individuals, before IN orexins could be used clinically to successfully treat ARCD.

4. Conclusion and perspectives

There is currently a paucity of efficacious treatments for ARCD, which is concerning due to the rapidly expanding aging population. As aging and AD involve dysregulated neuropeptide signaling in the brain, IN administration of neuropeptides may serve as a potential therapeutic strategy by directly targeting the brain and restoring signaling, and ultimately, cognition. Insulin and orexins, in particular, are two neuropeptides that hold promise as successful treatments for ARCD.

Because aged or AD brains have revealed reductions in various insulin signaling proteins, there is provocative evidence to support the concept that IN insulin may restore insulin signaling and therefore slow the progression of ARCD. While these studies demonstrated improved performance on various cognitive tasks following IN insulin, the results were influenced by sex, age, APOE genotype, strain of animal (if applicable), dose, length and timing of dose, drug formulation, and type of memory task. As such, more studies are needed to fully understand the impact of each of these factors on cognition, as well as treatment strategies that could include how frequently IN insulin should be administered. Additionally, more preclinical studies are necessary to elucidate the mechanism underlying the memory-enhancing effects of IN insulin. Although the hypothesis that IN insulin stimulates brain insulin receptor signaling seems to be widely supported, precisely what happens downstream to elicit pro-cognitive effects is still unknown. Like IN insulin, IN administration of orexins may also restore signaling, albeit through a different pathway. Only a few preclinical studies have examined the effects of IN orexins, despite the emerging literature suggesting that IN orexins may enhance cognition through the restoration of basal forebrain cholinergic activity and/or reduction of neuroinflammation. Future studies will be essential in the discovery of the mechanistic basis of orexin-induced neuroprotection, as well as to determine the most efficacious dose and dosing paradigm.

In summary, IN administration of insulin and orexins are non-traditional approaches to restore endogenous peptide signaling in the aged CNS, and thus may represent novel approaches for prevention or treatment of ARCD.

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Highlights

- A more effective treatment for age-related cognitive decline is needed
- Aging and Alzheimer's disease involve dysregulated neuropeptide signaling
- Intranasal insulin and orexin may restore peptide signaling and thus cognition

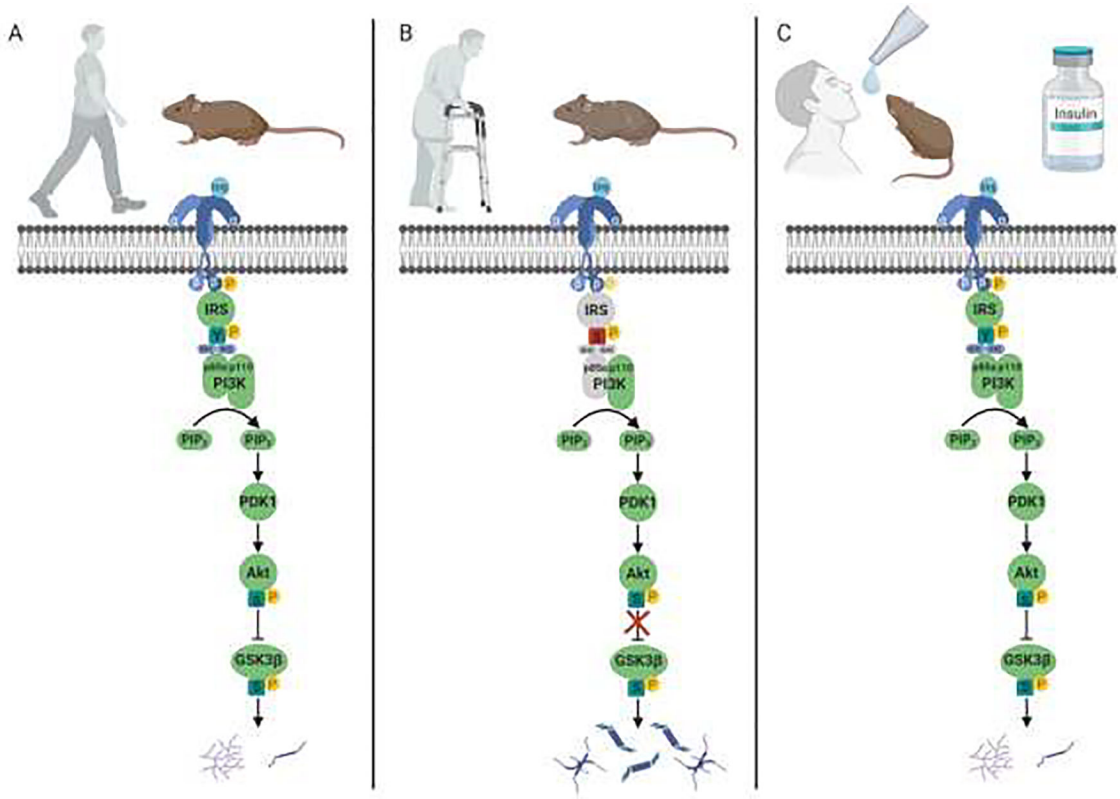

Figure 1:

Intranasal insulin administration may treat age-related cognitive decline via restoration of brain insulin signaling to overcome insulin resistance. A) In the healthy young adult brain, insulin binds to the insulin receptor, activating the intrinsic tyrosine kinase activity and initiating a phosphorylation cascade. The activated insulin receptor phosphorylates insulin receptor substrate (IRS) proteins at tyrosine residues (Y), which bind to Src homology 2 (SH2) domains of the p85α subunit of phosphoinositide-3 kinase (PI3K). This catalyzes the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3). This then stimulates phosphoinositide-dependent kinase (PDK1), which phosphorylates and activates Akt at Serine (S) 473. Akt phosphorylates, and thus inhibits, glycogen synthase kinase 3β (GSK3β) at Serine (S) 9, thereby stimulating glycogen synthesis (purple) and the phosphorylation of tau protein (dark blue). B) A reduction in tyrosine kinase and levels of insulin receptor subtrate proteins, as shown here in gray, has been shown with aging and Alzheimer's disease Post-mortem analysis of brains of patients with Alzheimer's disease has also revealed increased phosphorylation at serine residues on IRS-1. Unlike tyrosine phosphorylation of IRS-1, phosphorylation at serine residues (red S) can inhibit IRS-1 activity. A decrease in IRS-1 binding to the p85α subunit of PI3K (shown in gray) has also been demonstrated in AD brains. Finally, dysregulated insulin signaling may lead to the overactivation of GSK3β, thus inhibiting glycogen synthesis and promoting the hyperphosphorylation of tau activity and aggregating into neurofibrillary tangles. C) The intranasal administration of insulin may increase insulin receptor and IRS-1 activation to enhance PI3K/Akt activity, ultimately restoring insulin signaling in the brain to overcome age-related cognitive decline.

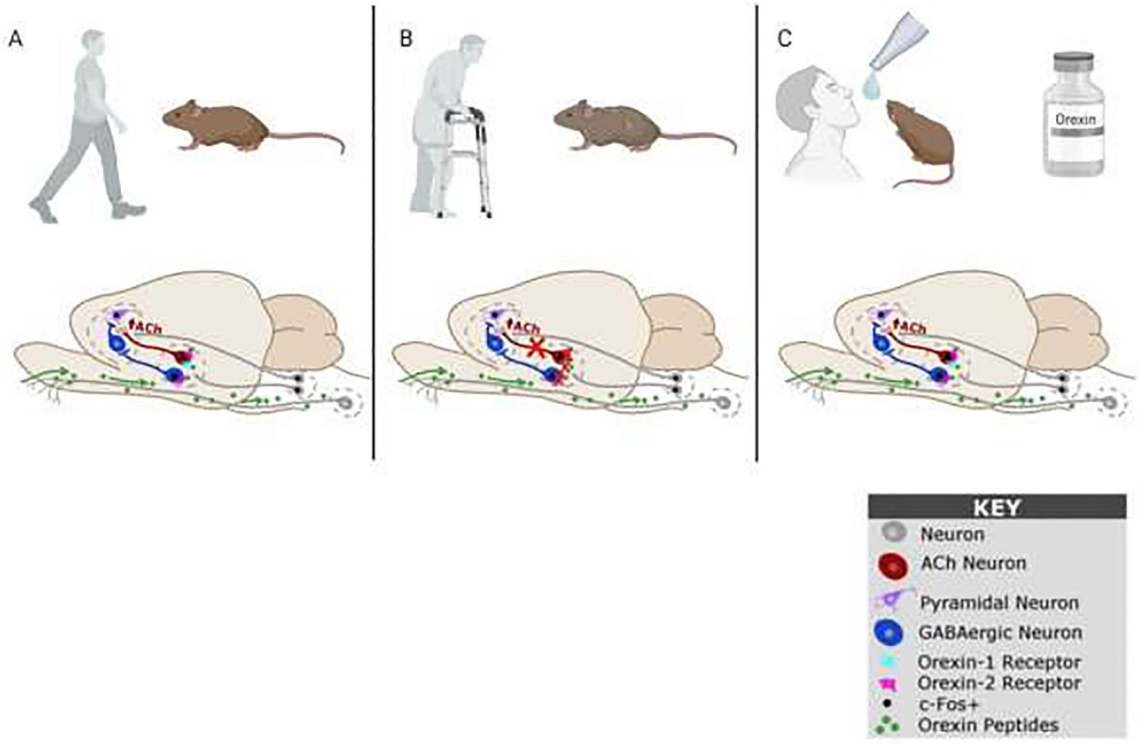

Figure 2:

Intranasal orexin administration may treat age-related cognitive decline via activation of the basal forebrain cholinergic system. A) In the healthy young adult brain, orexins bind to the orexin-1 or orexin-2 receptors to activate basal forebrain cholinergic neurons to increase acetylcholine (ACh) efflux within areas important for cognitive functions, including the prefrontal cortex and hippocampus. Orexins can also increase the activation of excitatory pyramidal neurons and decrease the activation of inhibitory GABAergic interneurons in the prefrontal cortex, as well as activate brainstem neurons of the pedunculopontine nucleus to modulate basal forebrain activity B) Aging and neurodegenerative diseases such as Alzheimer's disease may be accompanied by a change in orexin neuron morphology and decreases in orexin neuron expression and number of orexin receptors. Loss of orexin inputs may contribute to basal forebrain cholinergic dysfunction and cognitive decline in aging. C) The intranasal administration of orexins may restore an important source of afferent regulation of basal forebrain cholinergic neurons and thereby improve cholinergic-dependent cognitive processes such as attention and memory.

Table 1

Studies examining the cognitive effects of IN insulin in young healthy volunteers.

Duration	Dose	Type	Patient Population	Outcome	Citation
Acute and 8 weeks	4 × 40 IU/day	Regular human insulin (Insulin Actrapid)	38 young healthy volunteers (24 men; 18–34 years)	8 weeks of IN regular human insulin improved long-term declarative memory (delayed recall of word lists) and enhanced mood. No effect on wordstem priming (non-declarative memory) or attention (Stroop test). Acute regular human insulin did not affect declarative memory but did enhance feelings of well-being and self-confidence.	[141]
Acute and 8 weeks	4 × 40 IU/day	Regular human insulin (Insulin Actrapid) or insulin aspart (NovoLog)	36 young healthy men (18–35 years)	8 weeks of both IN regular human insulin and insulin aspart improved long-term declarative memory (delayed recall of word lists), but insulin aspart did so to a greater extent. Acute treatment, of either regular human insulin or insulin aspart, did not have effects on declarative memory.	[142]
Acute	160 IU	Regular human insulin (Insulin Actrapid)	32 young healthy volunteers (14 men; M: 22.29 ± 0.62 years, F: 22.44 ± 0.63 years)	Decreased food intake from a breakfast buffet in men and improved performance on spatial learning (two-dimensional-object location) and working memory (digit span) tasks in women.	[143]
Acute	160 IU	Regular human insulin (Insulin Actrapid)	14 healthy postmenopausal women (51–62 years)	Enhanced working memory (digit span task). No effect on food intake or a visuospatial object location task. No difference between postmenopausal and young women.	[144]
Acute	40 IU	Regular human insulin (Insulin Actrapid)	16 young healthy men (24.69 ± 1.04 years)	No effect on declarative or spatial memory performance (olfactory or visual mazes).	[145]
Acute	40 IU	Regular human insulin (Insulin Actrapid)	18 young healthy men (24.28 ± 0.80 years)	Improved delayed odor-cued recall of spatial memory but had no effect on immediate odor-cued recall of spatial memory performance (olfactory or visual mazes).	[146]
Acute	160 IU	Regular human insulin (Insulin Actrapid)	32 young healthy volunteers (16 men; 18–30 years)	Impaired the acquisition of new declarative (word recall) and procedural (finger sequence tapping) information but did not affect retrieval of original memories learned before sleep.	[153]
8 weeks	160 IU	Regular human insulin (Insulin Actrapid)	36 young healthy men (18–40 years)	Evening IN insulin improved delayed word recall after 5 weeks of treatment, and morning IN insulin reduced serum cortisol levels after 2 weeks of treatment. No differences in body weight or composition were seen between placebo, morning, or evening IN insulin.	[154]

Table 2

Studies examining the cognitive effects of IN insulin in patients with MCI or AD.

Duration	Dose	Type	Patient Population	Outcome	Citation
Acute	20 or 40 IU	Regular human insulin (Novolin R)	13 MCI and 13 probable AD patients lacking the APOE ε4 allele and 35 cognitively normal controls (all in their 70s)	Both 20 and 40 IU IN insulin improved verbal memory (story recall) in MCI and AD patients lacking the APOE ε4 allele. 40 IU also facilitated contextual verbal memory (total word list recall). These doses had no effect on attention (Stroop task), visual working memory (Self-Ordered Pointing Task), or speed of target identification on a visual search task. Individuals with the APOE ε4 allele did not have memory enhancement following IN insulin.	[155]
Acute	10, 20, 40, 60 IU	Regular human insulin (Novolin R)	20 MCI and 13 probable AD patients lacking the APO ε4 allele and 59 cognitively normal controls (all in their 70s)	10, 20 and 40 IU IN insulin improved verbal declarative memory (story recall); 60 IU did not in the memory-impaired patients lacking the APOE ε4 allele. 20 IU led to greatest improvement in verbal memory. Individuals with the APOE ε4 allele showed declines in memory scores following IN insulin.	[157]
Acute	20 IU	Regular human insulin (Novolin R)	14 MCI and 11 probable AD patients in their 70s and 80s	Improved verbal memory (story recall), selective attention (Stroop task), and functional status (Dementia Severity Rating Scale). iv	[158]
4 months	20 or 40 IU/day	Regular human insulin (Novolin R)	64 MCI and 40 probable AD patients (56.7% male; in their 60s and 70s)	Both doses of IN insulin preserved general cognition and functional ability (Dementia Severity Rating Scale, AD Assessment Scale-cognitive subscale, and AD Cooperative Study-Activities of Daily Living); 20 IU/day also improved delayed memory (delayed story recall).	[159, 160]
4 months	20 or 40 IU/day	Regular human insulin (Novolin R)	Same patient population as [116]; stratified by whether or not they were carriers of the APOE ε4 allele	In individuals lacking the APOE4 allele, the 20 IU/day dose of IN insulin improved delayed memory (story recall) for both men and women, but only men showed improved cognitive performance with the 40 IU/day dose. Those with the APOE ε4 allele remained cognitively stable. Functional abilities (AD Cooperative Study-Activities of Daily Living) appeared preserved for women with either dose compared to men.	[161]
12 months	40 IU/day	Regular human insulin (Humulin R)	244 MCI and probable AD patients (123 men, 55–85 years)	No cognitive or functional benefits were observed (AD Assessment Scale-cognitive subscale 12, AD Cooperative Study Activities of Daily Living Scale for MCI, the Clinical Dementia Rating Scale Sum of Boxes, and a memory composite evaluation) with IN insulin.	[162]
Acute	20 IU	Zinc-free rapid-acting insulin analogue (Glulisine/ Apidra)	12 probable mild to moderate AD patients with the APOE ε4 allele (9 men; 65–85 years)	Did not improve performance on learning, memory, executive function, language, or visuospatial function tasks (Repeatable Battery for the Assessment of Neuropsychological Status, Wechsler Adult Intelligence Scale-Fourth Edition digit span subtest, Trail-Making Test, Boston Naming Test) in mild to moderate AD patients with the APOE ε4 allele.	[163, 164]
3 weeks	20 or 40 IU/day	Long-lasting insulin analogue (Detemir/ Levemir)	60 MCI and probable mild to moderate AD patients with the APOE ε4 allele (37 men; 50–89 years)	40 IU/day of IN insulin detemir improved verbal memory composite scores (immediate story recall, delayed story recall, immediate word list recall, delayed word list recall) only in patients with the APOE ε4 allele. 40 IU/day also improved visuospatial (Benton Visual Retention Test) and verbal working memory (Dot Counting N-back), but did not affect daily (Dementia Severity Rating Scale) or executive functioning (Stroop task) in patients, regardless of APOE ε4 status. 20 IU/day did not affect cognitive outcomes.	[165, 166]
4 months	40 IU/day	Long-lasting insulin analogue (Detemir/ Levemir) and regular human insulin (Novolin R)	36 MCI and probable mild to moderate AD patients (17 men; 50–89 years)	Regular IN insulin treatment was associated with better delayed memory composite scores (delayed story recall and delayed Selective Reminding Test recall) after 2 and 4 months, preserved volume of the left superior parietal cortex, right middle cingulum, left cuneus, and right parahippocampal gyrus, and reduction in the tau-P181/ Aβ42 ratio compared with placebo-treated subjects. IN insulin detemir did not elicit significant effects.	[167, 168]

Table 3

Studies examining the cognitive effects of IN insulin in rodents.

Duration	Dose	Type	Animals	Outcome	Citation
1 week	100 μg/day	Insulin-FITC reconstituted in 0.01 N HCl	Male C57BL/6 mice (2 months)	Restored insulin signaling in the hippocampus, ability to build a nest, and spatial working memory (Y-maze) impaired with chronic restraint stress.	[174]
6 weeks	1 IU/day	Regular human insulin (Humulin R)	Female APPswe/PS1dE9 double-transgenic mice (4.5 months)	Decreased anxiety-related behaviors (open-field test), improved spatial memory plasticity (reversal Morris Water Maze task), promoted brain insulin signaling, reduced levels of amyloid β and plaque deposits, and enhanced neurogenesis.	[175]
2 weeks	2 IU/day	Regular human insulin (Humulin R)	Adult male Sprague Dawley rats with ICV streptozotocin injections	Restored spatial memory (Morris Water Maze), prevented downregulation of the insulin receptor and insulin degrading enzyme, restored disturbed phosphorylation of insulin signaling proteins, and prevented astrocytic and microglial activation in streptozotocin-treated animals.	[176]
2 weeks	2 IU/day	Regular human insulin (Humulin R)	Adult male Sprague Dawley rats with ICV streptozotocin injections	Restored spatial memory (Morris Water Maze), prevented a reduction in cerebral blood flow, increased Nrf-2 and pCREB expression, BDNF level, and ATP content, and attenuated cholinergic dysfunction in streptozotocin-treated animals.	[177]
2 weeks	0.1, 0.2, or 0.3 IU/day	Regular human insulin (Exir Pharmaceuticals)	Wistar rats unilaterally injected with amyloid β_{25-35}	0.2 and 0.3 IU/day of IN insulin restored spatial memory (Morris Water Maze) that was impaired by amyloid β.	[178]
6 weeks	2 IU/day	Regular human insulin (Humulin R)	Adult male Sprague Dawley rats with ICV streptozotocin injections	Restored spatial memory (Morris Water Maze), reduced tau phosphorylation and tau kinases, attenuated microglial activation, and enhanced neurogenesis in streptozotocin-treated animals.	[179]
1 week	450 μg twice daily	Human recombinant insulin (Roche)	C57BL/6 mice (postnatal days 41–60)	Increased short- and long-term object memory recognition (novel object recognition task), anxiolytic behavior (light/dark box, marble burying, elevated plus maze), and odor discrimination. It also increased Kv1.3 tyrosine phosphorylation and interactions with insulin receptor and post-synaptic density 95. No general anosmia or damage to the olfactory epithelium was observed.	[180]
4 and 8 weeks	450 μg twice daily	Human recombinant insulin (Roche)	Male C57BL/6 male mice (beginning at 4 months)	No effect on olfactory ability (computerized olfactometry), object memory recognition (novel object recognition task), or physiological properties (body weight, food or water intake, energy expenditure, and respiratory exchange ratio).	[181]
1 week	1.75 IU/day	Regular human insulin (Humulin R)	Female C57BL6/129 mice (17–18 months)	Prevented an anesthesia-induced deficit in spatial learning in memory (Morris Water Maze), attenuated hyperphosphorylation of tau, enhanced the level of synaptic proteins in the brain, and promoted brain insulin signaling.	[182]
26 days	1.75 IU/day	Regular human insulin (Humulin R)	Male C57BL/6 mice (17–18 months)	Prevented long-term anesthesia-induced impairments (novel object recognition and contextual-dependent fear conditioning tasks), attenuated the hyperphosphorylation of tau, increased expression of Post-synaptic Density Protein 95 and Microtubule-associated Protein-2 in the hippocampus, and restored insulin signaling.	[183]
4 weeks	0.024 IU/day	Regular human insulin (Humulin R)	Male C57BL/6 mice (18 months)	Significantly improved memory (radial arm water maze) and increased *PKCδII*, *Bcl2*, and *bcl-xL* expression in the hippocampus.	[184]
1 week	450 μg twice daily	Human recombinant insulin (Roche)	Male mice (15 months) following 12	Elicited anxiety-like behaviors in the aged animals on the control diet. In the diet-induced obese animals, IN insulin did not improve object memory	[180]

Duration	Dose	Type	Animals	Outcome	Citation
			months of a high-fat or regular control diet	performance (novel object recognition task), elicited an anxiogenic response (light-dark box and elevated plus maze), and induced significantly less phosphorylation of Kv1.3 in the olfactory bulbs.	
8–11 days	0.0715, 0.143, 0.286 IU/day	Long-lasting insulin analogue (Detemir/ Levemir) and short-acting insulin lispro (Humalog)	Male Fischer 344 rats (3 and 21 months)	0.0715 IU restored memory recall (Morris Water Maze) to that of a young cohort; this was not seen with the higher Levemir doses.	[185]
Acute and 9 days	0.0715 IU/day	Zinc-free rapid-acting insulin analogue (Glulisine/Apidra)	Male Fischer 344 rats (3 and 21 months)	Neither acute nor chronic IN Apidra improved memory or recall of the platform on the Morris Water Maze; chronic IN Apidra appeared to have a negative impact on learning in the aged animals.	[186]
3 months	0.0715 IU 5× per week	Insulin Aspart (NovoLog)	Male Fischer 344 rats (2 and 18 months)	Did not significantly enhance learning (Morris Water Maze), but the aged animals showed a 30% (although non-significant) increase in time spent in the correct quadrant.	[187]

Table 4

Studies examining the effects of IN orexins.

Duration	Dose	Type	Animals	Outcome	Citation
Acute	1 ug/kg	Orexin A	Adult male rhesus monkeys (n=8)	Significantly improved performance of sleep-deprived animals on a short-term memory task (multi-image delayed match-to-sample), to a greater extent than IV administration. IN orexin A also restored local cerebral glucose metabolism in the dorsolateral PFC, striatum, and thalamus to non-sleep deprived levels, which was also a greater effect than that seen with IV administration.	[287]
Acute	0.8 nmol	Orexin A	Adult male and female wild-type and orexin/ataxin-3 C57BL/6 mice (3–6 months)	Partially restored social memory (two-enclosure homecage test) in orexin/ataxin-3 mice to that of wild-type counterparts.	[288]
Acute	50 μl of a 100 μM solution	Orexin A	Young (3–4 months) male Fischer 344/Brown Norway F1 rats	Increased neuronal activation (cFos expression) in the PFC and in subpopulations of basal forebrain cholinergic neurons. Additionally, IN orexin A increased ACh and glutamate efflux in the PFC.	[227]
Acute	50 μl of a 100 μM solution	Orexin A	Young (3–4 months) and aged (26–28 months) male Fischer 344/Brown Norway F1 rats	Significantly increased neuronal activation (cFos expression) in several telencephalic brain regions, PFC ACh efflux, and attentional function (attentional set-shifting task) in aged animals.	[228]
Acute	50 μl of a 100 μM solution	[Ala^{11},D-Leu^{15}]-Orexin B	Young (3–4 months) male Fischer 344/Brown Norway F1 rats	Increased neuronal activation (cFos expression) in cortical and basal forebrain regions, and within cholinergic neurons of the medial septum.	[212]
Acute	10 or 50 μM	Orexin A	Adult male Wistar rat model of cardiac arrest	Significantly reduced the levels of pro-inflammatory markers and increased production of orexin receptors in the hypothalamus. IN orexin A also resulted in early arousal (EEG analysis) and improved neurological behaviors (Neurologic Deficit Scale).	[289]
Acute	20, 60, and 200 ng/μl	Recombinant Orexin A peptide	Adult (56 ± 5 days old) male CD-1 mice subjected to intracerebral hemorrhage	Dose-dependently reversed the neurological deficits observed 24 and 72 h after ICH (Garcia test, forelimb placement, corner turn). IN Orexin A treatment also improved mid- and long-term (1–4 weeks) neurological outcomes after ICH (foot fault test, rotarod, Morris Water Maze). Orexin A significantly increased the expression of anti-inflammatory cytokines and decreased the downstream pro-inflammatory markers, effects that were reversed by a CaMKKβ inhibitor (STO-609) and OxR2 inhibitor (JNJ-10397049), but not an OxR1 inhibitor (SB-334867).	[290]