Universitat Autònoma de Barcelona

Modulation of sleep and cortical activity through the orexin and cholinergic axes: role of the IGF-I

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TESIS DOCTORAL

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Modulation of sleep and cortical activity through the orexin and cholinergic axes: role of the IGF-I.

Memoria presentada por Jonathan Adrián Zegarra Valdivia para optar al título de Doctor por la Universidad Autónoma de Barcelona (UAB) en el Programa de Doctorado en Neurociencias de acuerdo con el trabajo realizado bajo la dirección del Prof. Ignacio Torres Alemán y del Prof. Ángel Núñez Molina en el Instituto Cajal (CSIC) y en el Dpto. de Anatomía, Histología y Neurociencia de la Facultad de Medicina de la UAM.

Don Ignacio Torres Alemán, profesor de Investigación en el Achucarro Basque Center for Neuroscience,

Don Ángel Núñez Molina, profesor de Investigación en la Universidad Autónoma de Madrid,

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CERTIFICAN:

Que Don Jonathan Adrián Zegarra Valdivia ha realizado en el Instituto Cajal y en la Universidad Autónoma de Madrid, bajo su dirección, el presente trabajo de investigación correspondiente a su Tesis doctoral titulada "*Modulation of sleep and cortical activity through the orexin and cholinergic axes: role of the IGF-I*".

Consideramos que esta memoria reúne todos los requisitos necesarios y una vez revisada, manifestamos nuestra conformidad con su presentación para ser juzgada.

Fdo. Ignacio Torres Alemán

Fdo. Ángel Núñez Molina

Fdo. José Rodríguez Álvarez

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Todos los procedimientos con animales de experimentación realizados durante el desarrollo de la Tesis Doctoral cumplen con las normas Europeas de Bienestar Animal (2010/63/UE) y han sido previamente aprobados por el Comité de Ética de la Universidad Autónoma de Madrid (CEI72-1286-A156), y el Comité de Ética Experimentación Animal de la Comunidad de Madrid según el RD 53/2013.

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Abbreviations

ACh	Acetylcholine
AD	Alzheimer's disease
AKT1	Serine/Threonine Kinase 1
Aps	Action potentials
BBB	Blood-brain-barrier
BF	Basal forebrain
BNST	Bed nucleus of the stria terminalis
ChAT	Cholinergic acetyltransferase
ChR2-YFP	Channelrhodopsin-2, tagged with a fluorescent protein mouse
CNS	Central nervous system
CNO	Clozapine N – oxide (CNO)
CSF	Cerebrospinal fluid
DREADD	Receptors Exclusively Activated by Designer Drugs
E/I	Excitatory-inhibitory activity
EC	Entorhinal cortex
ECoG	Electrocorticogram
EEG	Electroencephalographic
FIROC	Floxed IGF-IR/Orexin Cre mice
FOXO	Forkhead box protein O
GABA	Gamma-aminobutyric
GH	Growth Hormone
GPCRs	G -protein-coupled receptors

GSK3	Glycogen synthase kinase 3
HDB	Horizontal nucleus of the diagonal band of Broca
hIGF-I	Human IGF-I
IGFBP	Insulin-like growth factor binding proteins
IGF-I	Insulin growth factor-I
IGF-IR	Insulin growth factor-I receptor
IR	Insulin receptor
IRS-1	Insulin receptor type 1 substrate
IRS-2	Insulin receptor type 2 substrate
JNK	N-terminal c-Jun kinase
LC	Locus coeruleus
LDT	Laterodorsal tegmental nuclei
LH	Lateral hypothalamus
LID	Liver-IGF-I deficient mice
LRP1	Low-density lipoprotein receptor type 1
LRP2	Low-density lipoprotein receptor type 2
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
mPFC	Medial prefrontal cortex
mTOR	Mammalian target of rapamycin
MUA	Multiunit activity
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor

nBM	Nucleus basalis of Meynert
NCX	Sodium-calcium exchanger
non-REM	non-Rapid Eye Movement
NSCCs	Non-selective cation channels
nvlDBB	Nucleus of the vertical limb of diagonal band of Broca
OX1R	Orexin receptor 1
OX2R	Orexin receptor 2
PeF	Perifornical area
PI3K	Phosphatidyl-inositol-3 kinase
POA	Hypothalamic preoptic area
PPT	Pedunculopontine nuclei
PVN	Paraventricular nucleus of the hypothalamus
PVT	Paraventricular nucleus of the thalamus
REM	Rapid Eye Movement
SHC1	Src homology 2 domain-containing transforming protein 1
SI	Substantia innominata
SWS	Slow-wave sleep
TBN	Tuberomammillary nucleus
TNFα	Tumor necrosis factor-alpha
VTA	Ventral tegmental area

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Supplementary figure 1. Diverse control studies

Summary

BACKGROUND

The study of insulin-like growth factor-I (IGF-I) has reached relevance in recent years for its pleiotropic activity, role in brain pathology, and potential therapeutic applications. IGF-I is a circulating hormone mainly produced by the liver under growth hormone (GH) signaling that can cross the blood-brain-barrier (BBB) into the central nervous system (CNS). However, its extensive role in aging and Alzheimer's disease is not entirely understood. Some studies suggest that IGF-I is involved in adaptive changes during aging, and there is some evidence that supports the notion that Alzheimer's disease (AD) is also associated with perturbed metabolic function affecting insulin and insulin-like growth factor.

Aging and AD are closely related to cortical, information processing, and sleep/wakefulness disturbances, common comorbidities of metabolic diseases. Besides, aging is frequently accompanied by a decline in cognition, and changes in sleep duration are paralleled by changes in IGF-I levels. Furthermore, aging is associated with lower serum IGF-I levels that may contribute to this deterioration. Nonetheless, the underlying processes linking both are not yet fully defined. Here, we take advantage of a specific group of cells involved in sleep regulation and cortical activity. The first group, the orexin neurons, is a discrete cell population in the lateral hypothalamus involved in the circadian sleep/wake cycle, metabolic, energy expenditure, and arousal. The second group, cholinergic cells from the basal forebrain, plays an essential role in cortical function, information processing, and sleep/wake status.

GENERAL AIMS

- To understand the mechanisms relationships between IGF-I and sleep/wake cycle in orexinergic neurons (Study 1).
- To evaluate if reduced IGF-I input to orexin neurons during aging contributes to age-associated sleep and cortical activation disturbances (Study 2).
- To analyze if IGF-I facilitates cholinergic activity modulates arousal and cortical activation by BF neurons (Study 3).

METHODS

Study 1

We use a novel transgenic mice model under Cre/Lox system (Floxed IGF-IR/Orexin Cre: Firoc mice) and immunofluorescence, ELISA, hybridization *in situ*, and other techniques to validate our transgenic model. Furthermore, electrocorticographic (ECoG), electrophysiology (recordings in anesthetized animals), and optogenetic studies confirm the role of the IGF-IR in orexinergic neurons.

Study 2

We use young (3-6 months old) and old (>18 months old) C57BL/6J57 mice. Besides, electrocorticographic (ECoG) analysis and electrophysiology (recordings in anesthetized animals) are used to determine the sleep architecture and their cortical electrical activity. Furthermore, we use immunofluorescence and hybridization *in situ* to determine the IGF-IR/IGF-IR mRNA. Additionally, we use a novel IGF-R sensitizer (Aik3a305) to treat old mice and recover IGF-IR signaling.

Study 3

We use young (3-6 months old) and old (20-22 months old) C57BL/6J57 mice and transgenic mice that express the light-activated cation channel, channelrhodopsin-2, tagged with a fluorescent protein (ChR2-YFP) under the control of the choline acetyltransferase promoter in cholinergic neurons. Besides, we use immunofluorescence to identify IGF-IR and OX1R in ChAT cells. Once again, we use electrophysiology (recordings in anesthetized animals) and optogenetic to confirm the role of the IGF-IR in cholinergic neurons. Additionally, we treated old mice with hIGF-I chronically.

RESULTS

Study 1

We now report that the activity of orexin neurons, a discrete cell population in the lateral hypothalamus (LH), is involved in the circadian sleep/wake cycle and arousal, which IGF-I modulates. Moreover, mice with blunted IGF-I receptor activity in orexin neurons have lower orexin levels in the hypothalamus, show altered ECoG patterns with predominant slow-wave activity, and reduced onset-sleep latency. Besides, our transgenic mice have reduced response to IGF-I i.p., or intraparenchymal injected.

Study 2

Older mice show altered sleep structure compared to young animals, with changes in both slow and fast electrocorticographic (ECoG) activity and fewer transitions between sleep and wake stages. Hence, ECoG stimulation and activation of orexin neurons by systemic IGF-I is abrogated in old mice. Additionally, stimulation of orthodromically activated LH neurons by either systemic or local IGF-I in young mice is absent in old mice. As orexin neurons of old mice show markedly increased IGF-I receptor (IGF-IR) levels, suggesting loss of sensitivity to IGF-I, we treated old mice with AIK3a305, a novel IGF-IR sensitizer, and observed restored responses to IGF-I and rejuvenation of sleep patterns.

Study 3

We studied the effect of IGF-I in neurons of the horizontal diagonal band of Broca (HDB) of young (≤6 months old) and old (≥20-month-old) mice. Young mice respond to local injection of IGF-I in the HDB nucleus, increasing their neuronal activity and inducing fast oscillatory activity EcoG, where old mice have decreased response. Furthermore, IGF-I facilitated tactile responses in the primary somatosensory cortex elicited by air-puffs delivered in the whiskers. Immunohistochemistry showed that cholinergic HDB neurons express IGF-I receptors and that IGF-I injection increased the expression of c-fos in young but not in old animals. Besides, the effects of aging were partially ameliorated by chronic IGF-I treatment in old mice.

CONCLUSIONS

The results of this thesis confirm the pleiotropic role of IGF-I in brain modulation. Specifically, orexinergic and cholinergic neurons in the lateral hypothalamus and the basal forebrain display IGF-IR´s. IGF-I signaling on these cells modulates their activity and suggests that IGF-I/orexin/cholinergic axes orchestrate cortical information processing and sleep/wake status. The present findings suggest that reduced IGF-I activity in old animals, either by low IGF-I availability or IGF-I signaling disruption, participates in age-associated changes in cortical activity and disturbed sleep architecture in aging mice. Furthermore, hIGF-I or Aik3a305, a novel IGF-IR sensitizer, recovers cholinergic and orexinergic functions.

Resumen

ANTECEDENTES

El estudio del factor de crecimiento similar a la insulina tipo I (IGF-I) ha adquirido relevancia en los últimos años por su actividad pleiotrópica, su papel en la patología cerebral y sus posibles aplicaciones terapéuticas. IGF-I es una hormona circulante producida principalmente por el hígado bajo la señalización de la hormona del crecimiento (GH) que puede cruzar la barrera hematoencefálica (BBB) hacia el sistema nervioso central (SNC). Sin embargo, no se comprende del todo su importante papel en el envejecimiento y la enfermedad de Alzheimer. Algunos estudios sugieren que el IGF-I está involucrado en los cambios adaptativos durante el envejecimiento, y existe evidencia que respalda la noción de que la enfermedad de Alzheimer (EA) también se asocia con una función metabólica alterada que afecta a la insulina y al IGF-I.

El envejecimiento y la EA están estrechamente relacionados con las alteraciones corticales, del procesamiento de la información y del sueño/vigilia, comorbilidades comunes de las enfermedades metabólicas. Además, el envejecimiento suele ir acompañado de una disminución de la cognición, y los cambios en la duración del sueño van acompañados de cambios en los niveles de IGF-I. Además, el envejecimiento se asocia con niveles más bajos de IGF-I en suero que pueden contribuir a este deterioro. No obstante, los procesos subyacentes que vinculan a ambos aún no están completamente definidos. En este trabajo estudiamos un grupo específico de células involucradas en la regulación del sueño y la actividad cortical. El primer grupo, las neuronas orexinérgicas, es una población de células discretas en el hipotálamo lateral involucrado en el ciclo circadiano de sueño/vigilia, metabolismo, gasto energético y arousal. El segundo grupo, las células colinérgicas del prosencéfalo basal, desempeñan un papel esencial en la función cortical, el procesamiento de la información y el estado de sueño/vigilia.

OBJETIVOS GENERALES

• Comprender las relaciones de los mecanismos entre el IGF-I y el ciclo sueño / vigilia en neuronas orexinérgicas (Estudio 1).

• Evaluar si la entrada reducida de IGF-I a las neuronas de orexina durante el envejecimiento contribuye a las alteraciones del sueño y la activación cortical asociadas a la edad (Estudio 2).

• Analizar si el IGF-I facilita la actividad colinérgica modula la excitación y la activación cortical por las neuronas BF (Estudio 3).

MÉTODOS

Estudio 1

Utilizamos un nuevo modelo de ratones transgénicos bajo el sistema Cre/Lox (Floxed IGF-IR / Orexina-Cre: ratones Firoc) e inmunofluorescencia, ELISA, hibridación *in situ* y otras técnicas para validar nuestro modelo transgénico. Además, los estudios electro-corticográfico (ECoG), electrofisiológico (registros en animales anestesiados) y optogenético confirman el papel del IGF-IR en las neuronas orexinérgicas.

Estudio 2

Usamos ratones C57BL / 6J57 jóvenes (3-6 meses) y viejos (> 18 meses). Además, un análisis electro-corticográfico (ECoG) y electrofisiológico (registros en animales anestesiados) se utilizaron para determinar la arquitectura del sueño y su actividad cortical. También utilizamos inmunofluorescencia e hibridación *in situ* para determinar la cantidad de IGF-IR y IGF-IR ARNm. Además, utilizamos un nuevo sensibilizante del IGF-R (Aik3a305) para tratar ratones viejos y recuperar la señalización de IGF-IR.

Estudio 3

Usamos ratones C57BL / 6J57 jóvenes (3-6 meses) y viejos (20-22 meses) y ratones transgénicos que expresan el canal catiónico activado por luz, canalrodopsina-2, etiquetado con una proteína fluorescente (ChR2-YFP) bajo el control del promotor colina acetiltransferasa en neuronas colinérgicas. Además, utilizamos inmunofluorescencia para identificar IGF-IR y OX1R en células ChAT. Una vez más, utilizamos electrofisiología (registros en animales anestesiados) y optogenética para confirmar el papel del IGF-IR en las neuronas colinérgicas. Además, tratamos a ratones viejos con hIGF-I de forma crónica.

RESULTADOS

Estudio 1

Descubrimos que la actividad de las neuronas de orexina, una población celular discreta en el hipotálamo lateral (LH) involucrada en el ciclo circadiano de sueño/vigilia y arousal, se encuentra modulada por el IGF-I. Además, los ratones con una actividad atenuada del receptor de IGF-I en neuronas orexinérgicas tienen niveles más bajos de orexina en el hipotálamo, muestran patrones de ECoG alterados con actividad predominante de ondas lentas y una latencia reducida del inicio del sueño. Además, nuestros ratones transgénicos tienen una respuesta reducida al IGF-I inyectado de forma sistémica o intraparenquima.

Estudio 2

Los ratones viejos muestran una estructura del sueño alterada en comparación con los jóvenes, con cambios en la actividad electrocorticográfica (ECoG) lenta y rápida. Presentan además menos transiciones entre las etapas de sueño y vigilia. Por tanto, los registros ECoG y la activación de neuronas de orexina por IGF-I sistémico se encuentra alterado en ratones viejos. Además, la estimulación de las neuronas del hipotálamo lateral, activadas ortodrómicamente por IGF-I sistémico o local, en ratones jóvenes está ausente en ratones viejos. Como las neuronas de orexina de ratones viejos muestran niveles marcadamente aumentados del receptor de IGF-I (IGF-IR), esto sugiere una pérdida de sensibilidad a IGF-I, por lo que tratamos a los ratones viejos con AIK3a305, un nuevo sensibilizador de IGF-IR, y observamos respuestas restauradas al IGF-I y el rejuvenecimiento de los patrones de sueño.

Estudio 3

Estudiamos el efecto del IGF-I en neuronas de la banda diagonal horizontal de broca (HDB) de ratones jóvenes (≤6 meses) y viejos (≥20 meses). Los ratones jóvenes respondieron a la inyección local de IGF-I en el núcleo HDB, aumentando su actividad neuronal e induciendo una actividad oscilatoria rápida en el ECoG, mientras que los ratones viejos tienen una respuesta disminuida. Además, el IGF-I facilitó las respuestas táctiles en la corteza somatosensorial primaria provocadas por bocanadas de aire administradas en los bigotes. La inmunohistoquímica mostró que las neuronas HDB colinérgicas expresan receptores de IGF-I y que la inyección de IGF-I aumentaba la

expresión de c-fos en animales jóvenes, pero no en animales viejos. Además, los efectos del envejecimiento mejoraron parcialmente con el tratamiento crónico con hIGF-I en ratones viejos.

CONCLUSIONES

Los resultados de esta tesis confirman el papel pleiotrópico del IGF-I en la modulación cerebral. Específicamente, las neuronas orexinérgicas y colinérgicas en el hipotálamo lateral y el prosencéfalo basal muestran receptores de IGF-I. La señalización de IGF-I en estas células modula su actividad y sugiere que los ejes de IGF-I/orexina/colinérgico orquestan el procesamiento de información cortical y el estado de sueño/vigilia. Los presentes hallazgos sugieren que la actividad reducida de IGF-I en animales viejos, ya sea por baja disponibilidad de IGF-I o por alteración de la señalización de IGF-I, participa en cambios asociados con la edad en la actividad cortical y alteración de la arquitectura del sueño en ratones envejecidos. Además, el tratamiento con hIGF-I o Aik3a305, un novedoso sensibilizador del receptor de IGF-I, recupera las funciones colinérgicas y orexinérgicas.

INTRODUCTION

1. INTRODUCTION

1.1 Insulin-like Growth Factor-I in brain physiology

Insulin growth factor-I (IGF-I), initially named "somatomedin C," is a neuropeptide composed of 70 amino acids and has a weight of 7.5 kDa, mapped on chromosome 12 in humans and chromosome 10 in mice (1,2). These peptides share 48% structural homology with insulin and 50% to its amino acid precursor proinsulin (1,3,4). IGF-I is produced in a paracrine and endocrine manner by different tissues such as the brain, muscle, cartilage, pancreas, and others and then released into interstitial fluids. Nonetheless, its primary source is the liver, where about 75-80% of the total available IGF-I is produced by hepatocytes (5,6). See Figure 1.

Its production is controlled in response to growth hormone stimulation (GH) and then is released to the bloodstream where, with the help of the insulin-like growth factor binding proteins (IGFBP), it extends its availability, half-life, and transport to distant sites through the blood circulation (7).

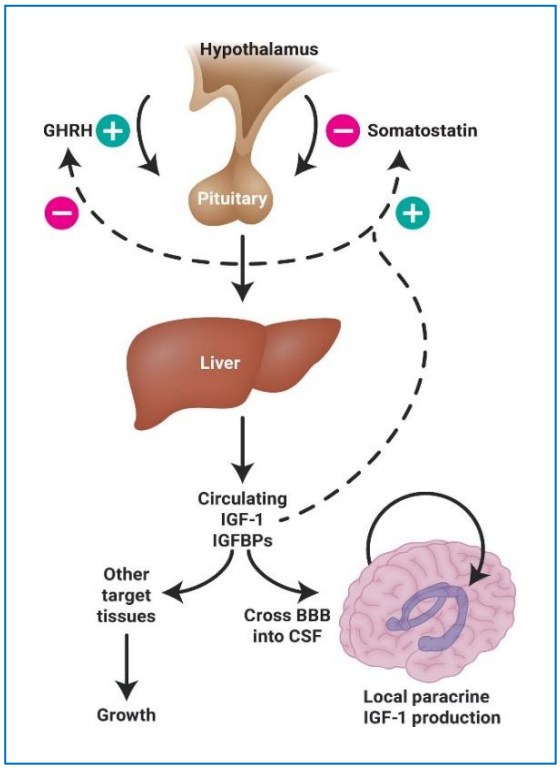

Figure 1. IGF-I's primary source is the liver under Growth Hormone signaling. Taken from Frater et al., (8).

There are 6 high-affinity IGFBPs, where IGFBP2 is the most abundant in the cerebrospinal fluid (CSF) and the IGFPB3 in the body (9). Also, IGFBP-4 and IGFBP-5 are highly expressed in the brain (10). In the central nervous system (CNS), IGF-I fulfills a multitude of actions, including neurotropic, neuromodulatory, and neuroendocrine actions, and also during the different developmental stages (11). For example, cell growth and development, differentiation, synaptogenesis, and mitogenesis (1).

1.1.1 IGF-I Receptor and signaling mechanisms

IGF-I exerts its physiological effects, mainly by binding its receptor (IGF-IR) but also using the insulin receptor (IR) due to its structural homology. Besides, IGF-IR and IR might form hybrid heterodimers receptors (12). The IGF-IR is a tetramer composed of two extracellular α chains and two intracellular β chains containing a tyrosine kinase domain (2). The binding of IGF-I to its receptor originates in a cysteine-rich region of the receptor α subunit and generates a conformational change which allows the activation of its tyrosine kinase domain, phosphorylating the corresponding sites of the β subunit (13). The IGF-IR remains in reduced catalytic activity in its non-phosphorylated state until its binding with IGF-I, allowing autophosphorylation of the receptor (See Figure 2).

The activation also promotes the phosphorylation of different substrates, including one of the principal ones, insulin receptor type 1 substrate (IRS-1) (13), but also includes others such as the insulin receptor type 2 substrate (IRS-2), Src homology 2 domain-containing transforming protein 1 (SHC1) (9). The activation of IRS-1/IRS-2 substrates are essential elements of the receptor activation since they promotes several intracellular signaling pathways (14). Among the different downstream pathways activated, the best studied are through the phosphatidyl-inositol-3 kinase (PI3K)- AKT Serine/Threonine Kinase 1 (AKT1) - mammalian target of rapamycin (mTOR), and inhibition of GSK3 (glycogen synthase kinase-3) pathway (15), the PI3K-AKT-forkhead box protein O (FOXO) pathway, and through the mitogen-activated protein kinase (MAPK) pathway (9). The activation of these canonical pathways initiates intracellular signaling related to cell growth, metabolism, and inhibition of apoptosis (PI3K pathway), and mitogenesis, cell differentiation (MAPK pathway) (1).

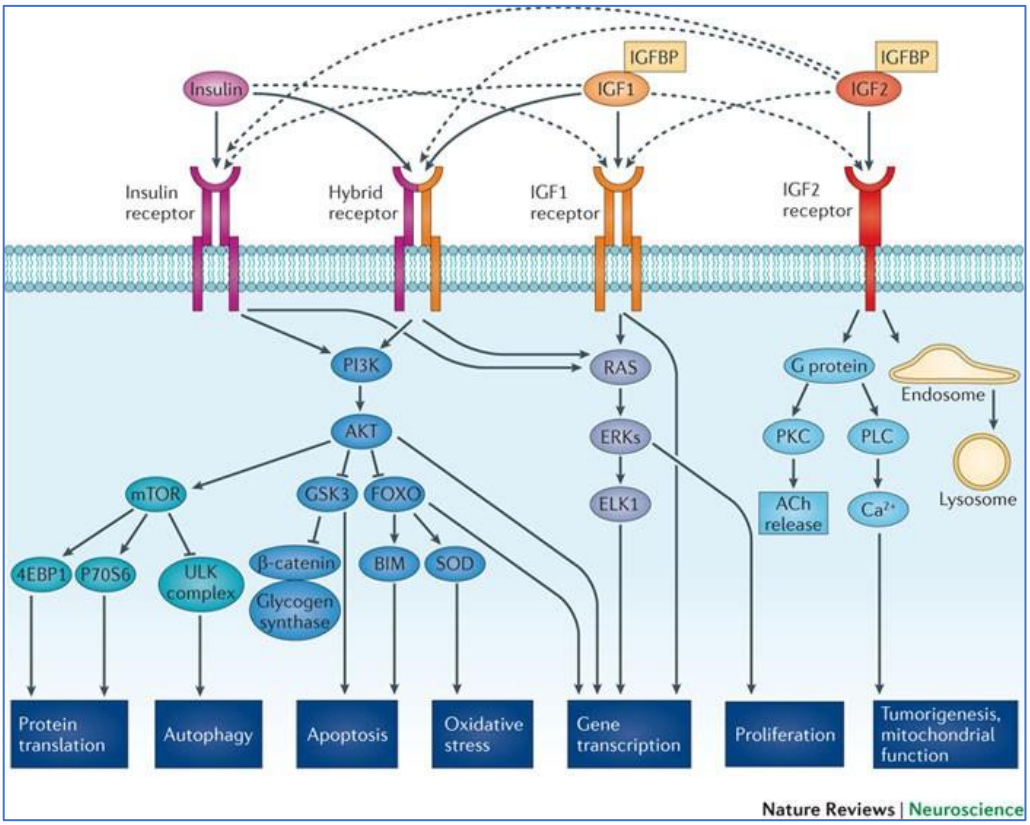

Figure 2. IGF-I signaling pathways in the brain. Taken from Fernandez and Torres-Aleman (1).

1.1.2 IGF-I activity on the brain

It was believed that Insulin and IGF-IR signaling have the same effects in peripheral cells as in neurons, but not in brain cells insulin (1). Nowadays, existing evidence is that insulin, associated with the IGF-I, modulates brain glucose metabolism through concerted action on astrocytes (16). Besides, several effects of the IGF-IR are mediated by the canonical pathways previously seen and through the direct gene transcription detected in neuronal nuclei, modulating the transcription process (15) and probably, explaining the divergent biological effects of the IGFIR to IR.

Regarding the access of IGF-I to the brain, this process is mediated by the blood-brain-barrier (BBB), choroid plexus, and blood vessels (1). One of the main mechanisms of crossing the BBB is the transcytosis process, where the IGF-I is transported through the endothelial (BBB) or epithelial (choroid plexus barrier) cells for its release to the interstitial space or CSF.

The BBB regulates the flow of nutrients and metabolites that pass from the blood to the CNS, controlling its availability by different transport systems (17), including the IGF-IR and the low-density lipoprotein receptor type-1/2 (LRP1, LRP2). The entrance to the CNS reaches the cerebrospinal fluid and the parenchyma areas such as the hypothalamus and hippocampus (18). As previously mentioned, IGF-I plays an essential role in brain development (pre and post-natal) by its mediation of mitogenesis, cell growth, differentiation, and synaptogenesis, but also in the adult brain, participating in brain homeostasis neurogenesis and response to trauma and brain pathology in general (19,20).

In the last decades, the study of IGF-I effects on the brain has been associated with aging, longevity, cognition, neurodegeneration, and neuropsychiatric disorders (5,21–23). As we can see, the IGF-I system comprises a complex regulatory network that operates throughout the body with multiple neurotrophic, metabolic, cell growth, and neuroprotection properties (24).

1.1.3 Aging, Alzheimer's disease, and IGF-I

It is known that serum IGF-I availability is reduced during aging (25), and its detriment has been associated with poor cognition through experimental studies in both rodents and humans (23,26). However, if IGF-I plays a detrimental or protective role in the aging brain is still controversial (27,28). It is possible that the critical modulation of the IGF-I system on the brain (i.e., cognition, sensory facilitation with acetylcholine neurons and astrocytes in the cortex (29), or even modulation of life-styles (30)), would be affected in different levels, systems, and brain mechanism as we are aging. Thus, its relationship with healthy aging is usually associated with increased serum levels and better cognition. On the contrary, poor levels should be related to aging pathology. In recent years, several studies are linking neurodegenerative diseases to the development of diabetes, metabolic disorders, and insulin resistance (14,31).

Both patients and mouse models of Alzheimer’s disease (AD) show decreased circulating IGF-I entrance to the brain, as evidenced by a lower ratio of cerebrospinal fluid/plasma IGF-I (5). The IGF-I deficiency in AD would originate broad changes in brain function

that closely resemble AD pathology and associated co-morbidities (32,33). In AD patients, IGF-I resistance might promote the appearance of neurofibrillary tangles and amyloidosis (31). Nonetheless, different studies show controversial results on the relation of IGF-I and AD (34), probably by the different methodologies, participants characteristics, and the not well-understood relation between IGF-I/binding proteins and absolute IGF-I levels, as the entrance to the brain through the Blood-Brain-Barrier (BBB) (1).

As mentioned above, studies in animal and human models show that reduced serum IGF-I levels are associated with cognitive dysfunctions, and it has been shown that these disorders may be reversible through prolonged systemic administration of IGF-I (35,36). Thus, decreased PI3K/Akt pathway activity, linked to low circulating levels of IGF-I (31), absence of their uptake by the brain, and IRS-1 alteration (37), produces increased amyloidosis and tau phosphorylation (31).

Insulin and IGF-I resistance have been linked to Alzheimer's disease and go beyond those with comorbidity with type 2 diabetes. For example, Talbot et al. (38), studying the hippocampus and cerebellar cortex in AD cases, found a substantial reduction of the IR/IRS1/PI3K signaling pathway and the minor response of the IGF-I/IR/IRS1/PI3K pathway. This resistance to IGF-I is related to IRS-1 dysfunction and larger A-Betha oligomers (38). Nevertheless, metabolic alterations, as IGF-I and insulin resistance processes, are a pathophysiological feature of Alzheimer's disease and other neurodegenerative processes (39–41).

Altered cognition associated with diminished IGF-I is not the only symptom produced because of its deficiency. For example, in our lab, we studied liver-IGF-I deficient (LID) mice, which have 75% less production of IGF-I in the liver by the Cre/Lox system (6). We analyze in their adult life (6-12 months old) the presence of epileptogenic activity, cognitive, mood, and sociability disturbances. We found that the reduced serum IGF-I availability in these mice promotes epileptogenic discharges, altered cognition, less resilience to stress events, and increased depressive-like and anxiety-like symptoms.

Interestingly, we also observed reduced social interactions and social preference. Thus, our results help to clarify the controversy of whether high or low serum IGF-I levels are related

to brain pathology by showing that prolonged IGF-I deficiency is deleterious for a wide array of brain functions. More importantly, we recovered cognitive and epileptogenic activity in LID mice through a chronic human IGF-I (hIGF-I) administration (32). Hence, not only cognitive but also emotional and social alterations may be related to IGF-I deficiency.

In humans, there is evidence of the relationship of ApoE-ε4 and tertiles of IGF-I receptor stimulating activity, where ApoE-ε4 homozygotes show lower levels of IGF-I receptor stimulating activity than heterozygotes and non-carriers (42). Also, low IGF-I serum levels are associated with increased atrophy and an increased risk of developing AD (31). Thus, many pathologies associated with aging and AD could be explained by IGF-I deficiency, such as brain amyloidosis, tau phosphorylation, inflammation, glucose uptake, brain blood flow, and others (14,43). Besides, a large human study found a significant relationship between plasma IGF-I and exposure to recent stressful life events, which indicates that circulating IGF-I appears to be involved in mood homeostasis (20).

Although IGF-I is reduced in serum during aging, healthy aging programs usually use constant exercise (in any modality), active brain tasks, and socialization to promote brain health related to the IGF-I pathway on the brain. Interestingly, exercise and environmental enrichment are also associated with cognitive performance, better mood, and social interaction (23,37–39), being both paradigms strongly associated with the neurotrophic support of IGF-I (21). Thus, insufficient cognitive and environmental enrichment is a risk factor for cognitive and emotional disorders at any age (44,45).

The affectation through aging is related not only to the IGF-I diminution but also to the resistance process. Resistance to IGF-I and insulin has been mainly addressed in neurodegeneration studies indicating alterations of the IGF-I system, such as a lower affinity of the IGF-IR to its substrate or IRS-1 altered signaling (46). Concerning the latter, higher IRS-1 phosphorylated serine 616 has been studied in tauopathies and AD (46). On the other hand, stress signaling pathways such as tumor necrosis factor-alpha (TNFα) and JNK (N-terminal c-Jun kinase); that are involved in neuronal plasticity, regeneration, and cell death can directly induce insulin/IGF-I resistance by inducing phosphorylating of IRS-1 (47), acting in this way as an antagonist pathway for IGF-I (48).

1.2 Hypocretin/Orexinergic System: Discovery

The neuropeptide hypocretin/orexin was the first discovery in 1998 by de Lecea (49) and Sakurai (50) almost simultaneously by two different approaches. De Lecea group use molecular biology and electrophysiology techniques, first studying and isolating cDNA clones in the hypothalamus that has not been express in other brain areas, and cloning these specific cDNAs to discover an entire coding region which encodes a secretory protein of 130 amino acids (51). de Lecea named these peptides hypocretin -1 and -2, considering their possible relation to the secretin hormone.

On the other hand, the Sakurai group performed a systematic biochemical search for endogenous peptides for multiple orphan G -protein-coupled receptors (GPCRs), using a cell-based reporter system (50). Thus, they found a neuropeptide family that bound two related orphan GPCRs. Considering their implication in stimulating feeding behavior after central administration of these peptides, they call them orexin -1R and -2R (OX1R and OX2R, respectively). After discovering hypocretin/orexinergic peptides, it was clear that orexin 1 and orexin 2 correspond to hypocretin 1 and 2, and both came from the polypeptide prepro-orexin.

Through this work, we would prefer to use the term orexin, considering a more precise origin. Both groups describe conjunction of cells located in the perifornical area of the lateral hypothalamus (LH) that express Orexin A and B as excitatory neuropeptides that play an essential role in sleep/wake homeostasis, along with other physiological processes such as thermoregulation, control of energy metabolism, cardiovascular responses, feeding behavior, and spontaneous physical activity (52). Also, in recent years the orexinergic system has been implicated in emotion, motivation, and cognitive processes (53).

1.2.1 Orexin Receptors, projections, and signaling pathways

As previously mentioned, orexin-A and -B are peptides derived from the same precursor called "prepro-orexin." This precursor has been identified in a discrete group of cells bilaterally in the perifornical area of the LH. With up to ~5000 cells in rodents; and between 20–50 000 cells in humans) (54). Both orexin peptides have a role in neuronal

activation (55). Orexin-B has between 10-100-fold higher potency at the OX2R than the OX1R, whereas orexin-A has the same affinity to both receptors (56).

Human OX1R is 1564 bp in length, whereas OX2R is 1843 bp, translated into proteins with 425 and 444 amino acids, respectively. In contrast, rat OX1R and OX2R are 2469 and 3114 bp in length, encoding proteins of 413aa and 460aa (57). The existence of two peptides and two receptors' subtypes gives us the idea of neuronal diversity activity and the importance of this system in the hypothalamus and brain regulation. Despite their specific location, orexin cells projects entirely to the brain, including the cerebellum (58,59), and this system could be modulated by multiple neuronal inputs (i.e., local orexin projections, glutamatergic transmission, decrease in PH by astrocytic-lactate-activity or even extracellular ATP (60)), or by distal afferents like the limbic system (52), basolateral and central amygdala, dorsal Raphe nucleus, nucleus accumbens (NAc), ventral tegmental area (VTA) and the hypothalamic preoptic area (POA), (61). Also, the distribution of both OX1R and OX2R are different and related to different processes (53). See Figure 3.

OX1R has a high expression in the amygdala and locus coeruleus (LC), whereas OX2R express in NAc and the paraventricular nucleus of the hypothalamus (PVN). Besides, both receptors are express in other regions such as the septum, medial prefrontal cortex (mPFC), bed nucleus of the stria terminalis (BNST), hippocampus, paraventricular nucleus of the thalamus (PVT), and (VTA) (53). Regarding the signaling pathway, when orexin-A or -B couples their respective receptors, it generates a change in membrane potential producing slow and long-lasting depolarization (62) which may generate or increase the neuronal firing. In this manner, orexin neurons are considered excitatory neuropeptides. Their study in narcolepsy and insomnia during the first decade of the 21st century dramatically increased research in this area. A few mechanisms underlying post-synaptic depolarization by orexin have been postulated, such as 1) closure of K+ channels active at rest, 2) activation of an electrogenic sodium-calcium exchanger (NCX), and 3) the activation of non-selective cation channels (NSCCs) (63).

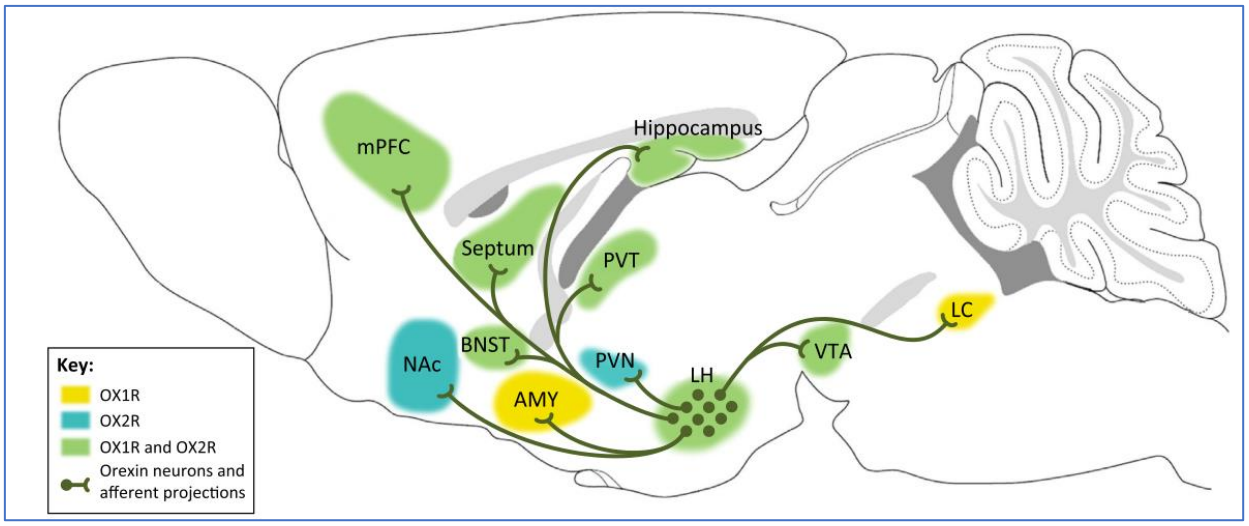

Figure 3. Representation of orexin projections and receptor distribution. Taken from Flores et al., (53).

1.2.2 Orexinergic neurons and brain physiology

As a result of these initial studies that demonstrated the implication of the orexinergic system and its receptors in critical physiological processes such as sleep-wake status, metabolism, and feeding behavior (54,64,65), in recent years, other hey processes have been linked to orexin function; namely learning and memory, motivation, emotion and fear response (66–68), and even a role on pathological processes of various neurological and neuropsychiatric diseases (69–71). Considering the large afferent and efferent projections of orexin cells, they play a crucial role in the internal/external regulation of states of vigilance and metabolism (feeding, energy expenditure, temperature, and others) (51,72,73).

In the case of sleep and circadian rhythm, orexin neurons send dense projections to the monoaminergic and cholinergic nuclei involved in sleep regulation, including the tuberomammillary nucleus (TBN) -histaminergic, LC - noradrenergic, Raphe –serotonin, and laterodorsal/pedunculopontine tegmental nuclei (LDT/PPT) –cholinergic (58). This research area has dramatically contributed to narcolepsy studies, caused by orexin deficiency, and provides an initial understanding of its physiology. Orexin potentiates wakefulness, suppresses non-Rapid Eye Movement (non-REM and REM sleep), and promotes electroencephalographic (EEG) desynchronization (74,75).

Regarding cognition, *in vitro* studies have shown that orexin induces cortical activation in mPFC, and injection in the same area induces an increased attention process in rats (54). Also, treatment studies with orexin-A reverse memory deficits in orexin/ataxin-3 mice (66). Other studies suggest orexin alteration and AD (i.e., the number of orexin cells is reduced by 40% in AD, and CSF concentration is reduced by 14% (76)). Besides, AD patients have increased sleep deterioration compared to controls, as a relation between sleep structure deterioration and cognitive dysfunction. Also, this study found a highly positive correlation between increased orexin and total tau protein levels, suggesting that overexpression of orexin results from an imbalance of the neurotransmitter networks regulating the wake-sleep cycle toward the orexinergic system.

1.2.3. Orexin and Cholinergic system

As mentioned above, orexin neurons project entirely to the brain, implicated in sleep/wake status and other processes. One of the main sites that receive projections from these cells in the lateral hypothalamus is the basal forebrain (BF) and the cholinergic system. Which coincidentally regulates similar processes in the brain. One of the first studies demonstrating orexin activation of cholinergic cells in BF was Eggermann (77). In this research, they use electrophysiological studies to confirm projections in the magnocellular preoptic nucleus of the BF, a major component of the BF-cortical projections. Orexin-A and -B stimulation produce responses in these cells, which later were confirmed to be cholinergic by immunofluorescence (77). However, previous anatomical reports suggest orexin-cholinergic innervation (78). Interestingly, disruption of orexin signaling in the basal forebrain impairs the cholinergic response to an appetitive stimulus (79), which strengthens the study of this other system, together with orexin.

In the past, it has been proposed that dysfunction in orexin-cholinergic interactions may play a role in arousal, wakefulness, and attentional deficits, which are associated with neurodegenerative conditions and cognitive decline (79). Nowadays, it is known that orexin-A microinjection in the BF induces a cholinergic response, increases acetylcholine release, and EEG desynchronization through orexin receptors (OX1R) (80). Besides, orexin current produces a significantly high frequency, including gamma, to cholinergic

and other cells (i.e., serotoninergic) (81). Orexin actions seem to reinforce thalamocortical states supporting arousal, REM sleep, and intracortical gamma patterns by boosting the encoding of high-frequency synaptic inputs to cholinergic neurons, helping their fire during EEG activation (81). Thus, promoting increased attention by orexin projections to cholinergic BF cells (82).

1.3 Cholinergic system

The cholinergic system has an essential role in cortical modulation, information processing, and cognition (83). The cholinergic acetyltransferase (ChAT) cells are part of local circuits (interneurons) and distal circuits (ChAT projections) (84). The first group of cholinergic interneurons plays a vital role in regulating brain substrates such as the striatal network, even if those cells represent a small amount (1-2%), this is possible due to the widely ramified morphology and a high release of acetylcholine (ACh), by single neurons (85).

The cholinergic system innervates most of the brain areas from two principal regions: the brainstem cholinergic projections, including the pedunculopontine nucleus and the laterodorsal pontine tegmental nucleus (86) and the basal forebrain (BF) area, which is the primary cholinergic output in the CNS to the cortex (87). The BF includes dispersing groups of ChAT cells (i.e., horizontal and vertical limb nucleus of diagonal band of Broca (HDB, and nvlDBB, respectively), medial septal, nucleus basalis of Meynert (nBM), pontine-mesencephalic nuclei, substantia innominata (SI), ventral pallidum and NAc). Given the widespread innervation from ChAT cells, it is not surprising that it modulates various neuronal functions, including learning and memory, attention, wakefulness and sleep, emotion, motivation, and sensory information (83,84,88–92).

1.3.1 Acetylcholine receptors

Cholinergic signaling is produced by the presynaptic release of ACh that activates two classes of transmembrane macromolecular ACh receptors, nicotinic (nAChR) and muscarinic (mAChR) (83,93). Both receptors are express pre, post, and peri-synaptically (94,95). The first group is an ionotropic receptor with fast neuronal actions by altering

cation channel currents. nAChRs are very widespread and non-uniform distributed and were the first described receptor (96). nAChRs are pentameric receptors formed from a combination of five membrane-spanning units consisting of nine isoforms of α subunits (α2–α10) and three isoforms of β subunits (β2–β4) and assembled either as heteromers or homomers (97). nAChR subtypes share a basic structure, but their biophysical and pharmacological properties depend on their subunit composition; the most predominant homomeric α7 and heteromeric α4β2, which are widely distributed nAChRs (86).

The second group (muscarinic acetylcholine receptors – mAChRs) are a class I heptahelical GPCRs with five distinct subtypes (M1-M5) encoded in separate genes (86). The mAChRs display unique but not exclusive expression patterns in the CNS (98). These mAChRs are integral membrane proteins with seven transmembrane segments that form a specific location for ACh binding, with high affinity and activating the intracellular G protein cascade (86). Using modified mouse models was possible to elucidate specific functions about mAChRs, for example, M1mediate ACh-induced MAP kinase activation, a process essential for memory, and its deficiency (M1 mAChR -KO mouse), shows elevated dopaminergic transmission in the striatum and increased response to amphetamine (98).

As mentioned above, the expression of mAChRs differs depending on brain areas, where M1, M2, M3, and M4 are expressed in the hippocampus. In contrast, M1, M2 are widely expressed in the neocortex of mice. M4 also express in the neocortex but with considerably lower expression (92,93). Regarding the human cortex, M1 and M2 are highly expressed in superficial layers (92). The existence of different AChRs combinations, and even the variations of expressions in one single cell, strongly suggest that nAChRs acts in a specific manner.

1.3.2 Basal Forebrain projections to the cortex

As previously mentioned, all cortical areas receive projections from BF containing cortically projecting cholinergic and non-cholinergic neurons. However, recent evidence supports that specific projections between different BF nuclei and their cortical targets are necessary to modulate precise cognitive functions in the cortex (89). Besides, these areas differ in the density of cholinergic projections; for example, limbic and paralimbic areas such as the amygdala, hippocampus, or sensory and motor cortex have a high density of

cholinergic projections (92). In the cortical modulation, the BF has two groups of cells (cholinergic and non-cholinergic), and its projections play a role in both excitatory/inhibitory variability, for example, promoting wakefulness by cholinergic activation. On the contrary, sleep and cortical inhibition are elicited by inhibitory projections of gamma-aminobutyric (GABA) neurons from BF (99). This variability is usually measured by EEG desynchronization in wakefulness and REM states, with a full ACh release and decreasing during slow-wave sleep (SWS) (93).

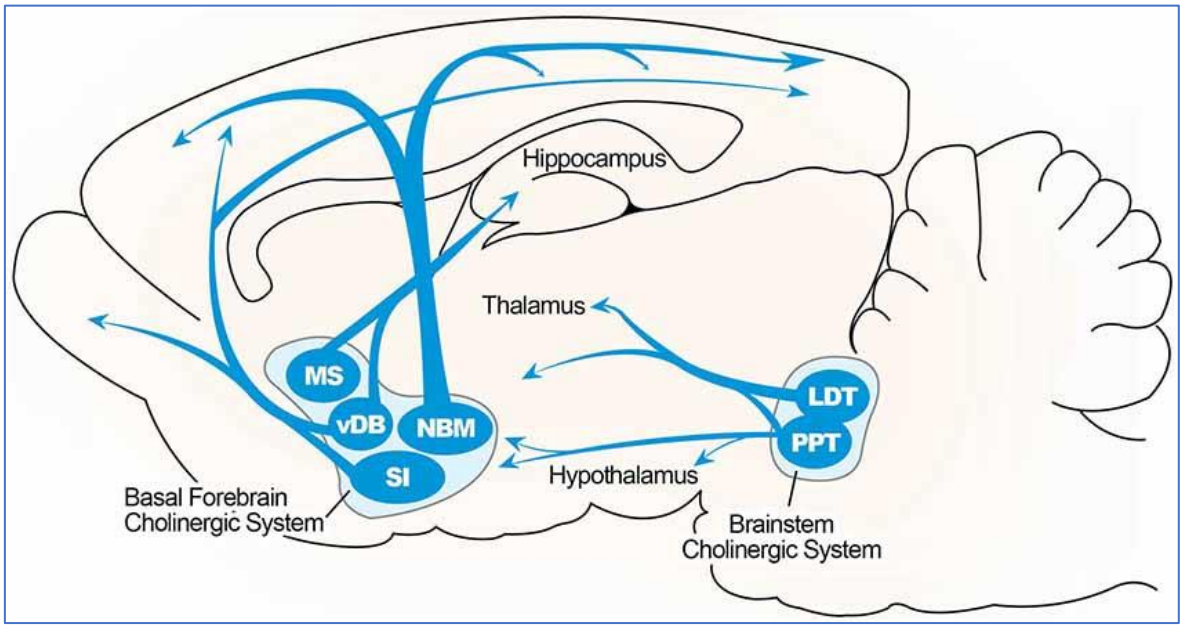

Figure 4. Representation of the basal forebrain and cholinergic pathway and projections. Taken from Paul et al., (100).

Also, retrograde tracer studies suggested a topographical organization of the BF projections to the cortex (101). ChAT cells exert a networking-coordinating signal in the cortex, but how ACh innervation modulates higher cortical functional organization is still unknown. Despite the innervation of ChAT cells in somatosensory -S1, visual -V1, and auditory -A1 areas, that seems to be related to specific BF projections and might have a similar firing rate in primary areas (89,102). Nonetheless, the cortex's secondary or tertiary association areas could have different firing rates of ACh, AChRs densities in cortical layers, and post-synaptic targets, complicating the cholinergic system's role in information processing and cognition or even have different disease vulnerabilities (103).

Laszlovsky et al. (104) discover two distinct cholinergic cell types in BF by recording *in*

vivo and *in vitro*. The first group was called "Burst-cells," an exhibited early firing in response to current injections *in vitro*, irregular action potentials (APs) *in vivo*, within-cell-type synchrony, and a strong correlation to cortical theta oscillation an auditory detection task. The second type with regular rhythmic activity was called "Reg-cells," located at the posterior BF. This type showed late firing in response to current injections *in vitro*, which could not be transformed into burst mode. Reg-cells showed largely asynchronous firing and synchronization with auditory cortical local field potentials (LFPs). Consequently, differences in the firing mode, synchrony, anatomical distribution of distinct cholinergic neuron types, and acetylcholine receptor densities might produce differential regulation of cortical activity and behavior (104).

This work studies the HDB area in mice, mainly related to primary sensory cortices with specific projections to somatosensory, visual, or auditory primary cortices according to the rostrocaudal neuronal distribution (93). Most HDB projections have many neurons that innervate the S1 cortex, indicating that this area is specialized in sensory processing of somatosensory stimuli (105). Previous studies also show that HDB projections are bilateral, orchestrating the modulation of cortical sensory responses, where BF may coordinate the neuronal activity of both hemispheres and consequently enhance sensory processing (88).

1.3.3 The cholinergic system, aging & Alzheimer's disease

Normal aging is accompanied by a gradual loss of cholinergic function caused by dendritic, synaptic, axonal degeneration, and decreased trophic support (106). Thus, cholinergic dysfunction in pathological aging is related to presynaptic ChAT alterations (i.e., degeneration in the nBM), as well as alterations in post-synaptic AChRs in the cerebral cortex (103). Besides, acetylcholine release plays an important role in memory function, whereas cholinergic neurons innervate the hippocampus facilitating hippocampal-dependent learning, memory consolidation, and increasing θ activity in hippocampus, enhancing memory encoding (107,108).

Nonetheless, ACh modulation of memory and hippocampus activity is complex. Showing changes accordingly of the memory stages (i.e., encoding vs. consolidation) reflects the

importance of constant cholinergic modulation to increase ACh release during memory encoding or reduce it during memory consolidation (108). Thus, decreased ACh synthesis during aging may affect processing information, and decrements in gene expression, impairments in intracellular signaling, trophic support, decrease in cholinergic boutons, and cytoskeletal transport could mediate cholinergic cell atrophy (106,109), promoting the age-related functional decline.

Therefore, ACh has been associated with being even more crucial in executive function, and the alteration of the cholinergic outputs to the cortex is related to attentional deficits and hippocampal inputs deficits with poor encoding processing (84,110). The cholinergic-dependent interaction between bottom-up and top-down processes (enhancing bottom-up process from thalamus in relevant stimuli and inhibiting top-down irrelevant stimuli from frontal modulation (110)) appears to be affected by aging, leading to difficulty in task-switching, working memory, and divided attention.

In this context, the cholinergic hypothesis of Alzheimer's disease came up in the '70s, where presynaptic cholinergic degeneration of BF nuclei, together with neurofibrillary tangles and amyloid-β deposition, became a hallmark of the disease (111). This hypothesis is sustained by the finding of depleted presynaptic cholinergic markers in the cerebral cortex, the discovery that the nucleus basalis of Meynert (NBM) has severe neurodegeneration in AD and the demonstration that cholinergic antagonists impair memory whereas agonists have the opposite effect (103).

Dumas and Newhouse (112) suggest that this hypothesis, under findings in the last 40 years with neuroimaging and psychopharmacological studies, deserves further consideration as new methodologies for evaluating its validity are increasingly being used. Interestingly, they propose a model where cholinergic modulates frontal/occipital and bottom-up and top-down processes, which may change from young to old, promoting and increasing the recruitment of frontal cholinergic projections (overactivation). But in mild cognitive impairment (MCI) and AD, cholinergic dysfunction leads to attentional deficits, which cholinergic recruitment could not compensate (112).
Consequently, this model put together the role of neuronal degeneration, cholinergic stimulation, and cognitive performance. In healthy older adults, BF volume is associated

with general cognition, and the integrity of this structure may contribute to brain resistance to cognitive decline in Alzheimer's disease (113).

Secondly, aging affects different characteristics of the cholinergic system, but all the pathology precursors are still unknown. Some studies suggest tau accumulation in BF starts early in AD, as amyloid-β deposition in the projections sites was observed (113). Besides, AD is characterized by a reduction in BF's nicotinic and muscarinic receptors in cholinergic cells (114). As previously mentioned, there are two types of abundant cholinergic receptors, the α7 nAChRs, and the α4β42 nAChRs. The first one is related to anti-inflammatory response, whereas the second one modulates cognition. Postmortem analysis has shown a reduction of both receptors in AD brains (115).

The cholinergic cells in BF (nBM) and their efferent targets in the entorhinal cortex (EC) exhibit particularly susceptibility to damage under neurofibrillary degeneration in the early stages of AD (106) but a debate of which area may precede to the other was under debate. Schmitz et al. (116) suggest that BF degeneration precedes and predicts further both entorhinal pathology and memory impairment, challenging the widely held belief that AD has a cortical origin. This result is fascinating, considering the relation with orexinergic inputs from the lateral hypothalamus. For example, the participation of the hypothalamus in AD is suggested by an increased volume of the third ventricle and synaptic loss and dendritic sensitivity of different hypothalamic nuclei, typically related to desynchronization of circadian rhythms (117). This suggesting that early alteration from hypothalamic projections may contribute to cholinergic disturbances, associating both to metabolic changes during aging, perturbed energy balance, sleep/wake alterations, and others.

1.4 IGF-I, Orexin & Cholinergic system participation in neurological diseases

Aging-associated pathology and Alzheimer's disease share common features such as cognitive impairments, memory loss, bioenergetic deficits, inflammation, and metabolic component (118). About the latter, metabolic alteration and AD manifest potential underlying mechanisms to support a link between both conditions (119). On the other hand, age is the more significant risk factor for AD (120). But other risk factors associated

with aging, such as hypertension, obesity, or diabetes, also display a link to metabolic function. Surprisingly, other important risk factors are related to life-styles factors (as less education, smoking, depression, sleep disturbances, and low social interaction) (121).

It is possible that all these risk factors engage insulinemic pathways that affect brain function, providing a potentially unifying thread for life-style and AD risk (30). Along this line, we postulated that insulin-like growth factor I (IGF-I), a hormone closely related to insulin, is also an important player in AD pathology (122). During our search for brain substrates of IGF-I actions on brain function, we observed an intriguing connection with orexin and cholinergic neurons that led us to propose that IGF-I actions onto these systems may provide a connection between cortical function, sleep disturbances, and cognition in aging and AD.

We hypothesized that IGF-I is a central process in brain homeostasis regulating peripheral and central metabolic communication in cortical activation and sleep, where IGF-I and orexin axis may form part of a feedback loop between the periphery and the hypothalamus to control the circadian pattern of activity. Besides, orexin neurons in the aged brain may also lose sensitivity to this hormone, which, coupled with age-associated reduced serum IGF-I, would aggravate IGF-I loss-of-function and eventually affect sleep/wake patterns. On the other hand, we also hypothesized that IGF-I facilitates cholinergic activity, modulating arousal and cortical activity by activating BF neurons, and this modulation decreases during aging. To probe our hypothesis, we want to determine the actions of IGF-I on orexin/cholinergic neurons, their impact on cortical areas, involvement in sleep regulation, and underlying mechanisms through aging. The latter is essential to identify specific perturbances in the future related to AD.

In this context, the present thesis represents an effort to elucidate the mechanism related to the IGF-I/orexinergic system and the IGF-I/cholinergic system. We studied both neuronal systems and their connections to IGF-I in aging, analyzed their interaction mechanisms, and tested possible treatments in the aging brain context.

HYPOTHESES AND OBJECTIVES

2. HYPOTHESES AND OBJECTIVES

2.1 MAIN HYPOTHESIS

1. IGF-I is a central process in brain homeostasis involved in peripheral and central communication. This interaction allows IGF-I to be a vital regulator of the orexinergic and cholinergic systems.

2. Aging and Alzheimer's disease pathology is related to insulin-like growth factor-I disturbance through aging. Its alteration in orexinergic and cholinergic systems affects cortical activation, sleep architecture, and information/sensory processing.

2.2 GENERAL & SPECIFIC OBJECTIVES

2.2.1 To understand the mechanisms relationships between IGF-I and sleep/wake cycle in orexinergic neurons.

- To validate a transgenic model of Cre/Lox mice lacking functional IGF-I receptors in orexin neurons (Floxed IGF-IR/Orexin Cre: Firoc mice).
- To determine how IGF-I modulates orexinergic activity.
- To investigate the role of IGF-I on sleep architecture.

2.2.2 To evaluate if reduced IGF-I input to orexin neurons during aging contributes to age-associated sleep and cortical activation disturbances.

- To examine changes in sleep architecture in old mice due to IGF-I disturbances.
- To characterized orexinergic neurons alteration in old mice due to IGF-IR disturbances.
- To determine if IGF-IR sensitization recovers orexin response to IGF-I in old mice.

2.2.3 To analyze if IGF-I facilitates cholinergic activity that modulates arousal and cortical activation by BF neurons.

- To examine cholinergic activity from the HDB area during aging in response to IGF-I.
- To determine how IGF-I specifically modulates cholinergic activity.
- To investigate the role of IGF-I on sensory processing.
- To determine if IGF-I chronic treatment recovers cholinergic response to IGF-I in old mice.
- To determine if IGF-I reduced effect during aging is specific to this peptide in cholinergic neurons.

RESULTS

3. RESULTS

3.1. Study 1

Zegarra-Valdivia, J., Pignatelli, J., Fernandez de Sevilla, M. E., Fernandez, A. M., Munive, V., Martinez-Rachadell, L., Nuñez, A., & Torres Aleman, I. (2020). Insulin-like growth factor I modulates sleep through hypothalamic orexin neurons. FASEB Journal, fj.202001281RR. https://doi.org/10.1096/fj.202001281RR

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RESEARCH ARTICLE

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Insulin-like growth factor I modulates sleep through hypothalamic orexin neurons

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Abstract

Although sleep disturbances are common co-morbidities of metabolic diseases, the underlying processes linking both are not yet fully defined. Changes in the duration of sleep are paralleled by changes in the levels of insulin-like growth factor-I (IGF-I), an anabolic hormone that shows a circadian pattern in the circulation and activity-dependent entrance in the brain. However, the specific role, if any, of IGF-I in this universal homeostatic process remains poorly understood. We now report that the activity of orexin neurons, a discrete cell population in the lateral hypothalamus that is involved in the circadian sleep/wake cycle and arousal, is modulated by IGF-I. Furthermore, mice with blunted IGF-I receptor activity in orexin neurons have lower levels of orexin in the hypothalamus, show altered electro-corticographic patterns with predominant slow wave activity, and reduced onset-sleep latency. Collectively, these results extend the role in the brain of this pleiotropic growth factor to shaping sleep architecture through the regulation of orexin neurons. We speculate that poor sleep quality associated to diverse conditions may be related to disturbed brain IGF-I input to orexin neurons.

KEYWORDS: IGF-I, orexin neurons, sleep

1| INTRODUCTION

Metabolism and sleep are functionally linked.[1,2] Anabolic signals of the somatotropic axis encompassing pituitary growth hormone (GH), and liver insulin-like growth fac- tors I and II (IGF-I and IGF-II), together with the other components of this axis, the hypothalamic peptides GHRH and somatostatin, have been reported to participate in sleep regulation in various ways. Thus, GH secretion is increased during early sleep phases,[3] but without affecting it.[4] IGF-I, together with GHRH and somatostatin, have been variously reported to either stimulate or decrease different stages of this universal homeostatic process,[5-8] while little is known of a possible role of IGF-II in sleep. Significantly, the link between IGF-I and sleep spans the evolutionary tree,[9-11] and at least in mammals, sound sleep increases circulating IGF-I, whereas reduced sleep impairs muscle IGF-I levels,[12-14] suggesting a bidirectional interaction between sleep and IGF-I (and probably other members of the somatotropic axis). However, the mechanisms underlying these relationships are still undefined.

In our search for a mechanistic understanding of the relationship of IGF-I with the sleep/wake cycle, we turned our attention to orexin neurons (also termed hypocretin neurons) located in the Perifornical area (PeF) in the lateral hypothalamus.[15,16] These neurons are key regulators of a wide range of physiological functions, such as the sleep/wake cycle[17] or physical activity,[18] and receive multiple endocrine modulatory inputs, including insulin, a hormone closely related to IGF-I.[19] Orexin neurons also express IGF-binding protein 3, a major regulator of IGF-I bio-availability,[20] suggesting that they are targeted by this growth factor.

Since orexin neurons are involved in the facilitation and/ or maintenance of arousal,[21-23] we hypothesized that IGF-I may form part of a feedback loop between the periphery and the hypothalamus to control the circadian pattern of activity by modulating orexinergic function. Indeed, circulating IGF-I enters into the brain in response to physical activity,[24] through an activity-dependent process.[25] Furthermore, recent evidence indicates that circulating IGF-I shows a circadian pattern,[26] and is a circadian entrain cue,[27] affecting hypothalamic clock genes.[28]

In this work we used in vivo recordings, and genetic in- activation of the IGF-I receptor (IGF-IR) in orexin neurons through the Cre/Lox system and determined that IGF-I stimulates their activity, ultimately shaping the sleep architecture. Our results help to explain the functional link between IGF-I and the sleep/wake cycle.

2| MATERIALS AND METHODS

Materials

Antibodies used in this study include rabbit polyclonal c-Fos (Abcam ab190289), rabbit polyclonal IGF-I Receptor-β (Santa Cruz 713/AC) and rabbit anti-IGF-I receptor β XP (9750, Cell Signaling Technology, USA), orexin polyclonal goat antibody (Santa Cruz 8070), orexin polyclonal rabbit antibody (Abcam ab 6214), orexin monoclonal mouse antibody (Santa Cruz 80263), cre recombinase monoclonal mouse antibody (Millipore MAB 3120, clone 2d8), as well as anti-pAkt (Cell Signaling, 9271S), and monoclonal antiphosphotyrosine (clone PY20, BD Transduction laboratories, USA). All antibodies used are thoroughly validated by the manufacturers (see supporting references for each antibody in the respective web sites). Human recombinant IGF-I was from Pre-Protech (USA).

Animals

Adult female and male C57BL/6J mice (Harlan Laboratories, Spain), Cre/Lox mice lacking functional IGF-I receptors in orexin neurons (Floxed IGF-IR/Orexin Cre: Firoc mice), and channelrhodopsin (ChR)/Orexin-Cre mice (Ox-ChR mice) were used (3-6 months old; 26-33 g). Littermates were used as controls. In a subset of electrophysiological experiments, wild-type mice were included in the control group since no differences were seen with littermates (see results). The estrous cycle of the female mice was not controlled. Experiments were done during the light phase, except when indicated.

Firoc mice were obtained by crossing Orexin-Cre mice (a kind gift of T Sakurai, Tsukuba Univ, Japan) a mouse model extensively used,29-31 with IGF-IRf/f mice (B6, 129 backgrounds; Jackson Labs; stock number: 012251; exonfloxed). Cre recombination of the floxed IGF-IR results in deletion of exon 3, functional inactivation of the receptor32 and substantial reduction of IGF-IR expression.33 ChR mice were obtained from L Menendez de la Prida (Cajal Institute) and crossed with Orexin-Cre mice. Genotyping of Firoc mice was performed using the primers: 5′-GGT TCG TTC ACT CAT GGA AAA TAG-3′ and

5′-GGTATCTCTGACCAG AGTCATCCT-3′ for Orexin- Cre and: 5′-CTT CCCAGCTTGCTACTCTAGG-3′ and 5′-AGGCTTGCAATGAGACATGGG-3′ for IGF-IRf/f. Ox-Chr mice were genotyped using the same Orexin-Cre primers, ChR primers: 5′-GCATTAAAGCAGCGTATCC-3′ and 5′-CTGTTCCTGTACGGCATGG-3′, and wild-type primers: 5′-AAGGGAGCT GCAGTGGAGTA-3′, and 5′-CCGAAAATCTGTGGGAAGTC-3′. We confirmed Cre-mediated recombination in the IGF-IR locus between the two sequences flanking the IGF-IR exon-3 only in the hypothalamus of Firoc mice using the previously de- scribed primer pair P3 (5′-TGAGACGTAGGGAGATT GCTGTA-3′) and P2 (5′-CAGGCTTGCAATGAGACATGGG-3′).[32] These primers amplify a fragment of 320 bp only when recombination has occurred. Furthermore, in situ hybridization using RNAscope (2.5 HD Detection kit—Red; #322350; ACD, USA) with an IGF-IR exon 3-specific probe combined with immunocyto- chemistry with anti-orexin antibodies was also performed to confirm the deletion of exon 3 in orexin cells.

DNA from brain tissue was isolated using TRIzol Reagent and ethanol precipitation. A total of 10 ng of genomic DNA was used in a PCR reaction containing 1X reaction buffer, 1 nM of each primer, 0.2 mM of dNTPS, and 0.75 µL of DFS- Taq DNA polymerase (Bioron, GmbH). The thermocycler program was 92°C, 3 minutes and 30 cycles of 94°C, 30 seconds; 65°C, 30 seconds; 72°C, 30 seconds, and after that, a final extension step at 72°C for 2 minutes was performed. Amplicons were analyzed in 3% agarose gels stained with SYBRsafe (Thermo fisher).

Animals were housed in standard cages (48 × 26 cm2, 5 per cage), and kept in a room with controlled temperature (22°C) under a 12-12 hours light-dark cycle. Mice were fed with a pellet rodent diet and water ad libitum. Animal procedures followed European guidelines (2010/63, European Council Directives) and were approved by the local Bioethics Committee (Government of the Community of Madrid).

Administration of IGF-I

IGF-I was dissolved in saline and intraperitoneally (ip) injected (1 µg/g body weight). In some experiments, mice were processed for immunocytochemistry (see below) 1 or 2 hours after ip IGF-I injection to allow the expression of phospho- Akt or c-fos, respectively, whereas in other experiments PeF recordings were carried out immediately

after ip injection. Alternatively, IGF-I was unilaterally delivered in the PeF (10 nM; 0.1 µL; coordinates from Bregma: A, −1.95; L, 1 and depth, 4.0-4.5 mm), and injected animals were thereafter submitted to unilateral electrophysiological recordings as de- scribed below. Doses were selected based on previous work using pharmacological injections in both systemic and local/ intraparenchymal administration.[25]

Recordings in anesthetized animals

Mice were anesthetized with isoflurane (2% induction; 1%- 1.5% in oxygen, maintenance doses), placed in a David Kopf stereotaxic apparatus (Tujunga, CA, USA) in which surgical procedures and recordings were performed, with a warming pad (Gaymar T/Pump, USA) set at 37°C. Local anesthetic (lidocaine 1%) was applied to all skin incisions and pres- sure points. An incision was made exposing the skull, and small holes were drilled in the skull. Tungsten macroelectrodes (<1 MΩ World Precision Instruments, USA) were used to record the local field potential and the evoked potential in the PeF (coordinates from Bregma: A, −1.95; L, 1 and depth, 4.0-4.5 mm). Recordings were filtered (0.3- 50 Hz) and amplified via an AC preamplifier (DAM80; World Precision Instruments). The LC was stimulated using 120 µm diameter stainless steel bipolar electrodes (World Precision Instruments, coordinates from Bregma: A, −5.4; L, 1 and depth, 4.0-4.5 mm). Electrical stimulation was carried out with single square pulses (0.3 ms duration and 20-50 µA intensity, delivered at 1 Hz; Cibertec Stimulator, Spain). After a basal recording, insulin or IGF-I were injected locally (Firoc = 13, control = 20) or systemically (Firoc = 10, control = 10).

Optogenetics

Optogenetic experiments were performed to identify orexin neurons through light activation using Ox-ChR mice (see Figure 5). Animals were anesthetized with isoflurane, positioned in the stereotaxic apparatus, and handled as above. The sagittal midline of the scalp was sectioned and retracted, and a small craniotomy was drilled over the perifornical (PeF) hypothalamic area (same stereotaxic coordinates as above). Optical stimulation of ChR-expressing neurons was achieved with light-emitting diodes (LED; Thomas Recording, Germany) delivered through an optrode, composed of a tungsten microelectrode of 0.5-0.8 MΩ attached to an optical fiber (core diameter 120 µm; Thomas Recording). Unit recordings were performed through the optrode, filtered (0.3-3 KHz), and amplified using a DAM80 preamplifier (World Precision Instruments). Single-unit activity

was extracted with the aid of Spike2 software (Cambridge Electronic Design, UK) and sampled at 10 KHz via an analog-to-digital converter built into the Power 1401 data acquisition unit and fed into a PC computer for off-line analysis with Spike 2 software. The LED was triggered with a square-step volt- age command. Stimulation was applied by a single long- lasting pulse of 473 nm light (16 stimuli with a duration of 300 ms) with an illumination intensity of <30 mW/mm2, which is below the damage threshold of ~100 mW/mm2 for blue light.34 The stimulation area was very restricted since total transmitted light power was reduced by 50%, after passing through 100 µm of neuronal tissue, and by 90% at 1 mm.35 When LED stimulation was applied to the cortex of Ox-ChR mice, no stimulation was observed.

Electrocorticogram (ECoG) recordings in freely moving animals

Mice were anesthetized as indicated above and placed in a stereotaxic device. The skin was cut along midline and a craniotomy made (0.5 mm diameter) in the area of the primary somatosensory area (S1). A stainless-steel macroelectrode of <0.5 MΩ was placed without disrupting the meninges to register the cortical electrical activity, using a DSI Implantable Telemetry device (Data Sciences International, USA). After surgery, mice remain in their cages a minimum of 4 days to recover. ECoG was registered for 60 minutes in 2 days (30 min per day, from 15:00-18:00). Signals were stored in a PC using DSI software and filtered off-line between 0.3- 50 Hz with Spike 2 software. ECoG segments of 5 minutes were analyzed with this software using the Fast Fourier Transform algorithm to obtain the power spectra. The mean power density was calculated for five different frequency bands that constitute the global ECoG: delta band (0.3-5 Hz), theta band (5-8 Hz), alpha band (8-12 Hz), beta band (12- 30 Hz), and gamma band (30-50 Hz). The total power of the five frequency bands was considered 100%, and the percent- age of each frequency band was calculated for the 60 minutes. To determine the global ECoG during the active phase we used the following animals: Firoc = 5, control = 5; during the passive phase: Firoc = 7, control = 12, and for the sleep pattern in the passive phase: Firoc = 7, control = 6.

To assess sleep/awake status, segments of 30 sec of the ECoG recording were analyzed according to the presence of slow waves (0.3-5 Hz), fast waves (>12 Hz), and mouse's movements. Every segment with an equal quantity of slow/ fast waves was considered a transition phase. The total % of events (sleep, transitions, or awakening state) were measured (Firoc = 6, control = 9). Latency to sleep-onset was measured as the time elapsed

between the moment the animal was placed on the recording platform until the appearance of continuous slow waves in the ECoG (Firoc = 6, control = 9). Thus, during mobility periods recordings show abundant high amplitude waves. Conversely, immobility was characterized by high occurrence of low amplitude or delta waves of less than 100 microvolts, and absence of movements as assessed by monitoring the animals. ECoG recordings were performed every 2 hours through a telemetry device.

Data analysis

Evoked potentials elicited by LC electrical stimulation (20- 50 μA; 0.3 ms duration; at 1 Hz) were calculated. The peak latency was calculated as time elapsed between the stimulus onset and the peak of the second evoked potential wave (orthodromic response, with a latency of 3.5 ± 0.81 ms). To quantify the orthodromic response, the area under the curve of the positive wave was measured from the beginning of the positive slope. Moreover, single-unit responses were measured from the peri-stimulus time histogram (PSTH; 1 ms bin width; 16 stimuli) as the number of spikes evoked during the 0-100 ms or 100-200 ms time-windows after stimulus onset (blue-light pulse). Plots of the unit activity show the percent- age of variation respect to basal period (5 min). In optogenetic experiments, outliers and recordings that did not elicit at least a 70% of increment activity were removed from the analysis to minimize the interference from non-orexinergic neurons.

Immunoassays

Immunocytochemistry

Animals were deeply anesthetized with pentobarbital (50 mg/kg) and perfused transcardially with saline 0.9% and then, 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). Coronal 50-μm thick brain sections were cut in a vibratome and collected in PB 0.1 N. Sections were incubated in permeabilization solution (PB 0.1N, Triton X-100, NHS 10%), followed by 48 hours incubation at 4°C with primary antibody in blocking solution (PB 0.1N, Triton X-100, NHS 10%). After washing three times in 0.1 PB, Alexa-coupled secondary antibodies (1:1000, Molecular Probes, USA) were used. Finally, a 1:1000 dilution in PB of Hoechst 33342 was added for 3 minutes. Slices were rinsed several times in PB, mounted with gerbatol mounting medium, and allowed to dry. Omission of primary antibody was used as control. Confocal analysis was performed in a

Leica (Germany) microscope. For double stained orexin/c-fos or orexin-cre counting, four sections per animal were scored using the Imaris software, as described.36

ELISA

Hypothalamus from control and Firoc mice were dissected on ice and immediately frozen on dry ice and kept at −80°C until use. Orexin concentration in hypothalamus was determined using Orexin-A ELISA (Phoenix pep- tides, Inc). Peptide extraction was performed following the manufacturer′s instructions. Briefly, tissue was homogenized in 1 N acetic buffer and boiled 20 minutes at 100°C. Samples were then filtered through a Seppak C-18 cartridge (Sep- Pak, Millipore, USA), eluted in 2 mL of methanol and dried using centrifugal concentration (SpeedVac). Each sample was rehydrated in 150 µL of ELISA buffer. Samples were assayed in duplicate. Values were normalized with respect to the total protein on each sample determined by the BCA method (Sigma-Aldrich).

Cell sorting of neurons from adult mouse brain

Hypothalami of >P30 mice were dissected, cut into small pieces in 1 mL of iced EBSS, and centrifuged at 300 g/4°C/5 min. Tissue was dissociated with papain for 15 minutes at 37°C, and mechanically disaggregated with a wide-tipped glass pipette for 10 times. The resultant cell suspension was incubated for 15 minutes more and further dissociated with smaller-tipped glass pipettes twice. The cell suspension was placed in a 70 µm strainer with 10 mL of EBSS, centrifuged at 600 g/4°C/5 min, and resuspended in 1 mL of PBS. Cells were placed in a 15 mL tube with a Percoll gradient, as described.37 After centrifugation at 430 g/4°C/3 min, the two top layers were removed, and the bottom layer resuspended with PBS. The tube was inverted three times and centrifuged at 550 g/4°C/5 min. Erythrocytes present in the cell suspension were lysed in 1 mL of Red blood cell lysis buffer (Miltenyi Biotec, 130-094-183; 1x) for 2 minutes at room temperature (RT) in a 1.5 mL tube, and centrifuged at 600 g/4°C/5 min.

For immunostainings, cells were fixed in 1% PFA in PBS at 4°C/10 minutes, and blocking buffer (10% FBS, 0.1% Triton, AzNa 0.01%, PBS) added for 15-30 minutes, at 4°C. Cells were then incubated for 1-2 hours with an anti-rabbit orexin antibody, washed twice with 1 mL blocking buffer and centrifuged at 600 g/5 min. The same incubation and washing conditions were applied for all subsequent antibodies. The pellet was resuspended in APC conjugate antibody in blocking buffer. After 45 minutes of incubation in ice and two

washings, cells were resuspended in 300 μL of PBS and sorted by cytometry in a FACS Aria cytometer (BD Biosciences). Two cell populations from the hypothalamus were sorted; that is: orexin+ and orexin−, DNA extracted and submitted to PCR using the primers P2-P3, as described above.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 soft- ware (San Diego, CA, USA), and R Package (Vienna, Austria). Depending on the number of independent variables, normally distributed data (Kolmogorov-Smirnov normality test), and the experimental groups compared, we used either Student's *t* test or two-way ANOVAs followed by Sidak's multiple comparison test. For non-normally distributed data, we used the Mann Whitney U test for comparing two groups, Kruskal-Wallis or Friedman test, with Dunn's multiple comparisons as a Post Hoc analysis, as well as Scheirer-Ray Test, a nonparametric alternative to multifactorial ANOVA. Sample size for each experiment was chosen based on previous experience and aimed to detect at least a $P < .05$ in the different tests applied, considering a reduced use of animals. Results are shown as mean ± standard error (SEM) and *P* values coded as follows: $*P < .05$, $**P < .01$, $***P < .001$. Animals were included in each experimental group randomly by the researcher, without blinding.

RESULTS

Inactivation of IGF-IR in orexin neurons

Since orexin neurons express IGF-I receptors (Figure 1), we inactivated them to determine their role in orexinergic function. We genetically ablated the IGF-IR activity using the Cre/Lox system following previous published procedures.[38] Mice with inactive IGF-IR in orexin neurons (Firoc mice) express a truncated IGF-IR in the hypothalamus (Figure 2A), specifically in orexin neurons, as determined by PCR detection of the truncated IGF-IR, combined with anti-orexin flow cytometry (Figure 2B and Supporting Figure S1A). We further confirmed that orexin neurons underwent Cre- recombination by in situ hybridization using an RNA probe that detects intact IGF-IR combined with orexin immucytochemistry. Indeed, orexin neurons did not express wild-type IGF-IR (Figure 2C). Cre-mediated recombination was produced mostly in orexin neurons, as double orexin/Cre immunocytochemical staining in Firoc mice shows that the majority of cells expressing Cre (Figure 2D) were orexin neurons; that is: >85 Cre+ cells were orexin+, while ~90% of

orexin+ cells were also Cre+. Furthermore, orexin responses to IGF-I were blunted in Firoc mice, as systemic injection of IGF-I resulted in Akt phosphorylation, a kinase downstream of IGF-IR,[39] in orexin neurons of littermate control mice but not in Firoc (Figure 2E). Number of orexin cells in Firoc mice and littermates was not significantly different (Figure 2F).

To confirm the functional inactivation of IGF-IR in orexin neurons of Firoc mice, we injected IGF-I (1 μg/g, ip), and 2 hours later we determined the number of orexin neurons ex- pressing c-fos. In control mice, IGF-I significantly increased the number of orexin neurons expressing c-fos (double la- beled orexin+/c-fos+ cells) as compared to saline-injected mice (Control: 4-7/group, saline: 36.61 ± 8.397; IGF-I: 54.49 ± 6.638, ***P* = .0076; see, Figure 3A, B, E). In Firoc mice a smaller, nonsignificant increase was seen (Firoc: 4-4/ group, saline: 35.68 ± 8.825; IGF-I: 47.89 ± 4.859, *P* = .1679; see, Figure 3C, D, E), indicating reduced sensitivity to IGF-I in orexin neurons. The increment in c-fos labeling of orexin neurons seen in Firoc mice after IGF-I administration probably is due to the stimulation by IGF-I of excitatory afferents to orexin neurons and/or the fact that Cre/Lox recombination of IGF-IR in mutant mice did not occur in 100% of orexin neurons (Figure 2B). Furthermore, Firoc mice had significantly lower levels of orexin in the hypothalamus (Figure 3F; **P* = .0211; Firoc = 7, Control = 9).

IGF-I modulates the activity of orexin neurons

Since in wild-type mice IGF-I significantly increases the number of orexin neurons expressing c-fos, a marker of cell activation, we determined the regulation of the activity of these neurons by IGF-I. We studied the activation of PeF neurons after locus coeruleus (LC) stimulation in Firoc mice and control littermates (Figure 4A). The LC, a major connection of orexin neurons,[40] provides feedback information to PeF neurons by facilitating its firing activity.[41] Electrical stimulation of the LC (20-50 μA; 0.3 ms duration; at 1 Hz) evoked a fast orthodromic potential in the PeF region. The orthodromic potential consisted of a positive wave with similar mean peak latency in both groups: 3.7 ± 0.7ms in control littermates (n = 22), and 3.1 ± 0.8 ms in Firoc mice (n = 11; *P* = .08). This indicates that the LC input to the PeF is unaffected in Firoc mice.

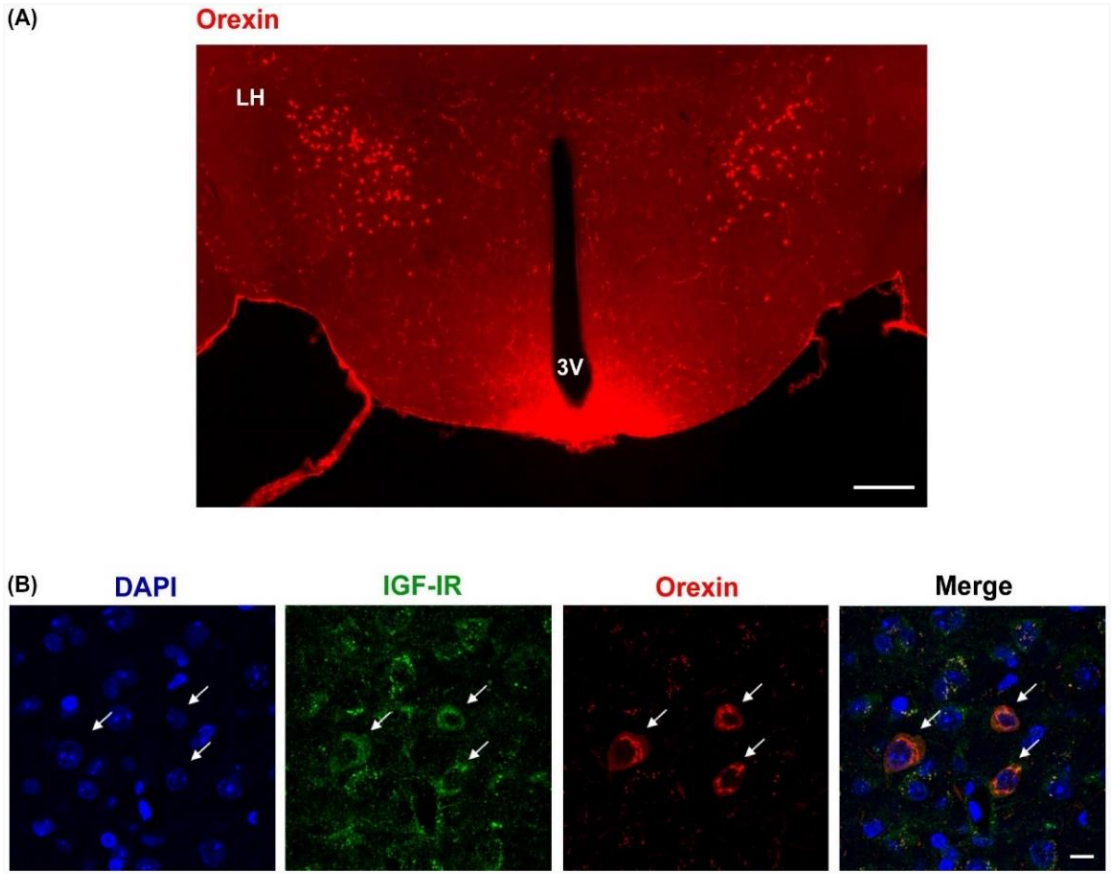

FIGURE 1 Orexin neurons express IGF-I receptors. A. Representative photomicrograph of the mouse hypothalamus showing staining of orexin neurons (red) in the PeF area of the lateral hypothalamus (LH). Note the presence of abundant orexin fibers in the median eminence, as reported by others.[78] 3v: third ventricle. Bar is 300 µm. B, Double immunocytochemistry in lateral hypothalamic sections at higher magnification show that orexin neurons (red) express IGF-IR (green). Note that many IGF-IR+ cells are not orexinergic, whereas not all hypothalamic cells (DAPI+, blue) express IGF-IR. Arrows indicate double stained orexin/IGF-IR cells. Bar is 10 µm

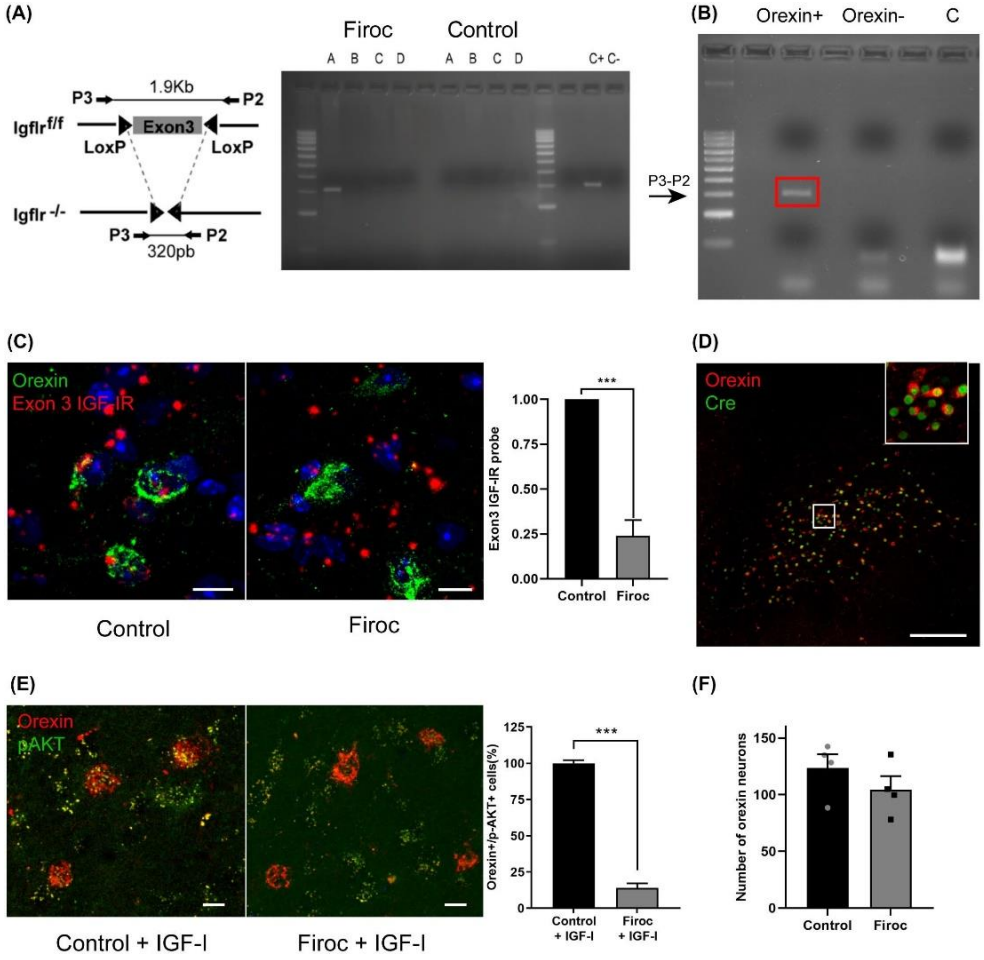

FIGURE 2 Characterization of mice expressing and inactive IGF-IR in orexin neurons (Firoc mice). A, Cre recombination in exon 3 of the IGF-IR was identified in different brain areas of Firoc and controls (littermates) using primers P3 and P2 that amplify a 320 bp fragment (left diagram) when exon 3 is deleted. Only the hypothalamus of Firoc, but not control mice, showed recombination. A: hypothalamus, B: ventral thalamus, C: dorsal thalamus, D: hippocampus. C+: positive control, astrocytes expressing IGF-IR truncated in exon 3; C-: negative control: wild-type astrocytes. B, PCR analysis using the P3-P2 primers in sorted hypothalamic cells from Firoc mice show the band of truncated IGF-IR in orexin+, but not in orexin- cells. C: negative control. C Only control littermates, but not Firoc mice, express the non-truncated IGF-IR (wild type) in orexin cells, as determined by RNAScope using an exon 3 IGF-IR-specific probe. Right upper corner: magnification showing the absence of IGF-IR exon-3 mRNA (red) in orexin cells (green). Bars are 25 µm. Right: quantification histogram showing a significant reduction in orexin neurons expressing wild-type IGF-IR including exon 3 ($Z = 3.823$; ***$P < .001$). D, Double immunocytochemistry confirmed that Firoc mice express Cre (green) in orexin neurons (red). Bar is 200 µm. E, Systemic injection of IGF-I (1 µg/g, ip) stimulates Akt in orexin neurons (red), as determined by immunoreactivity of phosphorylated Akt (pAkt, green) of control littermates, while in Firoc mice co-staining of pAkt and orexin (yellow), is significantly smaller ($t = 1.73$; ***$P < .001$). Representative micrographs and quantification histograms are shown. Bars are 10 µm. F, Number of orexin neurons was not significantly different in Firoc mice, as compared to control littermates (n = 4 animals in each group; 4-5 slices per animal)

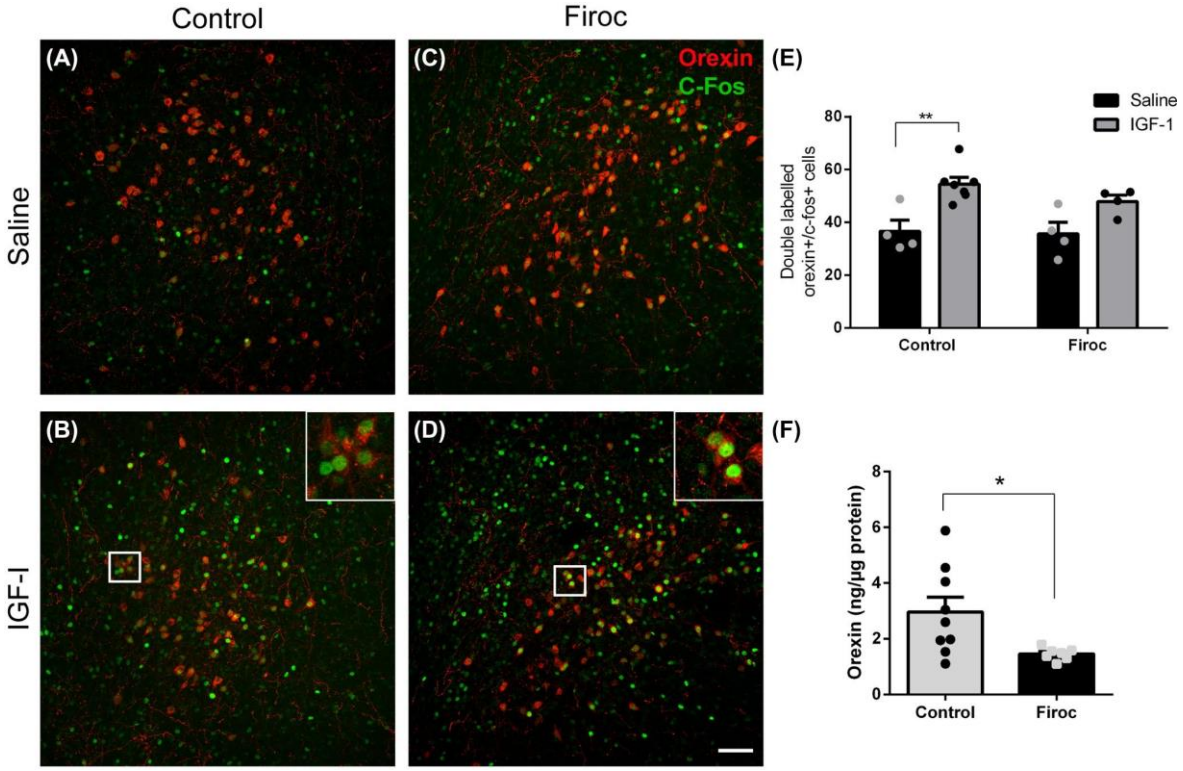

FIGURE 3 IGF-I stimulates orexin neurons. A-D, Double immunocytochemistry of orexin and c-fos in mice injected ip with IGF-I or saline (1 µg/g). Double-stained orexin/c-fos cells are shown in the upper right white square in panel B and D, at higher magnification. Bars are 100 µm. E, Quantification of orexin+/c-fos+ cells show that their number was significantly increased by IGF-I only in control littermates (F = 19.50; ***P* < .01; control = 4-7 mice/group, 4-5 slices per animal; two-way ANOVA and Tukey's test), but not in Firoc mice (*P* = .12; Firoc = 4/ group, two-way ANOVA and Tukey's test). F, Levels of orexin in the hypothalamus are significantly decreased in Firoc mice (t = 3.00; **P* < .05; Firoc = 7, control = 9; Unpaired *t* test).

However, while the latency of the evoked potential was normal in Firoc mice (Figure 4B), when we calculated its area to quantify changes in the amplitude and duration of PeF responses to LC stimulation, a decrease was observed. LC stimulation evoked a response area of 74.6 ± 11.9 µV2 (n = 22) in control mice, and a significantly lower in Firoc mice: 30.6 ± 2.7 µV2 (n = 11; **P* = .0315; Figure 4C).

Importantly, local injection of IGF-I (10 nM; 0.1 µL), but not of saline (Supporting Figure S1B,C), in the PeF region of control mice increased the response to LC stimulation (n = 22; ****P* < .001; Figure 4D). In contrast, Firoc mice (n = 11) were not affected by local injection of IGF-I in the PeF area (Figure 4D). To determine whether systemic IGF-I can also stimulate PeF responses to LC stimulation, we injected ip IGF-I (1 µg/g). While in

Firoc mice ip IGF-I did not affect LC responses in the PeF area (n = 10), in control animals ip IGF-I significantly increased the response area (n = 10; *P < .05, Figure 4E). IGF-I-evoked facilitation in control mice was specific for this growth factor because local application of a same dose of insulin (10 μM; 0.1 μL), a closely related hormone, did not facilitate an LC-evoked response (Supporting Figure S1B).

Since IGF-I stimulates cortical activity,[42] we performed ECoG recordings in S1 after local PeF injection of IGF-1 to determine if activation of orexin neurons by IGF-I would activate cortical activity differently in Firoc mice and littermate controls. We calculated the proportion of the different frequency bands in the power spectra of ECoG recordings 20 minutes after IGF-I application (Figure 4F). The ECoG showed faster activities in control mice, as compared to Firoc mice. The differences reached statistical significance in the θ activity compared with controls (n = 14; **P < .01). Conversely, Firoc mice showed larger δ activity than controls (n = 9; ***P < .0001, Figure 4F). Baseline activity was simi- lar in both groups of animals (Supporting Figure S1D).

A second approach to specifically determine whether orexin neurons are activated by IGF-I consisted in optogenetically identify orexin neurons using a mouse expressing ChR specifically in these cells (Ox-ChR mice, Figure 5A). A blue- light pulse (300 ms duration) able to stimulate a small volume of tissue (about 100-200 μm in radius35) was delivered to the PeF area of Ox-ChR mice using an optrode to perform unit recordings simultaneously with optical stimulation in the same place (Figure 5B,C).

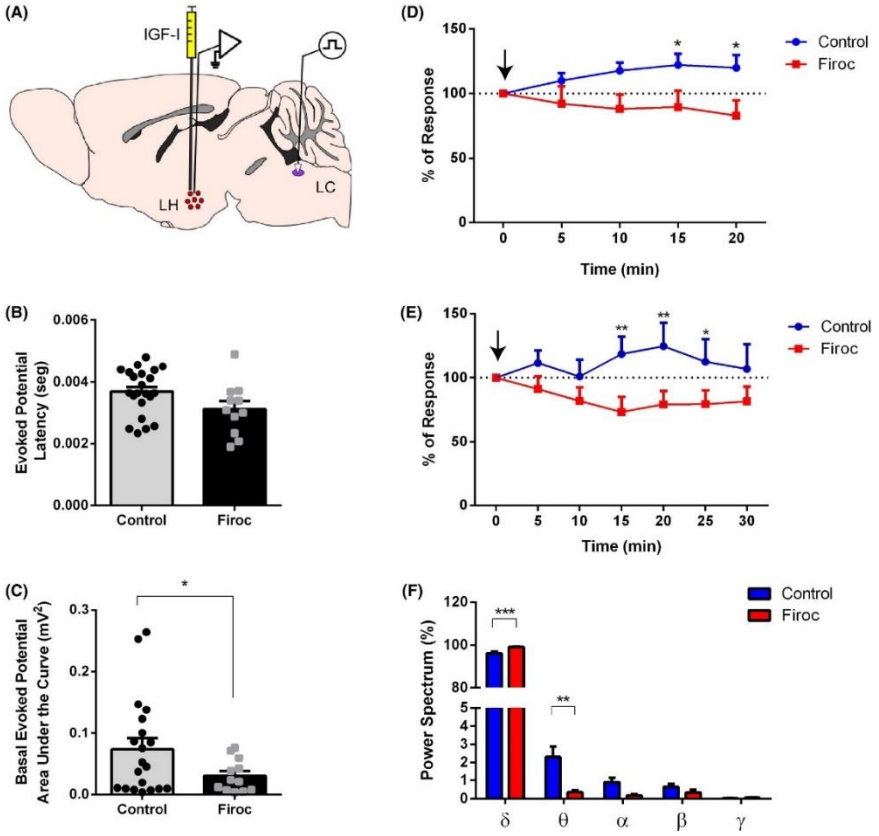

FIGURE 4 Responses of PeF neurons to Locus Coeruleus (LC) stimulation. A, Diagram of experimental design is shown. A stimulating electrode was placed in the LC, and a recording electrode in the PeF at the lateral hypothalamus (LH). B, No differences were seen in the latency of the evoked potential in control (a mixed group of wild-type mice and littermates), and Firoc mice after stimulation of the LC (Firoc = 10, control = 19; sex balanced). C, Area under the curve of the basal evoked potential after LC stimulation was significantly greater in control than in Firoc mice (t = 2.2; **P* = .0315; control = 22, Firoc = 11; sex balanced, Unpaired *t* test and Welch's correction). D, Control, but not Firoc mice, responded to local application of IGF-I in the PeF (arrow, 10 μM, 0.1 μL) after LC stimulation. Time course showing the evoked potential of orthodromic impulses after electrical stimulation of the LC in both experimental groups expressed as percentage of basal responses at time 0 (F = 18.08; ****P* < .001; control = 22, Firoc = 11; sex balanced, two-Way ANOVA, Sidak's Multiple comparison test). E, Intraperitoneal injection of IGF-I (arrow, 1 μg/g) increased neuronal activity in PeF orexin neurons after LC stimulation in control (n = 10; at 15 min ***P* = .0011, 20 min ***P* = .0011, and 25 min **P* = .0301), but not in Firoc mice (F = 5.47; n = 10, sex balanced, two-Way ANOVA, Sidak's Multiple comparison test). F, Local injection of IGF-I in the PeF area (10 μM, 0.1 μL) induces faster cortical activity in the ECOG in control mice recorded 20 min after injection. In the same conditions, Firoc mice show a larger proportion of δ frequency band, and a decrease of the proportion of faster activities in the power spectrum. These changes reach statistical significance in δ (n = 14; ****P* < .0001) and θ frequency bands (n = 14, F = 9.18; ***P* < .01, both sexes included) Correction Statement: Correction added on October 16, 2020, after first online publication: Figure 4 in PDF version has been updated.]

Neuronal activity was measured at 100 ms time intervals after light onset (0-100 ms and 100- 200 ms), and was compared with the previous 100 ms time interval before time onset (basal condition). Short-lasting blue LED stimuli-induced spike firing in neurons of Ox-ChR mice at 0-100 ms (36.25 ± 5.25 spikes/100 ms) vs basal conditions (22.53 ± 3.37 spikes/100 ms; n = 20-20/ group; $^{**}P < .0022$), as well as at 100-200 ms (36.30 ± 5.71 spikes/100 ms; 20-20/group; $^{*}P < .0320$, Figure 5D). The effect lasted 200 ms, recovering baseline activity later, even though the blue-light pulse lasted 300 ms. After orexin neurons were identified by their responses to the blue light, local application of IGF-I (10 nM; 0.1 μL) induced a 30.8% increase of their spontaneous firing rate at 10 minutes (SEM ± 9.56% spikes/100 ms; n = 12; $^{*}P = .026$), and of 43.6% at 15 minutes (SEM ± 15.54% spikes/100 ms; n = 14; $^{*}P < .012$), re- covering basal levels 20 minutes after (Figure 5E).

IGF-I modulates sleep through orexin neurons

The above results, coupled with the evidence that orexin levels may be regulated by IGF-I (Figure 3F), suggested that this growth factor stimulates orexin neurons. Since IGF-I shows a circadian cycle,[26] and activity-dependent entrance into the brain,[25] and orexin neurons contribute to the facilitation and/ or maintenance of arousal[43] in a close connection with the LC,[40] we studied if ECoG patterns are altered in Firoc mice (Figure 6A). We compared the ECoG signature in male Firoc mice and controls by calculating the mean percentage of each frequency band measured during the middle part of the dark period (ZT17-ZT19, n = 5-5/group). No differences in ECoG patterns were found (Figure 6B). However, during the light phase (ZT8-ZT11), Firoc mice displayed differences in δ and γ frequency bands compared to controls ($^{***}P < .001$ and $^{**}P < .01$, respectively, Figure 6C), indicating that Firoc mice show a slower ECoG than the control animals during the light period (their passive phase). This prompted us to determine in more detail if the lack of IGF-I receptor activity in orexinergic neurons favors a slowdown of the ECoG, and probably increased sleep periods. Thus, we measured the sleep-onset latency (time since the animal was placed in the recording place until the appearance of δ waves in the ECoG without movements) in Firoc vs control mice. As shown in Figure 6D, the latency was shorter in Firoc mice than in controls (n = 11/9 group; $^{**}P < .0058$).

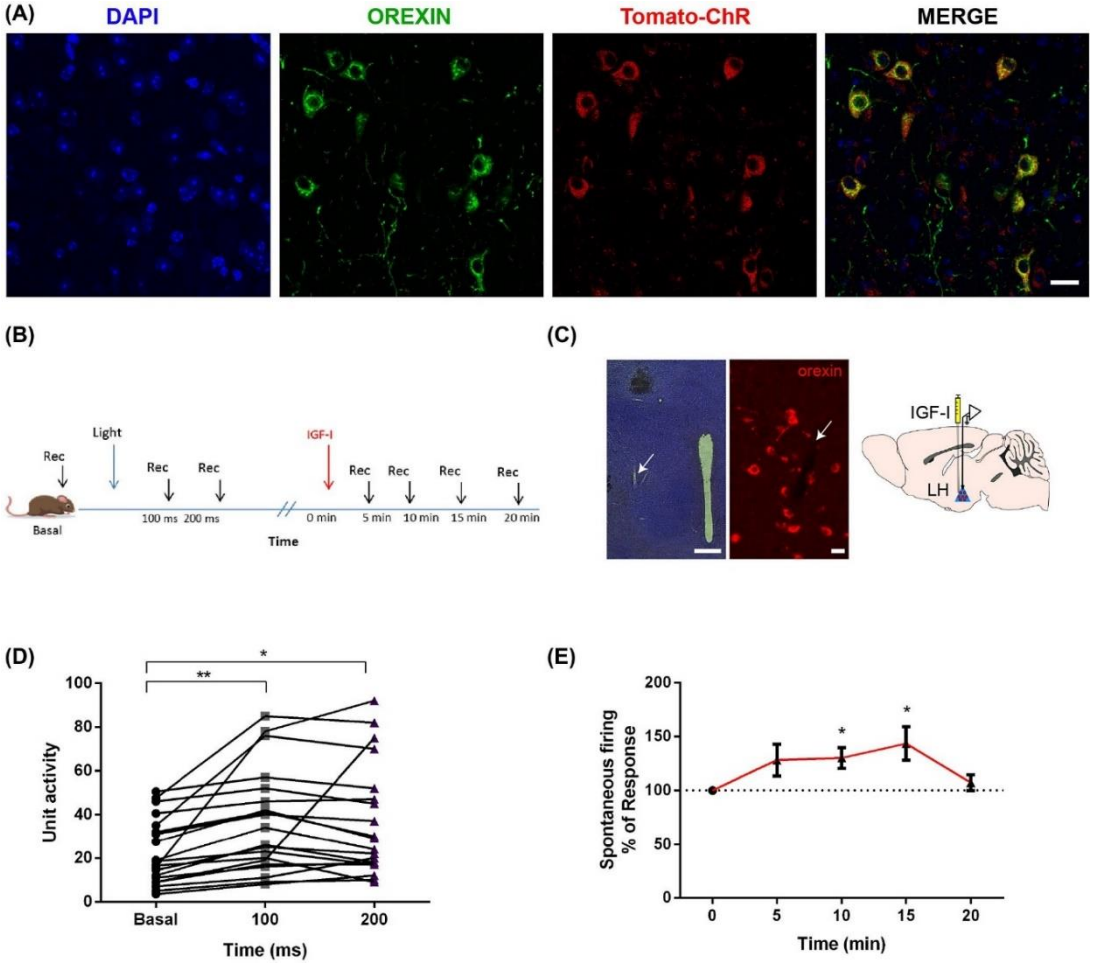

FIGURE 5 Orexin neurons are activated by IGF-I. A, Representative micrographs of orexin neurons (green) co-labeled with Tomato-ChR (red) in Ox-ChR mice. DAPI staining of cell nuclei is also shown. B, Diagram of experimental design used in optogenetic identification of orexin neurons. After recording (Rec) basal electrophysiological activity in the PeF area, light stimulation was delivered, and recordings continued thereafter. Once active neurons were identified, IGF-I was administered, and recordings continued for up to 20 min more. C, Left micrographs illustrate the positioning of the optrode (white arrow in left panel) in LH (Nissl staining). Scale bar is 200 µm. Orexin immunostaining show the presence of orexin neurons in the vicinity of the optrode (red). Scale bar is 20 µm. Right cartoon: Diagram of the positioning of the optrode and the cannula in the LH in optogenetic experiments. D, Unit recordings in the PeF area under optogenetic activation for a total of 16 light stimuli. Basal activity is the mean of unit activity between −200 ms and −100 ms. Values at 100 ms, and 200 ms represent unit activity during blue light stimulation ($F = 7.91$; $^{**}P = .0037$; n = 20-20-20/group; $^{***}P < .001$; Basal vs 100 ms: $^{**}P = .002$; Basal vs 200 ms: $^{*}P = .0320$; 100 ms vs 200 ms: $P = .999$; sex balanced, Repeated Measure one-Way ANOVA, Tukey's Multiple comparison test). E, Local injection of IGF-I also stimulated the spontaneous activity of optogenetically identified orexin neurons in the PeF area at 10 min ($^{*}P = .0261$), and 15 min ($^{*}P = .0121$). Ox-ChR mice = 16; sex balanced, Kruskal-Wallis test and Dunn's Correction test (K-W = 12.21)

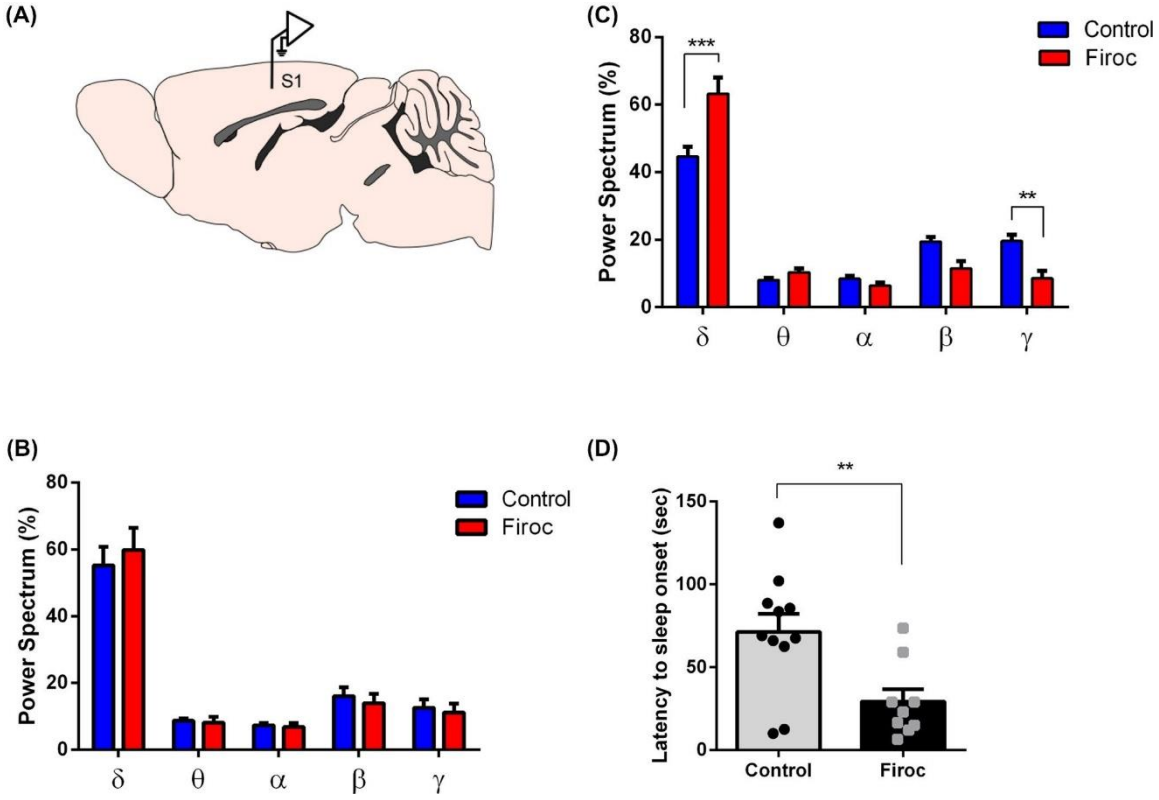

FIGURE 6 ECoG recordings during the light/dark cycle. A, Diagram of the intracranial localization of the electrodes in S1cortex; left hemisphere has the reference electrode in all cases. B, Power spectra analysis of ECoG bands obtained during the dark phase did not show any difference between groups. C, ECoG analysis during the light phase displays a predominant δ activity together with a reduced γ activity in Firoc mice, as compared to controls (F = 9.55; ***P < .001 and **P < .01; Firoc = 7, control = 12, males mice only, two-Way ANOVA, Sidak's Multiple comparison test). D, Latency to sleep-onset was markedly reduced in Firoc mice (t = 3.15; **P < .0058, Firoc = 9, control = 11, sex balanced, Unpaired *t* test and Welch's correction)

Since differences in ECoG patterns in Firoc mice were observed along the light period, we analyzed this phase in more detail. Firoc mice showed significantly higher sustained ECoG activity in the δ and θ frequency bands during the light phase (ZT3-ZT13; **P < .01, and ***P < .001, respectively; n = 7-6/group; Figure 7A, B). This slowing of the ECoG was accompanied by a significant decrease in α, β, and γ frequency bands (n = 7-6/group; *P < .05, **P < .01, and ***P < .001, respectively; Figure 7C-E).

DISCUSSION

These results indicate that reduced IGF-I signaling onto orexin neurons results in altered ECoG patterns with a predominance of slow waves, suggesting a direct effect of this anabolic hormone on sleep through these hypothalamic neurons. Specifically, we show that optogenetically identified PeF neurons are activated by IGF-I, facilitating in this way wakefulness and ECoG activation -although we cannot entirely rule out that other PeF, non-orexinergic neurons could be activated by IGF-I. However, blunted IGF-IR activity in the PeF area of Firoc mice induces changes in the ECoG. In turn, most of PeF neurons showing Cre recombination were orexin neurons (>85%), and until now only orexin neurons, among other PeF neurons, are known to affect the ECoG.41 Hence, we may conclude that the bulk of our results may be explained by a direct activation of orexinergic neurons by IGF-I, facilitating in this way wakefulness. Accordingly, Firoc mice showed an increase in δ waves as well as a de- crease in faster ECoG activities, especially during the light phase. This agrees with lower orexin levels in Firoc mice. However, basal activity of orexin neurons, as determined by c-fos immunoreactivity in saline-injected Firoc mice was not affected. This is probably reflecting different time-courses affecting neuronal activity, which is finely tuned by multiple stimuli and is highly state-dependent, and orexin levels, that are modulated at a slower rate.[44]

Given that IGF-I has been proposed to be a feeding-circadian entrainer,[27] and that sleep is regulated along phylogeny by nutrient availability,[45] our observations may provide an additional functional link between a metabolic signal and a multitasking hypothalamic circuit such as orexin neurons,[46] adding to recent observations in invertebrates on the molecular and cellular mechanisms linking sleep and metabolism.[45] Indeed, orexin neurons are known to be directly modulated by feeding signals, including glucose[47] and amino acids.[48]

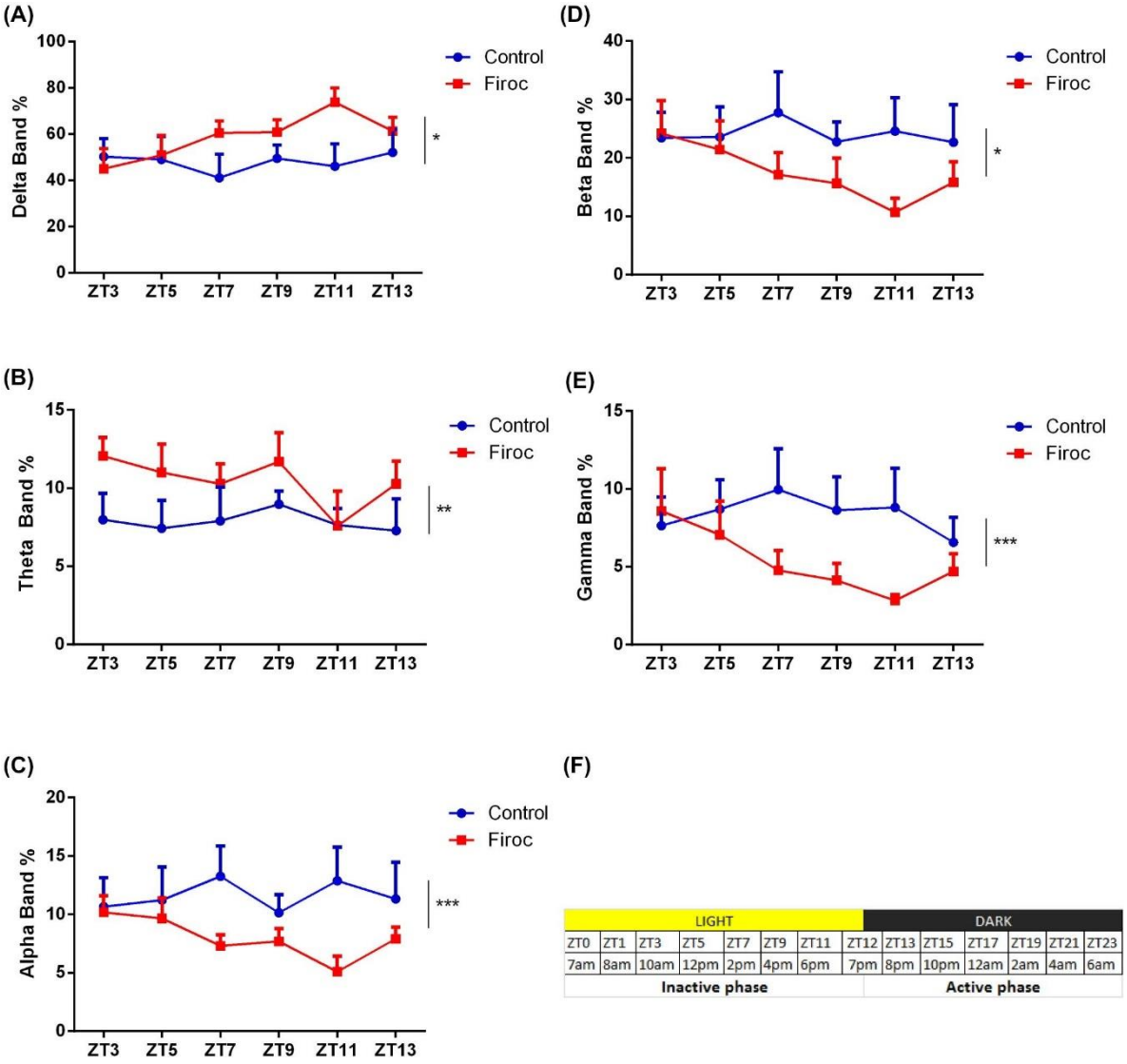

FIGURE 7 IGF-I shapes the sleep architecture through orexin neurons. A-E, Sleep architecture during the light phase (ZT 3-13) determined by δ (B), θ (C), α (D), β (E), and γ (F) activity patterns in Firoc (red line), and control mice (blue). An increase in slow activity (δ), and a decrease in fast waves was observed (Firoc = 7, control = 6; male mice only, Scheirer-Ray Test; **P* < .05, ***P* < .01, ****P* < .001; δ H = 4.72; θ H = 6.07, α H = 6.65, β H = 5.97, γ H = 11.03). F, Chart with the light and dark periods including the corresponding Zeitgeber time.

Of note, IGF-I shows a circadian pattern, with highest levels during the sleep period,[26] when orexin activity should be attenuated.17 Thus, it is unlikely that its circadian rhythm will dictate IGF-I signaling to orexin neurons. Rather, it seems more plausible that brain and physical activity,[24,25] and/or feeding schedule[27] will modulate orexin activity, since all these situations are accompanied by increased brain IGF-I input. In agreement with that, systemic injection of IGF-I- induced faster activity in the ECoG of mice and nonhuman primates.[42] Our findings also showed an induction of faster activities in the ECoG after local PeF injection of IGF-I that was not observed in Firoc mice. But more work is needed to determine the specific cues timing IGF-I input to orexin neurons.

While IGF-I is a highly conserved sleep regulatory signal throughout evolution, from invertebrates to vertebrates,[9-11,49] the mechanisms linking it to sleep-regulatory circuits remain poorly understood. Interestingly, reduced insulin-like signaling in *drosophila* produces also increased sleep,[50] as we have seen in Firoc mice. Thus, our results provide new in- sights into this connection in mice, although more studies are needed to clarify whether the functional link between IGF-I and orexin is present in other mammalian species, and even in lower taxa expressing orexin-like peptides.[51]

Significantly, genetic ablation of orexinergic neurons or mutation of orexin receptors results in a decrease in wake- fulness and altered sleep/wake cycles.[52-55] In turn, lack of IGF-IR activity in orexin neurons results in altered sleep architecture, and reduced sleep-onset latency, consistent with lower orexin levels in these mice. Orexinergic neurons are strongly activated during active wakefulness, decrease their activity during quiet wakefulness, and are silent during the different sleep phases.[56,57] These neurons are essential for maintaining wakefulness and regulate REM sleep, and their loss results in narcolepsy.[41,52,58,59] Moreover, orexin neurons are sensitive to metabolic signals,[60] as well as to peripherally derive the circulating factors whose fluctuations may provide information about homeostatic status. Our findings include IGF-I as an additional signal modulating orexin excitability. Thus, it seems that IGF-I input is necessary for orexin neurons to maintain their normal activity. In turn, since altered sleep modifies IGF-I activity, the way sleep influences IGF-I needs to be clarified.

A direct functional connection between IGF-I and orexin may be involved in sleep disturbances that take place during aging61 or in pathologies such as Alzheimer's dis- ease

(AD)[62,63] or acromegaly.[64] Thus, old age is associated with the "somatopause," a reduction in the GH-IGF-I axis resulting in lower serum IGF-I levels,[65] and impaired brain IGF-I activity.[66] In addition, old age is reportedly associated to lower number of orexin neurons.[67] Furthermore, sleep disturbances occur very early in the course of AD, which is consistent with the finding that brain regions involved in sleep and circadian control are affected early in the pathogenesis of the condition.[68] Moreover, long sleep duration in late-life, as well as sleep fragmentation or excessive daytime sleepiness have been associated to increase risk of dementia and poor cognitive performance.[69-73] Since IGF-I has been related to AD pathology,[74,75] regulation of orexin function by IGF-I may contribute to altered sleep patterns in AD patients.

This study contains several limitations. First, many of our results are based on a Cre/Lox transgenic mouse that may show ectopic Cre expression, which would result in a contribution of unidentified brain cells expressing IGF-IR in the observed changes. For instance, LH GABAergic neurons involved in arousal.[76] However, less than 15% of Cre+ cells were orexin- and since the threshold of detection of orexin immunoreactivity in orexin neurons may vary,[77] it is possible that part of this Cre+ population is still orexinergic. Also, while we did not observe any gross difference in orexin neurons in Firoc mutants (they show similar numbers than litter- mates), undetermined developmental effects due to lack of proper IGF-I signaling on orexin neurons cannot be entirely ruled out. Finally, Firoc mice show a nonsignificant increase in c-fos expression in orexin cells after IGF-I that may be related to their stimulation by unidentified IGF-IR-expressing cells and/or orexin cells still expressing fully active IGF-IR since Cre/Lox recombination of IGF-IR in mutant mice did not occur in 100% of orexin neurons (Figure 2B). Again, changes observed may also be contributed by these additional cells.

In summary, albeit with the above limitations, our results provide an explanation of the role of IGF-I in sleep architecture, pointing to orexin neurons as important mediators of its actions.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

J.A. Zegarra-Valdivia conducted experiments and prepared figures and results. J. Pignatelli conducted experiments and prepared figures, M.E. Fernandez de Sevilla conducted experiments and prepared figures, A.M. Fernandez conducted experiments and prepared figures, V. Munive conducted experiments and prepared figures, L. Martinez-Rachadell conducted experiments, A. Nuñez designed and conducted experiments and wrote part of the manuscript, I. Torres Aleman designed the study and wrote the manuscript.

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SUPPORTING INFORMATION

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Additional Supporting Information may be found online in the Supporting Information section.

3.2. Study 2 – See appendix 2

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3.3. Study 3

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Reduced Insulin-Like Growth Factor-I Effects in the Basal Forebrain of Aging Mouse

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It is known that aging is frequently accompanied by a decline in cognition. Furthermore, aging is associated with lower serum IGF-I levels that may contribute to this deterioration. We studied the effect of IGF-I in neurons of the horizontal diagonal band of Broca (HDB) of young (≤6 months old) and old (≥20-month-old) mice to determine if changes in the response of these neurons to IGF-I occur along with aging. Local injection of IGF-I in the HDB nucleus increased their neuronal activity and induced fast oscillatory activity in the electrocorticogram (ECoG). Furthermore, IGF-I facilitated tactile responses in the primary somatosensory cortex elicited by air-puffs delivered in the whiskers. These excitatory effects decreased in old mice. Immunohistochemistry showed that cholinergic HDB neurons express IGF-I receptors and that IGF-I injection increased the expression of c-fos in young, but not in old animals. IGF-I increased the activity of optogenetically-identified cholinergic neurons in young animals, suggesting that most of the IGF-I-induced excitatory effects were mediated by activation of these neurons. Effects of aging were partially ameliorated by chronic IGF-I treatment in old mice. The present findings suggest that reduced IGF-I activity in old animals participates in age-associated changes in cortical activity.

Keywords: cholinergic neurons, IGF-I, diagonal band of Broca, cortical activity, aging

INTRODUCTION

Aging is a physiological process accompanied by a decline in cognitive performance. In humans, aging has been associated with numerous and diverse changes in the EEG and on sleep, such as increased sleep fragmentation, decreased total sleep time, sleep efficiency, and in the frequency bands of the EEG (Landolt et al., 1996; Luca et al., 2015; Mander et al., 2017). Numerous studies have also provided important insights into the global age-dependent alterations in sleep-wake and EEG architecture in mice (Hasan et al., 2012; Panagiotou et al., 2017; McKillop et al., 2018). However, there are notable discrepancies between species concerning the effects of aging. For example, slow-wave sleep (SWS) is decreased in aged humans, whereas it is enhanced in aged mice (Panagiotou et al., 2017; McKillop et al., 2018). Despite these discrepancies, mouse studies have been considered an excellent model to investigate age-dependent physiological changes. Previous evidence suggests that aging may lead to specific changes in cortical activity. Both animal and human studies have shown that aging is associated with alterations in synaptic transmission and structural synaptic changes (Morrison and Baxter, 2012; Petralia et al., 2014) and a consistent loss of hippocampal synaptic connections (Burke and Barnes, 2006; Morrison and Baxter, 2012).

One of the most crucial structures controlling cortical activity is the basal forebrain (BF). Electrophysiological recordings in the BF combined with EEG recordings have indicated that cortical activation depends on BF inputs to the cortex (Metherate et al., 1992; Nuñez, 1996; Duque et al., 2000; Manns et al., 2000). Most of these effects have been explained by the release of acetylcholine (ACh) in the cortex during wakefulness as well as during rapid eye movement (REM) sleep (Celesia and Jasper, 1966; Jasper and Tessier, 1971; Golmayo et al., 2003; Fournier et al., 2004). Dysregulation of the cholinergic system is implicated in cognitive decline associated with aging and dementia, including Alzheimer's disease (Gallagher and Colombo, 1995; Grothe et al., 2010). For example, an impaired cholinergic transmission has been associated with age-related disorders in attention and memory storage and retrieval (Decker, 1987; Gallagher and Colombo, 1995; Hasselmo, 1999; Dumas and Newhouse, 2011). Since cholinergic neurons are involved in the facilitation and maintenance of arousal, we hypothesized that their activity might be altered in aged animals, particularly their response to modulatory inputs.

In addition, it has been demonstrated that insulin-like growth factor-I (IGF-I) can be a

potent stimulator of neuronal activity, participating in numerous brain processes (see for review Fernandez and Torres-Alemán, 2012; Fernandez et al., 2018). Indeed, IGF-I increases the spontaneous firing rate and response to afferent stimulation in target neurons (Carro et al., 2000; Nuñez et al., 2003; Gazit et al., 2016; Barros-Zulaica et al., 2019). We have recently demonstrated that IGF-I increases orexin neurons' activity, located in the lateral hypothalamus, involved in controlling the circadian sleep/wake cycle (Zegarra-Valdivia et al., 2020). Reduced serum IGF-I levels have been described during aging in all mammalian species studied (Kenyon, 2001; Trejo et al., 2004; Piriz et al., 2011; Muller et al., 2012; Junnila et al., 2013). Basal and IGF-I-induced activation of the brain IGF-I receptor/Akt/GSK3 pathway were markedly reduced in old mice even though they displayed high levels of brain IGF-I receptors (Muller et al., 2012). The reduction of IGF-I effects could be responsible for the decline of cognitive functions during aging. In the present work, we studied whether IGF-I modulates arousal and cortical activity by activation of BF neurons and whether this modulation decreases during normal aging. Specifically, we studied the effect on neurons located in the horizontal, diagonal band of Broca (HDB) nuclei that provide most of the cholinergic innervation to the sensory, motor, and prefrontal cortices and hippocampus (Duque et al., 2000; Zaborszky et al., 2015; Chaves-Coira et al., 2016, 2018). Our findings showed that IGF-I increased HDB neuronal firing, facilitating cortical activity, but this effect was reduced in old mice.

MATERIALS AND METHODS

Experiments were performed on B6Cg-Tg (Chat- COP4_H134R/EYFP, Slc18a3)5Gfng/J mice (The Jackson Laboratory). We used these transgenic mice because they express the light-activated cation channel, channelrhodopsin-2, tagged with a fluorescent protein (ChR2-YFP) under the control of the choline acetyltransferase promoter in cholinergic neurons (ChAT+ identified neurons). Thus, all ChAT+ identified neurons express the ChR2 and could be stimulated with blue-light in optogenetic experiments. C57BL/6J mice (Harlan Laboratories, Spain) of both sexes were also used. Animals were grouped into two age groups (young mice: 3–6 months old) and (old mice: 20–22 months old). All experimental groups were sex balanced.

Animals were housed under standard colony conditions with food and water supplied ad libitum and under a 12–12 h light-dark cycle. Animal procedures followed European

guidelines (2010/63, European Council Directives) and were approved by the local Bioethics Committee (Government of the Community of Madrid; PROEX: 189/16). Efforts were made to minimize animal suffering as well as to reduce the number of animals used.

Recordings and Tactile Stimulation

Animals were anesthetized with isoflurane (2% induction; 1–1.5% maintenance doses) and placed in a David Kopf stereotaxic apparatus (Tujunga, CA, USA). Body temperature was set at 37◦C through a water-heated pad (Gaymar T/Pump, Orchard Park, NY, USA). The sagittal midline of the scalp was sectioned and retracted. A small craniotomy was drilled over the HDB nucleus (coordinates from Bregma: 0.5 mm anterior 0.8 mm lateral, 5.5 mm–6 mm depth from the cortical surface).

Multiunit activity (MUA) was recorded in the HDB nucleus through a tungsten microelectrode (1–2 MΩ, World Precision Instruments, WPI, Sarasota, FL, USA). Electrocorticogram (ECoG) was also recorded in the primary somatosensory (S1) cortex (from Bregma 2 mm posterior, 3 mm lateral, 1 mm depth) through tungsten macroelectrodes (<1 MΩ, WPI). MUA and ECoG were filtered between 0.3 and 3 kHz and 0.3 Hz-100 Hz, respectively, and amplified using a DAM80 preamplifier (WPI). Signals were sampled at 10 or 1 kHz, respectively, through an analog-to-digital converter (Power 1401 data acquisition unit, Cambridge Electronic Design, Cambridge, UK) and fed into a PC for offline analysis with Spike 2 software (Cambridge Electronic Design).

Whisker deflections were evoked by brief air pulses using a pneumatic pressure pump (Picospritzer, Hollis, NH, USA; 1–2 kg/cm2, 20 ms duration), delivered through a 1 mm inner diameter polyethylene tube. All whiskers were first trimmed to a length of 5 mm to avoid complex responses due to multiple whiskers' deflections. The experimental protocol consisted of 60 air pulses delivered to the principal whisker at 0.5 Hz after a control period of 1 min for ECoG basal recording.

Optogenetic Stimulation and Recording

Optogenetic experiments were performed to identify cholinergic neurons (ChAT+ identified neurons) through blue-light activation, using the transgenic mice (see above). Animals were anesthetized and prepared as indicated above. Optical stimulation of ChR-

expressing neurons was achieved with light pulses from a light-emitting diode (LED; Thomas Recording, Germany) delivered through an optrode, composed of a tungsten microelectrode 0.5–0.8 MΩ attached to an optical fiber (core diameter 120 µm; Thomas Recording), stereotaxically positioned in the HDB nucleus. Optogenetic stimulation was applied by long-lasting pulses of 473 nm light (27 stimuli with a duration of 300 ms, each stimulus was repeated every 3s), with an illumination intensity of <30 mW/mm2. Unit recordings were performed through the optrode. They were filtered (0.3–3 kHz) and amplified using a DAM80 preamplifier (WPI). Single-unit activity was sampled at 10 kHz and extracted with the aid of Spike2 software.

Drugs

IGF-I was locally delivered in the HDB nucleus (10 nM; 0.2 µl; coordinates as above) employing a 1 µl Hamilton syringe. Besides, IGF-I was injected systemically (1 µg/g; i.p.) in other experiments. The muscarinic receptor antagonist atropine (1 mg/Kg in 0.9% NaCl i.p.) was administered 15 min before IGF-I systemic injection to assess whether IGF-I effects were due to activation of muscarinic receptors. Orexin A (Tocris, Spain) was also injected into the HDB nucleus (10 nM; 0.2 µl; coordinates as above).

In a set of experiments, we administrated human IGF-I (hIGF-I) through Alzet osmotic mini-pumps (Model 1004; USA) for chronic administration (Pre-Protech, USA; 50 g/kg/day) in ChAT-ChR2-YFP animals or the vehicle (saline solution). Pumps were implanted subcutaneously between the scapulae, following the manufacturer's instructions. Treatment lasted 28 days. After that, animals were submitted to electrophysiological recordings as described above.

Immunohistochemistry

Animals were euthanized with an overdose of pentobarbital (50 mg/kg) and perfused transcardially with saline 0.9% followed by 4% paraformaldehyde in 0.1 N phosphate buffer (PB), pH 7.4. Coronal 50-µm-thick brain sections were cut in a vibratome and collected in PB 0.1 N. Sections were incubated in permeabilization solution (PB 0.1N, Triton X-100, NHS 10%), followed by 24/48 h incubation at 4◦C with primary antibody (1:500) in blocking solution (PB 0.1N, Triton X-100, NHS 10%). After washing three times in PB, Alexa-coupled goat/rabbit polyclonal secondary antibodies (1:1,000, Molecular Probes, USA) were used.

Finally, a 1:1,000 dilution in PB of Hoechst 33342 was added for 5 min. Slices were rinsed several times in PB, mounted with gerbatol mounting medium, and allowed to dry. The omission of the primary antibody was used as a control. Confocal analysis was performed in a Leica (Germany) microscope. For double-stained ChAT/c-fos counting, five sections per animal were scored using Matlab and Imaris software. The antibodies used in this study include rabbit polyclonal c-Fos (Abcam, UK, ab190289), rabbit polyclonal IGF-I Receptor-β (Santa Cruz, USA, 713/AC), and rabbit anti-IGF-I receptor β XP (Cell Signaling Technology, USA, 9750), anti-Choline acetyltransferase polyclonal antibody (Merck-Millipore, USA, AB144P), and anti-pAkt (Cell Signaling Technology, USA, 9271).

In some experiments, mice were processed for immunocytochemistry 2 h after ip IGF-I injection to allow the expression of c-fos (see above). IGF-I (1 mg/ml) was first dissolved in acetic acid 1N and then prepared with saline to a final dose of 1 µg/g body weight.

Cell Image Analysis and Counting

Images (coordinates from Bregma: 0.5 mm anterior 0.8 mm lateral, 5.5 mm–6 mm depth from the cortical surface) were taken from the HDB in a confocal microscope (Leica, Germany). The analysis was carried out using the Imaris 9.4 software (the thickness of the images taken was 20 µm). In the ChAT/c-fos analysis, we used 20 magnification; images were taken from the HDB area. Spots of this area were selected with an estimated XY size of 20 µm to count ChAT+ neurons in the red channel. Then, spots were filtered with the c-fos channel in green to obtain the amount of spots ChAT+/c-fos+.

In the ChAT/IGF-1R analysis, we used 40x magnification to measure IGF-IR+ / ChAT+ cells by the signal intensity (%) after identifying the ChAT neurons in the red IGF-1R in the green channel. First, we create a 3D surface (taking the entire thickness) in the red channel using the ‘‘surface mode.’’ In this way, a threshold was set (it was the same for all pictures) to remove the background, and all ChAT neurons above it were selected. Then, we obtained a total number of neurons in the desired area. From the total number of ChAT neurons obtained, we select only those which coincide with the green channel’s maximum intensity, which is the corresponding color of the IGF-1R secondary antibody. To do that, we add a selection filter called ‘‘maximum intensity selection in the channel green.’’ In this way, we were able to distinguish only those neurons that colocalize with

the green channel maximum intensity, using an automatic threshold (corresponding to IGF-1R). Finally, we calculated the relationship between ChAT neurons colocalizing with the maximum intensity of IGF-1R.

Data Analysis

ECoG segments of 1 min every 5 min were analyzed by Spike 2 software, using the Fast Fourier Transform algorithm to obtain the power spectra. The mean power density was calculated for five different frequency bands that constitute the global ECoG: delta (0.3–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), beta (12–30 Hz), and gamma (>30 Hz) bands. The total power of the five frequency bands was considered 100%, and the percentage of each frequency band was calculated. Gamma frequencies were excluded from the results because their power was negligible in anesthetized mice.

Somatosensory evoked potentials (SEPs) were elicited in the S1 cortex by whisker deflections. The area under the curve of the first negative wave was calculated in each case. A control period of 10 min was recorded, and the mean SEP area was considered 100%. Recordings were also performed every 5 min for 30 min after local IGF-I injection in the HDB nucleus. The mean firing rate of MUA recordings was also calculated before (10 min) and after drug injections (every 5 min for 30 min). ChAT+ neurons were identified and included in this study when blue-light pulses induced an increase in their firing rate of at least 10%.

Statistics

Statistical analysis was performed using Graph Pad Prism 6 software (San Diego, CA, USA). Depending on the number of independent variables, normally distributed data (Kolmogorov–Smirnov normality test), and the experimental groups compared, we used either Student's *t*-test or two-way ANOVA followed by Sidak's multiple comparison test. For non-normally-distributed data, we used the Mann–Whitney U test to compare two groups, Kruskall-Wallis or Friedman test, with Dunn's multiple comparisons and a *Post Hoc* analysis such as Scheirer–Ray Test, a non-parametric alternative to multi-factorial ANOVA. The sample size for each experiment was chosen based on previous experience and aimed to detect at least a $p < 0.05$ in the different tests applied, considering a reduced use of animals. Results are shown as mean ± standard error (SEM) and p values coded as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

RESULTS

IGF-I Activates HDB Nerons in Young but Not in Old Mice Immunohistochemical studies were focused on the HDB area because it projects abundantly to the S1 cortex (e.g., Chaves- Coira et al., 2016, 2018; **Figure 1A**). Immunohistochemistry of the IGF-I receptor (IGF-IR) showed abundant staining in ChAT + cells in the HDB nucleus of young and old mice. The co-localization of IGF-IR signal to ChAT+ identified neurons strongly suggested that they expressed IGF-IR (**Figure 1B**). The expression of IGF-IR was also observed in the neuropil and in non-identified cells of HDB. We compared the number of ChAT+ cells per area in young and old mice. There was a slight increase of ChAT+ cells in old animals but differences were not statistically significant (n = 3 mice per group, $p > 0.05$; Unpaired t-test; **Figure 1C**). However, we found that the mean expression of IGF-IR in ChAT+ cells of old mice was reduced ($p = 0.0419$; Unpaired t-test; **Figure 1D**), suggesting a reduction of sensitivity to IGF-I in ChAT+ cells of old animals.

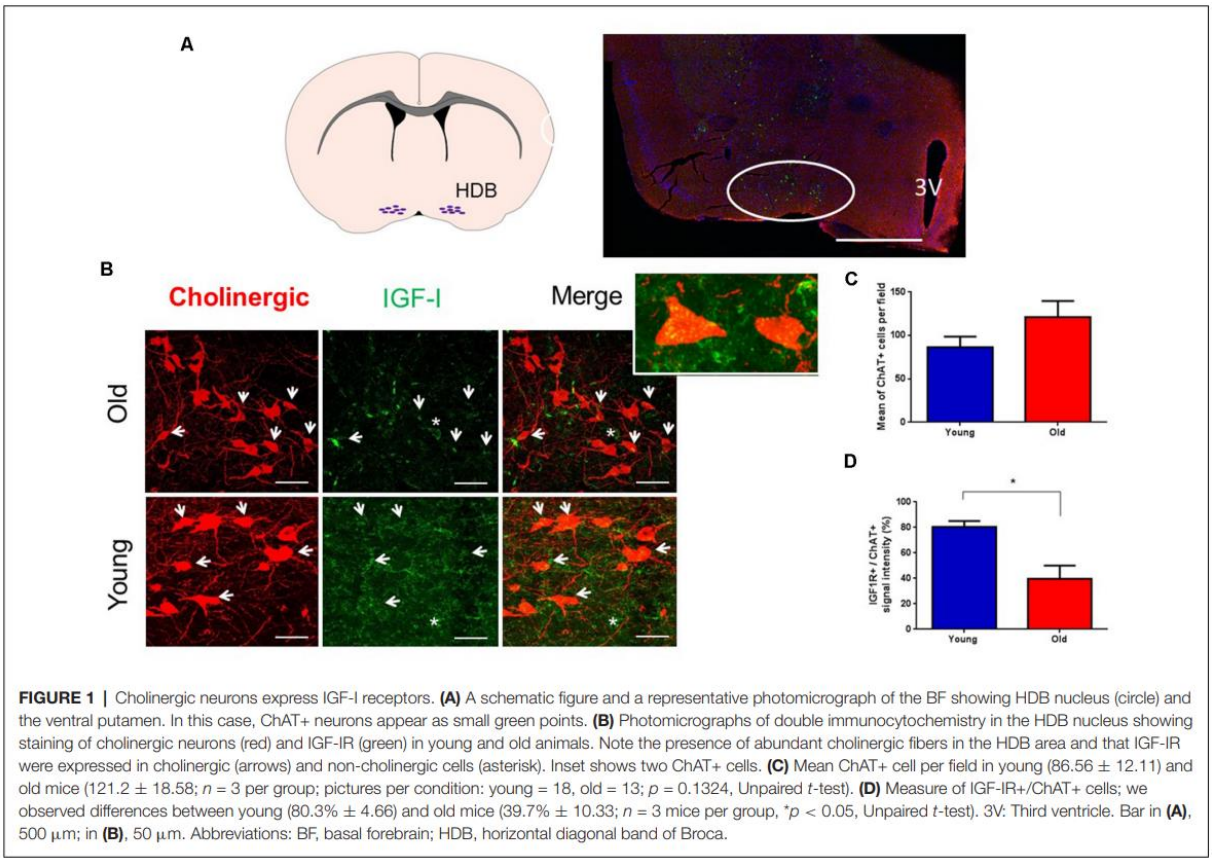

FIGURE 1 | Cholinergic neurons express IGF-I receptors. **(A)** A schematic figure and a representative photomicrograph of the BF showing HDB nucleus (circle) and the ventral putamen. In this case, ChAT+ neurons appear as small green points. **(B)** Photomicrographs of double immunocytochemistry in the HDB nucleus showing staining of cholinergic neurons (red) and IGF-IR (green) in young and old animals. Note the presence of abundant cholinergic fibers in the HDB area and that IGF-IR were expressed in cholinergic (arrows) and non-cholinergic cells (asterisk). Inset shows two ChAT+ cells. **(C)** Mean ChAT+ cell per field in young (86.56 ± 12.11) and old mice (121.2 ± 18.58; n = 3 per group; pictures per condition: young = 18, old = 13; p = 0.1324, Unpaired t-test). **(D)** Measure of IGF-IR+/ChAT+ cells; we observed differences between young (80.3% ± 4.66) and old mice (39.7% ± 10.33; n = 3 mice per group, $^*p < 0.05$, Unpaired t-test). 3V: Third ventricle. Bar in **(A)**, 500 μm; in **(B)**, 50 μm. Abbreviations: BF, basal forebrain; HDB, horizontal diagonal band of Broca.

Then, we determined the expression of the immediate early gene protein c-fos to test if IGF-I activates HDB neurons. We studied double-labeled ChAT+/c-fos+ cells in young and old animal groups after saline or IGF-I i.p. injections (**Figures 2A–D**). The plot of the percentage of ChAT+/c- fos+ cells showed a significant increase in old-saline over young-saline animals (p = 0.0087; Two-Way ANOVA, and Fisher's LSD comparison test; **Figure 2E**), suggesting a basal increase in neuronal activity in old animals. Furthermore, IGF-I injection increased the expression of c-fos in young animals (p = 0.045; Two-Way ANOVA, and Fisher's LSD comparison test). However, IGF-I reduced the number of ChAT+/c-fos+ cells in old animals although differences did not reach statistical significance (**Figure 2E**).

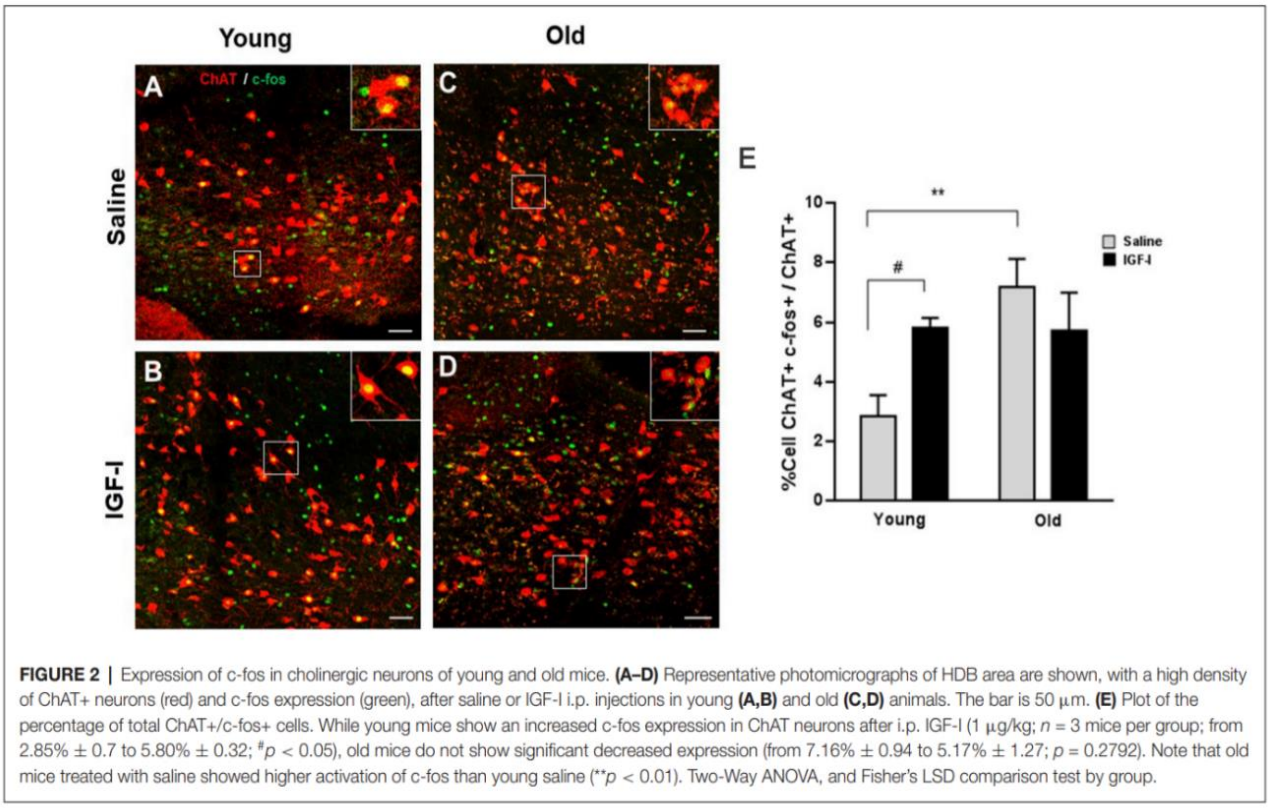

FIGURE 2 | Expression of c-fos in cholinergic neurons of young and old mice. **(A–D)** Representative photomicrographs of HDB area are shown, with a high density of ChAT+ neurons (red) and c-fos expression (green), after saline or IGF-I i.p. injections in young **(A,B)** and old **(C,D)** animals. The bar is 50 μm. **(E)** Plot of the percentage of total ChAT+/c-fos+ cells. While young mice show an increased c-fos expression in ChAT neurons after i.p. IGF-I (1 μg/kg; n = 3 mice per group; from 2.85% ± 0.7 to 5.80% ± 0.32; $^{\#}p < 0.05$), old mice do not show significant decreased expression (from 7.16% ± 0.94 to 5.17% ± 1.27; p = 0.2792). Note that old mice treated with saline showed higher activation of c-fos than young saline ($^{**}p < 0.01$). Two-Way ANOVA, and Fisher's LSD comparison test by group.

The above results suggest a reduced response of HDB neurons to IGF-I in old animals. Consequently, we studied HDB neuronal response to local injection of IGF-I (10 nM; 0.2 μl) through MUA recordings in young and old mice (**Figure 3A**). We used local injections to avoid a possible reduction of IGF-I entry in old mouse brains through the endothelium. At basal conditions, the spontaneous activity was similar in young and old mice (22.1 ± 6.4 spikes/s and 23.8 ± 4.6 spikes/s, respectively; $p > 0.05$, Unpaired t-test; **Figure 3B**).

Local IGF-I injection induced an immediate increase of their firing rate in HDB neurons of young mice (**Figure 3C**). However, the response to IGF-I was significantly lower in old animals. Five minutes after IGF-I injection, young mice showed an increase of the firing rate of 61.4%, while old animals displayed a reduction of 7.4%. Spontaneous activity continued to increase for at least 30 min (278.1% vs. 48.6% in young and old animals, respectively; young = 14, old = 6; $p = 0.0044$; Ordinary Two-way ANOVA; **Figure 3C**).

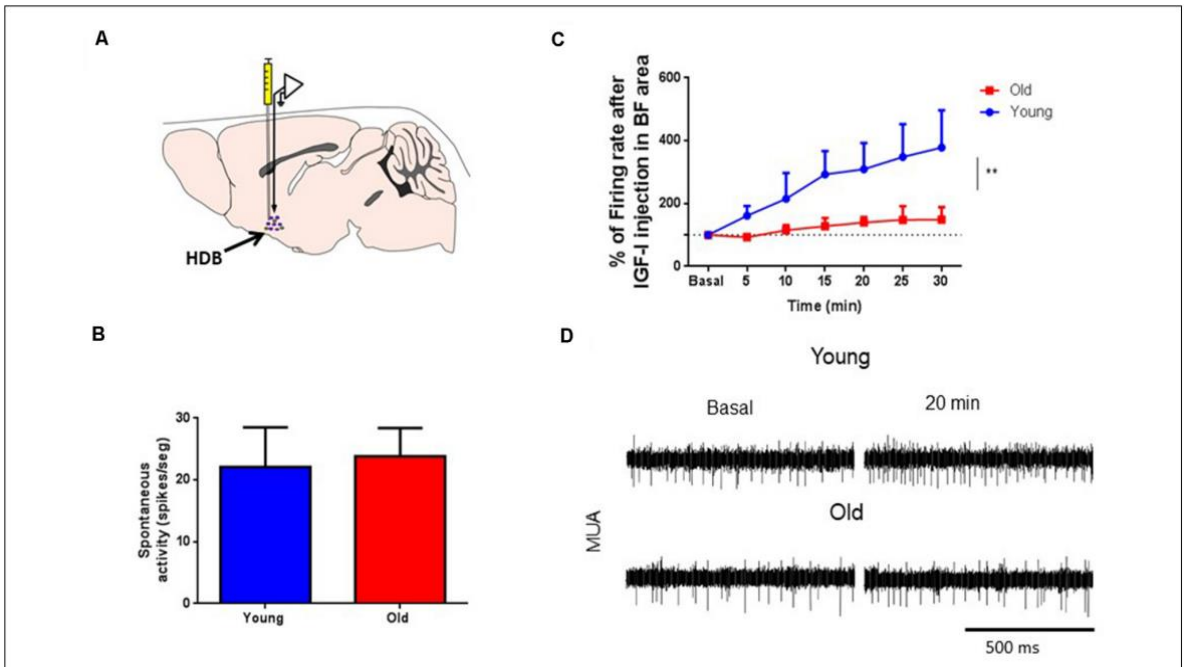

FIGURE 3 | IGF-I increased multiunit (MUA) activity in HDB neurons. **(A)** Schematic diagram of the experimental design. A recording microelectrode was sited in the HDB area (arrow); a cannula to inject IGF-I (10 nM; 0.2 μl) was also placed in the same area. **(B)** Plot of the spontaneous activity in young and old mice. No differences were found in MUA recordings at basal condition (young 22.09 ± 6.41 spikes/s, $n = 17$; old 23.81 ± 4.60 spikes/s, $n = 9$; $p = 0.2863$; sex balanced, Mann–Whitney test). **(C)** Time course of the HDB firing rate after IGF-I local injection. The firing rate of MUA is expressed as a percentage of basal responses at time 0 in both experimental groups. Young but not old mice responded to IGF-I in HDB nucleus (young, $n = 14$; old, $n = 6$; $F_{(1,122)} = 8,433$; $^{**}p = 0.0044$; Ordinary Two-way ANOVA). **(D)** Representative Multi-unitary recordings (MUA) at basal and 20 min after IGF-I in both young and old mice. The bar represents 500 ms.

Since cortical projections of HDB neurons induce cortical activation (Duque et al., 2000; Zaborszky et al., 2015; Chaves- Coira et al., 2018), we tested if the injection of IGF-I in HDB could induce changes in the ECoG recorded in the S1 area of young and old anesthetized mice. We studied the IGF-I effect on the ECoG analyzing its power spectrum and the proportion of the frequency bands. In basal conditions, delta waves were the predominant frequency band due to anesthesia. In these conditions, there were no differences between young and old mice ($n = 9$ per group; $F_{(4,32)} = 0.04674$; $p = 0.9957$, Two-way RM ANOVA; **Figure 4A**). However, local IGF-I injection in the HDB nucleus (10 nM; 0.2 μl) increased fast ECoG activities in young but not in old mice (**Figures 3B–D**). θ waves gradually increased their power after IGF-I injection in young animals while old animals only showed a rapid increase at 5 min after injection and returned to basal

values, showing a statistically significant difference throughout the 30 min of recording (young = 13, old = 9; $F_{(1,138)}$ = 6,436; p = 0.0123; Ordinary Two-way ANOVA; **Figure 4B**). Similarly, α waves increased their power after IGF-I application in young but not in old animals (young = 13, old = 9; $F_{(1,138)}$ = 11,85; ∗∗∗p = 0.0008; Ordinary Two-way ANOVA; **Figure 4C**). β waves also slightly increased after IGF-I injection in young animals, but differences in this frequency band were small, probably due to anesthesia, and did not reach statistical significance (young = 13, old = 8; $F_{(1,145)}$ = 1,833; p = 0.1779; Ordinary Two-way ANOVA; **Figures 4D,E**).

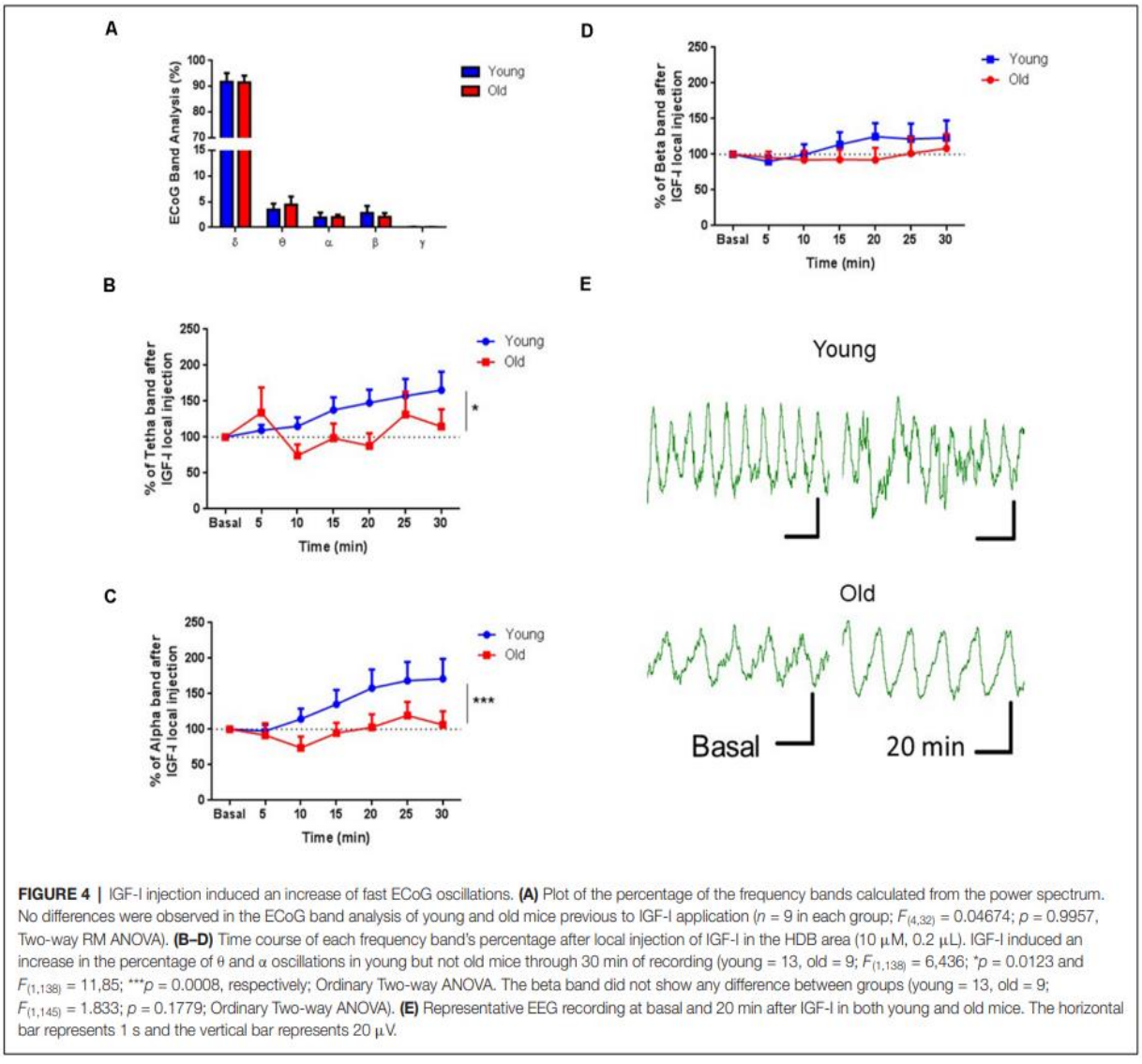

FIGURE 4 | IGF-I injection induced an increase of fast ECoG oscillations. **(A)** Plot of the percentage of the frequency bands calculated from the power spectrum. No differences were observed in the ECoG band analysis of young and old mice previous to IGF-I application (n = 9 in each group; $F_{(4,32)}$ = 0.04674; p = 0.9957, Two-way RM ANOVA). **(B–D)** Time course of each frequency band's percentage after local injection of IGF-I in the HDB area (10 μM, 0.2 μL). IGF-I induced an increase in the percentage of θ and α oscillations in young but not old mice through 30 min of recording (young = 13, old = 9; $F_{(1,138)}$ = 6,436; $^{*}p$ = 0.0123 and $F_{(1,138)}$ = 11,85; $^{***}p$ = 0.0008, respectively; Ordinary Two-way ANOVA. The beta band did not show any difference between groups (young = 13, old = 9; $F_{(1,145)}$ = 1.833; p = 0.1779; Ordinary Two-way ANOVA). **(E)** Representative EEG recording at basal and 20 min after IGF-I in both young and old mice. The horizontal bar represents 1 s and the vertical bar represents 20 μV.

Tactile Response Facilitation by IGF-I

Different studies have demonstrated that BF neurons facilitate cortical sensory responses by activating muscarinic receptors (Golmayo et al., 2003; Fournier et al., 2004; Chaves-Coira et al., 2016, 2018). Here, we want to test if local IGF-I injection in the HDB nucleus also facilitates cortical tactile responses. We analyzed the SEP induced in the S1 cortex by whisker deflections. The SEP area was calculated before and after local injection of IGF-I (10 nM; 0.2 µl) in the HDB nucleus (**Figure 5A**). We did not find differences in the SEP area between young and old mice at basal condition (young = 14; 0.1520 ± 0.0379 mV2; old = 13; 0.1402 ± 0.060 mV2; p = 0.8682, Unpaired t-test; **Figure 5B**).

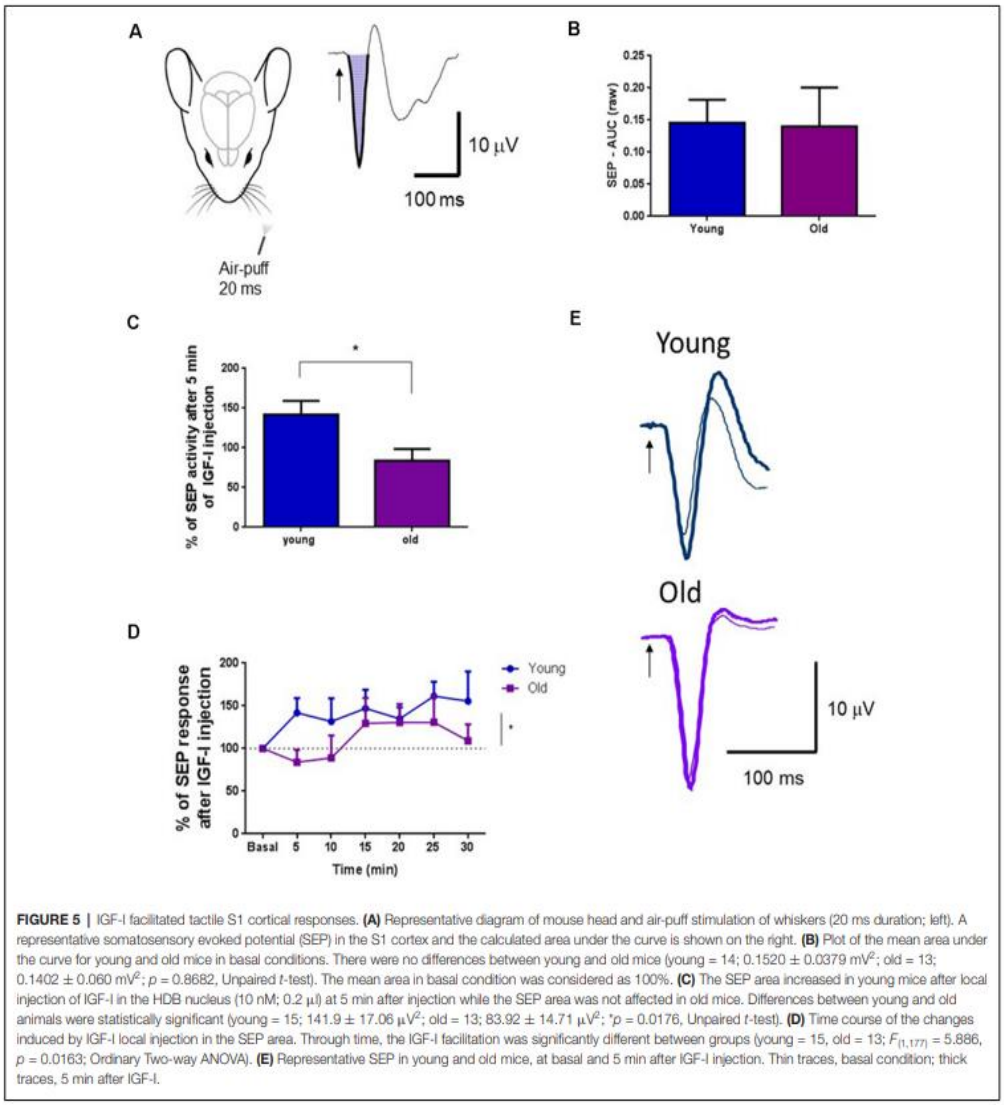

FIGURE 5 | IGF-I facilitated tactile S1 cortical responses. **(A)** Representative diagram of mouse head and air-puff stimulation of whiskers (20 ms duration; left). A representative somatosensory evoked potential (SEP) in the S1 cortex and the calculated area under the curve is shown on the right. **(B)** Plot of the mean area under the curve for young and old mice in basal conditions. There were no differences between young and old mice (young = 14; 0.1520 ± 0.0379 mV^2; old = 13; 0.1402 ± 0.060 mV^2; p = 0.8682, Unpaired t-test). The mean area in basal condition was considered as 100%. **(C)** The SEP area increased in young mice after local injection of IGF-I in the HDB nucleus (10 nM; 0.2 µl) at 5 min after injection while the SEP area was not affected in old mice. Differences between young and old animals were statistically significant (young = 15; 141.9 ± 17.06 μV^2; old = 13; 83.92 ± 14.71 μV^2; *p = 0.0176, Unpaired t-test). **(D)** Time course of the changes induced by IGF-I local injection in the SEP area. Through time, the IGF-I facilitation was significantly different between groups (young = 15, old = 13; $F_{(1,177)}$ = 5.886, p = 0.0163; Ordinary Two-way ANOVA). **(E)** Representative SEP in young and old mice, at basal and 5 min after IGF-I injection. Thin traces, basal condition; thick traces, 5 min after IGF-I.

However, IGF-I injection in the HDB nucleus induced fast facilitation of tactile S1 responses in young mice that increased their response up to 41% over basal response at 5 min after IGF- injection, while IGF-I did not modify tactile responses in old mice at that time (young = 15, old = 13; $p = 0.0176$, Unpaired *t*-test; **Figures 5C,E**). The time course of tactile responses showed a rapid increase in young mice. Nevertheless, tactile responses slowly increased in old mice, reaching a percentage similar to young animals at 15–25 min after IGF-I application (young = 15, old = 13; $F_{(1,177)} = 5.886$, $p = 0.0163$; Ordinary Two-way ANOVA; **Figure 5D**).

Response of Optogenetically Identified Cholinergic Cells to IGF-I

It is well known that cholinergic projections from the BF are responsible for the cortical activation and facilitation of sensory responses (see above references). Consequently, we determine the specific effect of IGF-I on ChAT cells using an optogenetic approach. We used mice expressing channelrhodopsin (ChR) specifically in cholinergic neurons (ChAT-ChR2-YFP animals; see ‘‘Materials and Methods’’ section). We used optogenetic stimulation for selective activation of cholinergic neurons in the HDB nucleus through an optrode that performed unit recordings and optical stimulation simultaneously in the same place. Long-lasting blue-light pulses (300 ms duration) were applied, which stimulated a small volume of tissue (about 200 µm in radius; **Figure 6A**). Unit activity was measured at 100 ms time intervals after the light onset (0–100 ms, 100–200 ms, and 200–300 ms intervals) and compared with the previous 100 ms time interval before light onset (basal condition). Neurons were considered cholinergic (ChAT neurons) when blue-light pulses induced an increase of at least 10% of their unit activity.

Blue-light pulses induced similar responses in ChAT neurons of either young or old mice (**Figures 6B, C**, respectively). In young animals, blue-light pulses increased 33.3% the firing rate over the basal level at 100 ms after light onset ($p = 0.0001$), and 22.2% at 200 ms over the basal level ($F_{(2.040,42.85)} = 9.216$; $p < 0.0004$; $n = 26$ neurons; One-way Repeated Measure ANOVA; at basal vs. 0–100 ms: $p < 0.0001$, or 100–200 ms: $p = 0.0078$, Dunnett’s Multiple Comparison test; **Figure 6B**). Old animals also displayed a blue-light response, which increased at 100 ms (25.4%; $p = 0.0010$) and 200 ms (14.9%; $p = 0.0277$) over basal levels ($F_{(1.908,40.06)} = 6.702$, $p = 0.0035$, $n = 24$; One-way Repeated Measure ANOVA; $p < 0.0010$ at 0–100 ms vs. basal activity, $p < 0.0277$ at 100–200 ms; $n = 24$ neurons; Dunnett’s Multiple Comparison test; **Figure 6C**). The increment of the

firing rate evoked by the blue-light pulse returned to basal levels at 300 ms after light onset in both young and old animals.

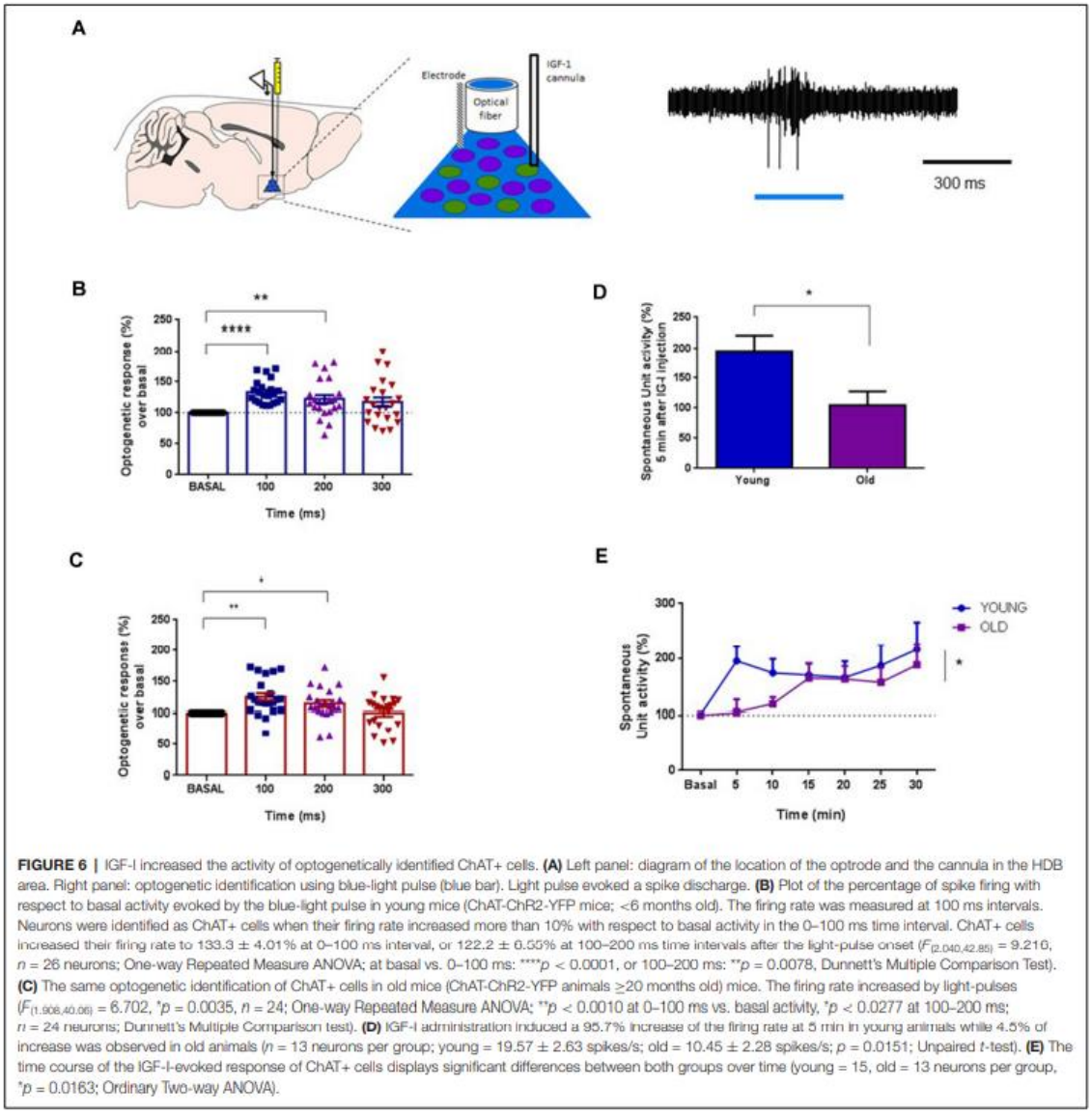

FIGURE 6 | IGF-I increased the activity of optogenetically identified ChAT+ cells. **(A)** Left panel: diagram of the location of the optrode and the cannula in the HDB area. Right panel: optogenetic identification using blue-light pulse (blue bar). Light pulse evoked a spike discharge. **(B)** Plot of the percentage of spike firing with respect to basal activity evoked by the blue-light pulse in young mice (ChAT-ChR2-YFP mice; <6 months old). The firing rate was measured at 100 ms intervals. Neurons were identified as ChAT+ cells when their firing rate increased more than 10% with respect to basal activity in the 0–100 ms time interval. ChAT+ cells increased their firing rate to 133.3 ± 4.01% at 0–100 ms interval, or 122.2 ± 6.55% at 100–200 ms time intervals after the light-pulse onset ($F_{(2.040,42.85)} = 9.216$, $n = 26$ neurons; One-way Repeated Measure ANOVA; at basal vs. 0–100 ms: $^{****}p < 0.0001$, or 100–200 ms: $^{**}p = 0.0078$, Dunnett's Multiple Comparison Test). **(C)** The same optogenetic identification of ChAT+ cells in old mice (ChAT-ChR2-YFP animals ≥20 months old) mice. The firing rate increased by light-pulses ($F_{(1.908,40.06)} = 6.702$, $^{*}p = 0.0035$, $n = 24$; One-way Repeated Measure ANOVA; $^{**}p < 0.0010$ at 0–100 ms vs. basal activity, $^{*}p < 0.0277$ at 100–200 ms; $n = 24$ neurons; Dunnett's Multiple Comparison test). **(D)** IGF-I administration induced a 95.7% increase of the firing rate at 5 min in young animals while 4.5% of increase was observed in old animals ($n = 13$ neurons per group; young = 19.57 ± 2.63 spikes/s; old = 10.45 ± 2.28 spikes/s; $p = 0.0151$; Unpaired t-test). **(E)** The time course of the IGF-I-evoked response of ChAT+ cells displays significant differences between both groups over time (young = 15, old = 13 neurons per group, $^{*}p = 0.0163$; Ordinary Two-way ANOVA).

After neuronal identification, we studied the effect of local application of IGF-I (10 nM; 0.2 µl) in the HDB nucleus on those neurons. The response pattern of ChAT neurons to IGF-I was substantially different in both animal groups. IGF-I induced an immediate 95.7% increase in the firing rate in young animals at 5 min, whereas only a 4.5% increase was seen in old animals (n = 13 neurons per group; young = 19.57 ± 2.63 spikes/s; old = 10.45 2.28 spikes/s; p = 0.0151; Unpaired t-test; **Figure 6D**). The time course showed a rapid increase of the ChAT firing rate in young animals while the firing rate increased slowly in old animals (n = 13 neurons per group; p = 0.0163; Ordinary Two-way

ANOVA; **Figure 6E**).

Muscarinic Blockade Prevents the Effects of IGF-I in ChAT+ Neurons

There is some evidence that IGF-I may interact with muscarinic receptors in various brain systems (Batty et al., 2004; Granja et al., 2019). Thus, we explore if the muscarinic cholinergic receptor antagonist atropine could prevent IGF-I effects in young animals. We firstly identified ChAT cells through optogenetic stimulation, as above. Blue-light pulses increased their firing rate in 16 recorded neurons at 100 ms (53.3%; $p < 0.001$) as well as at 200 ms (34.3%; $p = 0.0345$; **Figure 7A**). After that, we compared the IGF-I effect on ChAT neurons in control conditions (saline solution i.p.; $n = 7$ neurons) or when atropine was i.p injected 15 min before the local injection of IGF-I in the HDB nucleus ($n = 9$ neurons). In both cases, IGF-I induced a fast increase in the firing rate. However, the long-lasting effect evoked by IGF-I was blocked (saline/IGF-I = 7 neurons, atropine/IGF-I = 9 neurons, $F_{(1,151)} = 4.878$, $p = 0.0287$; Two-Way ANOVA; **Figure 7B**). This local effect of atropine on the activity of ChAT neurons may be due to the existence of local collaterals within the BF, as has been observed previously (Zaborszky and Duque, 2000; Zaborszky, 2002). Thus, atropine may affect ChAT neurons, reducing spontaneous activity in the HDB as well as the response to IGF-I local application.

In addition, atropine also blocked the SEP facilitation evoked by IGF-I (**Figure 7C**). IGF-I increased SEP area in control animals (saline-injected animals), while tactile facilitation was blocked in atropine-injected animals (saline/IGF-*I* = 13 animals, atropine/IGF-*I* = 9 animals; $F_{(1,160)} = 26.38$, $p < 0.0001$; Ordinary Two-Way ANOVA).

Chronic IGF-I Injection Partially Restores Neuronal Responses in Old Mice

The above findings showed a reduction of IGF-I effects in old animals. To study if a chronic IGF-I application may restore these effects, we administered IGF-I or saline solutionfor 28 days through Alzetr mini-pumps in old animals.

We compared the spontaneous activity of ChAT+ cells in young and old mice and with ChAT+ cells recorded in IGF- I-treated old mice. Although no differences were observed between the spontaneous firing rate of young and old mice according to the MUA recordings (see **Figure 3B**), old mice had a higher firing rate (11.19 ± 0.89 spikes/s $n = 10$) than young mice when ChAT+ neurons were specifically recorded (7.12 ± 0.71

spikes/s; *n* = 14 neurons *p* = 0.0119; **Figure 8A**). Furthermore, ChAT+ neurons in IGF-I-treated old animals showed a greater spontaneous firing rate (21.17±1.19 spikes/s; *n* = 16 neurons) than old untreated (*p* = 0.0001) or young mice (*p* = 0.0001; One-Way ANOVA, *p* < 0.0001, Holm–Sidak's multiple comparisons test).

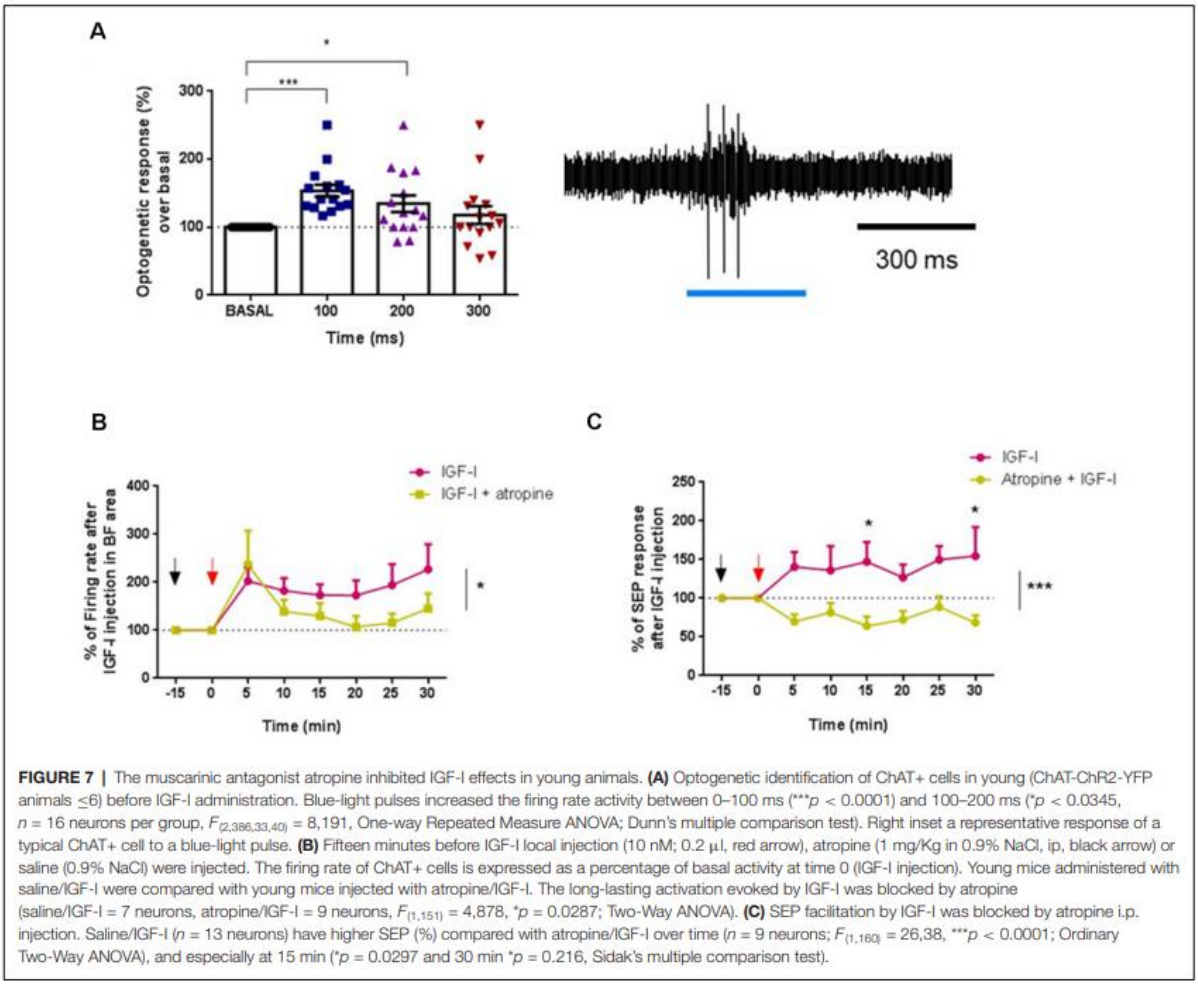

FIGURE 7 | The muscarinic antagonist atropine inhibited IGF-I effects in young animals. **(A)** Optogenetic identification of ChAT+ cells in young (ChAT-ChR2-YFP animals ≤6) before IGF-I administration. Blue-light pulses increased the firing rate activity between 0–100 ms (***$p < 0.0001$) and 100–200 ms (*$p < 0.0345$, $n = 16$ neurons per group, $F_{(2,386,33,40)} = 8,191$, One-way Repeated Measure ANOVA; Dunn's multiple comparison test). Right inset a representative response of a typical ChAT+ cell to a blue-light pulse. **(B)** Fifteen minutes before IGF-I local injection (10 nM; 0.2 µl, red arrow), atropine (1 mg/Kg in 0.9% NaCl, ip, black arrow) or saline (0.9% NaCl) were injected. The firing rate of ChAT+ cells is expressed as a percentage of basal activity at time 0 (IGF-I injection). Young mice administered with saline/IGF-I were compared with young mice injected with atropine/IGF-I. The long-lasting activation evoked by IGF-I was blocked by atropine (saline/IGF-I = 7 neurons, atropine/IGF-I = 9 neurons, $F_{(1,151)} = 4,878$, *$p = 0.0287$; Two-Way ANOVA). **(C)** SEP facilitation by IGF-I was blocked by atropine i.p. injection. Saline/IGF-I ($n = 13$ neurons) have higher SEP (%) compared with atropine/IGF-I over time ($n = 9$ neurons; $F_{(1,160)} = 26,38$, ***$p < 0.0001$; Ordinary Two-Way ANOVA), and especially at 15 min (*$p = 0.0297$ and 30 min *$p = 0.216$, Sidak's multiple comparison test).

Local IGF-I injection in the HDB area (10 nM; 0.2 µl) increased the firing rate in both untreated and IGF-I-treated- old mice. The effect of IGF-I was faster in treated animals; however, differences did not reach statistical significance (*n* = 15 neurons, $F_{(1,182)}$ = 0.6303; *p* = 0.4283; Ordinary Two-Way ANOVA; **Figure 8B**). The IGF-I-facilitatory effect on tactile responses was also faster in IGF-I-treated old mice than in old untreated animals (old = 13 neurons, old treated = 15 neurons, $F_{(1,180)}$ = 5.868, *p* = 0.0164, Ordinary Two-Way ANOVA; **Figure 8C**). In addition, IGF-I-treated old mice show greater fast oscillatory ECoG than untreated old mice after local IGF-I injection in the HDB nucleus (**Figures 8D–F**). The θ, α, and β frequency bands increased; power of θ band (old = 13, old

treated = 15; $F_{(1,180)}$ = 8.075; p = 0.005; **Figure 8D**), α band (old = 13, old treated = 15; $F_{(1,166)}$ = 6,790; p = 0.001; **Figure 8E**), and β band (young = 15, old = 13; $F_{(1,182)}$ = 14.08; p < 0.0002, Ordinary Two-Way ANOVA; **Figure 8F**) over 30 min recording. In conclusion, chronic IGF-I treatment improves basal cholinergic activity and overall cortical activity in old mice.

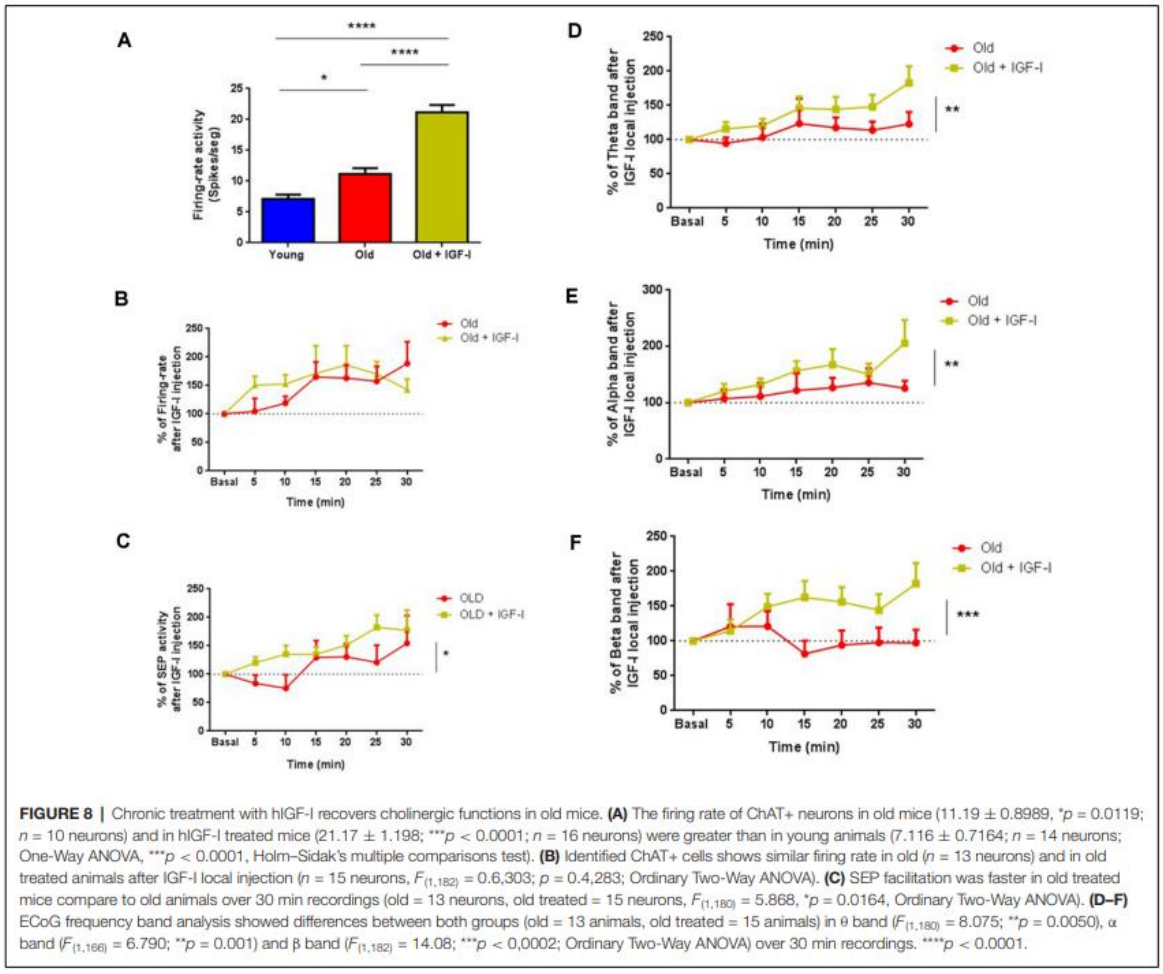

FIGURE 8 | Chronic treatment with hIGF-I recovers cholinergic functions in old mice. **(A)** The firing rate of ChAT+ neurons in old mice (11.19 ± 0.8989, *p = 0.0119; n = 10 neurons) and in hIGF-I treated mice (21.17 ± 1.198; ***p < 0.0001; n = 16 neurons) were greater than in young animals (7.116 ± 0.7164; n = 14 neurons; One-Way ANOVA, ***p < 0.0001, Holm–Sidak's multiple comparisons test). **(B)** Identified ChAT+ cells shows similar firing rate in old (n = 13 neurons) and in old treated animals after IGF-I local injection (n = 15 neurons, $F_{(1,182)}$ = 0.6,303; p = 0.4,283; Ordinary Two-Way ANOVA). **(C)** SEP facilitation was faster in old treated mice compare to old animals over 30 min recordings (old = 13 neurons, old treated = 15 neurons, $F_{(1,180)}$ = 5.868, *p = 0.0164, Ordinary Two-Way ANOVA). **(D–F)** ECoG frequency band analysis showed differences between both groups (old = 13 animals, old treated = 15 animals) in θ band ($F_{(1,180)}$ = 8.075; **p = 0.0050), α band ($F_{(1,166)}$ = 6.790; **p = 0.001) and β band ($F_{(1,182)}$ = 14.08; ***p < 0,0002; Ordinary Two-Way ANOVA) over 30 min recordings. ****p < 0.0001.

Orexin Stimulates Cholinergic Neurons in Young and Old Mice

Finally, we wanted to know whether reduced IGF-I action on ChAT neurons of old animals is a general phenomenon. We tested the effect of orexin in the HDB nucleus because orexin neurons contribute to ECoG activation in the awake state (Sakurai and Sasaki, 1998; de Lecea et al., 1998; Zegarra- Valdivia et al., 2020). Orexinergic receptors are expressed in the BF region and can activate cholinergic and non-cholinergic neurons (Marcus et al., 2001; Arrigoni et al., 2010; Villano et al., 2017). In agreement with that,

we observed orexin-A receptors in ChAT+ neurons (**Supplementary Figure 1A**) and non-identified neurons of the HDB nucleus. Recordings in ChAT neurons of the HDB nucleus of young and old animals (**Supplementary Figure 1B**) showed an increase in the firing rate in both animal groups after local orexin-A injection (10 nM; 0.2 µl), indicating that orexin responses were not affected by aging (young = 12, old = 8, p = 0.6680, **Supplementary Figure 1C**).

DISCUSSION

It is well known that healthy aging is frequently accompanied by a decline in cortical activity and with impairment in cognitive information processing. Furthermore, aging is associated with a reduction in the GH-IGF-I axis activity, resulting in lower serum IGF-I levels (Breese et al., 1991) and impaired brain IGF-I activity (Muller et al., 2012). The present study shows that cholinergic-identified and non-identified neurons in the HDB nucleus showed a decreased response to IGF-I in old mice that provoked a reduction of cortical activation and the response to tactile stimuli. We have focused our experiments on the HDB nucleus of the BF because it has been demonstrated that these neurons project to several sensory cortical areas and the prefrontal cortex, controlling their activity (Zaborszky et al., 2015; Chaves-Coira et al., 2016, 2018). Specifically, optogenetic stimulation of cholinergic neurons located in the HDB area facilitated whisker responses in the S1 cortex through activation of muscarinic receptors (Chaves-Coira et al., 2018). The reduction of cholinergic responses with aging effects was partially ameliorated after chronic IGF-I treatment in old mice. Consequently, the present findings suggest that reduced IGF-I responses in the HDB nucleus may explain the cognitive decline observed in old subjects.

IGF-I exerts many actions in the brain, including protection against injury, modulation of neuronal excitability, angiogenesis, or neurogenesis (see for review Fernandez and Torres-Alemán, 2012; Fernandez et al., 2018). IGF-I enhances neuronal activity in many brain areas (Carro et al., 2000; Gonzalez de la Vega et al., 2001; Kelsch et al., 2001; Nuñez et al., 2003; Barros-Zulaica et al., 2019; Zegarra-Valdivia et al., 2020). IGF-I has also been implicated in brain neurotransmitter release regulation, such as ACh neurotransmission. Nevertheless, contradictory results have been observed in *in vitro* experiments. IGF-I decreased ACh release in the hippocampus (Araujo et al., 1989; Seto

et al., 2002), while other authors showed an increase of the potassium-induced release of ACh in rat cortical slices by IGF-I (Nilsson et al., 1988). The present results *in vivo* showed that IGF-I application increased the activity of optogenetically identified cholinergic HDB neurons and induced an increase of cFos expression in them. In addition, IGF-I application in the HDB induced fast (>4 Hz) ECoG oscillations and increased tactile responses in the S1 cortex, effects that are mainly due to ACh release in this brain area (Celesia and Jasper, 1966; Jasper and Tessier, 1971; Buzsaki et al., 1988; Golmayo et al., 2003; Fournier et al., 2004). These effects were further supported by the presence of IGF-IR in cholinergic and non-cholinergic HDB neurons. In agreement with that, IGF-I's facilitatory effect on cortical activity was blocked by systemic injection of the muscarinic receptor antagonist atropine. Note that the IGF-I-evoked increase of cholinergic-identified neurons was also blocked by systemic injection of atropine. This effect may be due to the existence of extensive local collaterals of cholinergic neurons within the BF (Zaborszky and Duque, 2000; Zaborszky, 2002) that may boost the IGF-I local action and thus, blocked by atropine. Thus, atropine may affect local cholinergic action, reducing spontaneous activity in the HDB. Therefore, IGF-I's effect on cortical activity is, at least in part, due to its action on cholinergic HDB neurons. These results are of great interest because the entry of serum IGF-I into the CNS is more significant during periods of increased neuronal activity or physical activity (Carro et al., 2000; Nishijima et al., 2010). As shown here, systemic IGF-I is known to enhance fast activity in the EEG of mice and non-human primates (Trueba-Sáiz et al., 2013).

Besides, IGF-I increases orexinergic neuronal activity in the lateral hypothalamus, facilitating wakefulness (Zegarra-Valdivia et al., 2020). Therefore, IGF-I facilitates cortical activity in those periods where there is a greater demand for neuronal activity, such as wakefulness, through enhancing cholinergic neurotransmission. The present findings also may explain the reduction of IGF-I effects on cortical activity in an animal model of Alzheimer's disease (APP/PS1 mouse) in which a reduction of ACh neurotransmission is a hallmark of this disease (Trueba-Sáiz et al., 2013). Therefore, reducing cholinergic neuronal activity by a decrease of IGF-I brain entry/activity can cause the aggravation of different neurodegenerative diseases such as Alzheimer's, Schizophrenia or Parkinson's diseases, or aging.

Impairments of cortical activity during aging have been explained by reduced BF neuronal

activity, mainly observed in the cholinergic system (Decker, 1987; Gallagher and Colombo, 1995; Hasselmo, 1999; Dumas and Newhouse, 2011). Studies in animals have shown that pharmacological inhibition or neurotoxic lesions of this region cause dramatic impairments in cortical activity, increasing ECoG slow waves and decreasing attention (Buzsaki et al., 1988; Cape and Jones, 1998; Burk and Sarter, 2001; Alenda and Nuñez, 2007; Kaur et al., 2008; Ishii et al., 2017). It has been reported that IGF-I signaling is deteriorated in the brain of aged animals. With increasing age, IGF-I levels decline substantially both centrally (30% decline) and peripherally (70% decline) in both humans and rodents (Bando et al., 1991; Sonntag et al., 2005, 2013; Deak and Sonntag, 2012; Ashpole et al., 2015). Basal and IGF-I-induced activation of the brain IGF-I receptor/Akt/GSK3 pathway is markedly reduced in the old brain (Muller et al., 2012).

The present findings show that there is an important change in the BF activity during aging. We found that multiunitactivity of HDB area remained equal in young and old animals (**Figure 3B**); however, when we studied specifically ChAT neurons we found an increase in the spontaneous activity of these neurons recorded in old animals with respect to young animals (**Figure 8A**). This finding was in agreement with a larger expression of c-fos in ChAT neurons of old animals than in young animals when a saline solution was i.p. injected (**Figure 2E**). It is well known that there is a reduction of cholinergic functions with aging, but this increase of spontaneous activity of ChAT neurons may not be effective and could be due to a reduction of inhibition from GABAergic cells, as has been described in other neuronal systems (Ling et al., 2005; Ouda and Syka, 2012). In addition, we observed a slight increase of ChAT+ cells in old animals although differences were not statistically significant. It has been indicated previously that the reduction of cholinergic functions is not due to a loss of cholinergic cells (Schliebs and Arendt, 2011). However, the expression of IGF-IR was clearly reduced in old animals. According to these findings, the IGF-I-evoked responses were clearly reduced in old mice.

Consequently, the facilitation of tactile responses in the S1 cortex and the activation of ECoG induced by IGF-I was also reduced in old mice. The effect of aging on the IGF-I responses looks specific since responses of HDB neurons to orexin remained intact in old animals (see **Supplementary Figure 1**).

Therefore, the present results suggest that reduced IGF-I activity may hinder

information processing in the cortex, thus explaining the cognitive deficits observed in aging. Mainly, the response of neuronal activity, ECoG, and sensory responses were lower in old animals with respect to young ones. These lower effects may be due to a reduction of the molecular pathways evoked by IGF-I or by a reduction of IGF-IR on ChAT neurons, as has been demonstrated in the present work. Prolonged systemic administration of IGF-I in old mice partially restored IGF-I effects on HDB neurons and cortical activity, as shown here and by other authors (Markowska et al., 1998; Trejo et al., 2007).

These beneficial effects may be due to the activation of all available receptors. Certainly, IGF-I administration can prevent loss of cognitive performance in humans (Lupien et al., 2003). Another possibility to increase IGF-I activity is physical exercise. It is known that exercise stimulates brain entrance of circulating IGF-I, which mediates many beneficial exercise actions in the brain (Carro et al., 2000; Trejo et al., 2001). Indeed, our previous findings indicated that physical exercise activates the EEG, increasing the hippocampal theta rhythm and improving memory in healthy mice, suggesting that the exercise-evoked increase of IGF-I may favor cortical activity and memory processes (Miki Stein et al., 2017). In addition, there are also pathological situations in which circulating IGF-signaling is decreased, such as diabetes and Alzheimer's disease (Torres- Aleman, 2005; Fernandez et al., 2018). Our findings open the possibility for the development of new therapeutic strategies based on increasing circulating IGF-I levels or mimetic drugs of this peptide, potentiating neuronal activity, and specifically the reinforcement of cholinergic activity during pathological conditions or normal aging.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal procedures followed European guidelines (2010/63, European Council Directives) and were approved by the local Bioethics Committee (Government of the Community of Madrid; PROEX: 189/16).

AUTHOR CONTRIBUTIONS

JZ-V conducted experiments, prepared figures, results, and wrote part of the manuscript. IC-C, MS, LM-R, and JE conducted immunohistochemistry studies and cell image analysis. IT-A wrote part of the manuscript. AN designed and conducted experiments and wrote part of the manuscript. All authors contributed to the article and approved the submitted version.

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DISCUSSION

4. GENERAL DISCUSSION

The different objectives set out in this doctoral thesis would be discussed to argue and respond to the role of IGF-I/orexin/cholinergic axes in regulating sleep and cortical activity through aging.

- *To understand the mechanistic relationships between IGF-I and the sleep/wake cycle in orexinergic neurons.*

In the first study, we explored a transgenic mice model of Cre/Lox mice lacking functional IGF-I receptors in orexin neurons (Floxed IGF-IR/Orexin Cre: Firoc mice), to assess how the ablation of the IGF-I receptor in orexinergic neurons affects their function. Previously, through immunofluorescence, we detected that different cells in the lateral hypothalamus express IGF-IR (Study 1 – Figure 1), providing one mote instance of the ample role of the IGF-I system in the peripherical and central nervous system (35,123). Also, we quantified the response of orexin neurons to an i.p. injection of IGF-I and found an increase of c-fos expression in control mice, whereas Firoc mice seem to remain unaffected (Study 1 – Figure 3).

Through electrophysiology and optogenetic techniques, we corroborate that IGF-I, either locally or systemically injected, activated perifornical (PeF) neurons facilitating wakefulness and electrocorticogram (ECoG) activation -although we cannot entirely rule out that other PeF, non-orexinergic neurons could be activated by IGF-I (Study 1 – Figure 4-7). But most of the PeF neurons showing Cre recombination were orexin neurons (>85%), and it is not known that other PeF neurons modulate the ECoG (124). Besides, analyzing the architecture of sleep through different time points, we observed that Firoc mice have an increase in δ waves and a decrease in faster ECoG activities, especially during the light phase, which agrees with lower orexin levels in Firoc mice.

This finding is similar to Tsunematsu's study (125), with orexin/Halo mice (expressing halorhodopsin, an orange light-activated neuronal silencer). Orexin neurons were silenced by optogenetic orange light in vivo, induced synchronization of the electroencephalogram, and a reduction in amplitude of the electromyogram characteristic of SWS. Compared to our results, functional inactivation of orexin by IGF-IR ablation produces similar

characteristics of SWS. Nonetheless, specific patch-clamp studies might confirm this idea. Besides, genetic ablation of orexinergic neurons, mutation of orexin receptors results, or even chemogenetic inhibition of these neurons with Designer Receptors Exclusively Activated by Designer Drugs (DREADD), under clozapine N – oxide (CNO) inhibitor (See annexed study 1 – Supp Fig 2) results in a decrease in wakefulness and altered sleep/wake cycles (126–129),

Considering all this, the role of IGF-IR in orexinergic neurons seems to be important to the modulation of its activity, and IGF-I signaling exerts a direct activation of orexinergic neurons, resulting in FIROC mice altered ECoG patterns with a predominance of slow waves, suggesting a direct effect of this anabolic hormone on sleep through these hypothalamic neurons. Nonetheless, other possible functional links between the IGF-I / orexin axis in other processes such as cognition or emotion remain to be elucidated.

- *To evaluate if reduced IGF-I input to orexin neurons during aging contributes to age-associated sleep and cortical activation disturbances.*

Different studies show that serum IGF-I diminishes during aging (23,106,107), and the IGF-I/insulin system is associated with poor cognition, increased dementia prevalence, cardiovascular and metabolic disease (23,30,130,131). Besides, sleep disturbances are common (132,133), that whether they are an inherent component of the aging process is under debate (134). Nonetheless, it has been seen that alterations in sleep during aging are related to orexin loss (135) and in the case of AD pathology, for example, caused by tau accumulation (136). Together, these associations of aging, sleep and the IGF-I/orexin axis are intriguing (66).

Changes in sleep/wake status have been reported during aging, with fragmented and increased daytime sleep, but the overall SWS and REM decreases (137). Considering our previous result that confirms the participation of IGF-I axis in orexin modulation, we studied if aged brain may lose sensitivity to IGF-I in these cells. Using ECoG recordings, we corroborate the existence of sleep architecture changes in old mice (>18 months old) compared to young mice (3-6 months old). Showing typical age-related decrease in δ activity (132,133,138) and increased fast-waves -θ, -α, and -β bands. Thus, aged mice display sleep alterations, as previous studies reported.

Then, we observed that systemic injection of IGF-I to old mice resulted in reduced c-fos expression in orexin neurons compared to young mice. Also, responses of LH neurons to either systemic or local IGF-I were abrogated in old mice in electrophysiology studies, which suggested the loss of sensitivity of LH neurons to this growth factor. This orexin absence response to IGF-I may be related to metabolic and bioenergetic modulation alterations during aging and decreased orexin tone activity. Orexin deficiency caused the age-related development of impaired glucose tolerance and insulin resistance (139), closely related to IGF-I resistance.

In contrast, designer receptors exclusively activated by designer drugs (DREADD) study by Stanojlovic et al. (140) suggest that increased orexin tone by DREAD activation in middle-age and aging mice could ameliorate the effect of aging on behavioral and metabolic alterations. Bearing in mind the reduced sensibility of IGF-I in old mice, we use immunofluorescence and *in situ* hybridization to explore the role of IGF-IR signaling in orexin neurons and to discover increased express levels of IGF-IR, but reduced expression of IGF-IR mRNA, hinting to disturbed brain IGF-I activity. These results suggest that increases in brain IGF-I receptors and the capacity to take up serum IGF-I were ineffective because IGF-I function is reduced (2,141). Besides a dysregulated control of IGF-IR activity, IGF-IR protein and mRNA do not necessarily change parallel and may reflect post-receptor disturbances in IGF-I signaling (142). Again, these results combine with the previous reports of insulin resistance during aging promoted by orexin dysregulation and impaired circadian patterns (139,143).

Then, taking advantage of a novel IGF-IR sensitizer (AIK3a305) that crosses the BBB and acts through inhibiting the p38 MAPK pathway (144), a kinase involved in feedback inhibition of IGF-I signaling (145), we treated old mice chronically (four weeks) with this compound and found that sleep patterns were rejuvenated, and c-fos responses to IGF-I were recovered to youthful levels. The present observations reinforce previous ones indicating that the brain loses sensitivity to insulin peptides and IGF-I during aging, together with lower serum IGF-I levels (2), produce an overall reduction of IGF-I effects. This interpretation is reinforced by the practical therapeutic actions of Aik3a305 drug.

Thus, reduced IGF-I input to orexin neurons during aging contributes to age-associated cortical and sleep disturbances are due to decreased activation of orexinergic neurons by reduced IGF-I input and opens a gate to study further systems disruptions related to the IGF-I/orexin axis in aging. It is plausible that energy metabolism, sleep/wake alterations, cognitive decline, and other disturbances related to aging are directly related to orexin loss or decreased tone or indirectly via energy expenditure disturbances, including insulin and IGF-I resistance. Besides, it opens those other neurotransmitters and nuclei in the brain, which contributes to cortical and sleep disturbances.

- *To analyze if IGF-I facilitates cholinergic activity to modulate arousal and cortical activation by BF neurons.*

Considering the pleiotropic actions of IGF-I in the brain and its regulation of many neurotransmitters (146–149), it is not surprising that cholinergic neurons may respond to it (150). But also, seeing the cholinergic contribution in arousal, cortical activity, and sleep, as the BF projections to the entire cortex (151), and receiving afferents from different brain areas (152,153), including the orexinergic cells from LH (154), it might be that the BF is a site or hub through which collaborate with orexins cells to activated the cortex and promotes behavioral arousal (155).

Then, it is plausible that IGF-I/orexin/cholinergic link is orchestrating these processes. Thus, we explore if cholinergic cells in BF express IGF-IR and orexin receptor (OX1R) (see Study 3 – Figure 1 and suppl. Fig 1). We also analyzed their response to IGF-I and orexin-A in BF in young (3-6 months) and old mice (>18 months). We take advantage of electrophysiological and optogenetic studies, finding impaired IGF-I response in old animals (see Study 3 – Figures 3-6), manifested in reduce multiunit activity (MUA), reduced fast-waves (θ and α bands). Optogenetic studies identified specific ChAT cells using a light-activated cation channel (channelrhodopsin-2) tagged with a fluorescent protein (ChR2-YFP) under the control of the choline acetyltransferase promoter in cholinergic neurons (ChAT+ identified neurons). These studies confirmed that most of the IGF-I induced excitatory effects were mediated by activation of these neurons and were reduced in old mice.

On the contrary, responses of ChAT+ identified neurons to orexin-A were kept normal in old mice (see Study 3 – Suppl Fig. 1). These findings suggest that the reduction of activity in BF neurons may be due to a specific reduction of IGF-IR in cholinergic neurons while other neurotransmitter receptors such as orexin receptors are not affected. It is necessary to bear in mind that although the machinery that generates the response to orexin in cholinergic neurons is not affected in aging, the activity of these hypothalamic neurons is reduced, as we have previously shown.

Contemplating a similar impairment as previously see (Study 2) in the IGF-I signaling onto orexin cells, we measured the IGF-IR in ChAT cells by inmunocytochemistry (see Study 3 – Figure 1). In this case, the IGF-IR+/ChAT+ ratio was reduced in aged mice. One possible explanation is that the brain's reduced entrance and IGF-I signaling are insufficient to modulate IGF-IR expression (142) in cholinergic neurons of old mice. This reduction may affect essential sensorial/cortical processing and could explain the reduction of sensory perception with aging. For example, BF neurons demonstrated their participation in facilitating cortical sensory responses by activating muscarinic receptors (88,105,156). In our results, IGF-I increases cortical activation and the response to tactile stimuli only in young mice. Whereas old mice have a delayed response probably due to a reduction of the number of IGF-IR and/or reduction in intracellular machinery activated by IGF-I.

Then we try to recover cholinergic function with human IGF-I (hIGF-I) treatment chronically diffuse (see Study 3 – Figure 8). Old mice treated with hIGF-I display partially improved tactile response and IGF-I response in HDB neurons from BF. This finding agrees with the idea that in old animals, there is a reduction of the number of IGF-IR and/or a reduction in intracellular machinery activated by IGF-I that cannot be totally recovered by chronic application of IGF-I. This way, we again confirm the importance of IGF-I regulating brain function, specifically in cholinergic cells from BF.

In conclusion, it is well known that healthy aging is frequently accompanied by a decline in cortical activity and impairment in cognitive information processing. Furthermore, aging is associated with reducing the GH/IGF-I axis activity, resulting in lower serum IGF-I levels. The reduction of cholinergic responses with aging effects was partially ameliorated

after chronic IGF-I treatment in old mice. The pleiotropic role of IGF-I seems to regulate different brain areas (1,30), neurotransmitters (146,157), and participation/response to brain insults (20,158). Our results expand previous observations suggesting that IGF-I is a neuroendocrine regulator of neuronal plasticity, which may be affected through aging. Previous research suggests the implication of IGF-I in aging and AD through different mechanisms (5,122,159,160). Here we provide evidence of specific IGF-I/cholinergic axis modulation *in vivo,* suggesting that reduced IGF-I responses in the HDB nucleus may explain the cognitive decline observed in old subjects.

4.1. Final comments

The fact that a pleiotropic neurotrophic factor such as IGF-I (1) modulates the activity of orexin and cholinergic neurons, also considered a multitasking system (161), contributes to explaining the diversity of IGF-I actions in the brain, especially regarding its regulatory activity of circadian patterns, metabolism, arousal, and sleep. The direct projections to the cholinergic neurons from cells in the LH and orexin neurons and the orexin receptor expression in ChAT suggest a strong link, participating together in wakefulness, arousal, and cortical activation. All together with IGF-IR expression, increased firing rate, and ECoG activation in response to IGF-I in the present results, expose intricate participation of this peptide in brain system regulation, which offers new understanding mechanisms related to brain pathology, but also reveals therapeutic possibilities to ameliorate brain changes during aging, for example with IGF-I or IGF-IR sensitizer (144,162–166).

But not only IGF-I exerts a modulation of brain activity. For example, orexin interacts with the somatotrophic axis (167), suggesting a feedback loop between these systems, as orexin also expresses IGFBP-3, a significant factor controlling circulating IGF-I availability (168) in turn regulates orexin activity. Also, orexin shows to activate the mTOR pathway, dependent on extracellular Ca^{2+} influx (169). As previously mentioned, the mTOR pathway is activated in cell growth, metabolism, and energy expenditure, a key element in the IGF-I pathway. Thus, different results display a link between IGF-I and the orexin system, suggesting that a direct action of IGF-I onto orexin neurons may be essential for their proper function.

On the other hand, like other vital areas, BF receives orexin afferents, expressing OX1R and OX2R (79), which indicates participation in BF cholinergic and non-cholinergic processes. At the same time, BF cells in HDB express IGF-IR, and as our results suggest, exert regulatory actions on ChAT cells and possible other non-cholinergic neurons (such as HDB GABAergic cells (170)), which may play a role in excitatory-inhibitory (E/I) activity in BF.

The excitatory participation of both orexin-A and IGF-I suggests significant modulation of both. Nonetheless, during aging, the alteration of IGF-I but not of orexin-A indicates that IGF-I should be a key regulator of both orexin and cholinergic cells in cortical activity and wakefulness. Interestingly, ChAT cells increase ACh expression in the cortex under IGF-I treatment (171). Besides, ACh may modulate the GH/IGF-I axis, suggesting a feedback loop between these two during brain development (172) and probably in the adult brain.

During aging, diverse factors modulate synapse density, including E/I balance or synaptic pruning and sprouting. The loss of synapses (i.e., 10-15%) occurs during normal aging, and cognitive functioning is dependent on synapse density in the brain (173). Metabolic alterations could lead transition between normal to pathological aging, and dependent on which neural systems are involved, different symptoms could appear. Regarding this work, we focus on two main areas involved in sleep/wake regulation, cortical activity, and information processing. Curiously, both areas seem to be modulated by IGF-I, which may suggest its participation in pathology, and both neuronal groups seem in turn to modulate IGF-I.

However, it is essential to point out that IGF-I function is necessary but insufficient to trigger AD pathology alone. Altered IGF-I may reflect the impact of life-styles factors on pathological changes. However, also, the relationship of old age with the reduction of GH/IGF-I or "somatopause" promoting reduced serum levels in the blood as impaired signaling/resistance in the brain may suggest a functional connection of IGF-I/orexin/cholinergic cells involved in sleep disturbances and daytime drowsiness that take place during aging, or in pathologies such as AD.

4.2. Limitations

Throughout these experiments, different limitations have emerged in the different studies presented. We can mention, for example, in ECoG studies on Study 1, the difficulty to measure REM and non-REM stages, considering possible functional characteristics on sleep disturbance in Firoc mice, which would reflect a better understanding of their sleep architecture perturbance, and considered later in old mice studies. Another significant limitation in study 1 was the use of Cre/Lox transgenic mouse. Many of our observations are based on this method that may show ectopic Cre expression, which would result in a contribution of unidentified brain cells expressing IGF-IR in the observed change and also in developmental effects of IGF-IR deletion in orexin cells. Despite this, less than 15% of neurons showing Cre recombination were not orexin neurons, whereas parallel studies in the laboratory using viral-mediated deletion on IGF-IR in adult orexin cells confirm an identical phenotype. Thus, undetermined developmental effects on orexin neurons can be reasonably ruled out.

Study 2 observed an increased c-fos activation response in old mice under basal conditions and a null response to IGF-I. One limitation was the impossibility to study more in depth this alteration to elucidate this c-fos pattern. However, one possible explanation is that the increment of c-fos may reflect decompensations in the excitatory/inhibitory (E/I) ratio process that insulin peptides (174) and IGF-I plays to regulate brain networks (175). Similarly, in humans, E/I alterations have been seen in hyper-synchronization resting-state analysis, a hallmark of increased activity of brain circuits underlying synaptic dysfunction by neuronal loss in the elderly and AD (176,177). Other limitations are related to studying sleep architecture in the ECoG *in vivo* studies, especially in the treated mice with Aik3a305 drug. Thus, we could provide further insight of the functional recovery of sleep patterns.

On the other hand, a confirmation of IGF-I/orexin signaling disruption in old mice through optogenetic studies would have strengthened our claims, as well as the use of IGF-I or insulin resistance mice models, to confirm orexinergic alteration, for example, using a model of low IGF-I bioavailability, with produce insulin resistance (178). Further analysis

of how AIK3a305 drug improves functioning of other brain areas and cells that contribute together with orexin on sleep patterns regulation would also be interest.

Another significant limitation in study 3 was the impossibility of simultaneously analyzing the involvement of orexinergic and cholinergic areas using an electrophysiological approach, which may explain distinct E/I interactions between brain regions, primarily through IGF-I serum reduction or resistance during aging. Another interesting study would have been to analyze if AIK3a305 drug could affect recovering multi unitary activity or firing rate of HDB and cholinergic cells. Thus, a direct contrast between hIGF-I and Aik3a305 treatment would have elucidated possible mechanisms (IGF-I resistance vs. low IGF-I bioavailability) and better treatment opportunities.

Finally, some limitations are common to the three studies. First, the limited use of animals and small groups has prevented us from designing further studies, especially with older animals due to the difficulty which represents (i.e., premature death, long wait for the appropriate age of mice, obesity, diseases typical of the age, and others). This may also be reflected in the absence of other in vivo experiments, exploring other contributions of IGF-I/orexin/cholinergic axes in the process, such as cognition or emotion (learning and memory, working memory, copying behavior).

CONCLUSIONS

5. CONCLUSIONS

In this section, we will evaluate the achievement of the main objectives defined in section 2. The first main objective was to understand the mechanisms relationships between IGF-I and the sleep/wake cycle in orexinergic neurons.

We confirm IGF-I modulation of orexin neurons taking advantage of transgenic Firoc mice obtained through Cre/Lox system. Firoc mice have blunted IGF-IR in orexinergic cells. After validating by different techniques, the IGF-IR inactivation, we use electrophysiology and immunofluorescence confirming reduced response to IGF-I systematic (i.p.) or locally injected in LH. Specifically, we show that optogenetically identified PeF neurons are activated by IGF-I, facilitating in this way wakefulness and ECoG activation. We also discover sleep alterations in Firoc mice during the inactive/active phase. These results are explained by IGF-I's direct activation of orexinergic neurons, facilitating different sleep/wakefulness regulation processes.

The second main objective was to evaluate if reduced IGF-I input to orexin neurons during aging contributes to age-associated sleep and cortical activation disturbances.

We confirm that old C57BL/6J57 mice have altered sleep structure compared to young animals, with slow and fast ECoG activity changes and fewer transitions between sleep and awake states. The local and systemic response to IGF-I in LH was altered, analyzed by electrophysiological or c-fos immunofluorescence. Our data suggest impaired IGF-IR signaling with loss of sensitivity to IGF-I. This idea was confirmed after treating the old mice chronically with a new IGF-I sensitizer (Aik3a305), which restored IGF-I responses and rejuvenated sleep patterns.

The third main objective was to analyze if IGF-I facilitates cholinergic activity that modulates arousal and cortical activation by BF neurons.

We confirmed the IGF-I excitatory modulation of cholinergic cells in HDB/BF area. Local injection of IGF-I in the HDB nucleus increased cholinergic activity, induced fast oscillatory activity in the ECoG, and facilitated tactile responses in the primary

somatosensory cortex elicited by air-puffs delivered in the whiskers. These excitatory effects decreased in old mice. Cholinergic cells express IGF-IR in cholinergic HDB neurons, but IGF-R reduced its expression in old mice. Aged mice lose their response to IGF-I in cholinergic neurons, measured by reducing c-fos expression and confirmed by IGF-I injection and electrophysiological/optogenetic studies. IGF-I excitatory effects were partially recovered by chronic hIGF-I treatment (4 weeks). Thus, we confirmed that reduced IGF-I activity in old animals participates in age-associated changes in cortical activity.

5.1 Future directions

This work verified the importance of IGF-I and its pleiotropic activity in the brain and its implications during aging. In the future, it would be necessary, for example, to investigate how other processes are affected by the absence of orexin activity, mediated by the ablated IGF-I receptor (i.e., cognition, emotion, and motivation). It has been seen that orexin knock-out mice display reduced voluntary exercise (179,180) and less dopaminergic activity in the VTA, another site of orexin action where mediates reward and motivational effects (181,182).

Some initial studies suggest that the Firoc model simulates similar alterations in exercise motivation and mood (183). Thus, altering the orexinergic tone modulated by IGF-I would affect sleep/wakefulness, emotion, and motivation. Other pending studies would explore how orexinergic projections to cortical areas would be involved with cognitive processes and whether the functional alteration of orexins in the Firoc model also decreases. If it is verified that the IGF-I/orexin axis modulates other critical processes both at the dopaminergic or serotonergic level and with the affectation of cognitive processes, it would be interesting to study how orexinergic projections to these centers are affected in aged mice; for example, in the prefrontal cortex, promote improvements on cognitive performance in old mice. Following this line, it has been found that orexin improves memory performance when it is microinjected (66).

Likewise, we have explored how the IGF-I enables coping behaviors by balancing E/I input onto orexin neurons in a context-dependent manner (Fernandez de Sevilla et al.,

2020). Previous data would complement the study of orexin and confirm its crucial for the consolidation of conditioned fear (53). On the other hand, IGF-I is also an interesting treatment for post-traumatic stress models since IGF-I favors fear extinction memory—showing novel functional consequences of IGF-I signaling, revealing IGF-I as a critical element in controlling the fear extinction memory (184). The next step, knowing that the IGF-I / orexin axis modulates other cognitive and emotional processes, or knowing that it also modulates the cholinergic activity of BF, would be to examine how the mechanisms identified are found in transgenic AD mice to determine if they are affected.

Besides, it would be essential to know how other neural centers participate in the regulation of sleep/wakefulness or involved in the regulation of mood and intellect, such as the locus coeruleus (LC) of the brainstem and the medial prefrontal cortex (mPFC), respectively, and are highly interconnected with the lateral hypothalamus and the basal forebrain, and if they are also modulated or not by IGF-I. It is possible that exploring these areas through the expression of IGF-I receptors is the first step. Another approach would be to know if, compared to the injection of IGF-I i.p., either under physical exercise, they show increased activity of c-fos, this because we know that physical exercise promotes the entry of IGF-I into the brain, or through environmental enrichment, another method to promote the entry of IGF-I (36,142).

6. LIST OF PUBLICATIONS DERIVED FROM THE DOCTORAL PERIOD

1. Munive, V., **Zegarra-Valdivia, J. A.,** Herrero-Labrador, R., Fernandez, A. M., & Aleman, I. T. (2019). Loss of the interaction between estradiol and insulin-like growth factor I in brain endothelial cells associates to changes in mood homeostasis during peri-menopause in mice. Aging, 11(1), 174–184. https://doi.org/10.18632/aging.101739

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1. Fernandez de Sevilla, M., Pignatelli, J., **Zegarra-Valdivia, J. A.,** & Torres Alemán, I. (2020). Insulin-Like Growth Factor I Modulates Vulnerability to Stress Through Orexin Neurons. BioRxiv, 2020.02.10.941377. https://doi.org/10.1101/2020.02.10.941377

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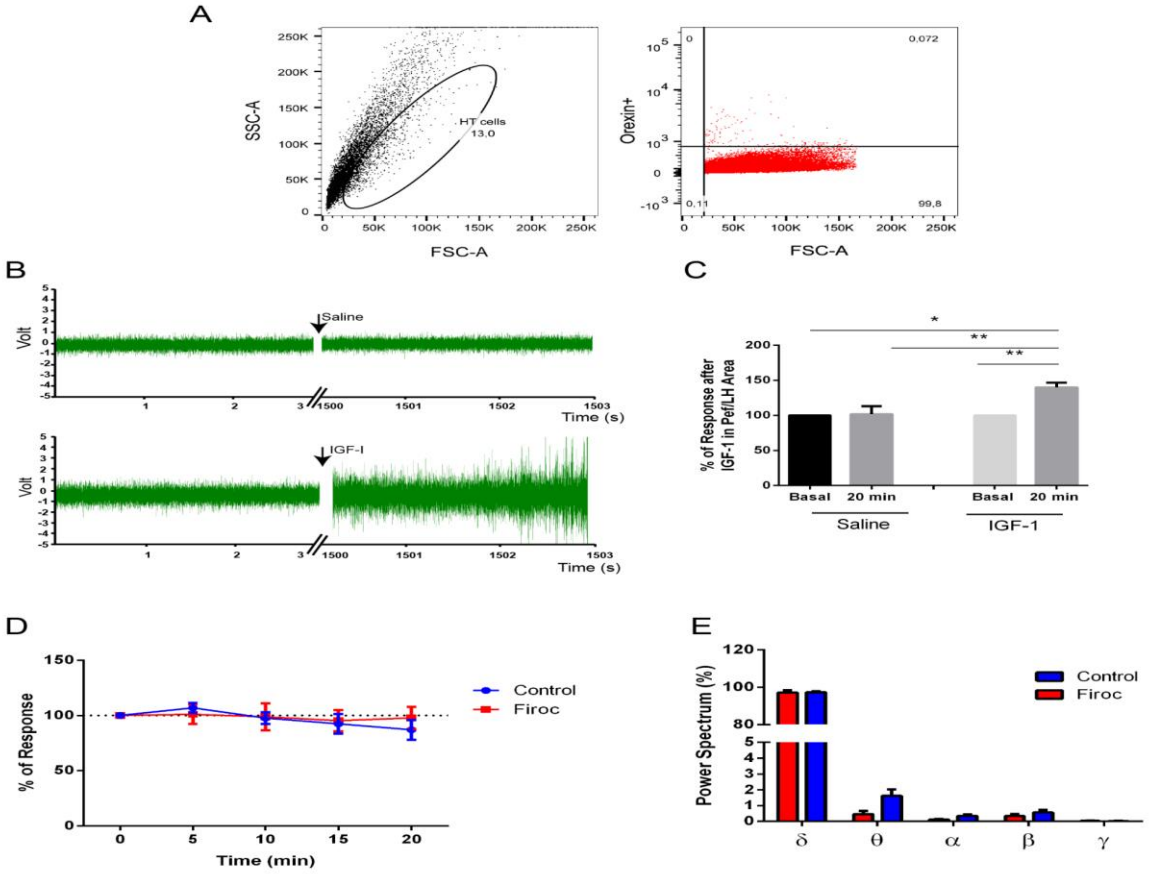
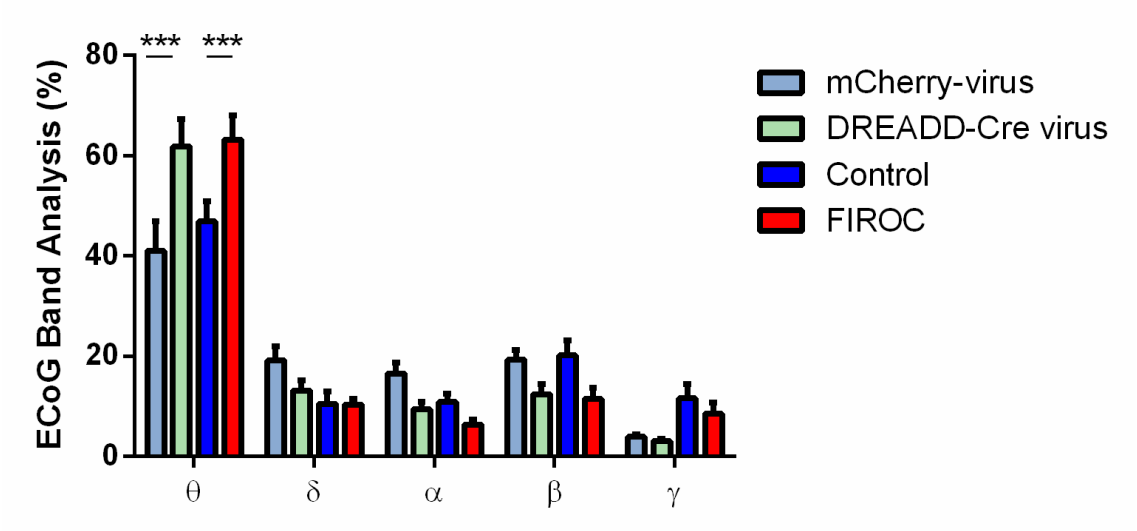
APPENDIX 1

Study 1 - Supplementary figure 1.



Supplementary Figure 1: A, Flow cytometry charts showing isolation of orexin^{+} cells. Left panel: Scatter plot isolating hypothalamic (HT) cells from debris. Forward scatter (FSC) and side scatter (SSC) indicate size and granularity of each event (representing as a dot), respectively. Right: Fluorescent plot to sort orexin^{+} and orexin^{-} cell populations from the selected cluster (HT cells). A threshold in the y-axis (APC fluorescent intensity channel) is determined to sort the different populations. **B,** Local injection of insulin (10 µM; 0.1µl) does not affect neuronal activity in PeF orexin neurons after LC stimulation (Firoc=9, control=17; sex balanced). **B,** Saline injection did not alter PeF responses to LC stimulation. Representative traces are shown. Saline or IGF-1 were injected after 300 sec of basal recording. **C**, Quantification histograms of multi-unitary activity in basal vs IGF-1conditions at 20min (Saline = 6; IGF-1= 9; Kruskal-Wallis test; ***p<0.0007, Dunn´s Multiple Comparison Test, 20 min IGF-1 vs 20 min saline **p=0.0042, 20 min IGF-1 vs Basal IGF-1 **p=0.0055). **D,** Local injection of insulin (10 µM; 0.1µl) does not affect neuronal activity in PeF orexin neurons after LC stimulation (Firoc=9, control=17; sex balanced). **E,** Power spectra analysis of ECoG bands obtained at baseline in both Firoc and control littermates during electrophysiological recording in S1 (Ordinary Two-Way ANOVA).

Study 1 - Supplementary figure 2.



Supplementary Figure 2: A posterior study was performed using Designer Receptors Exclusively Activated by Designer Drugs (DREADD), under clozapine N – oxide (CNO) inhibitor. For chemogenetic experiments using DREADD, a viral construct (AAV-hSyn-DIOhM4D(Gi)-mCherry; AAV5; 8.6 x 1012 viral infective units/ml) was locally injected bilaterally to inactivate orexin-cre neurons in Orexin-Cre mice. As a control virus, AAV-hSyn-DIO-mCherry (AAV5). Both viral constructions were obtained from Addgene (pAAV-hSyn-DIO-hM4D(Gi)-mCherry (AAV5), # 44362-AAV5 and pAAV-hSyn-DIO-mCherry (AAV5) # 50459-AAV5) (See in Fernandez de Sevilla et al., (185)). Clozapine N-Oxide (CNO, 2mg/kg dissolved in saline 0.9%) was administered ip, and 40 min later, ECoG activity was assessed. DREADD-Cre and mCherry-virus mice were compared to previous Firoc (n=7) and littermates' mice (n=8). In both cases, orexin inactivated mice by CNO or blunted IGF-IR display an increase θ activity and SWS. (Two-Way ANOVA, Sidak´s Multiple comparison test ($F(4,115)=167.8$, ***$p>0.0001$).

APPENDIX 2

Study 2

INSULIN-LIKE GROWTH FACTOR I SENSITIZATIONREJUVENATES SLEEP PATTERNS IN OLD MICE

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ABSTRACT

Sleep disturbances are common during aging. Compared to young animals, old mice show altered sleep structure, with changes in both slow and fast electrocorticographic (ECoG) activity and fewer transitions between sleep and wake stages. Insulin-like growth factor I (IGF-I), which is involved in adaptive changes during aging, was previously shown to increase ECoG activity in young mice and monkeys. Furthermore, IGF-I shapes sleep architecture by modulating the activity of mouse orexin neurons in the lateral hypothalamus (LH). We now report that both ECoG activation and excitation of orexin neurons by systemic IGF-I is abrogated in old mice. Moreover, orthodromical responses of LH neurons are facilitated by either systemic or local IGF-I in young mice, but not in old ones. As orexin neurons of old mice show dysregulated IGF-I receptor (IGF-IR) expression, suggesting disturbed IGF-I sensitivity, we treated old mice with AIK3a305, a novel IGF-IR sensitizer, and observed restored responses to IGF-I and rejuvenation of sleep patterns. Thus, disturbed sleep structure in aging mice may be related to impaired IGF-I signaling onto orexin neurons, reflecting a broader loss of IGF-I activity in the aged mouse brain.

Keywords: Aging, IGF-I, orexinergic neurons, sleep.

INTRODUCTION

Sleep disturbances are so common during aging, that whether they are an inherent component of the aging process is under debate [1]. Importantly, these alterations present together with metabolic and cognitive impairments frequently found in aged individuals, but their contribution to pathology is unclear [2, 3]. Therefore, knowledge of the mechanisms underlying these age-associated changes is of great interest.

We recently documented that circulating IGF-I, a hormone that participates in the aging process all along phylogeny [4], is also involved in the sleep/wake cycle, as mice with reduced serum IGF-I present altered circadian electrocorticographic (ECoG) activity, among many other disturbances [5]. Significantly, aging is associated to reduced serum IGF-I levels in all mammalian species studied [6]. More recently, we observed that IGF-I shapes sleep architecture by modulating the activity of hypothalamic orexin neurons [7], a group of neurons in the lateral hypothalamus (LH) involved in the regulation of the sleep/wake cycle [8]. However, whether IGF-I plays a detrimental or protective role in the aging brain is controversial [9, 10.] At any rate, loss of IGF-I activity in the aging brain has been previously documented by us [11], and many others [12, 13, 14.] We hypothesized that orexin neurons in the aged brain may also lose sensitivity to this hormone, which coupled with age-associated reduced serum IGF-I, would aggravate IGF-I loss-of-function and eventually affect sleep/wake patterns.

We observed that systemic injection of IGF-I to old mice resulted in reduced c-fos expression in orexin neurons, as compared to young mice. Also, responses of LH neurons to either systemic or local IGF-I were abrogated in old mice, which suggested loss of sensitivity of LH neurons to this growth factor. Since orexin neurons in old mice express altered levels of IGF-IR, hinting to disturbed brain IGF-I activity, we treated old mice with a novel IGF-IR sensitizer and found that sleep patterns were rejuvenated. Thus, reduced IGF-I input to orexin neurons during aging contributes to age-associated sleep disturbances.

RESULTS

Changes in sleep architecture in old mice

We first confirmed that changes in sleep structure during normal aging documented in humans [15] are also present in aged mice (Hasan et al 2012). Using ECoG recordings

during the light period (corresponding to the inactive phase in mice, Figure 1A) we observed that old mice (>18 months old) have markedly different patterns of ECoG activity in both slow and high-frequency bands, as compared to young animals (4-6 months old). Thus, the typical age-related decrease in δ activity [15] was observed (young=8, old=4; ***p<0.0001; Figure 1B), together with increases in θ, α, and β bands (young=8, old=4; ***p<0.0001; Figure 1C-E). The γ band was significantly depressed specifically at Zeitgeber time (ZT)19 (**p=0.0084; Figure 1F). ECoG analysis during the inactive phase shows a total mean decrease of δ activity (young=13, old=8, **p=0.0004; Figure 1G). Next, we analyzed sleep onset latency and found that old mice show a shorter latency to sleep (young=11, old=7; **p=0.0099; Figure 1H). In these experiments we combined both sexes, as ECoG patterns are similar in males and females (Suppl Figure 1A, B).

Orexin neurons in old mice show reduced sensibility to IGF-I

As already reported, old mice show reduced serum IGF-I levels and loss of sensitivity to this hormone [11]. Accordingly, while systemic administration of IGF-I (1 µg/g, ip) to young mice results in enhanced ECoG activity [16], in old mice, ECoG responses are significantly attenuated (Figure 2A,B).

Orexin neurons are a discrete cell population in the lateral hypothalamus that participate in sleep/wake regulation 8. Since IGF-I shapes sleep architecture through them [7], we speculated that orexin neurons might be involved in age-associated sleep disturbances due to impaired sensitivity to IGF-I. Indeed, after systemic IGF-I injection young mice responded with increased c-fos expression in orexin neurons, whereas old mice, that had significantly higher basal c-fos immunoreactivity in orexin neurons, showed slightly decreased c-fos expression in response to IGF-I (Figure 3).

To elucidate whether altered responses to systemic IGF-I are due to locally disturbed IGF-I signaling or reduced entrance of IGF-I from the periphery [17, 18,] we recorded responses to IGF-I in lateral hypothalamic (LH) neurons in young and old mice. First, we stimulated LH neurons through the locus coeruleus (LC) following previously published procedures 7, and then we administered IGF-I either systemically or locally. While orthodromic stimulation of LH neurons elicited comparable evoked potentials in young and old mice, and similar latency (Suppl Fig 1 C), this was not the case for

their responses to IGF-I. Either after local (Figure 4A; young=15, old=13), or systemic (ip) IGF-I administration (young=9, old= 7, Figure 4B), LH neurons of old mice responded to this growth factor with slightly reduced activity, whereas young mice responded with significantly increased activity.

Since orexin neurons affect overall brain activity due to their broad projections throughout the brain [19], we analyzed ECoG activity and observed that before local injection of IGF-I, old mice show a significant increase in δ waves compared to young ones (Figure 4C; ***p>0.0001; young=19, old=13). After local IGF-I administration, the α band increased only in young mice, suggesting that IGF-I does no longer influence LH projections to the cortex in old mice (young=19, old=12, **p=0.0094, Figure 4D). On the other hand, orexin levels in the hypothalamus of old mice (n=6) were reduced compared to young mice (Figure 4E; n=5, *p=0.0129).

Orexin neurons in old mice show dysregulated expression of IGF-I receptors.

Altered responses to local administration of IGF-I suggest changes in orexin sensitivity to this growth factor. Thus, we examined IGF-IR expression in orexin neurons using immunocytochemistry and in situ hybridization (Figure 5A,C). Quantification of IGF-IR immunoreactivity in orexin neurons of young and old animals revealed increased IGF-IR levels in the latter (Figure 5B; young=32 cells, old=26 cells), while the mean number of orexin neurons/field was similar in both age groups (Supple Fig 1F). Conversely, in situ hybridization showed decreased expression of IGF-IR mRNA in orexin neurons of old mice (n=20 cells), as compared to young ones (n=19 cells) (Figure 5C; ***p=0.0004).

IGF-IR sensitization recovers orexin responses to IGF-I.

To confirm that dysregulated IGF-IR expression in orexin neurons of old mice reflects perturbed sensitivity to IGF-I, and at the same time assess a potential treatment for age-associated sleep disturbances, we treated aging mice with AIK3a305, a novel IGF-IR sensitizer (Suppl Fig 2A) that crosses the blood-brain-barrier (Suppl Fig 2B). We found that c-fos responses to IGF-I were recovered to youthful levels after treatment of old mice with AIK3a305 (Figure 6A,B).

Furthermore, prolonged treatment with AIK3a305 restored sleep patterns in old mice to those seen in young animals, with δ wave diminishing after treatment (compare

Figure 6C with Figure 4C; old=10, old+AIK3=15, **p=0.0085). During the active phase, no differences were seen between old treated and untreated groups (Suppl Fig 2C). The sleep-onset latency was also recovered by AIK3a305 treatment (n=7 group, ***p<0.0001, Figure 6D). In addition, the sensibility of LH neurons to IGF-I was re-established compared to untreated old mice (n=13 group, Figure 6E). The evoked potential showed no significant differences under basal conditions between the three groups (Suppl Figure 2D).

Considering the broad projections of orexinergic neurons from LH to the cortex, we analyzed the ECoG activity both before and after IGF-I injection as above. Before IGF-I administration, old animals treated with AIK3a305 show a δ band activity similar to young mice (Figure 6F; young=19, old=13, old+AIK3a305=10, ***p<0.0001). In the θ band, old-AIK3a305 mice display an increase over untreated old mice (old=13, old-AIK3a305=10, *p=0.0308, Figure 6F). After IGF-I, the θ band of old-AIK3a305 mice kept the difference over old untreated mice (old=12, old-AIK3a305=9, **p=0.0095, Figure 6G), while the α band displayed an increase, similar to young mice (old=12, old-AIK3=10,**p=0.0066, Figure 6H).

DISCUSSION

The present observations reinforce previous ones indicating that during aging the brain loses sensitivity to IGF-I and extend them by showing that this loss includes hypothalamic orexin neurons. Since these neurons are involved in the sleep/wake cycle, sleep activity is disturbed. Indeed, dysregulation of IGF-IR levels in aging orexin neurons suggests impaired IGF-I signaling due to resistance, deficiency, or both [20], a possibility reinforced by the observed therapeutic actions of AIK3a305, a novel sensitizer of IGF-IR. The combined loss of sensitivity to IGF-I in orexin neurons and elsewhere, together with lower serum IGF-I levels 11, will produce an overall reduction of IGF-I effects on the aged brain.

Intriguingly, higher brain IGF-IR protein levels have been associated with reduced longevity in rodents [21]. This increase, in conjunction with the observed reduction of IGF-R mRNA, points to a dysregulated control of IGF-IR activity in orexin cells of old mice, although brain IGF-IR protein and mRNA do not necessarily change in parallel [22]. At any rate, since IGF-I regulates the expression of its own receptor in the

brain [22], this mismatch between protein and mRNA may reflect post-receptor disturbances in IGF-I signal transmission [23]. Dysregulated IGF-IR expression suggests a less efficient IGF-I system in the brain, like this and previous [24] observations suggest. Other age-associated changes leading to aberrant IGF-IR function are likely involved, as reported for the brain insulin receptor [25].

It is known that IGF-I induces cortical activation [16], and that its effect on cortical activity is, at least in part, due to excitation of LH neurons [7]. Our present findings show that this excitatory mechanism is reduced in aging mice; that is, IGF-I-evoked c-fos expression in orexin neurons and orthodromical responses in LH by locus coeruleus stimulation are blunted. These findings may explain the reduction in sleep-onset latency observed in old mice since LH is involved in the induction of the awake state [26]. Furthermore, effects of IGF-I on the ECoG were recovered in old mice after treatment with AIK3a305, suggesting that some ECoG alterations in aging animals may be due to a reduction of IGF-IR signaling.

The precise role of IGF-I in the aging process is controversial, albeit mainstream thinking supports the notion that its function, together with other members of the insulin family, is detrimental [27, 28, 29, 30, 31]. While many observations of the beneficial actions of IGF-I indicate that this notion is not univocal [32, 33, 34, 35, 36,] and is probably simplistic [37, 38,] in the particular case of brain aging, the situation is even more confusing, with both detrimental [39, 40] and beneficial actions of IGF-I [41, 42, 43, 44, 45] profusely supported by data. Nevertheless, while these observations indicate that loss of IGF-I activity in the aging mouse brain disturbs sleep patterns, we cannot rule out potential beneficial effects of this loss in other aspects of brain function.

In summary, we observed a loss of sensitivity to IGF-I during brain aging, leading to dysregulated orexin function, which impacts onto sleep patterns. Since an IGF-IR sensitizer recovered brain responses to IGF-I, including sleep patterns, these results may provide new therapeutic avenues for age-related sleep disturbances.

MATERIAL AND METHODS

Materials

Antibodies used in this study include rabbit polyclonal c-Fos (Abcam ab190289), rabbit polyclonal IGF-I Receptor-β (Santa Cruz 713/AC) and rabbit anti-IGF-I

receptor β XP (Cell Signaling Technology, USA), orexin polyclonal mouse antibody (Santa Cruz 80263), orexin polyclonal rabbit antibody (Abcam ab 6214), anti-pAkt (Cell Signaling, 9271S) and monoclonal anti-phosphotyrosine (clone PY20, BD Transduction Laboratories, USA). Human recombinant IGF-I was from Pre-Protech (USA).

Animals

Adult female and male C57BL/6J mice (3-5 months and 18-20 months old; 28-34g, Harlan Laboratories, Spain) were used. The estrous cycle of the female mice was not determined. Experiments were done during the light phase, except when indicated. Animals were housed in standard cages (48 × 26 cm2, 5 per cage) and kept in a room with controlled temperature (22°C) under a 12-12h light-dark cycle. Mice were fed with a pellet rodent diet and water ad libitum. Animal procedures followed European guidelines (2010/63, European Council Directives) and were approved by the local Bioethics Committee (Government of the Community of Madrid, Proex 112/16).

Drug administration

IGF-I was dissolved in saline, and intraperitoneally (ip) injected (1µg/g body weight). In some experiments, mice were processed for immunocytochemistry (see below) one or two hours after ip IGF-I injection to express phospho-Akt or c-fos, respectively, whereas, in other experiments, PeF recordings were carried out immediately after ip injection. Alternatively, IGF-I was locally delivered in the PeF (10 nM; 0.1 µl; coordinates from Bregma: A, −1.95; L, 1 and depth, 4.0 - 4.5mm), and injected animals were after that submitted to electrophysiological recordings as described below. Doses were selected based on previous work using pharmacological injections in both systemic and local/ intraparenchymal administration. AIK3a305 (Allinky Biopharma, Spain) or the vehicle (DMSO + saline) were injected intraperitoneally (ip) at a dose of 20 mg/kg/day for four weeks to old mice. This new compound shows good blood-brain-barrier penetration (Supple Fig 2B).

Recordings in anesthetized and freely moving animals

Mice were anesthetized with isofluorane (2% induction; 1–1.5% in oxygen, maintenance doses), placed in a David Kopf stereotaxic apparatus (Tujunga, CA, USA) in which surgical procedures and recordings were performed, with a warming

pad (Gaymar T/Pump, USA) set at 37ºC. Local anesthetic (lidocaine 1%) was applied to all skin incisions and pressure points. An incision was made exposing the skull, and small holes were drilled in the skull. Tungsten macroelectrodes (<1 M□World Precision Instruments, USA) were used to record the local field potential and the evoked potential in the PeF (coordinates from Bregma: A, −1.95; L, 1 and depth, 4.0 - 4.5mm).

Recordings were filtered (0.3–50 Hz) and amplified via an AC preamplifier (DAM80; World Precision Instruments). The LC was stimulated using 120 µm diameter stainless steel bipolar electrodes (World Precision Instruments, coordinates from Bregma: A, −5.4; L, 1 and depth, 4.0 - 4.5mm). Electrical stimulation was carried out with single square pulses (0.3 ms duration and 20-50 µA intensity, delivered at 1 Hz; Cibertec Stimulator, Spain). After a basal recording, IGF-I was injected locally (young=13, old=13) or systemically (young= 9, old=7), both sex included.

For recordings in freely moving animals, mice were anesthetized as indicated above and placed in a stereotaxic device. The skin was cut along the midline, and a craniotomy was made (0.5 mm diameter) in the primary somatosensory area (S1). A stainless-steel macro-electrode of <0.5 M□was placed without disrupting the meninges to register the cortical electrical activity (ECoG), using a DSI Implantable Telemetry device (Data Sciences International, USA). After surgery, mice remain in their cages for a minimum of 4 days to recover.

ECoG recordings were performed for 15 minutes from ZT1-ZT19 every 2 hours/day. Signals were stored in a PC using DSI software and filtered off-line between 0.3–50 Hz with Spike 2 software. ECoG segments of 5 minutes were analyzed using the Fast Fourier Transform algorithm to obtain the power spectra. The mean power density was calculated for five different frequency bands that constitute the global ECG: delta band (0.3–5 Hz), theta band (5–8 Hz), the alpha band (8–12 Hz), the beta band (12–30 Hz), and gamma band (30–50 Hz). The total power of the five frequency bands was considered 100%, and the percentage of each frequency band was calculated for the 15 minutes in each time point. We used five young and four old mice to determine the ECoG profile during the active and passive phases.

To assess sleep onset-latency, segments of 30 sec of the ECoG recording were analyzed according to the presence of delta waves (0.3–4 Hz), fast waves (>12 Hz), and mouse movements. We measured the latency to sleep onset from the time the animal was placed on the platform until the absence of movement and the appearance of delta waves. (young=11, old=7). ECoG recordings were performed at different time points through a remote computer.

Data Analysis

Evoked potentials elicited by LC electrical stimulation (20-50 μA; 0.3 ms duration; at 1 Hz) were calculated. The peak latency was calculated as time elapsed between the stimulus onset and the peak of the second evoked potential wave (orthodromic response, with a latency of 3.5 ± 0.81 ms). To quantify the orthodromic response, the area under the positive wave curve was measured from the beginning of the positive slope. The unit activity plots show the percentage of variation concerning the basal period (5 mins). Outliers and recordings that did not elicit at least 70% of increment activity were removed from the analysis to minimize non-orexinergic neurons' interference**.**

Immunoassays

Immunocytochemistry. Animals were deeply anesthetized with pentobarbital (50 mg/kg) and perfused transcardially with saline 0.9% and then 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). Coronal 50-μm-thick brain sections were cut in a vibratome and collected in PB 0.1 N. Sections were incubated in permeabilization solution (PB 0.1N, Triton X-100, NHS 10%), followed by 48 hours incubation at 4°C with primary antibody (1:500) in blocking solution (PB 0.1N, Triton X-100, NHS 10%). After washing three times in 0.1 PB, Alexa-coupled mouse/rabbit polyclonal secondary antibodies (1:1000, Molecular Probes, USA) were used. Finally, a 1:1000 dilution in PB of Hoechst 33342 was added for 3 minutes. Slices were rinsed several times in PB, mounted with gerbatol mounting medium, and allowed to dry. The omission of the primary antibody was used as a control. Confocal analysis was performed in a Leica (Germany) microscope. For double-stained orexin/c-fos counting, four sections per animal were scored using the Imaris software, as described 46.

ELISA.

Orexin in hypothalamus was determined using orexin Elisa (Phoenix peptides, Inc), as described in detail elsewhere 7. Samples were assayed in duplicate. Hypothalamus were dissected and frozen at -80ºC until used.

WB. Assays were performed as described in detail elsewhere 47. Densitometry analysis of blots was performed using the Odyssey system (Lycor Biosciences, USA). A representative blot is shown from a total of at least three independent experiments.

In situ hybridization

In situ hybridization assays were performed with RNAscope (2.5 HD Detection kit – Red; #322350; ACD, USA) using a mouse IGF-IR probe combined with immunocytochemistry with anti-orexin antibodies, as described 7.

Image Analysis

Images were segmented via k-means clustering with the number of clusters set to 5 (k=5) using MATLAB 48. The red channel (Orexin antibody) was further processed by individualizing unconnected regions, putative neurons and eliminating all connected components with areas smaller than 25 μm2. The resulting binary masks were visually inspected to confirm proper segmentation, and the number of segmented neurons in each image was manually counted. The green channel (IGF-IR antibody) was also visually inspected to confirm the validity of the segmentation. The co-localization ratio was defined as the fraction of each segmented neuron area in the red channel that superposed with the segmented green channel. Lastly, the final ratio for each sample was calculated as follows:

$$R_A \equiv \frac{1}{n}\sum_{i=1}^{n} R_{A,i}$$

where R_A is the ratio of condition A, and R_(A,i) is the ratio for the image i of condition A.

Images of secondary antibody were used to check the secondary antibody's specificity 49 and segmented as described above. The secondary antibody's specificity was evaluated by measuring the segmented receptor's area and the number of segmented

neurons and comparing the results with tissue images that underwent complete immunohistochemistry protocol.

Astrocyte cultures

Astroglial cultures were prepared as described in detail elsewhere 50 from postnatal (day 1-2) brains. After the forebrain was removed and mechanically dissociated, the mixed cell suspension was centrifuged and plated in DMEM/F-12 (Life Technologies) with 10% fetal bovine serum (Life Technologies) and 100 mg/ml of antibiotic-antimycotic solution (Sigma-Aldrich, Spain). Cells were maintained for 2 weeks at 37ºC, 5% CO¬¬¬2 and re-plated at 105 cells/cm2 in a 12-multiwell plate and grown until 80% confluency. On the day of the experiment, cells were washed twice with warm PBS and medium without FCS was added. Then, LPS was added (1µg/ml) and cells incubated for 12h at 37ºC. After LPS treatment, cells were washed twice with PBS, and a fresh medium without FCS was added. AIKa305 was added (27nM) in DMSO for 3 hours at 37ºC. Controls received DMSO alone. After AIK treatment, IGF-I (1 nM) was added, and cells incubated for 1h at 37ºC. Then, plates were placed on ice for 5 minutes, washed and 100 µl of Lysis buffer containing proteases and phosphates inhibitors (Merck) with Laemmli loading buffer 1X, was added and cells scrapped on ice. Samples were frozen at -20ºC until use.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software (San Diego, CA, USA) and R Package (Vienna, Austria). Depending on the number of independent variables, normally distributed data (Kolmogorov-Smirnov normality test), and the experimental groups compared, we used either Student's t-test, two-way ANOVAs, or Two-way repeated measure ANOVA, followed by Sidak's multiple comparison test. For non-normally distributed data, we used the Mann-Whitney U test to compare two groups, Kruskal-Wallis or Friedman test, with Dunn's multiple comparisons as a Post Hoc analysis as Scheirer-Ray Test, a non-parametric alternative to multi-factorial ANOVA. The sample size for each experiment was chosen based on previous experience and aimed to detect at least a $p<0.05$ in the different tests applied, considering a reduced use of animals. Results are shown as mean ± standard error

(SEM) and p values coded as follows: *p< 0.05, **p< 0.01, ***p< 0.001. Animals were included in each experimental group randomly by the researcher.

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AUTHOR CONTRIBUTIONS

JAZV conducted experiments, prepared figures, results and wrote part of the manuscript. JF, ATS, MEFS, JP, AMF conducted experiments and prepared figures. KS and LMR conducted experiments, MN provided experimental expertise, JE analyzed data, MV provided reagents and data on AIK3a305, AN designed and conducted experiments and wrote part of the manuscript, ITA designed the study and wrote the manuscript.

COMPETING INTERESTS

MV and ITA have shares in Allinky BioPharma, manufacturer of AIK3a

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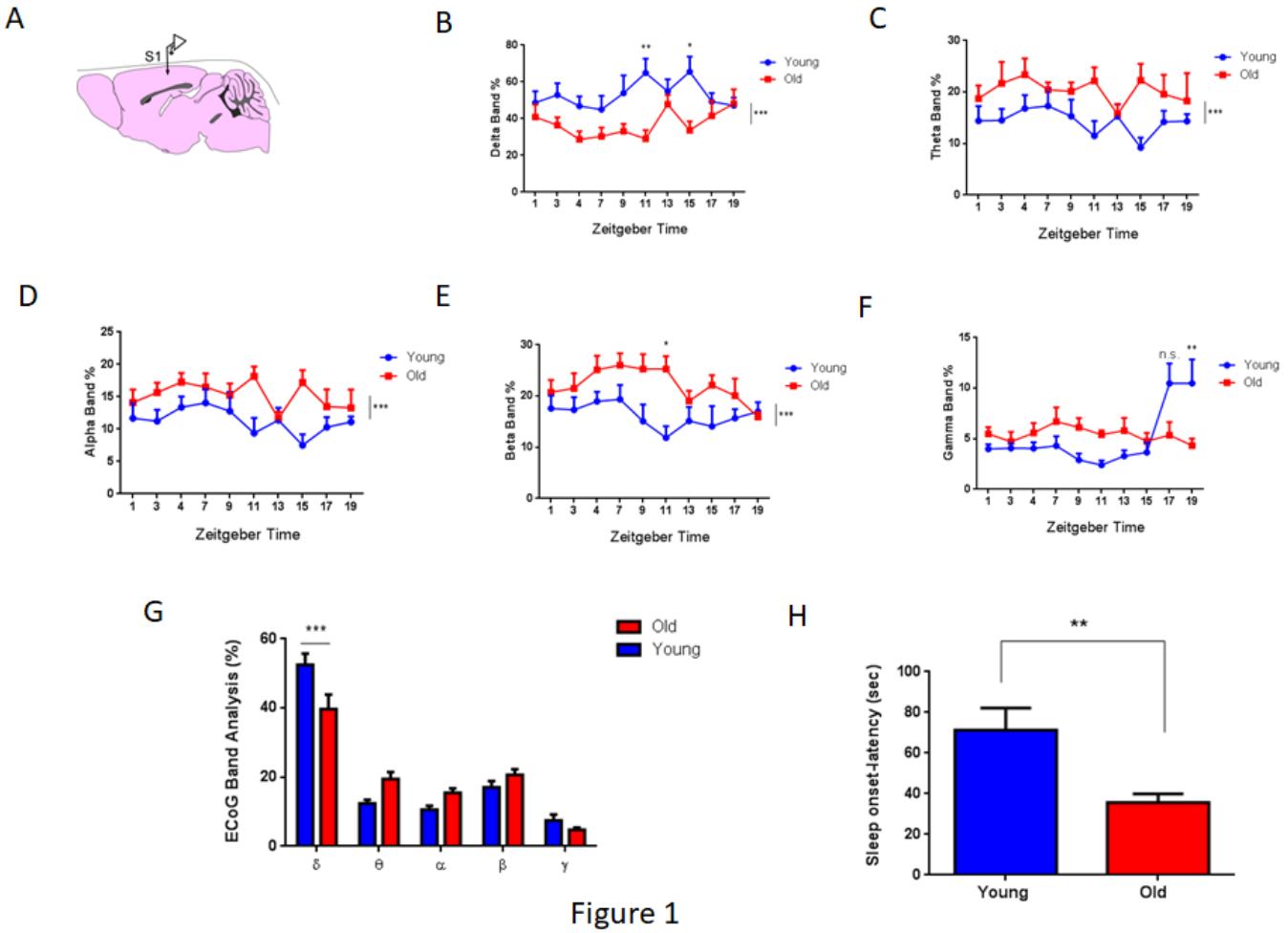
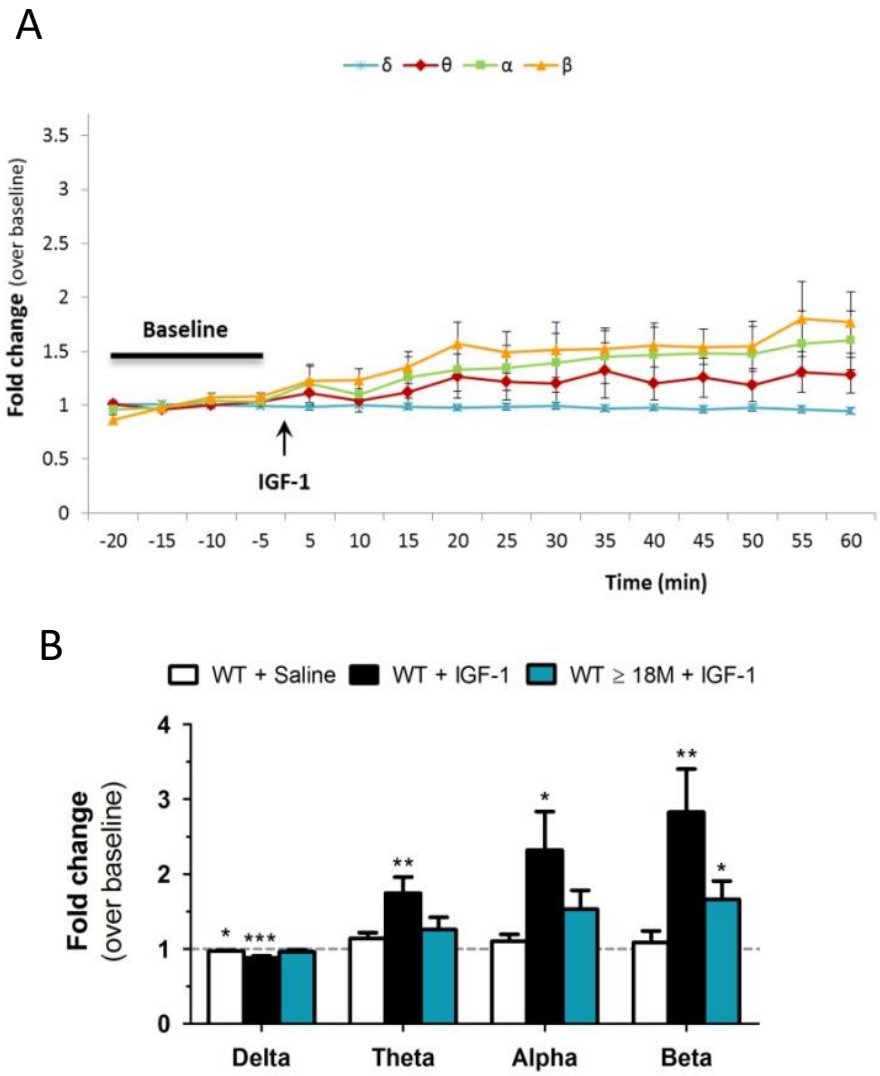


Figure 1

Figure 1: Altered sleep structure in old mice. A, Diagram of the intracranial localization of the electrodes in S1cortex; left hemisphere has the reference electrode in all cases. **B-F,** Sleep architecture during the light and dark period (ZT 1-19) determined by δ (C), θ (D), α (E), β (F), and γ (G) activity patterns in Old mice (red line), and young mice (blue line). Old mice display lower delta activity, as compared to young mice, and an increase in fast-wave activity was observed (Young=8, Old=4; male mice only, Two-Way ANOVA, and Sidak's multiple comparison test; *p<0.05, **p<0.01, ***p<0.001). **G,** Average changes in ECoG bands during the passive phase (ZT 9 – 11) display significant differences in δ band in old mice (***p<0.0004, Young=13, Old=8, sex balanced, Two-Way ANOVA, and Sidak's multiple comparison test). **H,** Latency to sleep-onset was markedly reduced in old mice (**p<0.0099, Young=11, Old=8, sex balanced, Unpaired t-test, and Welch's correction).



Figure

Figure 2: Loss of sensitivity to systemic IGF-I in aged mice. A, Average changes in ECoG bands 20 to 60 min after administration of IGF-I (1 µg/kg, ip) were compared with average baseline activity**. B,** ECoG response to IGF-I (ip) is lost in theta and alpha bands in old mice (> 18 months; n = 7), compared to young ones (3-4 months, n = 10). Differences between young and old mice injected with IGF-I are maximal within the frequency range of the delta and beta wave (*p<0.05; **p<0.01; ***p<0.001, One-Way ANOVA).

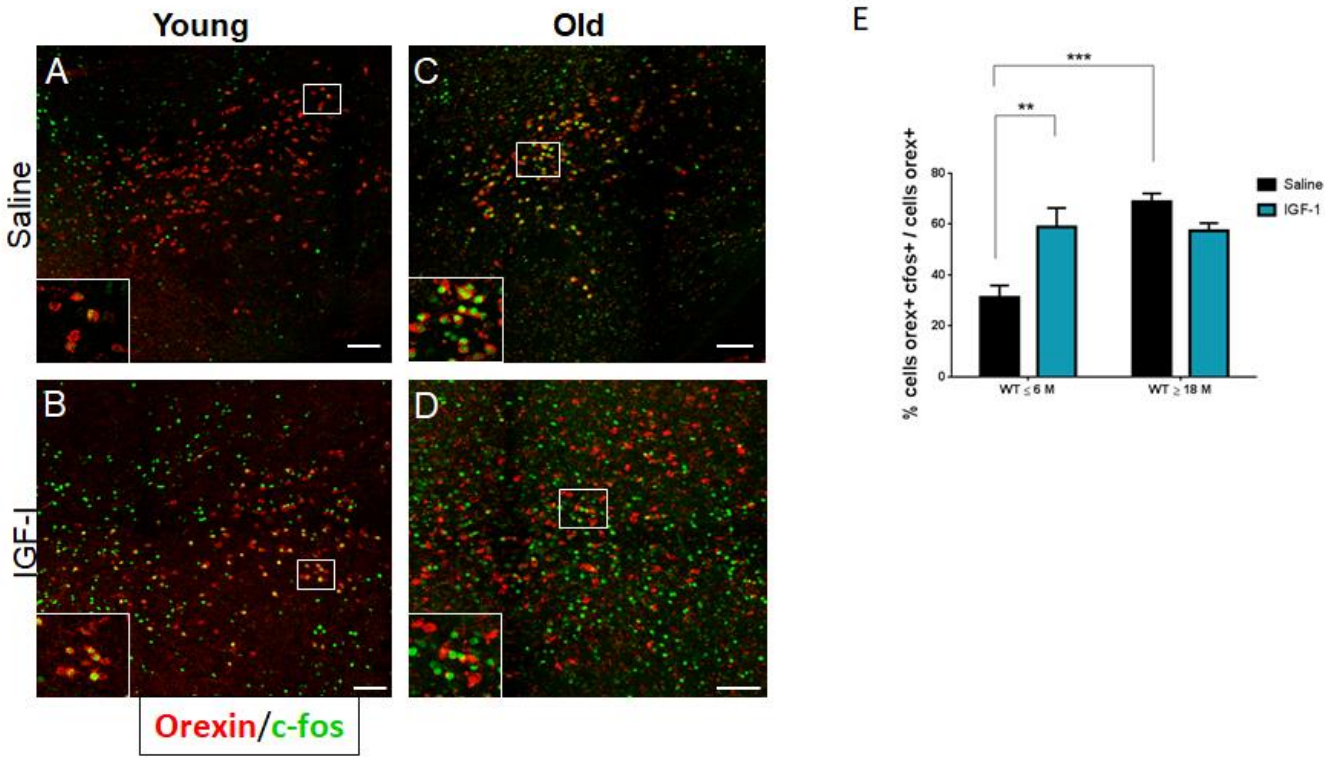


Figure 3

Figure 3: Systemic IGF-I does not promote the expression of c-fos in orexin neurons of old mice. A, Representative photomicrographs of double-labelled c-fos^{+}/orexin^{+} cells in young and old mice after saline and IGF-1 administration. **B,** Young mice show increased c-fos expression in orexin neurons after ip injection of IGF-I (1 µg/kg), while old ones show decreased expression. Old mice treated with saline (n=6) express increased activation of c-fos than young ones (n=6, **p<001; ***p<0.001, sex balanced, Two-Way ANOVA, and Sidak's multiple comparison test.

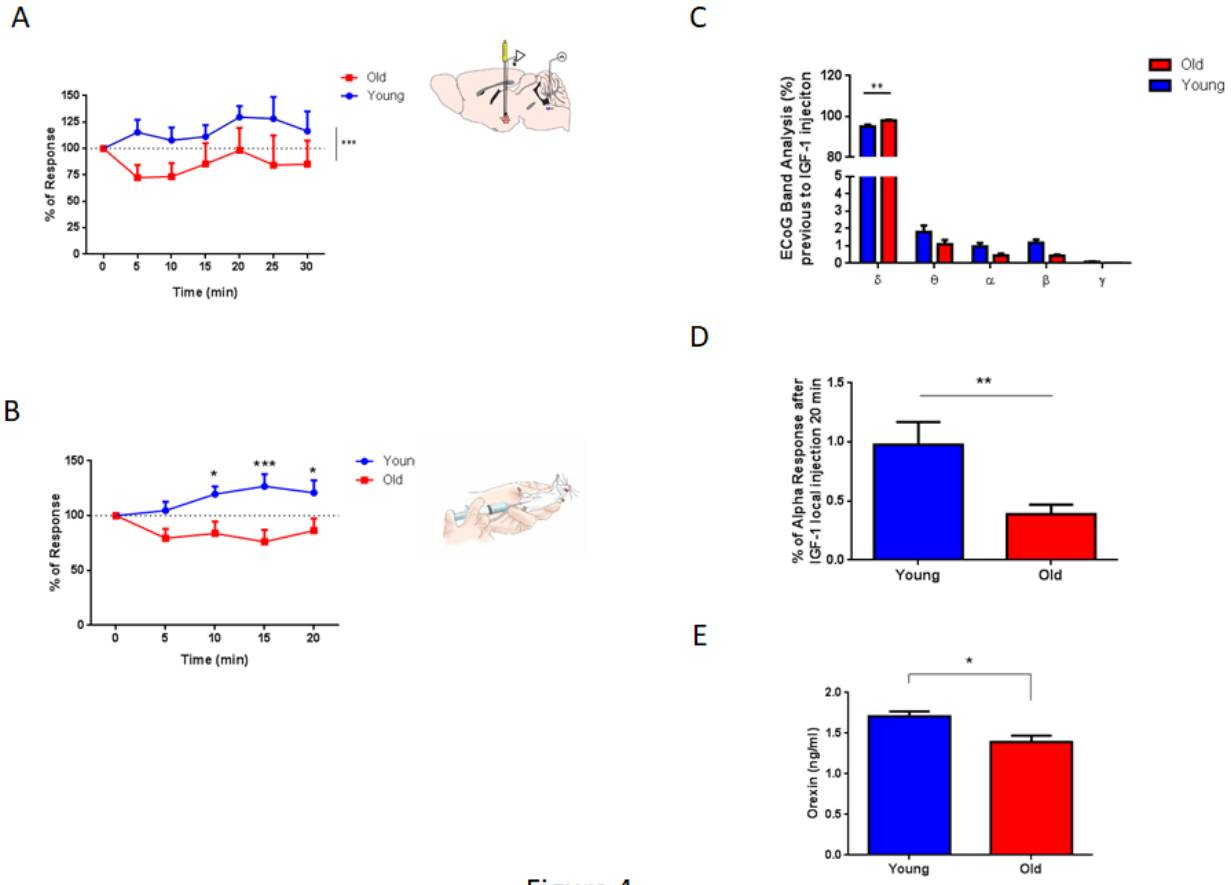


Figure 4

Figure 4: Lateral hypothalamic neurons lose sensitivity to IGF-I in old mice. A, Right cartoon: a stimulating electrode was placed in the LC and a recording electrode in the PeF at the lateral hypothalamus (LH). After LC stimulation, LH neurons of young, but not old mice, increased their activity in response to local application of IGF-I (at time 0) in the PeF (arrow, 10 µM, 0.1 µl). Time course of evoked potentials expressed as a percentage of basal responses at time 0 (at 5 min **p< 0.0053; at 10 min ***p<0.001, 15 min ***p<0.001, and 20 min ***p<0.001; n=13 per group; sex balanced, Two-Way Repeated Measure ANOVA, Sidak's Multiple comparison test). **B,** Intraperitoneal injection of IGF-I (1µg/g, right cartoon) increased neuronal activity in LH neurons after LC stimulation in young but not old mice (***p=0.0003; young=9; old=7; sex balanced, Ordinary Two-Way ANOVA, Sidak's Multiple comparison test)**.** Time course as in previous panel. **C,** Before local IGF-I administration, analysis of ECoG bands revealed increased amount of δ waves in old mice (***p>0.0001); no other differences were observed. **D,** ECoG band analysis 20 min after IGF-I injection indicate an increase of α band only in young mice (young = 19, old = 12, sex balanced, Unpaired t-test, and Welch's correction). **E,** Levels of orexin in the hypothalamus were reduced in old mice (n=6) compared to young ones (n=5). *p=0.0129; sex balanced, Unpaired t-test.

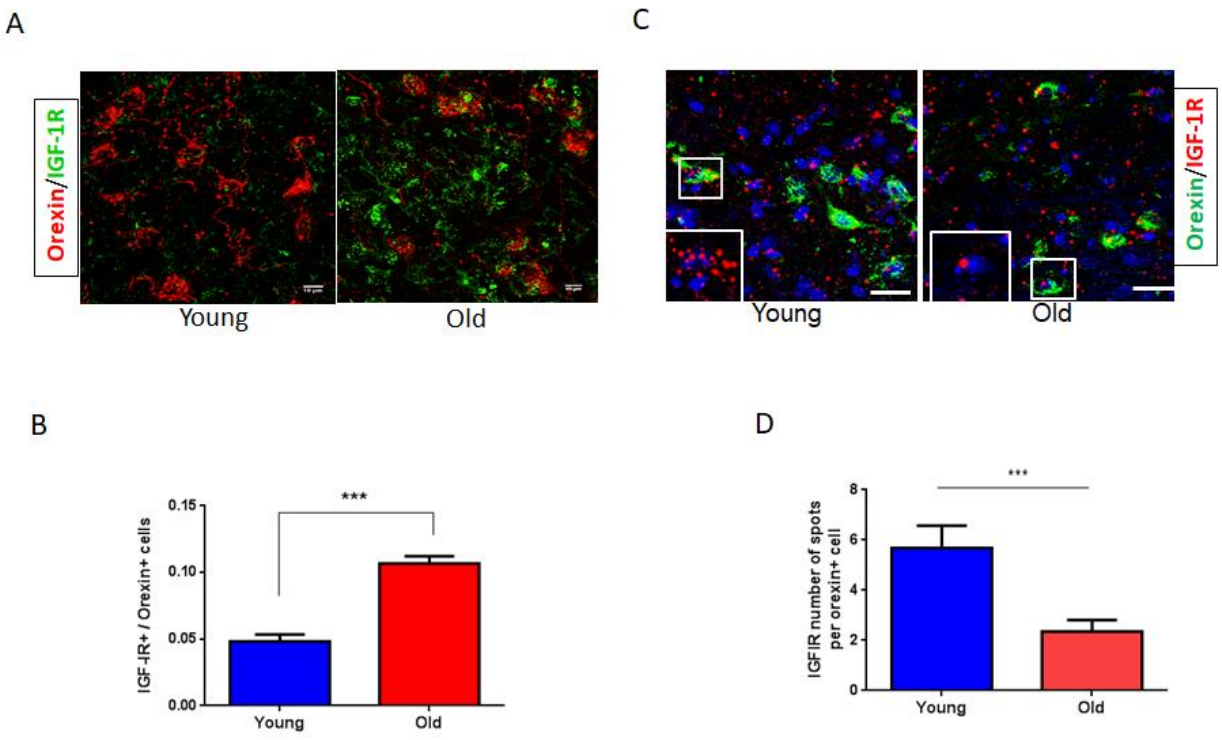


Figure 5

Figure 5: Dysregulation of IGF-IR expression in orexin neurons of old mice. A, Representative photomicrograph of the mouse hypothalamus showing staining of orexin neurons (red) in the PeF area of the lateral hypothalamus (LH) and IGF-IR (green). Bars are 10 µm. **B,** Co-localization ratio of double-stained IGF-IR/orexin cells in young and old mice revealed greater levels of IGF-IR immunoreactivity in old mice (***p=0.0001, young=32 cells, old=26 cells; male-only, Unpaired t-test). **C,** Representative photomicrograph of combined in situ hybridization by RNAScope for IGF-IR mRNA (red) and orexin immunocytochemistry (green) in the mouse PeF area of the lateral hypothalamus (LH). Bars are 10 µm. Large white squares: magnifications detailing IGF-IR mRNA (red) signal seen in the smaller white squares showing both IGF-IR and orexin signal (green). **D,** Quantification analysis showed that expression of IGF-IR mRNA was increased in orexin cells of old mice compared to young ones (young=19, old=20 cells; ***p<0.001, Mann-Whitney test).

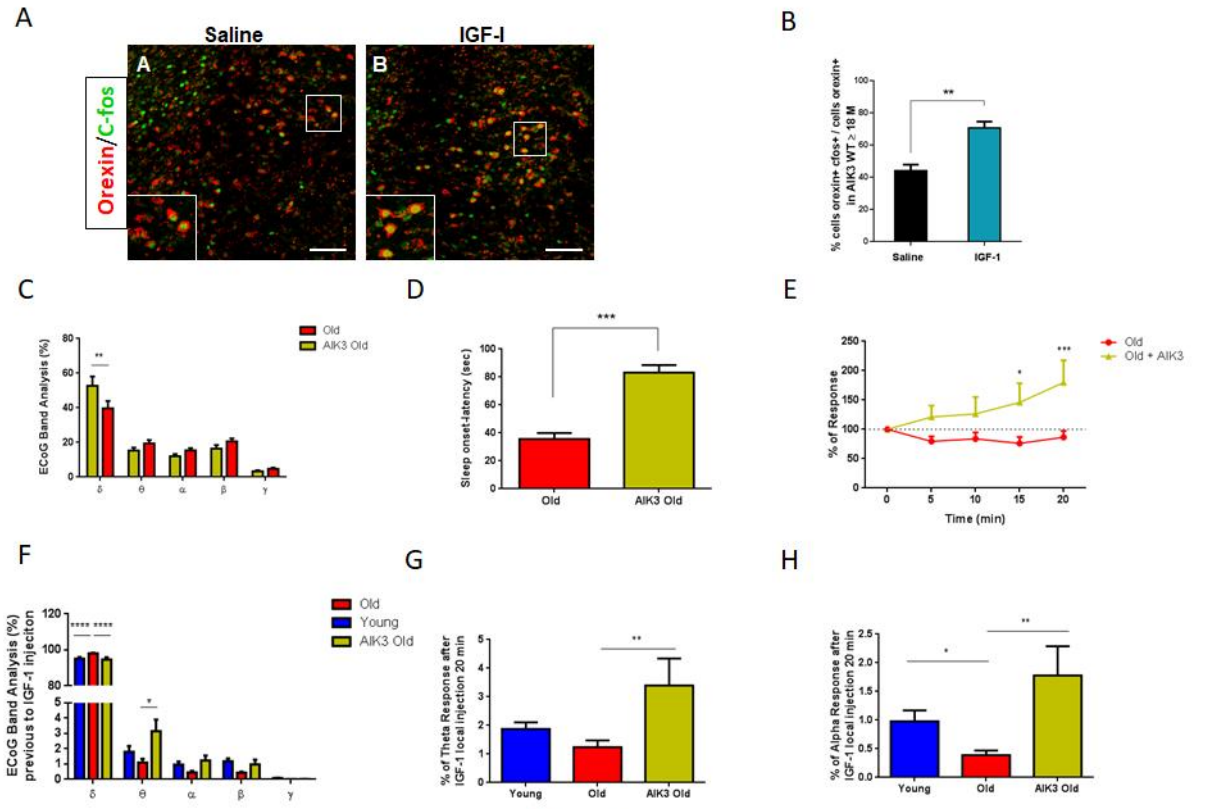
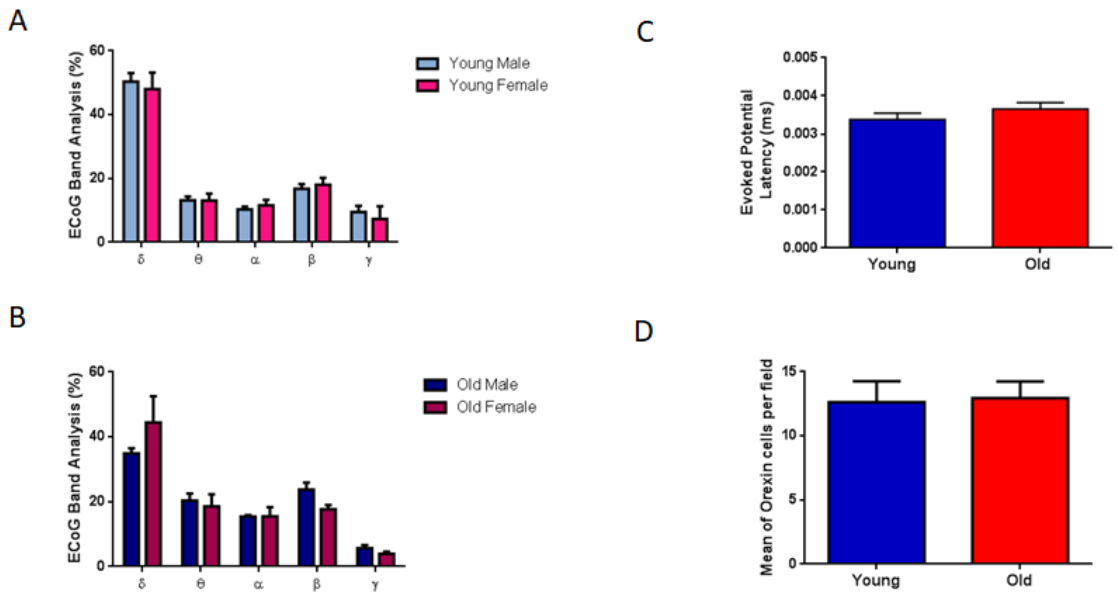


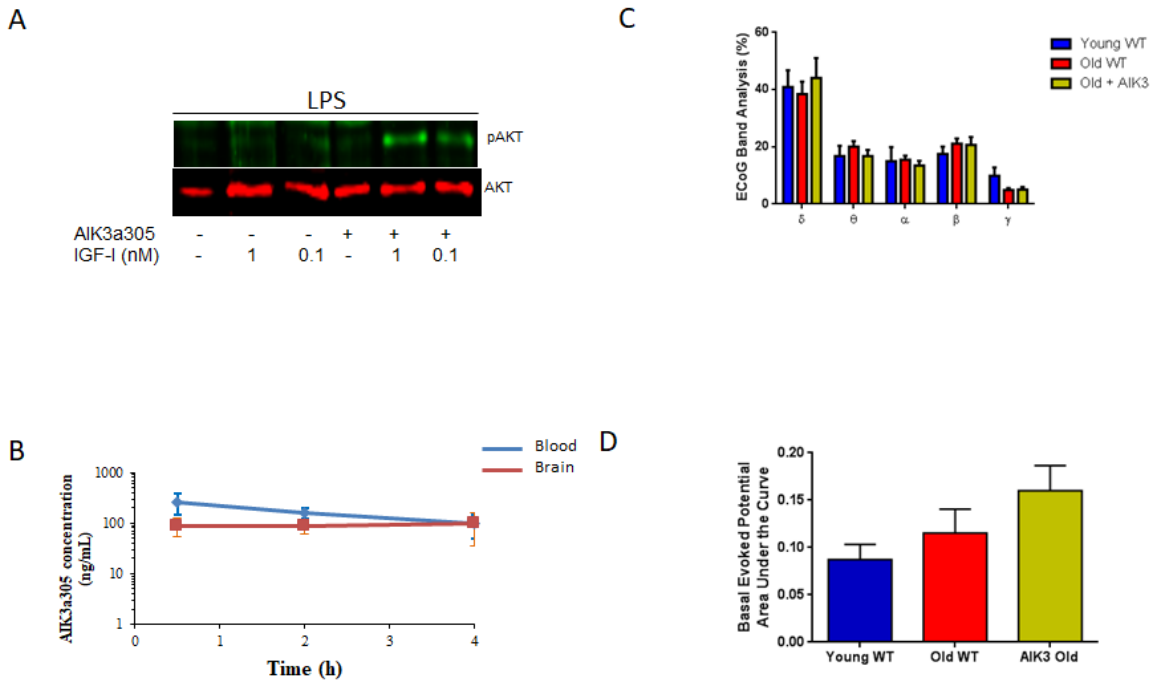
Figure 6

Figure 6: IGF-IR sensitization with AIK3a305 recovers orexin responses to IGF-I and rejuvenates ECG patterns in old mice. A, Representative photomicrograph of orexin$^+$ (red) and c-fos$^+$ (green) cells under saline and IGF-I condition in old-AIK3 treated mice. **B,** Quantification of double-stained c-fos$^+$/orexin$^+$ cells after AIK3 treatment shows a recovery of the c-fos response to IGF-I (n=3 per group, **p=0.0082, sex balanced, Unpaired t-test). **C,** Average ECoG bands during the passive phase (ZT 9 – 11) display significant differences in the δ band in old AIK3 treated mice compared to old untreated mice (**p=0.0085, old+AIK3=14, old=8, sex balanced, Two-Way ANOVA, and Sidak's multiple comparison test). **D,** Treatment with AIK3 normalized sleep-onset latency (***p<0.001, n=7 per group, Unpaired t-test). **E,** Old AIK3 mice recover responses to local application of IGF-I in LH (10 μM, 0.1 μl) after LC stimulation. Time course showing the evoked potential of orthodromic impulses after electrical stimulation of the LC in both experimental groups expressed as a percentage of basal responses at time 0 (at 15 min *p=0.0109, and 20 min ***p=0.0004; n=13 per group; sex balanced, Two-Way Repeated Measure ANOVA, Sidak's Multiple comparison test). **F,** Treatment of old mice with AIK3 rejuvenated the level of δ band. ECoG analysis was carried out previously to local IGF-I administration (***p>0.0001). AIK3 old mice also display an increase of θ band (*p=0.0308, young= 19, old= 13, old AIK3= 10, sex balanced, Ordinary Two-Way ANOVA, Sidak's Multiple comparison test). **G,H,** After 20 min of local IGF-I injection, ECoG band analysis reveals an increase of θ (G) and α (H) band in old AIK3 treated mice (young = 19, old = 12, old AIK = 10, sex balanced, *p<0.05; **p<0.01; ***p<0.001, One-Way ANOVA and Kruskal-Wallis test, with Dunn's multiple comparison test, respectively).



Supplementary Figure 1

Supplementary Figure 1: A, No differences were seen in the ECoG band analysis of young male and female mice (WT ≤ 6). **B,** No differences were seen in the ECoG band analysis of old male and female mice (WT ≥ 18). **C,** No differences between young and old mice were seen in the latency of evoked potentials registered in the PeF after stimulation of the LC. **D,** Mean number/field of immunoreactive orexin neurons in young and old WT mice (young=32, old=26; p= 0,8766, Unpaired t-test).

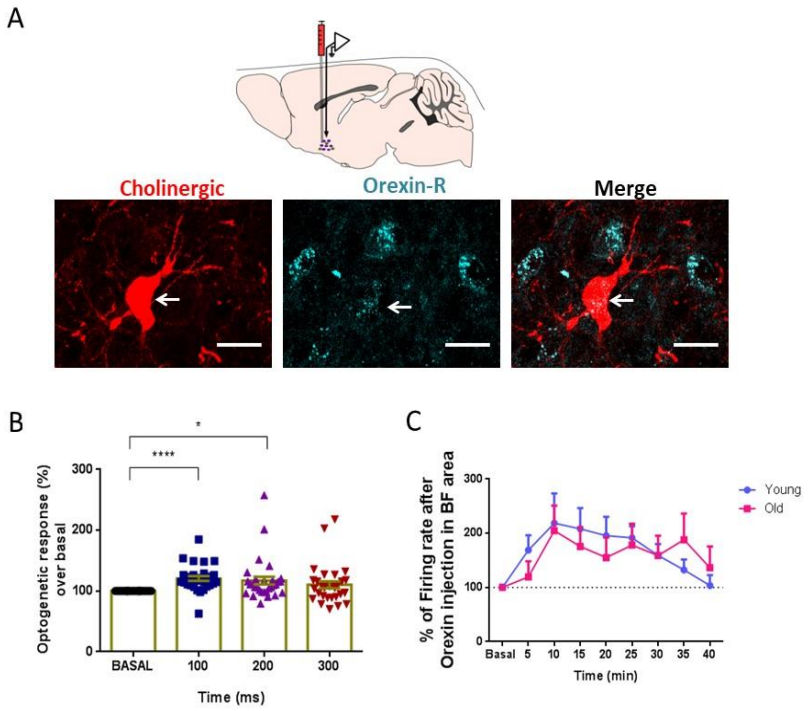


Supplementary Figure 2

Supplementary Figure 2: A, AIK3 sensitizes astrocytes to IGF-I. After treatment with LPS (1 µg/ml), astrocytes become unresponsive to IGF-I, as determined by lack of phosphorylated Akt (pAkt) after 0.1 or 1 nM IGF-I. Astrocytes simultaneously receiving AIK3a305 (27 mM) regain sensitivity to IGF-I, as phosphorylated Akt was readily detected. A representative blot is shown. **B,** Concentration along time in the blood (blue trace) and brain (red trace) after oral administration of AIK3a305 (10 mg/kg) in adult male mice (Swiss albino). **C,** No differences were seen in the ECoG band analysis of young mice (WT ≤ 6 months old), old mice (WT ≥ 18 months old), and old mice treated with AIK3. **D,** No differences between groups in the basal evoked potential in LH after LC stimulation (One-way ANOVA, p=0.1028).

APPENDIX 3

Study 3 - Supplementary figure 1.



Supplementary 1

Supplementary Figure 1 (A) Diagram of the optrode and the cannula location in the HDB nucleus (upper inset). Lower: Representative photomicrograph of ChAT+ cell (red) and orexin receptor-A. Cholinergic neurons expressed orexin receptor-A. The bar in **(A)** is 50 μm. **(B)** Optogenetic identification of ChAT+ cells in old mice (ChAT-ChR2-YFP animals; ≥18 months old). ChAT+ identified neurons increased their active during light pulse (at 0–100 ms, 120.6 ± 3.916; ***$p < 0.001$, and at 100–200 ms, 117.2 ± 6.345; **$p = 0.0044$, $n = 42$ neurons; over basal, 100 ± 0; Friedman test, Dunn's multiple comparison test. **(C)** Orexin-A injection in HDB (10 nM; 0.2 μl) increased both young and old mice's firing rate. The firing rate in both experimental groups is expressed as a percentage of basal activity at time 0 (100%), does not find differences between groups (F (1,159) = 0.1847; $p = 0.6680$; Ordinary Two-way ANOVA.