METABOLIC MEDIATORS AND SYNAPSES: LINKING BODY PERIPHERY TO NEURAL PLASTICITY

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METABOLIC MEDIATORS AND SYNAPSES: LINKING BODY PERIPHERY TO NEURAL PLASTICITY

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EDITORIAL
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Editorial: Metabolic Mediators and Synapses: Linking Body Periphery to Neural Plasticity

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Editorial on the Research Topic

Metabolic Mediators and Synapses: Linking Body Periphery to Neural Plasticity

Neural circuits are endowed with the unique capability to tailor their activity and connectivity in response to incoming stimuli, in order to orchestrate appropriate behavioral responses. The plethora of molecular and functional changes serving these adaptive processes is collectively referred to as "synaptic plasticity." In addition to sensory stimuli, synaptic plasticity can be also triggered and modulated by internal messengers, epitomized by neurotrophins (Reichardt, 2006; Begni et al., 2017). Hormonal regulators of metabolism show similar properties. For instance, administration of leptin to *ob/ob* mice (a natural loss-of-function mutant for the *obese* gene, encoding leptin) induces rewiring of synaptic inputs onto hypothalamic neurons (Pinto et al., 2004). Strikingly, this action extends outside of nuclei involved in metabolic homeostasis; indeed, leptin treatment affects synaptic transmission and long-term potentiation in the hippocampus, the brain's hub for learning and memory (Mainardi et al., 2013). Analogous capabilities have also been observed for insulin, ghrelin and glucagon-like peptide-1 (GLP-1; Mainardi et al., 2015). On the other hand, the synaptic plasticity master controller Brain-Derived Neurotrophic Factor (BDNF) can be also considered a metabolic regulator, as it represses food intake via its action on the ventromedial hypothalamus (VMH; Xu et al., 2003). Interestingly, compounds that stimulate synaptic plasticity in cortical areas by increasing BDNF levels, such as fluoxetine (Maya Vetencourt et al., 2008), have similar effects on the hypothalamus (Barone et al., 2018), which can lead to enhanced leptin sensitivity via signaling mediated by TrkB, the main receptor for BDNF (Scabia et al., 2018).

Starting from these interesting functional parallels between metabolic hormones and neurotrophins, this Research Topic has the goal of providing an update on the rapidly developing field of the interaction between metabolism and brain plasticity.

Understanding how hormones interact with hypothalamic circuits is the primary target of research on brain-metabolism interactions. In this regard, dos-Santos et al. used multi-electrode array and patch-clamp recordings plus single-cell rtPCR to elucidate how ghrelin can regulate the activity of the paraventricular hypothalamic nucleus, leading to decreased secretion of TRH and CRH. Their results fit into the general picture delineated by Serrenho et al., who discuss the effects of ghrelin on synaptic plasticity of the integrated feeding behavior regulatory system, composed of brain regions controlling homeostasis, motivation and memory.

Metabolic hormones bind their receptors to trigger signal transduction processes that are, in principle, sufficient to support synthesis or modification of proteins involved in synaptic transmission. However, the balance of metabolically active molecules can *per se* affect neuronal activity, which can be applied to ameliorate pathological conditions, such as epilepsy (Testa et al., 2019). Within this context, Calì et al. summarize our current knowledge on the

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central role of L-lactate as the energetic exchange currency between astrocytes and neurons. Strikingly, the Authors show that L-lactate also acts as a signal that regulates synaptic plasticity (Calì et al.). We envisage that future research should take into account this fact by dissecting between actions of hormones centered on signaling and on regulation of energy substrate synthesis and availability.

An apparently secondary target of metabolic hormones is the hippocampus, the brain's hub for formation and recall of episodic memories. As discussed by McGregor and Harvey, leptin exerts neurotrophin-like actions on this structure, by enhancing synaptic plasticity and, in turn, learning and memory. The implications of this idea extend to brain pathology, namely age-related cognitive decline and Alzheimer's disease, whose severity can be exacerbated by leptin insufficiency or resistance (McGregor and Harvey). This hypothesis is in accordance with data showing that a hyperlipidic diet leads to impaired plasticity (Spinelli et al., 2017) and leptin resistance in the hippocampus (Mainardi et al., 2017).

Insulin also takes part in the enlarging network of interactions that maintains neural circuit homeostasis (Mainardi et al., 2015). Indeed, Gonçalves et al. provide an overview on the relationship between insulin and Tau protein, and on their implications for tauopathy and Alzheimer's disease. Accordingly, a dietary regimen centered on a high intake of fats is now recognized to negatively affect hippocampal function, as highlighted in the review by Del Olmo and Ruiz-Gayo.

Interestingly, the consequences of deficiency or resistance to metabolic hormones on learning and memory are accompanied by direct effects on basic homeostatic functions, such as feeding and circadian regulation, which, as Hiller and Ishii discuss, indicate that the hypothalamus is an early target of neurodegeneration. This concept could suggest fresh approaches to the early diagnosis and therapy of Alzheimer's disease and dementia (Hiller and Ishii) and, to further strengthen its potential, recent evidence indicates that the hypothalamus responds to stimuli that induce synaptic plasticity (Mainardi et al., 2010; Barone et al., 2018) also in the adult age.

An apparently straightforward development of all these findings is the design of therapies aimed at counteracting neurodegeneration by restoring metabolic hormone-associated signaling in the brain. Indeed, Sanguinetti et al. first demonstrate that a hyperlipidic diet worsens the severity of the phenotype in the 3xTg mouse model of Alzheimer's disease, and then that intranasal insulin treatment attenuates the signs of neurodegeneration, in addition to ameliorating metabolic dysregulation. However, metabolic hormones activate multiple signaling pathways with possible opposite effects on synaptopathy. Cisternas et al. suggest the feasibility of a more precise approach, by using a Wnt inhibitor, andrographolipide, whose administration to the J20 mouse AD model ameliorated both metabolic and cognitive aspects of the disease.

This collection of contributions supports the idea that metabolic hormones are key players in the delicate regulation of synaptic plasticity. Moreover, experimental work indicates that altered metabolic homeostasis is a strong determinant of the severity of age-related cognitive decline and neurodegeneration. In addition to prompting further research on the physiological aspects of the matter, we hope that this Research Topic will succeed in stressing the importance of harnessing the potential of metabolic hormones to counteract neurodegeneration and dementia.

AUTHOR CONTRIBUTIONS

MMai and MMaf discussed the manuscripts submitted to the Research Topic, the current literature, and wrote the manuscript.

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ORIGINAL RESEARCH
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Electrophysiological Effects of Ghrelin in the Hypothalamic Paraventricular Nucleus Neurons

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The paraventricular nucleus (PVN) is involved in the control of sympathetic tone and the secretion of hormones, both functions known to be influenced by ghrelin, suggesting direct effect of ghrelin in this nucleus. However, the effects of ghrelin on the excitability of different PVN neuronal populations have not been demonstrated. This study assessed the effects of ghrelin on the activity of PVN neurons, correlating the responses to subpopulations of PVN neurons. We used a 64 multielectrode array to examine the effects of ghrelin administration on extracellular spike frequency in PVN neurons recorded in brain slices obtained from male Sprague-Dawley rats. Bath administration of 10 nM ghrelin increased (29/97, 30%) or decreased (37/97, 38%) spike frequency in PVN neurons. The GABAA and glutamate receptors antagonists abolish the decrease in spike frequency, without changes in the proportion of increases in spike frequency (23/53, 43%) induced by ghrelin. The results indicate a direct effect of ghrelin increasing PVN neurons activity and a synaptic dependent effect decreasing PVN neurons activity. The patch clamp recordings showed similar proportions of PVN neurons influenced by 10 nM ghrelin (33/95, 35% depolarized; 29/95, 30% hyperpolarized). Using electrophysiological fingerprints to identify specific subpopulations of PVN neurons we observed that the majority of pre-autonomic neurons (11/18 -61%) were depolarized by ghrelin, while both neuroendocrine (29% depolarizations, 40% hyperpolarizations), and magnocellular neurons (29% depolarizations, 21% hyperpolarizations) showed mixed responses. Finally, to correlate the electrophysiological response and the neurochemical phenotype of PVN neurons, cell cytoplasm was collected after recordings and RT-PCR performed to assess the presence of mRNA for vasopressin, oxytocin, thyrotropin (TRH) and corticotropin (CRH) releasing hormones. The single-cell RT-PCR showed that most TRH-expressing (4/5) and CRH-expressing (3/4) neurons are hyperpolarized in response to ghrelin. In conclusion, ghrelin either directly increases or indirectly decreases the activity of PVN neurons, this suggests that ghrelin acts on inhibitory PVN neurons that, in turn, decrease the activity of TRH-expressing and CRH-expressing neurons in the PVN.

Keywords: corticotropin-releasing hormone, thyrotropin releasing hormone, vasopressin, oxytocin, appetite regulation, neuroendocrinology

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INTRODUCTION

The brain constantly monitors energy balance, changing behavior and energy expenditure as necessary to maintain metabolic status. Hunger is a sensation that arises from caloric deficit and induces food intake in order to re-establish homeostasis (Yi and Tschöp, 2012). One of the modulators of hunger is ghrelin, an orexigenic hormone secreted by the stomach during situations of caloric deficit (Müller et al., 2015). While ghrelin-induced food intake has been demonstrated in rodents and humans (Sato et al., 2012) this peptide also affects functions not directly related to food intake, and has been shown to decrease sympathetic activity (Matsumura et al., 2002; Tanida et al., 2007). In addition, intra-cerebro ventricular (ICV) ghrelin increases plasma vasopressin (AVP) (Ishizaki et al., 2002), and adrenocorticotropic hormone (ACTH) (Wren et al., 2000, 2002), and decreases thyroid-stimulating hormone (TSH) (Wren et al., 2000). These neuroendocrine effects suggest effects on corticotropin releasing hormone (CRH) and thyrotropin releasing hormone (TRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus. *In vitro*, ghrelin induces AVP and CRH release in hypothalamic explants (Wren et al., 2002; Mozid et al., 2003), and oxytocin (OT) and AVP release in neurohypophyseal cell culture (Gálfi et al., 2016). Food deprivation, which increases ghrelin, reduces TRH (Mori et al., 1988; Blake et al., 1991), and TRH-mRNA levels in the brain decrease after peripheral ghrelin injection, suggesting that TRH neurons are affected by ghrelin (Pekary and Sattin, 2012).

The effects of ghrelin on blood hormone levels and autonomic regulation indicate the PVN as a target nucleus where central ghrelin may act to elicit these effects. The PVN is composed of magnocellular and parvocellular neurons. Magnocellular neurons project to the neurohypophysis where, on depolarization, they release AVP and OT into the systemic circulation. Parvocellular neuroendocrine neurons secrete CRH and TRH into the hypophyseal portal circulation at the median eminence which act in the adenohypophysis to cause release of ACTH and thyrotropin into the general circulation (Stern, 2015). Pre-autonomic parvocellular neurons projecting to the brainstem and spinal cord play critical roles in the regulation of autonomic systems (Stern, 2001) while those that project to other brain regions mediate diverse physiological processes (Knobloch et al., 2012). Thus, the PVN controls sympathetic tone and the secretion of hormones, both functions known to be influenced by ghrelin, suggesting direct effect of ghrelin in this nucleus. In accordance with this hypothesis the growth hormone secretagogue receptor (GHSR), the only described ghrelin receptor (Kojima et al., 1999), is present in the PVN (Cowley et al., 2003; Harrold et al., 2008). In addition, ICV ghrelin binds in the PVN (Cabral et al., 2013) and both ICV (Lawrence et al., 2002; Olszewski et al., 2007; Cabral et al., 2012; Stevanovic et al., 2014) and peripheral (Rüter et al., 2003; Kobelt et al., 2008; Cabral et al., 2012) injections of ghrelin increase the activity of PVN neurons while intra-PVN injection of ghrelin induces feeding (Wren et al., 2001; Melis et al., 2002; Olszewski et al., 2003; Shrestha et al., 2004). Electrophysiological studies have shown that ghrelin reduces inhibitory (Cowley et al., 2003) and excitatory (Kola et al., 2008) post-synaptic currents in the PVN. It has also been suggested that ghrelin inhibits γ-aminobutyric acid (GABA) releasing PVN neurons, in turn increasing the activity of CRH PVN neurons, an effect that is independent of the arcuate nucleus (ARC) (Cabral et al., 2016).

Taken together, these observations suggest that the PVN mediates, at least some of the central effects of ghrelin, however, the effects of ghrelin on the excitability of different PVN neuronal populations have not been demonstrated. We hypothesized that ghrelin would influence the activity of PVN neurons, and may exert different effects on different subpopulations of neurons within this hypothalamic nucleus.

MATERIALS AND METHODS

Ethical Approval

All animal protocols were approved by the Queen's University Animal Care Committee, conformed to the standards of the Canadian Council on Animal Care and were in accordance with the "Guide for the Care and Use of Laboratory Animals: Eighth Edition, NIH, 2011."

Animals

We used 25–30 days old Male Sprague-Dawley rats (Charles River, Quebec, Canada) (50–100 g) for all experiments. Since healthy viable neurons are harder to obtain from brain slices taken from adult animals, our lab (Loewen and Ferguson, 2017) and others (Luther and Tasker, 2000; Ma and Liu, 2012) have previously used juvenile rats to measure the effects of peptides on PVN neurons. However, brain functions may differ between juvenile and adult rats, thus, the age of the animals is a methodological limitation of the study. Animals were housed in a room maintained at 22°C under a 12:12 h light-dark cycle with food and water *ad libitum*. A total of 53 rats were used in these experiments from which hypothalamic brain slices were obtained either for extracellular recordings (9 slices, obtained from 4 rats), or patch clamp (49 rats – 122 neurons, 45 of which were used for RT-PCR analysis). Anesthesia influences PVN neuronal activity (Tanaka et al., 1989) and AVP release (Reed et al., 2009), therefore decapitation was carried out in non-anesthetized animal.

Electrophysiology

Rats were decapitated and the brains were removed and immersed in ice-cold slicing solution (87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.25 NaH_2PO_4, 25 glucose and 75 sucrose, in mM) and continuously aerated with 95% O_2/5% CO_2. The brain was blocked and 300 μm coronal slices containing the PVN were cut using a vibratome (VT1000 S; Leica, Nussloch, Germany). Slices were then incubated at 32°C for at least 1 h in artificial cerebrospinal fluid (aCSF) (124 NaCl, 2.5 KCl, 20 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgSO_4$, 1.24 KH_2PO_4 and 10 glucose, in mM), after which they were used for extracellular or intracellular recordings.

Extracellular Recordings

Extracellular recordings were performed on planar-multielectrode array (MED-64 system; Alpha MED Sciences, Osaka, Japan). An 1 mm 8 × 8 electrode arrangement, with 50 μm × 50 μm electrodes and a 150 μm distance between electrodes was used. Signals were recorded at a sampling rate of 20 kHz and 16-bit resolution. Slices were placed on the probe, submersed in carbogenated aCSF and held in place by a small weight with a net. The correct placement of the slice was assessed on a microscope, a representation of the PVN placement over the probe is shown (**Figure 1**). Then the probe was connected to the MED64 system, in which a flow of 1–2 ml/min of carbogenated aCSF, heated to 35°C was maintained. After a stabilization period of 10 min, baseline activity was recorded for at least 5 min. Then, 10 nM ghrelin, diluted in aCSF, was applied for 2 min and recording proceeded for an additional 30 min. Channels positioned over the PVN that showed activity during the experiment were analyzed. Mobius software (Alpha MED Sciences, Osaka, Japan) was used to automatically sort spike shapes and identify neurons within each channel, the threshold of detection was set above noise levels for each channel (ranging from |10| to |13| μV) and spikes with a similarity greater than 60% that appeared a minimum of 100 times were considered. Spike frequency for individual neurons was plotted in bins of 30 s. 4 min immediately before ghrelin hits the bath and 4 min of peak effect were compared and neurons were classified as affected by ghrelin when application changed firing rate by more than ± 20% of mean baseline (Yousheng et al., 2008).

In order to assess whether ghrelin effects were direct or dependent on prior activation of glutamatergic or GABAergic neurons, the effects of ghrelin were assessed in the presence of 10 μM bicuculline methiodide and kynurenic acid, respectively, GABAA and glutamate receptor antagonists (Latchford and Ferguson, 2004). After 10 min of stabilization, baseline was recorded for 5 min after which aCSF was changed to aCSF with antagonists. After 5 min, ghrelin 10 nM diluted in aCSF with antagonists, was applied for 2 min and the responses were analyzed as previously described.

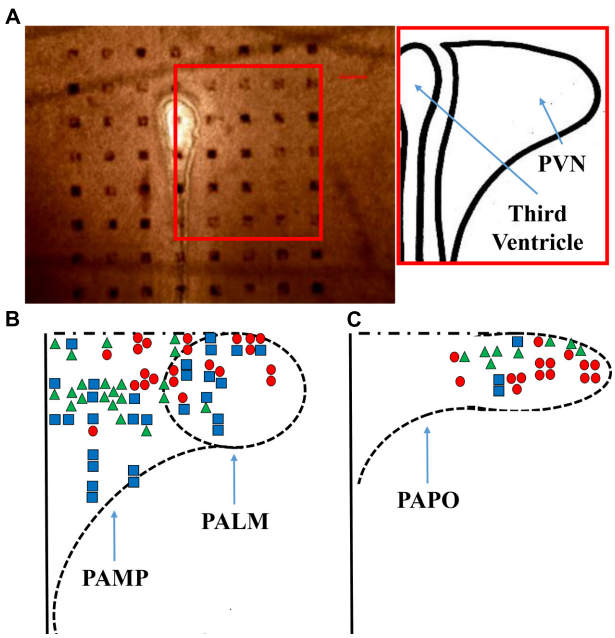

FIGURE 1 | Probe positioning. **(A)** A representative micrograph of the PVN slice positioning over the probe is shown, the bar indicates 100 μm and the inset shows a representation of the nucleus. A diagram **(B,C)** shows all the responses obtained from the application of ghrelin. PAMP = medial parvocellular; PALM = lateral magnocellular; and PAPO = posterior parvocellular portions of the paraventricular nucleus. Triangles, squares, and circles represent, respectively, increased, unchanged, and decreased firing frequency.

Intracellular Recordings

Patch electrodes were manufactured with borosilicate glass (World Precision Instruments, Sarasota, FL, United States) pulled by a micropipette puller (P-97; Sutter Instrument, Novato, CA, United States) and filled with intracellular solution (125 potassium gluconate, 10 KCl, 2 MgCl2, 0.1 CaCl2, 5.5 EGTA, 10 HEPES, 2 NaATP, and adjusted to pH 7.2 with KOH, in mM). Micropipettes with resistances between 3 and 5 MΩ were used. Slices containing the PVN were transferred to a recording chamber continuously perfused with carbogenated aCSF, heated to 35°C at flow between 1.5 and 2 ml/min and used for whole-cell current clamp recording. Neurons were visualized using a 40× water immersion objective mounted on an upright microscope with differential interference contrast optics (Scientifica, East Sussex, United Kingdom). A seal of at least 1 GΩ resistance was achieved, then brief suction was applied to rupture the membrane and obtain whole cell access. Whole cell recordings were obtained using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, United States), with a sampling rate of 10 kHz, filtered at 2.4 kHz, using a Micro1401 mk II interface and Spike2 software (Cambridge Electronic Design, Cambridge, United Kingdom) for offline analysis. Neurons were classified in accordance with membrane potential responses to hyperpolarizing pulse protocols. Neurons with a large A-current were classified as magnocellular (Tasker and Dudek, 1991), neurons with low-threshold calcium spikes were classified as pre-autonomic (Stern, 2001) and neurons with neither of these characteristics were classified as neuroendocrine (Luther et al., 2002). Following a minimum 5 min stable baseline recording of membrane potential ghrelin diluted in aCSF was applied to the slices for 2 min and the responses were recorded until a return to baseline or loss of GΩ seal. Effects of ghrelin were assessed by comparison of the mean membrane potential during the 100 s immediately prior to ghrelin application with the 100 s of peak effect within 10 the minutes following peptide application. The effects of ghrelin on membrane potential were considered significant if the change was greater than 2 mV and two times baseline SD. A liquid junction potential of 15 mV was subtracted from all reported membrane potentials.

Single Cell RT-PCR

Single cell RT-PCR was carried out as previously described (Pires da Silva et al., 2016). In short, immediately after

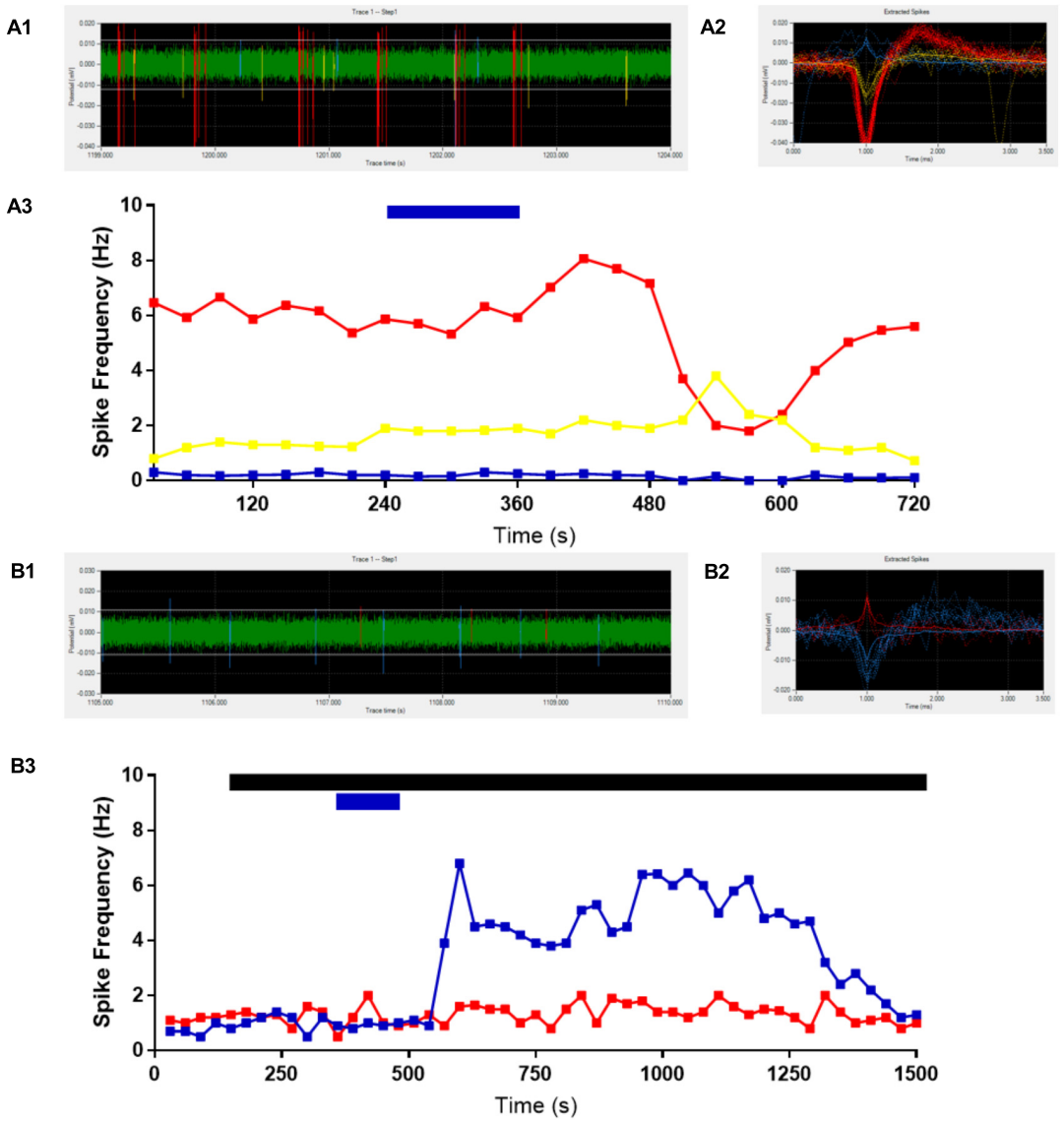

FIGURE 2 | Representative extracellular recordings. Channels with detectable spikes and correct positioning were selected for analysis. One channel is shown as representative **(A)**, 3 neurons were identified **(A1,A2)** and ghrelin application decreased (red line), increased (yellow line) or did not change (blue line) firing frequency (1/3 each) **(A3)**. The addition of GABAA and Glutamate receptor antagonists to the bath (black bar) abolishes the decreases in activity due to 10 nM ghrelin application (blue bar). Another representative recording is shown **(B)**, in which two neurons were identified in the trace **(B1,B2)**. Ghrelin increased firing frequency in one (blue line) and did not affect the other (red line) **(B3)**.

electrophysiological recording the content of the micropipette was collected and used for cDNA generation with High Capacity cDNA Reverse transcription kit (Invitrogen, Carlsbad, CA, United States), followed by a pre-amplification step with TaqMan Pre-Amp Master Mix Kit (Invitrogen, Carlsbad, CA, United States), and the probes for vasopressin (Rn00566449_m1), oxytocin (Rn00564446_g1), CRH (Rn01462137_m1), TRH (Rn00564880_m1), and β-Actin (Rn00667869_m1). Then, RT-PCR was performed in simplex and triplicates, using the aforementioned probes and TaqMan Universal PCR Master Mix kit (Invitrogen, Carlsbad, CA, United States). All reactions were carried out according to manufacturers recommendations and β-Actin was used as an endogenous control. Samples in which β-Actin was not present were removed from the analysis and the remainder were classified by the presence of mRNA for the target genes. The mRNA was considered as present when the amplification threshold occurred before cycle 36.

Chemicals and Drugs

All solutions were prepared on the day of the experiment. Ghrelin was purchased from Phoenix Pharmaceuticals (Belmont, CA, United States); all RT-PCR reagents were purchased from Applied Biosystems (Foster City, CA, United States); bicuculline methiodide, kynurenic acid and all salts used for the

preparation of aCSF, slicing solution and intracellular solution were purchased from Sigma Pharmaceuticals (Oakville, ON, Canada).

Statistical Analysis

GraphPad Prism v. 7.0 was used for all statistical analyses. Proportions were compared with Chi-Square test. Magnitudes were compared with one-way analysis of variance followed by Tukey's *post hoc* test. The duration of effects was compared with unpaired *t*-test or one-way analysis of variance where appropriate. The proportional changes in firing frequency were compared with two-way analysis of variance followed by Sidak *post hoc* test. Statistical significance was set at $p < 0.05$ and all data is described as mean ± standard deviation.

RESULTS

We first assessed the effects of ghrelin on extracellular spike frequency in PVN neurons in extracellular recordings obtained from 10 different hypothalamic slices. Only channels in which spikes were detectable and the PVN was correctly placed above the probes were analyzed. Channels outside of the PVN seldom showed spikes. A representative analysis (**Figure 2**) shows examples of 1 channel in one specific recording where extracellular spikes were detectable (**Figure 2A**); the detection threshold was set above noise values individually for each channel (**Figure 2A1**). The software then detects individual neurons based on the shape of the spikes (**Figure 2A2**). The firing frequency for each neuron was plotted in 30 s bins and the response to 10 nM ghrelin was observed (**Figure 2A3**). In the representative channel (**Figure 2A**), 3 neurons were recorded of which 1 neuron increased, 1 decreased, and 1 showed no change in spike frequency. In total (**Figure 3A**), 48 channels detected spikes in 7 slices, in which 1–3 neurons/channel were identified. A total of 97 neurons were recorded, of which 29 (30%) increased firing frequency and 37 (38%) decreased firing frequency in response to bath administration of 10 nM ghrelin. In a second series of recordings we assessed whether the responses to ghrelin were dependent on the modulation of glutamatergic or GABAergic neurotransmission by examining the effects of ghrelin in the presence of 10 μM bicuculline methiodide and kynurenic acid. In these recordings 27 channels detected spikes in 2 slices, in which 1–2 neurons/channel were identified, as illustrated in **Figure 2B**, single channels recorded neurons which were either depolarized or unaffected, but no cells in which spike frequency was inhibited (**Figure 2B3**). A total of 53 neurons were recorded (**Figure 3A**), of which 23 (43%) increased firing frequency and 30 (57%) were not affected by bath administration of ghrelin. These proportions of neurons responding to ghrelin were significantly different in the presence of glutamate and GABAA antagonists (Chi-square = 27.14, df = 2, $p < 0.001$). This indicates that GABAA and/or glutamate receptors activation is necessary for ghrelin to decrease the activity of PVN neurons. Additional representative channels showing the response to ghrelin in the absence (**Figures 4A–C**) or presence (**Figures 4D–F**) of GABAA and glutamate receptor antagonists demonstrate that

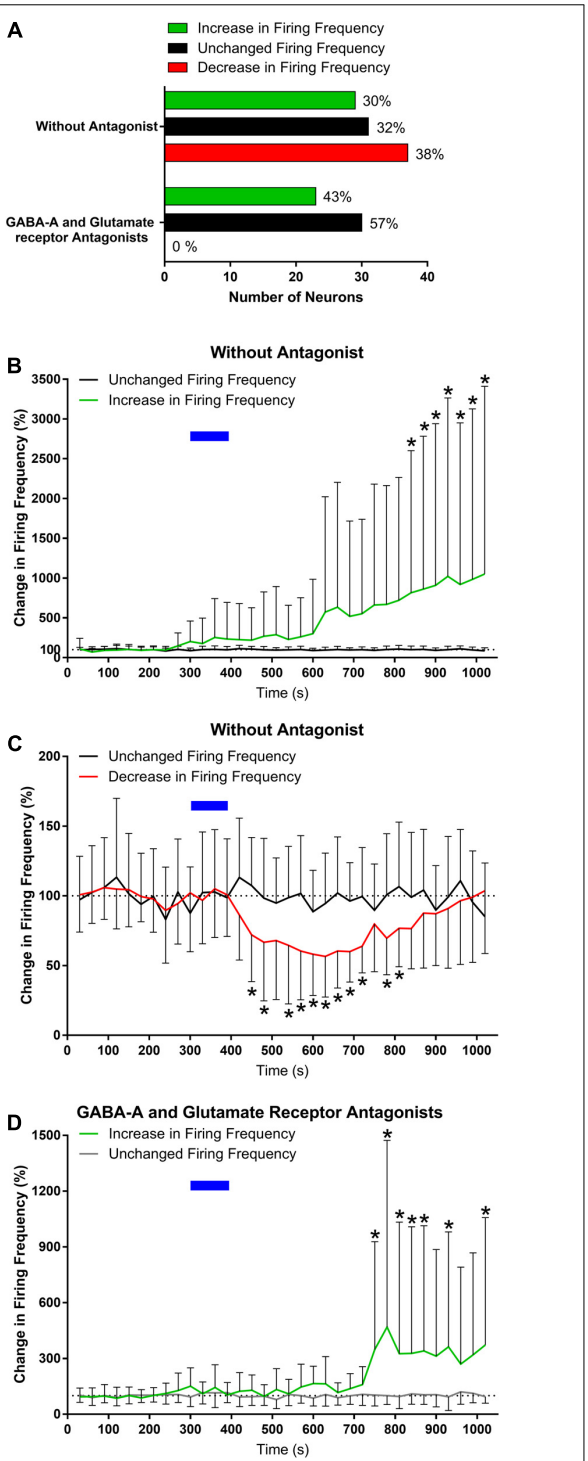

FIGURE 3 | Ghrelin affects the firing frequency of PVN neurons. Summary data from the extracellular recordings demonstrates that ghrelin affects most PVN neurons, either increasing or decreasing firing frequency; and that with the addition of glutamate and GABAA antagonists the decreases in activity are not present **(A)**. The proportion of change was significantly different in both the increases **(B)** and decreases **(C)** in firing frequency when compared to the group with unchanged firing frequency. Ghrelin effects were also significantly different in the presence of GABAA and glutamate receptor antagonists **(D)**. *$p < 0.05$ vs. unchanged firing frequency.

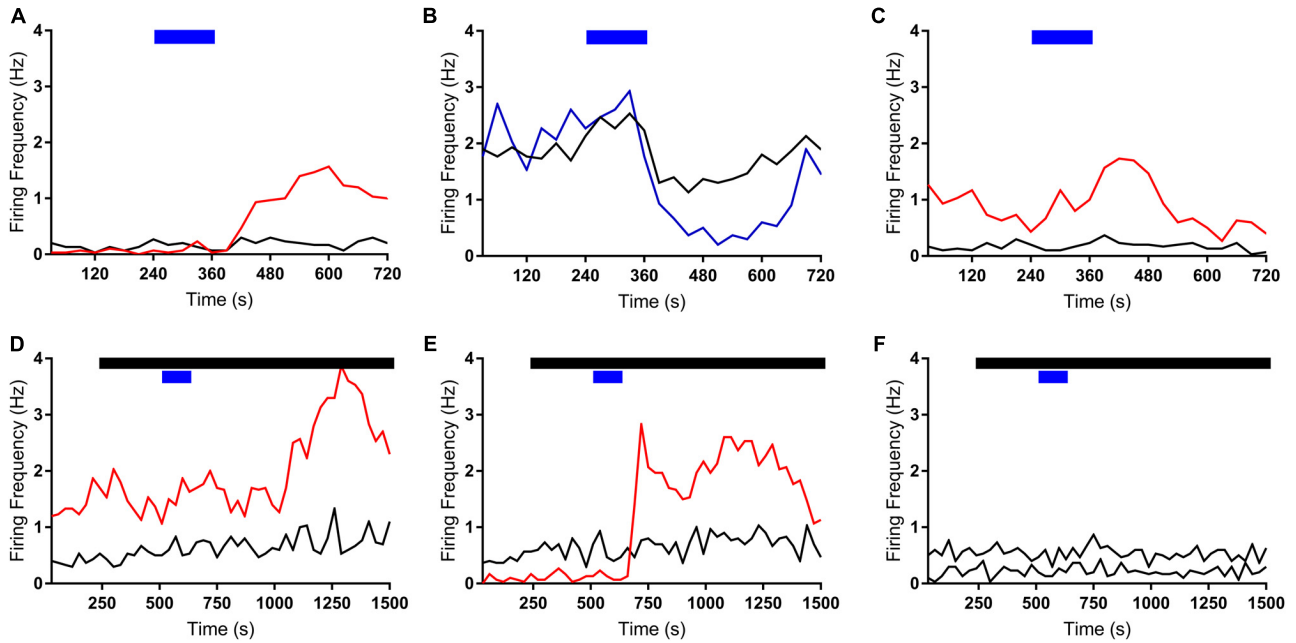

FIGURE 4 | Ghrelin effects vary within individual neurons. Additional representative recordings are shown, where each rate meter record **(A–F)** represents all data acquired from one specific channel and each line represents a single neuron. Bath application of ghrelin incurs both increases (red lines) and decreases (blue lines) in firing frequency. Black bar indicates application of GABAA and glutamate receptor antagonists; Blue Bar indicates ghrelin application.

individual neurons respond differently to ghrelin application. We hypothesize that these differences in response are due to the different subpopulations of neurons in this nucleus. In the neurons measured without the antagonists, the proportion of changes in firing frequency demonstrated a significant difference between neurons that increased firing frequency and neurons in which firing frequency was unchanged [$F_{(33,1881)} = 4.27$, $p < 0.001$, **Figure 3B**], and between neurons in which the firing frequency decreased and neurons that were unaffected [$F_{(33,2178)} = 4.44$, $p < 0.001$, **Figure 3C**]. Similarly, in neurons evaluated in the presence of antagonists, neurons that increased in firing frequency were significantly different from unaffected neurons [$F_{(33,1683)} = 3.45$, $p < 0.001$, **Figure 3D**].

While our extracellular recordings clearly demonstrate effects of ghrelin on spike frequency in PVN neurons, these data provide no direct insight as to either, the cellular mechanisms of action on single PVN neurons, or the specific subpopulations of PVN neurons affected. We therefore utilized whole-cell patch clamp recording techniques to examine specific effects of ghrelin on single PVN neurons. Bath administration of varying concentrations of ghrelin (1, 10, and 100 nM) also affected PVN neurons (**Figure 5**, 1 nM: 11% depolarized, 22% hyperpolarized, $n = 9$; 10 nM: 35% depolarized, 30% hyperpolarized, $n = 94$; 100 nM: 39% depolarized, 28% hyperpolarized, $n = 18$). Statistical analysis of the changes in membrane potential showed that both depolarizations [$F_{(2,44)} = 12.89$, $p < 0.001$, ONE-WAY ANOVA] and hyperpolarizations [$F_{(2,39)} = 16.81$, $p < 0.001$, ONE-WAY ANOVA] were significantly different between ghrelin concentrations; Tukey's *post hoc* analysis showed that all concentrations effects on membrane potential were significantly different between each other ($p < 0.05$). Therefore, although the proportion of ghrelin responsive neurons were similar in 10 and 100 nM (respectively, 65 and 67%), the magnitude of response is concentration-dependent. Notably, the proportion of responses to 10 nM ghrelin were not significantly different between intracellular and extracellular recordings (Chi-square = 1,27, df = 2, $p = 0.53$).

In regards to the duration of effects, 1 and 10 nM ghrelin induced similar responses (1 nM = 434 ± 250 s vs. 10 nM = 572 ± 312 s, $p = 0.45$, unpaired *t*-test). Administration of 100 nM ghrelin induced a long-lasting response that persisted until the seal was broken in 50% of ghrelin-responsive neurons (6/12). The remaining neurons showed a large variability in the duration of response, with two neurons in which effects lasted for a longer period (respectively, 1916 and 4024 s) and four that showed effects similar to the other concentrations [499.8 ± 126.7 s; $F(2,65) = 1.844$, $p = 0.16$, ONE-WAY ANOVA]. Thus, the 100 nM concentration extends the effects of ghrelin on most PVN neurons, whilst 10 and 1 nM cause effects of similar duration. Further analyzing 10 nM ghrelin, no significant differences were found between hyperpolarizing and depolarizing ghrelin-responsive neurons (Depolarizing = 572.7 ± 357.2 vs. hyperpolarizing = 571.8 ± 252.8, $p = 0.99$, unpaired *t*-test) or between different neuronal phenotypes [Neuroendocrine = 576.4 ± 268.6 s; pre-autonomic = 601.6 ± 438.6 s; magnocellular = 532.8 ± 271.3 s; $F(2,59) = 1.225$, $p = 0.30$, ONE-WAY ANOVA]. These results suggest that, within the same concentration, the duration of ghrelin effects are consistent in PVN neurons.

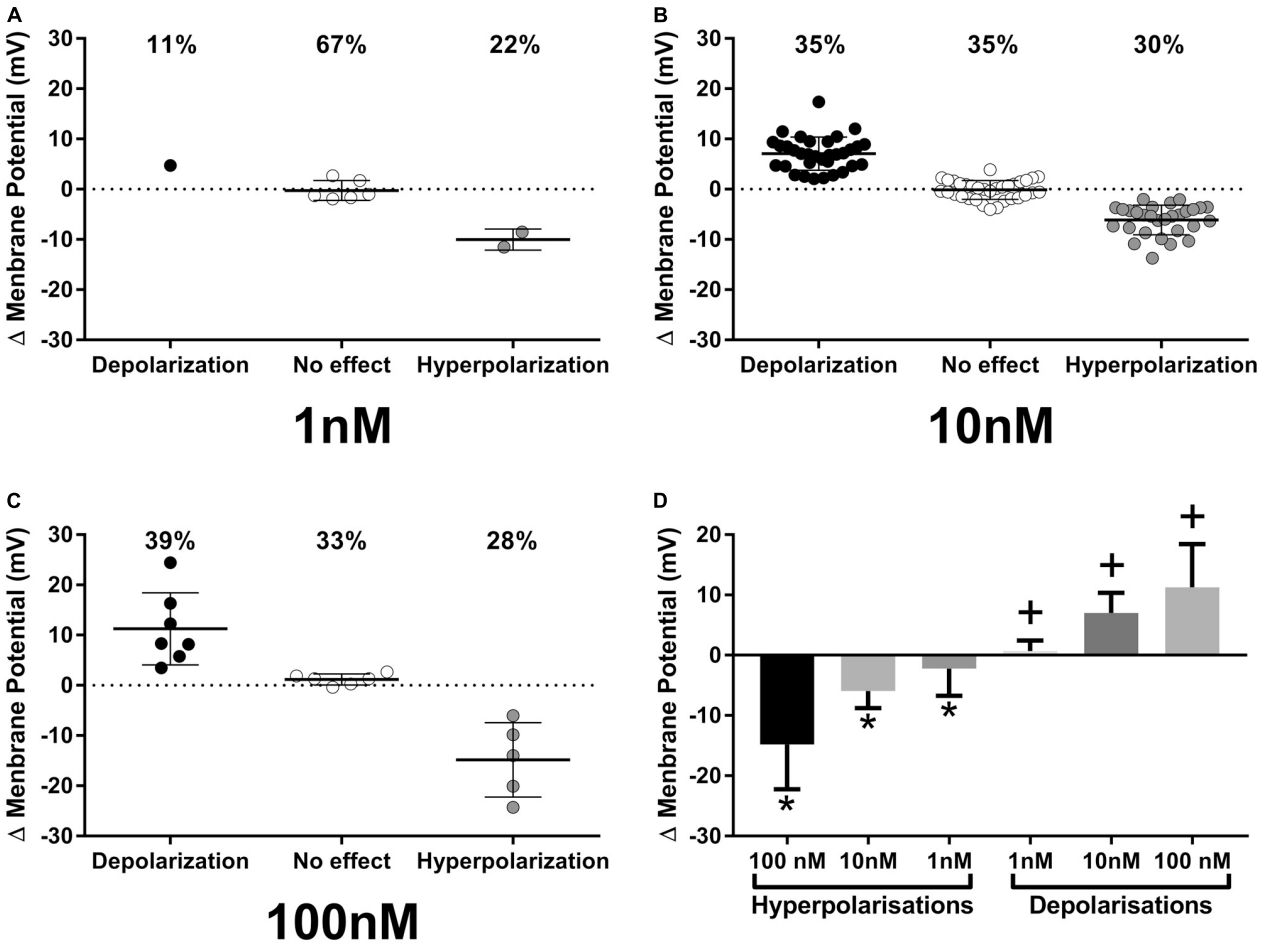

FIGURE 5 | Intracellular recordings of PVN neurons responses to ghrelin. Proportion and magnitude of PVN neurons responses to ghrelin in 1 **(A)**, 10 **(B)** and 100 **(C)** nM concentrations; Concentration-response of ghrelin effects **(D)**. Ghrelin affects PVN neurons in all concentrations tested and the effects are concentration-dependent. $^{*}p < 0.05$ against other hyperpolarizations; $^{+}p < 0.05$ against other depolarizations.

The PVN showed populations of neurons that depolarized, hyperpolarized or were unaffected by ghrelin and we speculated that these effects might be distributed to different functional subpopulations of PVN neurons. In order to assess if these differences in response were dependent on the neuronal phenotype, cells were sorted in accordance to the electrophysiological response to a hyperpolarizing pulse, as previously described (Tasker and Dudek, 1991; Stern, 2001; Luther et al., 2002). While significant proportions of neuroendocrine (29% depolarizations, 40% hyperpolarizations; **Figure 6**), and magnocellular neurons (29% depolarizations, 21% hyperpolarizations; **Figure 7**) responded to ghrelin, mixed effects were still observed in both groups of cells. In contrast, the majority of pre-autonomic neurons tested depolarized to ghrelin (61% depolarizations, 17% hyperpolarizations; **Figure 8**) suggesting primarily excitatory effects on these cells.

These data demonstrate, that even within these electrophysiologically defined subgroups within which different chemical phenotypes exist (e.g., magnocellular OT and AVP neurons), there is a heterogeneity in responsiveness to ghrelin. We therefore carried out additional recordings in which we correlated the electrophysiological responses to the mRNA expressed in individual neurons. We collected the mRNA content from 74 cells, of which 45 (61%) were positive for β-actin mRNA, and were used for this analysis. RT-PCR for AVP, OT, CRH, and TRH was performed on cytoplasm extracted from each neuron following assessment of ghrelin effects using whole cell recording (**Figure 9**). As illustrated in **Figure 10A**, this analysis demonstrated that both ghrelin responsive CRH (3/4 neurons) and TRH (4/5 neurons) expressing neurons demonstrated only hyperpolarizing responses. In contrast, both AVP- (20% depolarized, 40% hyperpolarized, $n = 35$), and OT- (11% depolarized, 44% hyperpolarized, $n = 9$) expressing neurons showed mixed responses to ghrelin, while OT showed a majority of hyperpolarizations. Interestingly, neurons that did not express any of the four tested genes ($n = 8$) showed a higher percentage of depolarizations (50%), with the remainder being either hyperpolarizations or unaffected (25% each).

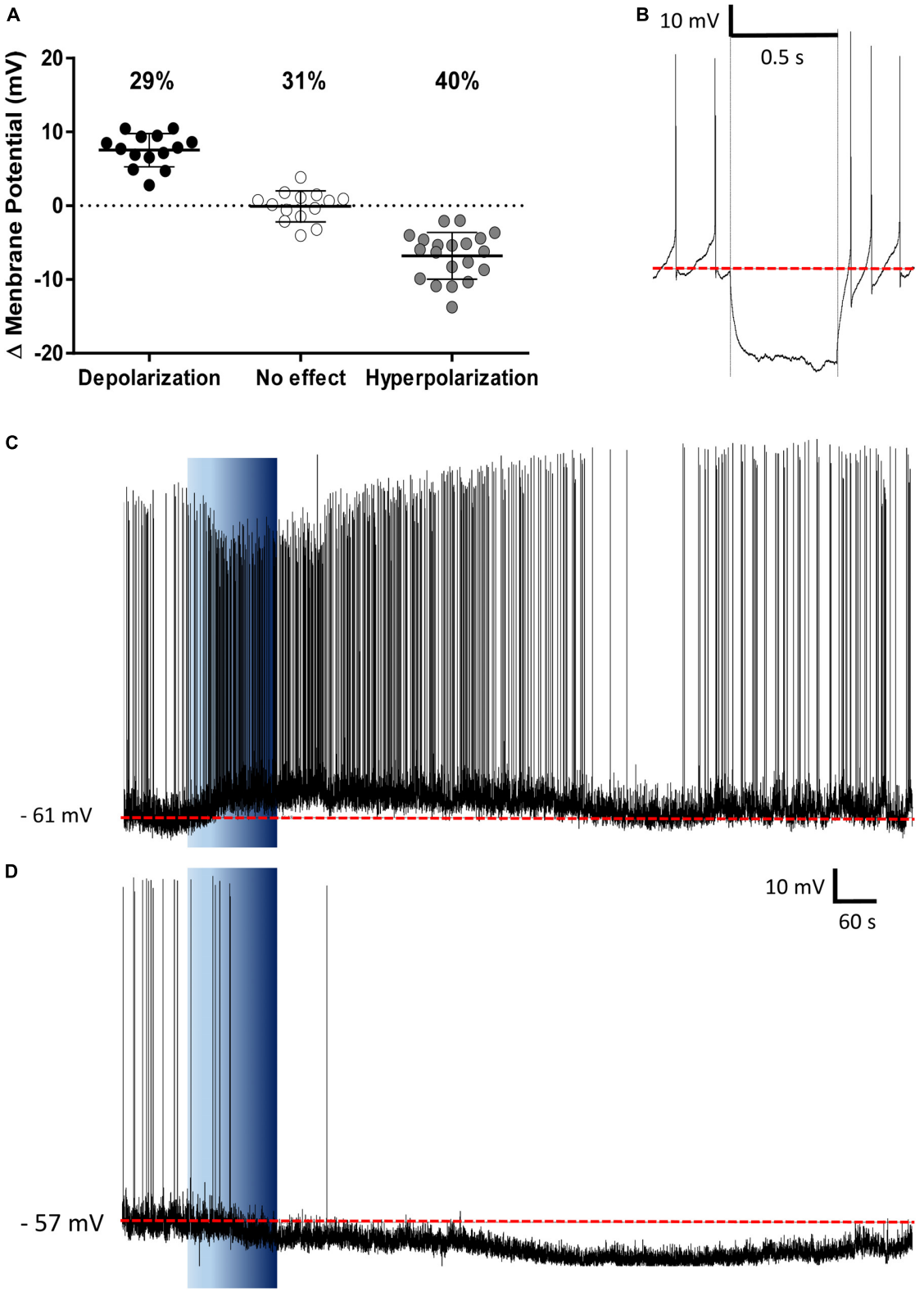

FIGURE 6 | Ghrelin affects neuroendocrine PVN neurons. Ghrelin changes membrane potential in neuroendocrine PVN neurons **(A)**, with populations of neurons either increasing or decreasing membrane potential in response to ghrelin. Neuroendocrine neurons were identified by the lack of an electrophysiological fingerprint in response to a hyperpolarizing pulse **(B)**. Representative traces of a depolarization **(C)** and a hyperpolarization **(D)** are shown. Background indicates 10 nM ghrelin application to the bath, dashed line represents mean baseline membrane potential.

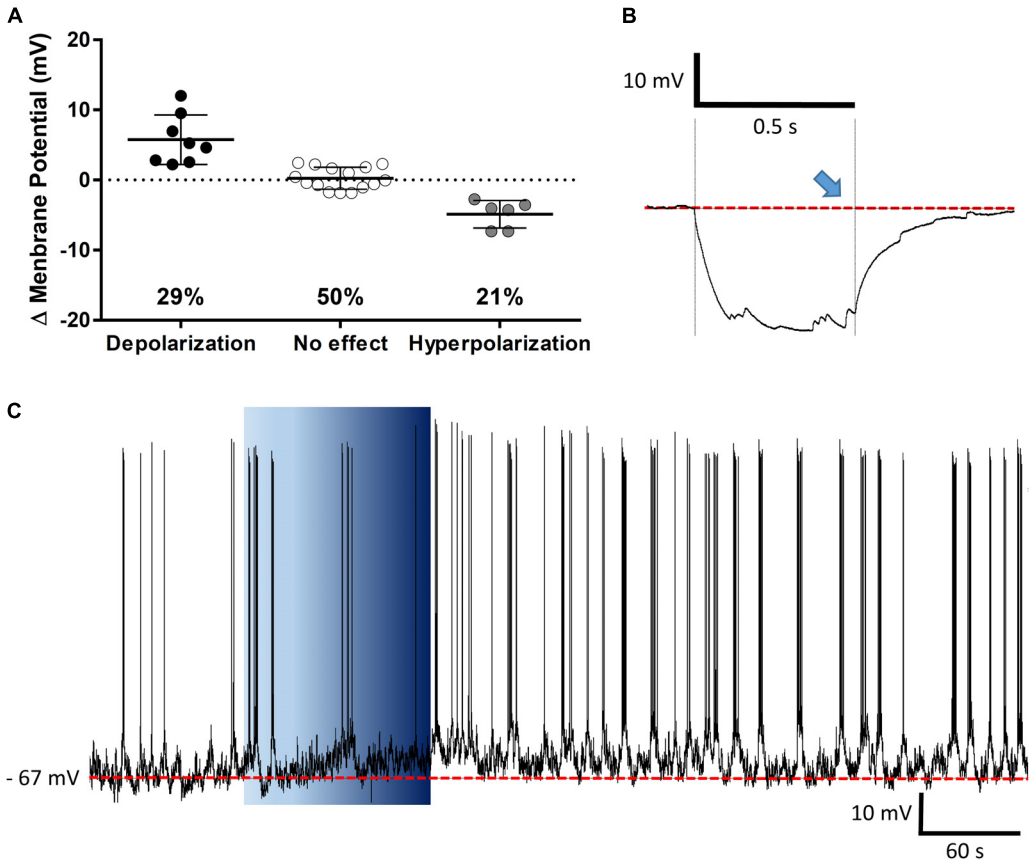

FIGURE 7 | Ghrelin affects magnocellular PVN neurons. Ghrelin does not affect half of PVN magnocellular neurons, while some neurons either depolarize or hyperpolarize in response to ghrelin **(A)**. Neurons were defined as magnocellular due to the presence of A-current, characterized by the delay to return to baseline (blue arrow) after a hyperpolarizing pulse **(B)**, a representative trace of a magnocellular neuron that increases membrane potential and firing frequency after ghrelin application is shown **(C)**. Background indicates 10 nM ghrelin application to the bath, dashed line represents mean baseline membrane potential.

We also have correlated mRNA expression with our electrophysiological classification of neuroendocrine (**Figure 10B**), pre-autonomic (**Figure 10C**) or magnocellular (**Figure 10D**) phenotypes. These data highlight the unexpected observation that AVP was expressed in the majority of PVN neurons (30/45). Most CRH-expressing neurons co-expressed AVP mRNA (3/4), while the only OT-expressing neuron that depolarized in response to ghrelin presented more AVP than OT mRNA.

DISCUSSION

The PVN is involved in the control of a series of physiological responses, including the response to osmotic stimuli, metabolic disturbances, cardiovascular and autonomic control, the stress response and secretion of pituitary hormones, and thus, unsurprisingly, this nucleus consists of distinct subpopulations of neurons (Stern, 2015). Ghrelin modulates all the aforementioned physiological responses (Müller et al., 2015), which suggests the PVN as a target for ghrelin actions. Previous studies have assessed the effects of ghrelin on neuronal excitability in the ARC (Cowley et al., 2003; Andrews et al., 2008), hippocampus (Diano et al., 2006; Ribeiro et al., 2014) and area *postrema* (Fry and Ferguson, 2009). However, to date studies examining the effects of ghrelin on the activity of PVN neurons have only reported effects on inhibitory (Cowley et al., 2003) and excitatory (Kola et al., 2008) post-synaptic currents. In this study we show that ghrelin affects the spike frequency of the majority of PVN neurons, either decreasing or increasing their activity. While inhibitory actions of ghrelin were also demonstrated in ARC neurons (Cowley et al., 2003; Yousheng et al., 2008), the fact that ghrelin acts through GHSR, a G_q coupled receptor that increases intracellular calcium (Cowley et al., 2003), supports the idea that direct actions of ghrelin would be primarily excitatory. Although the majority ghrelin effects are mediated by GHSR, some of ghrelin effects are maintained in GHSR knockout mice (Uchida et al., 2013), which suggests that another receptor may be involved in the responses to ghrelin. In order to evaluate whether the effects were dependent on direct effects on PVN neurons or mediated by previous activation of neurons within the slices, we assessed the effects of ghrelin in the presence of GABAA and glutamate antagonists, and demonstrated that the inhibitory actions of ghrelin were absent when the transmission

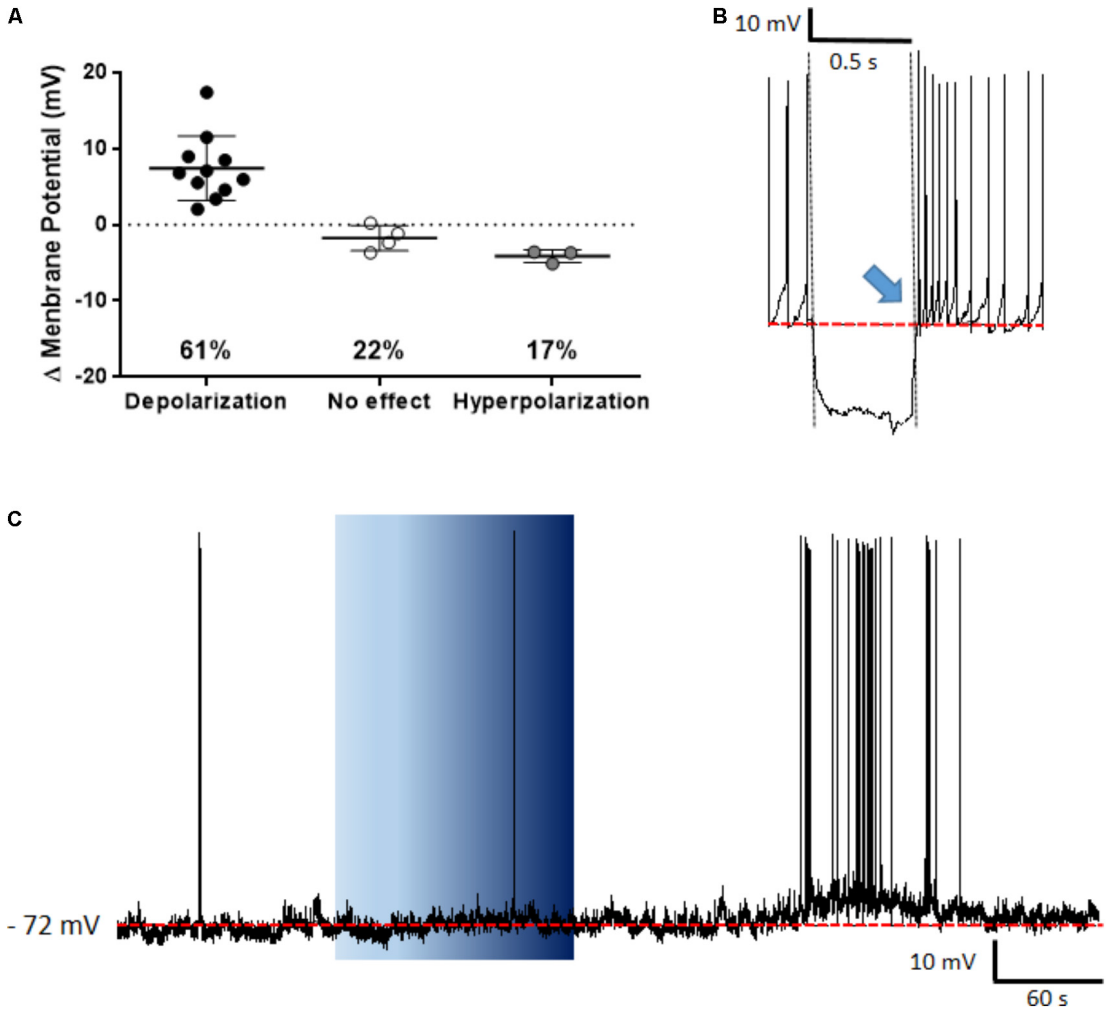

FIGURE 8 | Ghrelin affects pre-autonomic PVN neurons. Ghrelin depolarizes most pre-autonomic PVN neurons **(A)**. Neurons were defined as pre-autonomic due to the presence of low-threshold spikes (blue arrow) after a hyperpolarizing pulse **(B)**, a representative trace of a depolarization is shown **(C)**. Background indicates 10 nM ghrelin application to the bath, dashed line represents mean baseline membrane potential.

mediated by these rapid amino acids was blocked. Glutamate induces excitatory responses within the PVN, while GABA is mostly involved in inhibitory responses (Ferguson et al., 2008), suggesting that the different effects in the presence of the antagonists are due to the blockade of GABA actions. These results provide evidence that the direct effects of ghrelin on PVN neurons are excitatory, and that the inhibitory effects are dependent on the excitation of a GABAergic inhibitory circuit. These inhibitory afferents to the PVN may arise from inhibitory interneurons within the PVN or projections from other nuclei present in the slice, such as the ARC. The ARC is activated by ghrelin and sends projections to the PVN, however, some of the effects of ghrelin administration persist in ARC ablated mice, which indicates that the PVN is capable of responding directly to ghrelin (Cabral et al., 2016). Interestingly, both intra-PVN administration of ghrelin (Currie et al., 2005) and the activation of GABAA receptors in the PVN (Tsujii and Bray, 1991; Pu et al., 1999) increase food intake, and immunofluorescence studies demonstrated that ghrelin binds to GABA-expressing neurons in the PVN (Cabral et al., 2016). Therefore, we suggest that ghrelin directly activates GABAergic neurons in the PVN, that, in turn, inhibit other PVN neurons.

Our patch clamp recordings also show that ghrelin primarily depolarizes pre-autonomic PVN neurons, effects that presumably underlie some of the previously demonstrated effects of central ghrelin on autonomic output. ICV ghrelin affects sympathetic responses including, inhibition of renal sympathetic nerve activity (Matsumura et al., 2002; Tanida et al., 2007), increased fat mass and storage in white adipose tissue (Theander-Carrillo et al., 2006), and inhibition of fat oxidation in brown adipose tissue (Yasuda et al., 2003; Theander-Carrillo et al., 2006). Indeed, intra-PVN administration of ghrelin showed similar sympathetic-dependent inhibition of the brown-adipose tissue (Mano-Otagiri et al., 2009), and increases FOS in the nucleus of the solitary tract (Olszewski et al., 2003). Additionally, non-secretory PVN neurons may participate in ghrelin effects on other

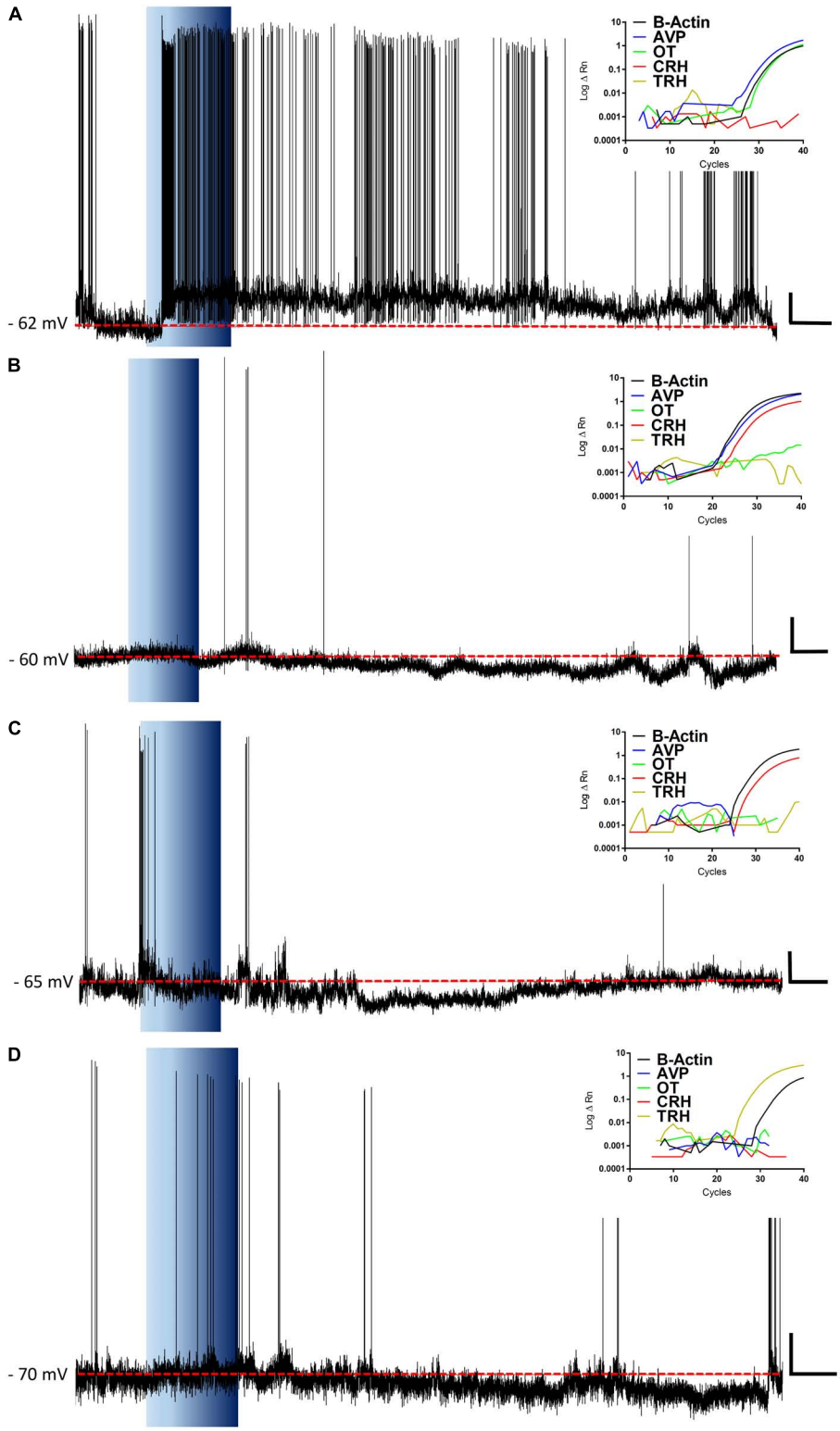

FIGURE 9 | Representative recordings and RT-PCRs. The figure shows representative traces for ghrelin responsive neurons and the insets show their respective amplification plots for β-Actin, AVP, OT, CRH, and TRH mRNA. Note, a neuron that depolarizes in response to ghrelin and expresses both AVP and OT mRNA, with the predominance of AVP **(A)**; hyperpolarizations in CRH expressing neurons, either with AVP-CRH coexpression **(B)** or with CRH only **(C)**; and a hyperpolarization in a TRH neuron **(D)**. Blue background indicates 10 nM ghrelin application; red dashed line represents mean membrane potential; vertical scale bar represents 10 mV; and horizontal scale bar represents 60 s.

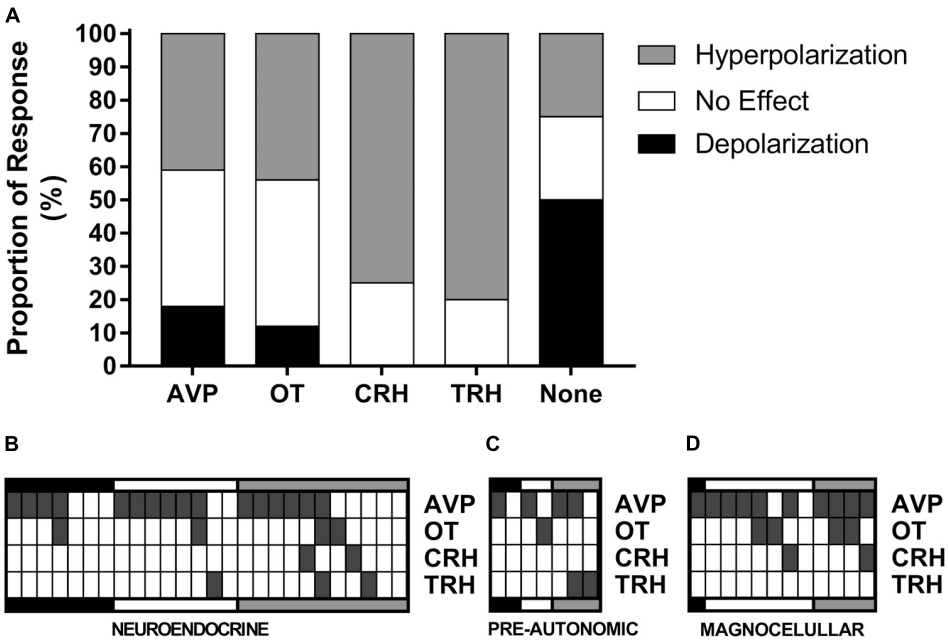

FIGURE 10 | Single-cell RT-PCR. The proportions of responses to ghrelin for each gene **(A)** showed that most TRH and CRH neurons hyperpolarize in response to ghrelin and most depolarizations come from neurons with none of the tested genes. Individual data from neuroendocrine **(B)**, pre-autonomic **(C)**, and magnocellular **(D)** neurons shows that AVP mRNA is expressed in most neurons and that the majority of CRH neurons co-express AVP.

physiological functions such as anxiety-like behavior (Currie et al., 2012; Wauson et al., 2015) and feeding (Wren et al., 2001; Melis et al., 2002; Olszewski et al., 2003; Shrestha et al., 2004) which are increased by intra-PVN administration of ghrelin.

Magnocellular and neuroendocrine neurons showed mixed responses to ghrelin, thus we attempted to further clarify the effects of ghrelin on magnocellular and neuroendocrine neurons using techniques to describe mRNA expression of AVP, OT, CRH, and TRH; all of which have been previously implicated in the responses to ghrelin (Müller et al., 2015), and correlate this with the effects of ghrelin on individual neurons. We have previously shown that the single cell mRNA expression closely relates to the protein expressed by immunofluorescence, and that the single cell mRNA analysis is a reliable technique to assess the neuronal phenotype (da Silva et al., 2015). Most notably, ICV administration of OT (Olson et al., 1991), CRH (Wang et al., 2007), and TRH (Steward et al., 2003; Schuhler et al., 2007) decreases food intake, and the majority of PVN neurons expressing these neurotransmitters were hyperpolarized in response to ghrelin, supporting the conclusion that this peptide inhibits PVN neurons that express anorexigenic neurotransmitters. Previous studies stated that in the hungry animal the ARC-PVN neurons that inhibit anorexigenic CRH, TRH, and OT neurons are activated, thus inducing food intake (Valassi et al., 2008).

Paraventricular nucleus TRH neurons control TSH and T3/T4 release, thus controlling thyroid function (Pekary and Sattin, 2012). Although the effects of ghrelin on the release of TRH have not been directly demonstrated, negative caloric balance decreases the release of TRH in the median eminence (Joseph-Bravo et al., 2015). Conversely, leptin – an anorexigenic hormone that opposes ghrelin actions – increases TRH release (Guo et al., 2004; Ghamari-Langroudi et al., 2010). This indicates that in the hungry animal the TRH axis is inhibited, thus decreasing T3 and T4 release and reducing energy expenditure. PVN CRH neurons also induce thermogenesis (Richard et al., 2002). Ghrelin is known to reduce energy expenditure (Tschop et al., 2000; Theander-Carrillo et al., 2006), possibly the inhibition of TRH and CRH neurons in the PVN contributes to this response.

Intra-cerebro ventricular ghrelin increases FOS expression on OT neurons in the PVN (Olszewski et al., 2007), and ghrelin induces OT release in hypothalamic cell culture (Gálfi et al., 2016). The release of OT in response to ghrelin, however, has not been demonstrated *in vivo*. Magnocellular OT neurons were mostly hyperpolarized by ghrelin, which suggests that ghrelin would not increase plasma levels of OT. Additionally, OT is involved in the modulation of other responses such as reproduction, social behavior and feeding (Spetter and Hallschmid, 2017). OT is present in pre-autonomic neurons (Sawchenko and Swanson, 1982), which indicates this peptide is involved in the control of these responses. The present results, together with the observations of effects caused by intra-PVN administration of ghrelin on food intake (Wren et al., 2001; Melis et al., 2002; Olszewski et al., 2003; Shrestha et al., 2004); and effects of ghrelin on neuroendocrine (Wren et al., 2002; Gálfi et al., 2016) and autonomic control (Matsumura et al., 2002; Yasuda et al., 2003; Theander-Carrillo et al., 2006; Tanida et al., 2007) indicate the PVN as a major site of action for ghrelin, therefore contributing to the response to caloric imbalance.

AVP mRNA, was co-expressed with all peptides assessed in this study. The cross-talk between AVP and CRH in the control of the hypothalamic-hypophysial-adrenocortical axis is well established (Keller-Wood, 2015), and AVP is essential for the increased CRH release in response to ghrelin in hypothalamic cell culture (Nemoto et al., 2011), which suggests an interplay between these peptides in ghrelin responses. The co-expression of AVP and OT, was previously shown in single-cell analysis of magnocellular neurons on the supraoptic nucleus (Pires da Silva et al., 2016) and PVN (Hoyda et al., 2009). The unexpected ubiquitous presence of AVP mRNA, indicates that this neurotransmitter is involved in more than AVP secretion to the circulation. AVP integrates neurosecretory and pre-autonomic populations of PVN neurons, through a mechanism deemed "volume transmission," in which the release of a neuromodulator by dendrites into the extracellular medium influences the activity of juxtaposed neurons that are not anatomically connected by axon-dendrite synapses (Stern, 2015). The dendro-dendritic AVP release by the PVN has been shown to be important for autonomic control (Lozić et al., 2016) and neurosecretory and pre-autonomic PVN neurons have been shown to interact in this manner (Son et al., 2013). Ghrelin influences adiposity and energy utilization (Theander-Carrillo et al., 2006), volume transmission could potentially mediate these effects, since they depend on long-term alteration in neurotransmission/neuromodulation. The influence of ghrelin on volume transmission poses an interesting question to be assessed in future studies. The majority of depolarizations in response to ghrelin were found in neurons that did not express any of the tested genes, effects which are therefore likely to be on other PVN neuronal subgroups such as those expressing other transmitters such as glutamate (Hrabovszky et al., 2005) and GABA (Park et al., 2007), indeed, previous studies demonstrated that icv ghrelin binds to GABA-expressing neurons in the PVN (Cabral et al., 2016). The observations that ghrelin inhibits several neuronal subgroups in the PVN, and that these inhibitions disappear in the presence of GABAA and glutamate antagonists suggest that ghrelin increases the activity of inhibitory neurons within the PVN, in turn decreasing the activity of TRH, CRH, and OT neurons. However, this hypothesis needs further testing, since the number of TRH and CRH expressing neurons obtained through this technique was low.

CONCLUSION

Ghrelin affects all types of PVN neurons, possibly increasing the activity of GABAergic neurons that inhibit anorexigenic OT-, CRH-, and TRH-expressing neurons, increasing food intake and reducing energy expenditure. Additionally, the ubiquitous presence of AVP mRNA, suggests a function of this peptide that surpasses that of a classical neurotransmitter, possibly being involved in the integration of the different neuronal populations within the PVN. Further studies are necessary to determine the neural circuitry involved in the PVN participation on the responses to ghrelin, with special attention to the different nuclei to which these neurons send efferent projections; and to the intra-PVN integration of these responses.

AUTHOR CONTRIBUTIONS

Rd-S, AF, and AM designed the study. All authors participated in data analyses or acquisition, drafted/revised the manuscript, approved the final version, and agreed to be accountable for all the aspects of the work.

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REVIEW
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The Role of Ghrelin in Regulating Synaptic Function and Plasticity of Feeding-Associated Circuits

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Synaptic plasticity of the neuronal circuits associated with feeding behavior is regulated by peripheral signals as a response to changes in the energy status of the body. These signals include glucose, free fatty acids, leptin and ghrelin and are released into circulation, being able to reach the brain. Ghrelin, a small peptide released from the stomach, is an orexigenic hormone produced in peripheral organs, and its action regulates food intake, body weight and glucose homeostasis. Behavioral studies show that ghrelin is implicated in the regulation of both hedonic and homeostatic feeding and of cognition. Ghrelin-induced synaptic plasticity has been described in neuronal circuits associated with these behaviors. In this review, we discuss the neuromodulatory mechanisms induced by ghrelin in regulating synaptic plasticity in three main neuronal circuits previously associated with feeding behaviors, namely hypothalamic (homeostatic feeding), ventral tegmental (hedonic and motivational feeding) and hippocampal (cognitive) circuits. Given the central role of ghrelin in regulating feeding behaviors, and the altered ghrelin levels associated with metabolic disorders such as obesity and anorexia, it is of paramount relevance to understand the effects of ghrelin on synaptic plasticity of neuronal circuits associated with feeding behaviors.

Keywords: ghrelin, synaptic plasticity, hypothalamus, ventral tegmental area, hippocampus, feeding

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INTRODUCTION

The capacity to seek and consume food is critical to survival. In nature, mammals need to optimize food searches, which requires a complex interaction of behaviors. On one hand, animals need to remember where to find food; on the other, animals may overeat to prevent future famine, which can be achieved by increasing the rewarding value of food. Nevertheless, in modern western society food is abundant, easily accessible and increasing its reward value can lead to an increase in food intake above the metabolic need. The complex interplay of behaviors associated with feeding can ultimately be prejudicial and induce metabolic disorders such as obesity and anorexia.

Behaviors associated with feeding are coordinated mainly by two inter-related neurobiological systems: the hypothalamic and mesolimbic systems. The hypothalamic system is mainly activated when energy store levels are low and drives feeding to replenish energy stores (homeostatic feeding), while the mesolimbic system, which connects the ventral tegmental area (VTA) to the striatum, is activated by pleasurable (hedonic) and incentive (motivational) aspects of food. However, all foods have a rewarding value that is influenced by hunger and food availability

(Perello and Dickson, 2015), suggesting that the mesolimbic and homeostatic pathways interact tightly to control feeding. Additionally, neuronal circuits that are involved in cognitive behavior, such as cortical and hippocampal circuits, are of paramount relevance in the control of feeding as food searches require memory and learning processes.

The regulation of feeding by the brain suggests that nutrients and peripheral signals released in the blood, such as glucose, free fatty acids, leptin, insulin and ghrelin can sense the energy status and reach the brain, where they modulate the activity of several neuronal circuits. Ghrelin is an orexigenic hormone affecting both energy homeostasis and higher brain functions. Ghrelin affects feeding-associated behaviors, which are accompanied by changes in synaptic strength and in the modulation of neuronal circuit function, a phenomenon termed synaptic plasticity. The effects of ghrelin on synaptic plasticity have been described in neurobiological circuits associated with feeding and cognitive behavior. In this review, we discuss the mechanisms for synaptic plasticity modulated by ghrelin in three main neuronal pathways previously associated with these behaviors: the hypothalamic, the mesolimbic and the hippocampal pathways (**Figure 1**). Ghrelin levels are changed in diseases linked to metabolism such as obesity and anorexia, which can lead to altered ghrelin signaling in the brain and be associated with defects on synaptic plasticity phenomena. Thus, understanding the effects of ghrelin on synaptic plasticity is critical both under physiological and pathological ghrelin signaling.

THE GHRELIN SYSTEM

Ghrelin is a 28 amino acid peptide mainly released from the empty stomach (Kojima et al., 1999) but also found in other peripheral tissues such as the testis, ovary, placenta, kidney, pituitary, small intestine, pancreas, lymphocytes (reviewed in Ferrini et al., 2009; Muller et al., 2015; Mani and Zigman, 2017). Ghrelin has gained attention due to its capacity to stimulate food intake (Nakazato et al., 2001; Wren et al., 2001), to induce fat storage (Tschop et al., 2000), to prevent falls in blood glucose (Broglio et al., 2001) and to increase memory retention (Carlini et al., 2002; Diano et al., 2006). In addition, ghrelin regulates other actions such as cell proliferation, gastric acid secretion and motility (Masuda et al., 2000).

Ghrelin is present in two major forms in the blood, the acyl (octanoylated) and non-acyl forms, but only acyl-ghrelin is able to bind to the growth hormone secretagogue receptor type 1 (GSH-R1a). In fact, ghrelin is the only known peptide that undergoes *n*-octanoylation at a serine residue, enabling it to bind to GHS-R1a (Kojima et al., 1999). Ghrelin octanoylation is a unique posttranslational modification accomplished by ghrelin *O*-acyl-transferase (GOAT; Gutierrez et al., 2008; Yang et al., 2008), a member of the membrane-bound *O*-acyltransferases. GOAT utilizes dietary medium-chain fatty acids as a substrate for ghrelin acylation (Nishi et al., 2005), and acts as a nutrient sensor signaling to the brain on the presence of dietary calories (Kirchner et al., 2009). The levels of acyl-ghrelin in the blood depend on GOAT activity, since mice deficient for GOAT lack acyl-modified forms of ghrelin (Gutierrez et al., 2008). Besides *n*-octanolylated ghrelin, other acyl-ghrelin forms which include modifications with longer saturated and unsaturated fatty acyl groups have been detected (reviewed by Nishi et al., 2011).

Ghrelin acts through the GHS-R1a, which was originally described in the pituitary gland and the hypothalamus as the target of growth hormone secretagogues (Howard et al., 1996). GHS-R1a is one of the two alternative splicing forms encoded by the *Ghsr* gene (McKee et al., 1997); the other isoform, GHS-R1b, is truncated at the C-terminus, does not bind to ghrelin and possesses no signaling activity known so far. The two GHS-R isoforms can form heterodimers, which reduces the cell surface expression of GHS-R1a (Chow et al., 2012). GHS-R1a is a G-protein coupled receptor expressed in the periphery (Papotti et al., 2000) and in the brain (Guan et al., 1997; Zigman et al., 2006; Mani et al., 2014), which can signal through G protein subunit $\alpha_{q/11}$ and activate phosphatidylinositol-specific phospholipase C, leading to protein kinase C (PKC) activation and the regulation of ion currents. The GSH-R1a can also be coupled to activation of the phosphatidylinositol 3 (PI3)-kinase signaling cascade in different cellular systems, and lead to protein kinase A (PKA) activation (Camina, 2006). The C-terminal region of GHS-R1a is critical for ligand-induced receptor internalization, recruitment of β-arrestin$_2$ and termination of GHS-R1a signaling (Evron et al., 2014). Interestingly, the GHS-R1a presents unusually high constitutive activity in the absence of the ligand (Holst et al., 2003). The physiological relevance of the GSH-R1a constitutive activity has not been fully clarified (reviewed in Mear et al., 2013), but the ligand-independent activity of the GSH-R1a is known to play a role in the control of food intake and regulation of body weight (Petersen et al., 2009; Els et al., 2012; McCoull et al., 2014; Fernandez et al., 2018), and in the acquisition of conditioned taste aversion (Li et al., 2018). Human mutations that lead to a selective loss of constitutive activity of GHS-R1a are associated with familial short stature (Pantel et al., 2006, 2009; Inoue et al., 2011). The GHS-R1a constitutive activity reduces presynaptic Ca_v2 currents and GABA release in hypothalamic and hippocampal neurons (Lopez Soto et al., 2015; Valentina et al., 2018), and reduces the cell surface expression of Ca_v2 channels (Mustafa et al., 2017).

Besides signaling in response to ghrelin, and in the absence of the ligand, the GSH-R1a has been shown to heterodimerize with and modulate signaling through other G-protein coupled receptors, such as dopamine D1 and D2 receptors (DR1R, DR2R), melanocortin 3 receptors and serotonin 2C receptors (Wellman and Abizaid, 2015). Recent studies provide evidence for further GSH-R1a heterodimerization with the orphan receptor G protein-coupled receptor 83 (Gpr83), which diminishes activation of GHS-R1a by ghrelin (Muller et al., 2013), and the oxytocin receptor, resulting in attenuation of oxytocin-mediated signaling (Wallace Fitzsimons et al., 2018).

In healthy humans, acute administration of ghrelin increases food intake, whether it is administered intravenously or infused (Wren et al., 2001) or subcutaneously applied (Druce et al., 2006). Similarly, in rodents, central or peripheral administration of ghrelin induces feeding/increases food intake (Tschop et al., 2000; Asakawa et al., 2001; Nakazato et al., 2001; Wren et al.,

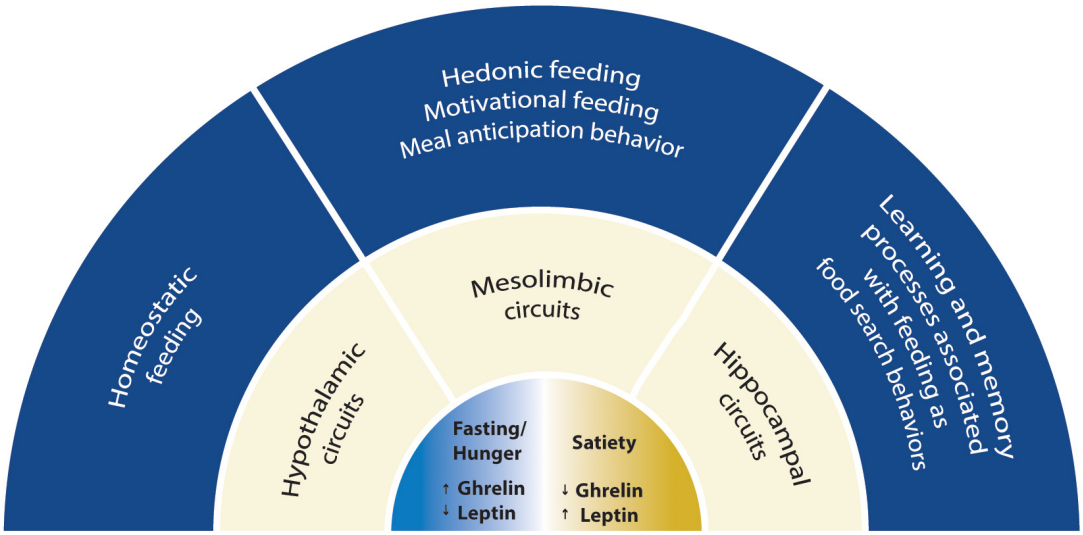

FIGURE 1 | Scheme with the scope of this review. The mechanisms for synaptic plasticity modulated by ghrelin are discussed in three main neuronal circuits associated with feeding: the hypothalamic, the mesolimbic and the hippocampal circuits. Two energy states are represented: fasting/hunger and satiety. Fasting or hunger conditions are associated with high ghrelin and low leptin levels, while satiety is associated with low ghrelin and high leptin levels. Hypothalamic circuitry drives food intake depending on energy store levels (homeostatic feeding), while the mesolimbic circuitry drives consumption of food with elevated rewarding properties (hedonic and motivational feeding). Hippocampal circuitry integrates feeding with cognitive behavior suggesting that ghrelin is relevant for food searches in nature and can impact cognitive functions.

2001). Circulating ghrelin binds neurons in the vicinity of fenestrated capillaries in the arcuate nucleus of the hypothalamus (Schaeffer et al., 2013). Ghrelin levels in the blood fluctuate throughout the day in humans, rising before a meal and decreasing upon food consumption (Cummings et al., 2001), which indicates that ghrelin works as a meal initiating peptide; however, the role for ghrelin oscillations in feeding behavior has not been elucidated. The levels of circulating ghrelin are elevated in negative energy balance conditions such as in anorexia and caloric restriction, and are decreased in positive energy balance conditions such as obesity (reviewed in Muller et al., 2015). Hence, disruption of ghrelin signaling in animal models by using loss of function or gain of function studies can provide important information regarding the pathological effects of ghrelin signaling in feeding and cognitive behaviors. Interestingly, pharmacological inhibition of the ghrelin system, either by neutralizing ghrelin, inhibiting GOAT or antagonizing the ghrelin receptor caused decreased body weight or reduced food consumption (Shearman et al., 2006; Zorrilla et al., 2006; Esler et al., 2007; Barnett et al., 2010; Landgren et al., 2011), suggesting that endogenous ghrelin contributes to food intake. However, genetic mouse models of ghrelin, GHS-R1a or GOAT manipulation provided conflicting results.

Ghrelin knock-out (KO) mice did not present differences in food intake or body weight in comparison to wild-type littermates (Sun et al., 2003, 2008; Wortley et al., 2004). However, ghrelin knock-out mice were protected from the weight gain triggered by exposure to a high-fat diet at early age (Wortley et al., 2005). In addition, mice overexpressing acyl-ghrelin displayed hyperphagia, glucose intolerance, decreased glucose-stimulated insulin secretion and reduced leptin sensitivity (Bewick et al., 2009). Ghrelin did not stimulate food intake in a GHS-R KO mouse model (Sun et al., 2004; Zigman et al., 2005), showing that ghrelin acts through GHS-R to influence food consumption.

However, food intake and body weight in GHS-R KO mice fed standard chow diet were similar to their wildtype littermates (Sun et al., 2004; Zigman et al., 2005; Pfluger et al., 2008). More recently, it was showed that GSH-R1a KO rats consume less food overall at basal conditions and weigh significantly less compared with wild-type littermates throughout development (Zallar et al., 2018). Nevertheless, young GHS-R KO animals fed with a high-fat diet ate less, preferentially used fat as an energy substrate, and presented reduced body weight, and reduced adiposity and glucose levels compared to wild-type littermates (Zigman et al., 2005). Adult GSH-R KO mice did not present changes in energy expenditure or body weight under conditions of positive or negative energy balance, but showed impairment in maintaining glucose homeostasis upon caloric restriction, suggesting a function for the ghrelin receptor in modulating glucose sensing and insulin sensitivity (Sun et al., 2008). On the other hand, in aged animals fed with regular diet ablation of the GHS-R decreased body weight and reduced adiposity, as well as improved insulin sensitivity (Lin et al., 2011), similarly to what was found in young GHS-R animals fed with high-fat diet (Zigman et al., 2005). These interesting observations suggest an age-dependent role for the GHS-R1 in regulating body weight, adiposity and insulin resistance. Collectively, the ghrelin and GSH-R1a loss-of and gain-of-function studies in rodents have also demonstrated that, albeit first described as an important stimulator of food intake, ghrelin functions on energy expenditure extend to glucose tolerance and insulin sensitivity (reviewed in Mani and Zigman, 2017).

Recently, liver-expressed antimicrobial peptide 2 (LEAP2) was described as the first endogenous non-competitive antagonist of the GSH-R1a. LEAP2 is produced in the liver and small intestine, and its secretion is suppressed by fasting. LEAP2 blocks ghrelin-induced food intake, GH release and maintenance of viable glucose levels during chronic caloric restriction (Ge et al., 2018). A recent study found that in addition to antagonizing the ghrelin-induced activity of GSH-R1a, LEAP2 behaves as an inverse agonist, blocking the constitutive ligand-independent activity of the receptor (M'Kadmi et al., 2019). Since LEAP2 interacts with the GSH-R1a, and plays a role in the regulation of energy homeostasis, it is a promising therapeutic target in the treatment of metabolic diseases (reviewed in Al-Massadi et al., 2018).

NEUROMODULATION OF SYNAPTIC PLASTICITY

Behavioral experiences such as learning or searching for food generate patterns of neuronal activity that can induce synaptic plasticity, a set of bidirectional changes in the strength of synaptic transmission (Citri and Malenka, 2008; Nicoll, 2017). During these processes, neuronal activity leads to a series of molecular and structural synaptic events, such as Ca^{2+} influx, changes in the release of neurotransmitters, alterations in receptor phosphorylation and expression at the post-synapse, changes in gene and protein expression, and modifications in the number and shape of dendritic spines. Overall, these changes are required for the expression of synaptic plasticity and learning (Citri and Malenka, 2008). In excitatory synapses, changes in the expression and biophysical properties of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors (AMPARs) are major mechanisms underlying various forms of synaptic plasticity (Diering and Huganir, 2018). N-methyl-D-aspartate-type glutamate receptors (NMDAR) play a pivotal role in plasticity by allowing calcium influx to the post-synaptic cell. Calcium binds kinases including calcium/calmodulin-dependent protein kinase II (CaMKII), which can in turn phosphorylate AMPAR subunits and alter their biophysical properties and synaptic traffic (Diering and Huganir, 2018). Other kinases such as PKA and PKC can, together with CaMKII, phosphorylate AMPARs subunits and other synaptic targets, ultimately contributing to synaptic plasticity (Diering and Huganir, 2018). It is believed that synapse-specific changes in synaptic strength form the cellular basis of learning, memory and other behavioral adaptations.

The brain integrates metabolic and environmental information which is transformed into neuronal and synaptic activity, to generate behavior that promotes energy balance and survival. It is therefore not surprising that synaptic function and plasticity in brain regions that participate in different aspects of feeding behavior are modulated by the action of hormones that regulate energy homeostasis. Neuromodulators such as ghrelin and other circulating hormones act in different brain regions to activate signaling pathways that impact on synaptic function and on the induction and/or expression of synaptic plasticity mechanisms, thus affecting behavior at different levels. Interestingly, the signaling pathways downstream of GHS-R1a activation crosstalk with synaptic plasticity pathways. The ghrelin receptor activity regulates calcium intracellular levels, through activation of both the phospholipase C-PKC pathway and adenylate cyclase-PKA signaling, and leads to the activation of phosphatidylinositol 3-kinase (PI3K) (reviewed in Castaneda et al., 2010), pathways with a role in synaptic plasticity mechanisms. Through this crosstalk, ghrelin may affect synaptic function and plasticity in the hypothalamus, midbrain and hippocampus, thereby regulating homeostatic, hedonic and cognitive aspects of feeding behavior.

GHRELIN SYNAPTIC SIGNALING IN THE HYPOTHALAMUS

The coordinated regulation of energy intake and energy expenditure sensed by peripheral signals as a response to changes in the body energy status requires the occurrence of synaptic plasticity in the hypothalamus, which is the main region for the control of energy balance in the brain. Thus, hypothalamic synaptic plasticity is a critical process for health and survival. In the hypothalamus, the arcuate nucleus (ARC) contains the Agouti-related peptide (AgRP) or neuropeptide Y (NPY)-expressing neurons which stimulate feeding and increase body weight, and the pro-opiomelanocortin (POMC)-expressing neurons which suppress feeding in mice (Aponte et al., 2011). AgRP and POMC neurons target downstream neurons that express the melanocortin receptor 4 (MC4R). AgRP neurons suppress MC4R signaling and POMC neurons produce POMC, which is cleaved producing alpha-melanocyte stimulating hormone (α-MSH), hence increasing MC4R signaling. Thus, the balance of the firing rate of these neurons regulates feeding behaviors (reviewed in Andermann and Lowell, 2017; Sternson and Eiselt, 2017).

In fact, the opposing levels of AgRP and POMC neuronal activity in fed versus fasted animals (increased activity of AgRP neurons and reduced activity of POMC neurons in fasted animals and reduced activity of AgRP neurons and increased activity of POMC neurons in fed animals) suggest that synaptic plasticity occurs in these neurons in response to different energy status. Additionally, AgRP and POMC neurons sense peripheral signals, such as ghrelin and leptin, which modulate their activity. Ghrelin is involved in the hypothalamic regulation of feeding. The GSH-R1a is expressed in the hypothalamus (Zigman et al., 2006), and ghrelin injection intra-cerebroventricularly (i.c.v.) or in the paraventricular hypothalamic nucleus (PVN) induced feeding and the expression of c-Fos, a marker of neuronal activation, in NPY and AgRP neurons (Nakazato et al., 2001; Olszewski et al., 2003). Antagonizing NPY or AgRP signaling abolished ghrelin-induced feeding (Nakazato et al., 2001). Consistently, the orexigenic effect of ghrelin was abolished by i.c.v. co-injection of Y1 receptor antagonist, suggesting that ghrelin increases food intake in part through the activation of the NPY/Y1 pathway in the hypothalamus (Shintani et al., 2001). To test the functional significance of the action of ghrelin on AgRP neurons, GHS-R1a was specifically re-expressed in AgRP neurons of GHS-R KO mice, using a tamoxifen-inducible AgRP-CreER(T2) transgenic mouse model. GHS-R1a re-expression specifically in AgRP neurons restored the orexigenic response to administered

ghrelin, suggesting that GHS-R1a-containing AgRP neurons are responsible for ghrelin's orexigenic effects (Wang et al., 2014). Altogether, these lines of evidence suggest that ghrelin acts through hypothalamic neurons to affect energy balance and feeding behaviors.

Electrophysiological assessment of the effect of ghrelin in hypothalamic slices revealed that ghrelin decreases the activity of POMC neurons and increases the activity of AgRP neurons (Cowley et al., 2003). Activation of AgRP neurons induced the release of NPY and GABA that bind to the NPY and GABA receptors on POMC neurons (Cowley et al., 2001), leading to their hyperpolarization. Interestingly, the inhibition of GABA receptors did not alter the ghrelin-induced hyperpolarization of POMC neurons, but inhibition of both GABA and NPY receptors reversed ghrelin's hyperpolarizing effects on POMC neurons (Cowley et al., 2003), suggesting that the effects of ghrelin in POMC hyperpolarization are induced by the upstream NPY neurons action on POMC neurons. In line with these results, a recent study showed that mice intraperitoneally (i.p.) injected with ghrelin display an increase in calcium signals in AgRP neurons *in vivo*. The POMC neurons showed the opposite response, with ghrelin injection inhibiting POMC activity (Chen et al., 2015). Altogether, these data suggest that ghrelin modulates neuronal activity in AgRP and POMC neurons. Thus, it is reasonable to hypothesize that ghrelin acts as a modulator of synaptic plasticity to regulate the activity of AgRP and POMC neurons to control feeding behaviors.

It has been hypothesized that ghrelin effects in AgRP and POMC activity can be induced by changes in the synaptic input onto these neurons. The frequency, but not the amplitude, of miniature excitatory postsynaptic currents (mEPSCs) recorded from AgRP neurons was increased in food-deprived animals (Yang et al., 2011), which show increased ghrelin circulating levels (Tschop et al., 2000). The increase in the frequency of mEPSCs onto AgRP neurons was blocked by i.c.v. injection of a GSH-R1a antagonist, suggesting that it is mediated by GSH-R1a signaling. Consistently, i.p. injection of ghrelin to fed mice (which have low levels of ghrelin) increased the frequency of mEPSC in AgRP neurons, and AgRP neuron firing (Yang et al., 2011). Additionally, ghrelin increased the frequency, but not the amplitude, of spontaneous GABAergic inhibitory postsynaptic currents onto POMC neurons *in vitro*, which was accompanied with a decrease in the activity of POMC neurons (Cowley et al., 2003). A presynaptic effect of ghrelin on both AgRP and POMC neurons suggests that ghrelin can modulate the activity of these neurons likely by targeting upstream neurons. In fact, initial work found that ghrelin-immunoreactive cell bodies are present in several hypothalamic nuclei, ghrelin is expressed in axons and is associated with dense-cored vesicles in presynaptic terminals that innervate several hypothalamic nuclei (Cowley et al., 2003). However, the detection of ghrelin in the central nervous system has been controversial, and more recent works suggest the ghrelin is produced in the brain at very low levels (reviewed in Cabral et al., 2017). It is possible that ghrelin effects on AgRP and POMC neurons are induced by peripheral ghrelin that targets GSH-R1a expressing neurons to regulate energy homeostasis.

Others mechanisms for ghrelin-induced presynaptic plasticity have been proposed. In fact, GSH-R1a is present at hypothalamic GABAergic presynaptic terminals and a recent work showed that the GHS-R1a activation elicits a strong impairment of voltage gated calcium channels $Ca_V2.1$ and $Ca_V2.2$ currents in hypothalamic neurons (Lopez Soto et al., 2015). Thus, ghrelin-mediated inhibition of Ca_V2 attenuates GABA release in hypothalamic neurons, which could contribute to downstream neuronal activation through the disinhibition of postsynaptic neurons (Lopez Soto et al., 2015).

Ghrelin-evoked feeding requires signaling through energy sensors such as AMP-activated kinase (AMPK), sirtuin1 (SIRT1) or mammalian target of rapamycin (mTOR) (reviewed in Al Massadi et al., 2017). Inhibition of AMPK blocked ghrelin-mediated effects on AgRP synaptic plasticity, through a presynaptic effect dependent on Ca^{2+}/calmodulin kinase kinase activation following ghrelin-triggered mobilization of Ca^{2+} from intracellular stores (Yang et al., 2011). Intriguingly, the frequency of AgRP mEPSCs was increased for hours upon ghrelin incubation, even after ghrelin removal or incubation with a GSH-R1a antagonist, and was reversed by administration of leptin and by opioid release from POMC neurons (Yang et al., 2011). This study showed that ghrelin induces long-lasting activation of AgRP neurons (a memory of the energy status), via a presynaptic AMPK-dependent mechanism that is reversed by opioids released by leptin-activated POMC neurons. Subsequent studies found that postsynaptic NMDARs in AgRP neurons are critical for fasting-induced AgRP activation and dendritic spinogenesis (Liu et al., 2012), and that inhibition of AMPK in AgRP neurons can block excitatory synaptic plasticity in AgRP neurons triggered by fasting (Kong et al., 2016). Fasting-activated AMPK phosphorylates and stimulates p21-activated kinase (PAK) signaling in AgRP neurons, which is required for fasting-stimulated synaptic plasticity (Kong et al., 2016).

SIRT1 is an energy sensor with NAD^+-dependent deacetylase activity, which is activated in response to caloric restriction and acts through the p53 tumor suppressor. Ghrelin has been shown to trigger the SIRT1/p53 pathway, and a SIRT1 inhibitor or the deletion of p53 blunted the ghrelin-induced food intake, and impaired the effect of ghrelin on hypothalamic AMPK phosphorylation (Velasquez et al., 2011). A recent study found that mice lacking p53 in the AgRP neurons are more likely to develop diet-induced obesity, and that c-Jun N-terminal kinase (JNK) mediates the effects of AgRP neurons p53 on energy balance (Quinones et al., 2018). p53 in AgRP neurons is essential for the ability of central ghrelin to induce food intake (Quinones et al., 2018).

mTOR is a cellular sensor of changes in energy balance and nutrients, and a component of mTOR complexes (mTORC) 1 and 2. mTORC1 phosphorylates serine/threonine ribosomal protein S6 kinase 1 (S6K1), which phosphorylates and activates the ribosomal protein S6, involved in translation. mTOR is expressed in NPY/AgRP neurons (Cota et al., 2006), and ghrelin elicits an upregulation of hypothalamic mTOR activity, and an increase in the phosphorylation of the downstream targets S6K1 and S6 (Martins et al., 2012). Inhibition of hypothalamic mTOR reversed the orexigenic effect of ghrelin, and ghrelin-induced expression of

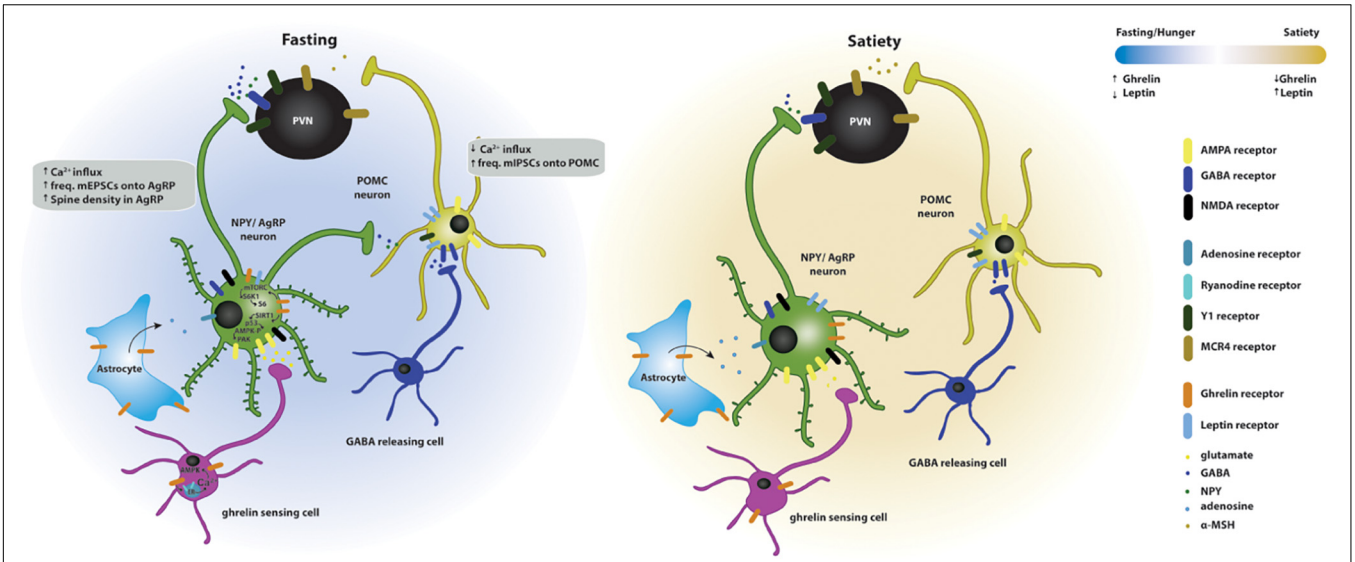

FIGURE 2 | Effects of ghrelin on synaptic plasticity of hypothalamic circuits associated with feeding. Two energy states are represented: fasting/hunger and satiety. In the fasted state, AgRP neuronal activity and dendritic spine density are increased, while POMC neuronal activity is decreased. The AgRP neurons release NPY and GABA and the POMC neurons release POMC, which is subsequently cleaved to produce α-MSH, and targets the melanocortin receptors in the paraventricular nucleus (PVN MCR4) to regulate feeding responses. The effects of fasting on hypothalamic synaptic plasticity can be mediated directly by the activation of the GSH-R1a in AgRP neurons or indirectly by the upstream regulation of AgRP and POMC neurons. Upstream neurons include ghrelin-sensing cells that release glutamate on AgRP neurons through a mechanism dependent on the release of calcium from internal stores and subsequent activation of AMPK, therefore increasing the excitatory synaptic input onto these neurons. Simultaneously, ghrelin leads to the activation of NMDA receptors, of the SIRT1 → p53 → AMPK → PAK and of the mTORC → S6K1 → S6 pathways, which drive state-dependent excitatory synaptic plasticity in AgRP neurons. In parallel, a population of neurons release GABA onto the POMC neurons therefore increasing the inhibitory tone on these neurons. In the fed state, the balance of excitatory versus inhibitory synaptic inputs in the AgRP and POMC neurons is reversed, increasing the anorexigenic melanocortin tone. Additionally, astrocytes express GSH-R1a and its activation promotes the release of adenosine that will bind to the adenosine A1 receptors expressed in the AgRP neurons, thus, decreasing their excitation threshold.

NPY and AgRP (Martins et al., 2012). These observations suggest that ghrelin also promotes feeding through upregulation of the mTOR pathway in the hypothalamus.

Astrocytes modulate neuronal synaptic inputs in the central nervous system, and have been recently shown to play a role in regulating ghrelin-induced activation of AgRP neurons. *In vivo* activation of medial basal hypothalamic astrocytes reduced food intake during the early dark period (when mice usually consume more) as well as ghrelin-induced food intake. Conversely, astrocyte inactivation enhanced and prolonged ghrelin-inducing feeding (Yang et al., 2015). These effects occur by a mechanism mediated by adenosine, which is released by astrocytes and negatively regulates synaptic transmission in AgRP neurons through activation of adenosine A1 receptors (Yang et al., 2015).

Overall, ghrelin promotes feeding by acting on hypothalamic circuits (**Figure 2**); the mechanisms that coordinate ghrelin's orexigenic action depend on ghrelin-induced synaptic plasticity and are starting to emerge, but need to be further clarified.

REGULATION OF SYNAPTIC FUNCTION IN THE VTA BY GHRELIN

The mesolimbic system activation increases the motivation for food-seeking and for consumption behaviors favoring high-sucrose or high-fat over chow diets. Disruptions of the mesolimbic system are associated with addictive behaviors such as overfeeding of palatable food or drug consumption. These behaviors are accompanied by plasticity phenomena occurring on mesolimbic pathways. For instance, exposure to drugs of abuse induces synaptic plasticity in the VTA (Luscher and Malenka, 2011). Thus, plasticity of mesolimbic pathways is of particular interest in the study of behaviors associated with motivational, hedonic consumption and with increased feeding. Ghrelin increases the incentive value for natural and chemical rewards, via activation of GHS-R1a (reviewed in Perello and Dickson, 2015), which is expressed in the VTA (Guan et al., 1997; Abizaid et al., 2006; Zigman et al., 2006; Perello and Dickson, 2015). Ghrelin recruits subsets of dopamine and GABA neurons of different VTA subnuclei (Cornejo et al., 2018). The mesolimbic pathway connects the VTA containing dopaminergic neurons projecting mainly to the nucleus accumbens (NAc) in the ventral striatum but also sending projections to the hypothalamus, prefrontal cortex and the hippocampus. The VTA also receives projections from these regions and from cholinergic neurons of the laterodorsal tegmental area (LTDg). In the scope of this review, we will focus mainly in the VTA-NAc projection, as this is one of the most studied pathways in the context of ghrelin effects on feeding.

In humans, functional MRI studies showed that peripheral ghrelin altered the response of the ventral striatum to visual food cues, suggesting that ghrelin can act through the mesolimbic

system to affect feeding behavior (Malik et al., 2008). In rodents, intra-VTA ghrelin injections increased food intake (Naleid et al., 2005; Abizaid et al., 2006), in particular of rewarding food (Egecioglu et al., 2010; Skibicka et al., 2012). Knock-out mice for the GHS-R1a, rats peripherally treated with a GHS-R1a antagonist, and VTA-lesioned rats showed suppressed intake of rewarding foods (Egecioglu et al., 2010). Consistently, ghrelin peripheral injection increased the motivation to consume high-fat diet when administered to *ad libitum*-fed mice, but both wild-type mice treated with a GSH-R1a antagonist and GSH-R KO mice failed to show the preference for a high-fat diet normally observed in animals under calorie restriction (Perello et al., 2010). This line of evidence suggests that ghrelin increases the motivation for calorie dense food through its actions on the VTA.

Interestingly, the reward value of food can compensate for defective feeding in mice in which AgRP neurons were selectively ablated (Denis et al., 2015). Ghrelin i.p. injection did not trigger chow intake in mice lacking AgRP neurons, but it significantly increased consumption in animals on a high fat and high sugar diet (Denis et al., 2015). Intra-VTA ghrelin administration was sufficient to trigger an increase in high fat and high sugar (but not chow) diet intake in AgRP-ablated mice, supporting the hypothesis that VTA ghrelin is orexigenic in mice fed a palatable diet in the absence of AgRP neuronal activity (Denis et al., 2015). Long-term exposure to high fat and high sugar diet results in ghrelin resistance in AgRP neurons (Briggs et al., 2010; reviewed in Zigman et al., 2016). In these circumstances, feeding behavior may result from the engaging of AgRP-independent but ghrelin-dependent neural circuits modulated by food palatability and dopamine signaling.

Natural and artificial rewards increase the activity of the VTA dopaminergic neurons, triggering the release of dopamine in the NAc and an increase in locomotor activity. Likewise, central or intra-VTA administration of ghrelin induced locomotor stimulation and dopamine-overflow in the NAc, whereas intra-VTA administration of a GHS-R1A antagonist suppressed this effect (Jerlhag et al., 2006, 2007). Moreover, peripheral ghrelin injections increased dopamine turnover in the NAc (Abizaid et al., 2006; Jerlhag et al., 2011), which was blocked by intra-VTA administration of a GHS-R1a antagonist (Jerlhag et al., 2011), indicating that activation of the mesolimbic dopamine system by ghrelin requires GHS-R1a signaling in the VTA. The overflow of dopamine is thought to increase the motivation to seek and consume food. Mechanistically, ghrelin was found to increase the frequency of action potentials (APs) in VTA dopaminergic neurons in brain slices, and peripheral ghrelin injection increased the number of excitatory synapses onto VTA dopaminergic neurons, while decreasing the number of inhibitory synapses. Accordingly, the frequency of mEPSCs recorded from dopaminergic neurons in VTA slices from mice peripherally injected with ghrelin was increased, whereas the frequency of miniature inhibitory postsynaptic currents (mIPSCs) was decreased (Abizaid et al., 2006). These results suggest that ghrelin induces synaptic rearrangements on the VTA dopaminergic neurons that result in an increase on the number of excitatory synapses.

The activity of VTA dopaminergic neurons is modulated by various afferents to the VTA, including glutamate-, opioids- and orexin-releasing neurons, suggesting that ghrelin-induced increase in VTA dopaminergic activity could be indirectly mediated by the activation of these pathways. Ghrelin increased the rewarding value of high-fat diet in an orexin-dependent manner, since both orexin-deficient mice and wild-type mice treated with an orexin receptor antagonist failed to show preference to rewarding food diet induced by ghrelin (Perello et al., 2010; Cone et al., 2014). However, intra-VTA administration of an NMDAR antagonist attenuated ghrelin-induced locomotor stimulation in mice, which was not affected by an orexin A receptor antagonist or peripheral injection of an opioid receptor antagonist (Jerlhag et al., 2011). Moreover, ghrelin did not affect the frequency of APs in VTA dopaminergic neurons in the absence of excitatory synaptic input, whereas it increased the frequency of APs in VTA dopaminergic neurons when ionotropic $GABA_A$ receptors were blocked (Abizaid et al., 2006). Collectively, these observations suggest that the ghrelin-mediated increase in the frequency of APs in dopaminergic VTA neurons requires excitatory inputs to these neurons. In fact, blockade of NMDAR in the VTA reduced both food- and ghrelin-induced NAc dopamine release and abolished ghrelin-induced locomotor stimulation (Abizaid et al., 2006; Jerlhag et al., 2011). The ghrelin effects on the reward pathway may also be mediated by cholinergic afferents from the LTDg to the VTA, which contribute to the regulation of motivated behaviors. Thus, another possibility is that the activation of nicotinic acetylcholine receptors expressed in cholinergic presynaptic neurons is involved in the ghrelin-induced rewards. This is supported by findings showing that the GSH-R1a is expressed in the cholinergic afferents coming from the LTDg and that peripheral injection of an unselective nicotinic antagonist blocks ghrelin−induced effects (Jerlhag et al., 2006). The cannabinoid system stimulates food intake and impacts on body weight, partially through modulation of the orexigenic effect of ghrelin. Rimonabant, an antagonist of the cannabinoid receptor type 1 (CB1), blocked the orexigenic effect of ghrelin, and ghrelin could not stimulate feeding in CB1 KO mice (Kola et al., 2008).

Taken together, these data suggest that ghrelin effects on synaptic function may be directly mediated by the GSH-R1a or indirectly by the activation of afferents from other brain regions. Moreover, neurotransmitters including acetylcholine and glutamate are required for ghrelin-induced reinforcement. These pathways in the VTA (**Figure 3**), that appear to be sensitive to cholinergic and glutamatergic input, may serve as a novel pharmacological target for treatment of ghrelin-induced addictive behaviors. Other studies have shown that ghrelin increases (whereas antagonists of the GHS-R1a antagonists decrease) the motivation to consume alcohol, and that ghrelin signaling is required for the rewarding properties of addictive drugs (reviewed in Panagopoulos and Ralevski, 2014). Modulation of the effect of ghrelin in the mesolimbic system may offer a potent therapeutic strategy to target the ghrelin-induced increase in the reward value of food and drugs of abuse. The recent discovery of the endogenous GHS-R1a antagonist LEAP2

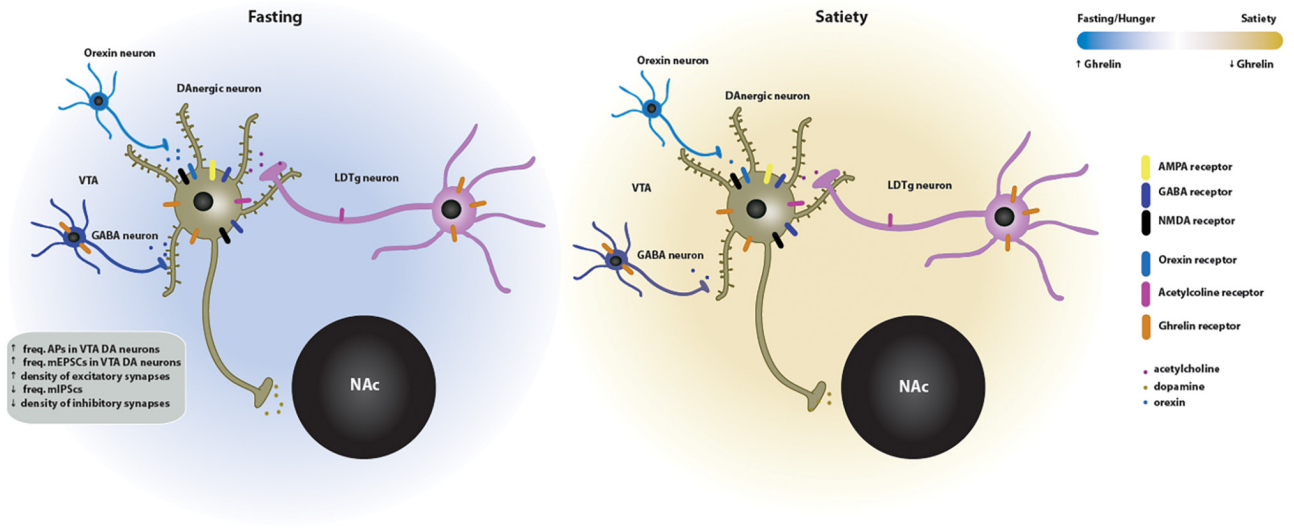

FIGURE 3 | Effects of ghrelin on synaptic plasticity in the VTA → NAc circuit of the mesolimbic system. Two energy states are represented: fasting/hunger and satiety. In the fasted state, ghrelin increases the neuronal activity and the density of excitatory synapses of dopaminergic neurons in the VTA. This increase in VTA dopaminergic activity triggers the release of dopamine onto the NAc. The ghrelin effects on the VTA synaptic plasticity may be directly mediated since the GSH-R1a is expressed in the VTA DA and GABA neurons. Indirect activation of the VTA-NAc pathway by ghrelin is mediated by orexin, which is released by upstream orexigenic neurons, and by cholinergic afferents from the laterodorsal tegmental area (LDTg), which contain both GSH-R1a and nicotinic acetylcholine receptors in cholinergic presynaptic neurons. GABAergic transmission is also involved in the ghrelin-induced effects on neuronal activity. In the fed state, the VTA neuronal activity and, subsequently, dopamine release on the NAc return to basal levels.

(Ge et al., 2018) opens the way to testing whether modulating its levels in the mesolimbic system can be of therapeutic value.

SYNAPTIC MODULATION BY GHRELIN IN THE HIPPOCAMPUS

Expression of the GHS-R1a in the hippocampus suggests that ghrelin is involved in hippocampal-dependent functions, and that this hormone may be a link between metabolism and cognition. In fact, intracerebroventricular (Carlini et al., 2002; Diano et al., 2006), intra-hippocampal (Carlini et al., 2004) or peripheral (Diano et al., 2006) injections of ghrelin increased hippocampal-dependent memory retention. In addition, ghrelin-null mice showed impairments in recognition memory (Diano et al., 2006), and GSH-R KO animals displayed spatial and contextual memory impairments (Davis et al., 2011; Albarran-Zeckler et al., 2012), suggesting that endogenous ghrelin signaling modulates cognitive behavior. The hippocampus is an anatomically defined structure in the brain composed of the Cornu Ammonis regions [I (CA1), II (CA2) and III (CA3)] and the dentate gyrus (DG). Synaptic plasticity paradigms of long-term potentiation (LTP) and long-term depression (LTD) are cellular correlates for learning and memory that have been described in detail in this brain region (reviewed in Nicoll, 2017).

Ghrelin receptor activation enhanced LTP at the Schaffer collateral-CA1 synapse (Diano et al., 2006; Ribeiro et al., 2014) in hippocampal slices, and led to the synaptic insertion of AMPAR through a mechanism dependent on PI3K, PKA and PKC activation (Ribeiro et al., 2014). *In vivo* a single infusion of ghrelin induced long lasting potentiation of synaptic transmission in the DG, and prevented decline in LTP (Chen et al., 2011). Ghrelin-induced potentiation of synaptic transmission in the DG involved both postsynaptic and presynaptic mechanisms, did not require NMDAR activation, and was dependent on activation of the PI3K pathway (Chen et al., 2011). Consistently, enhancement of spatial memory by hippocampal infusion of ghrelin was prevented by the blockade of PI3K (Chen et al., 2011). Synaptic plasticity is associated with changes at the microstructural level, specifically at dendritic spines. Ghrelin peripheral injections increased the density of dendritic spines in the hippocampus (Diano et al., 2006), and ghrelin incubation of hippocampal organotypic slices increased filamentous-actin, the main cytoskeleton component of dendritic spines (Berrout and Isokawa, 2012). Collectively, these findings suggest that the effects of ghrelin on synaptic and structural plasticity regulate hippocampal dependent-functions.

In the hippocampus, GSH-R1a-DR1R heteromers are involved in the regulation of hippocampal synaptic plasticity (Kern et al., 2015). In fact, activation of DR1Rs increased the intracellular levels of calcium, which was not observed in hippocampal neurons obtained from GSH-R KO mice. GHS-R1a-DR1R heteromers interact with $G\alpha_q$, and activation by a DR1R agonist led to phospholipase C activity in wildtype but not in GSH-R KO neurons, at the expense of canonical $G\alpha_s$ cAMP signaling downstream of DR1R (Kern et al., 2015). DR1R agonist-induced activation in hippocampal organotypical slices increased the phosphorylation of CaMKII and AMPAR subunits at serine residues relevant for plasticity, in a manner dependent on GHS-R1a. Consistently, DR1R agonist-induced activation increased the surface expression of AMPAR in wildtype

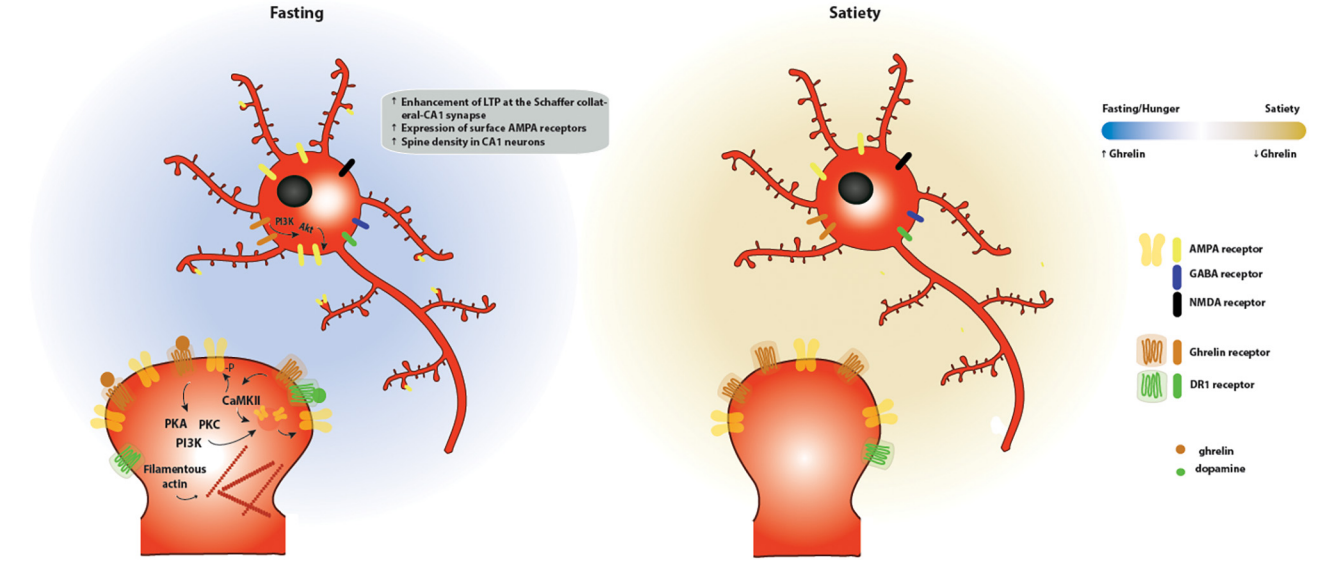

FIGURE 4 | Effects of ghrelin on synaptic plasticity in the hippocampus. Two energy states are represented: fasting/hunger and satiety. During fasting or hunger, ghrelin increases the expression of LTP at the Schaffer collateral-CA1 synapse promoting the insertion of AMPA receptors through a mechanism dependent on PI3K, PKA and PKC activation. These functional changes are accompanied by an increase in the density of dendritic spines, as a result of an increase in the levels of filamentous actin. One potential mechanism for the effects of ghrelin on hippocampal plasticity is through activation of GSH-R1a-DR1R heteromers by dopamine, which promotes an increase in the phosphorylation of CaMKII and of AMPA receptor subunits at serine residues relevant for plasticity, leading to the synaptic incorporation of AMPA receptors in a GHS-R1a-dependent manner.

but not GHS-R KO hippocampal neurons. In agreement with this molecular mechanism, DR1R activation modulated hippocampal-dependent behavior in a GHS-R1a-dependent manner (Kern et al., 2015).

Additionally, similarly to what was described in the hypothalamus (Lopez Soto et al., 2015), Ca_V2 voltage-gated calcium channels were found to be inhibited by GSH-R1a activity in the hippocampus. In fact, agonist-independent GHS-R1a activity inhibited $Ca_V2.2$ channels, which decreased GABA release in hippocampal cultures, suggesting a potential physiological role for GSH-R1a constitutive signaling in regulating synaptic transmission in the hippocampus (Martinez Damonte et al., 2018).

The hippocampus and the hypothalamus communicate to regulate high-order aspects of feeding. Although ghrelin-induced feeding has been associated with cognitive changes at the hippocampus, it has been proposed that ghrelin can act in the same circuitry in the opposite direction thus suggesting that ghrelin-induced cognitive effects can influence feeding (reviewed in Hsu et al., 2016). Injection of ghrelin in the ventral subregion of the hippocampus (VH, but not in the dorsal hippocampus) increased feeding primarily by increasing meal frequency and spontaneous meal initiation in *ad libitum* fed rats (Kanoski et al., 2013). The ghrelin-induced feeding was blocked by co-administration of a PI3K inhibitor suggesting that PI3K-Akt signaling in the VH is required for the hippocampal ghrelin effects on food intake (Kanoski et al., 2013). Interestingly, a recent study showed that ghrelin signaling in the VH is important for conditioned feeding behavior (Hsu et al., 2015). Ghrelin can stimulate meal-entrained conditioned appetite by acting in the VH, which GHS-R1a-expressing CA1 neurons provide input to neurons of the lateral hypothalamic area (LHA). Lesions in LHA block the hyperphagic response induced the GHS-R1a activation in VH neurons (Hsu et al., 2015), suggesting that this pathways is required for ghrelin-induced effects on entrained appetite. Furthermore, activation of downstream orexin-1 receptors was required for VH ghrelin-mediated hyperphagia (Hsu et al., 2015). These findings reveal a novel neurobiological circuitry regulating appetite through hippocampal GSH-R1a signaling. Social aspects of feeding behavior are also regulated by ghrelin action in the hippocampus. Ghrelin targeted to the VH CA1 region enhanced social transmission of food preference in rats, and this learning behavior was eliminated following knockdown of GHS-R1a in the VH (Hsu et al., 2018).

Taken together, these different lines of evidence support the hypothesis that ghrelin affects hippocampal-dependent functions (**Figure 4**). Nevertheless, a link between synaptic plasticity and ghrelin-dependent behavior is missing. Further manipulation of ghrelin signaling in the aforementioned circuits would be important to evaluate specific roles of ghrelin in hippocampal-dependent cognitive and feeding behavior. Understanding these ghrelin signaling pathways could have therapeutic value in cognitive deficits linked to metabolic disorders.

CONCLUDING REMARKS

Ghrelin signaling is strongly linked to alterations in synaptic function in circuits regulating homeostatic, hedonic and cognitive aspects of feeding behavior. The orexigenic effects of ghrelin depend on its actions in the hypothalamus, where it modulated neuronal activity in AgRP and POMC neurons.

The signaling downstream of the ghrelin receptor activation in the hypothalamus is complex, and may involve parallel pathways that include SIRT1/p53, AMPK and the mTOR pathways, which are crucial for the orexigenic action of ghrelin. It is now clear that besides its function in homeostatic feeding behavior, ghrelin plays a major role in hedonic and motivational aspects of feeding, through activation of dopaminergic signaling in the mesolimbic system. Additionally, the observed effects of ghrelin on cognitive behavior likely result from its actions in the hippocampus. The knowledge of the neuronal circuits impacted by ghrelin and of the molecular underpinnings of ghrelin's action in these systems has progressed in recent years, but further research is required to understand in an integrated manner how ghrelin regulates the multifactorial aspects of feeding behavior. In particular, when considering the ghrelin system as a therapeutic target in metabolic disorders it is important to ponder how interfering with ghrelin signaling may affect aspects of cognition. On the other hand, the dual role of ghrelin in metabolism and cognition poses an opportunity for targeting the ghrelin system in neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Shi et al., 2017). In modern societies plagued by an epidemics of overeating, weight gain and associated disorders, this understanding may be crucial to design better therapeutic interventions for patients with metabolic diseases.

AUTHOR CONTRIBUTIONS

DS, AC, and SS wrote the manuscript. DS made illustrations.

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REVIEW
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Influence of High-Fat Diets Consumed During the Juvenile Period on Hippocampal Morphology and Function

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The negative impact of obesity on neurocognitive functioning is an issue of increasing clinical interest. Over the last decade, a number of studies have analyzed the influence of high-fat diets (HFDs) on cognitive performance, particularly in adolescent individuals. Different approaches, including behavioral, neurochemical, electrophysiological and morphological studies, have been developed to address the effect of HFDs on neural processes interfering with learning and memory skills in rodents. Many of the studies have focused on learning and memory related to the hippocampus and the mechanisms underlying these processes. The goal of the current review article is to highlight the relationship between hippocampal learning/memory deficits and nutritional/endocrine inputs derived from HFDs consumption, with a special emphasis on research showing the effect of HFDs intake during the juvenile period. We have also reviewed recent research regarding the effect of HFDs on hippocampal neurotransmission. An overview of research suggesting the involvement of fatty acid (FA) receptor-mediated signaling pathways in memory deficits triggered by HFDs is also provided. Finally, the role of leptin and HFD-evoked hyperleptinemia is discussed.

Keywords: learning, memory, hippocampus, high-fat diets, obesity, leptin, fatty acids, PPAR

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INTRODUCTION

Elevated consumption of so-called western diets (WDs) is one of the main causes of overweight and obesity and a matter of concern for public health institutions. The damaging effect of these diets seems to be not only related to their content in terms of both saturated fat and easily assimilated carbohydrates, but also to the fact that they promote disorganized feeding patterns consisting of frequent energy-dense snacking and/or copious meals before bedtime (Corwin and Hajnal, 2005; Matheson et al., 2012, 2014). Closely related to this, the concept and term "comfort food" has been coined, referring to the consumption of palatable, high caloric food to mitigate stress and/or anxiety (Dallman et al., 2003).

Abbreviations: BBB, Blood-brain barrier; BMI, Body mass index; BW, Body weight; FA, Fatty acids; GLU, Glutamate; HFD, High-fat diet; LTD, Long-term depression; LTP, Long-term potentiation; NCAM, Neural cell adhesion molecule; NMDA, N-methyl-D-aspartate; WAT, White adipose tissue; WD, Western diet.

The effect of WD within the brain is multifactorial since such diets promote changes in the blood-brain barrier (BBB) and choroid plexus permeability (Hsu and Kanoski, 2014; Hargrave et al., 2016), inflammation (Pistell et al., 2010), biochemical processes compatible with neurodegeneration (Kanoski and Davidson, 2011; Boitard et al., 2012), changes in both dopaminergic (Naneix et al., 2017; Romaní-Pérez et al., 2017) and glutamatergic neurotransmission (Valladolid-Acebes et al., 2012) as well as neurocognitive deficits (Noble and Kanoski, 2016). In this regard, a pivotal issue that deserves particular attention is that WD consumption itself, in the absence of obesity/overweight, has the capacity to cause neurocognitive impairment (Beilharz et al., 2015). This suggests that dietary components (a certain fatty acid (FA), a particular saturated/unsaturated FA ratio, the presence of elevated mono- and disaccharides, etc.) may be sufficient to cause neurocognitive effects—and that these effects are not only the result of metabolic impairment, such as brain insulin resistance, caused by the regular consumption of these diets (Bomfim et al., 2012; Derakhshan and Toth, 2013).

The concept of WD applied to animal research is confusing as this term covers diets with different qualitative and quantitative composition. The most commonly used WD are the so-called high-fat diets (HFDs; Van Heek et al., 1997), which yield mostly lipid-derived calories from saturated fat (lard), but also contain an elevated proportion of unsaturated fat and sucrose. The proportion of lard in these diets usually ranges from 2% (this diet is often used as a low-fat, control diet) to 32%, and the lower the amount of fat, the higher the amount of sucrose, with these two constituents ranging between 33% and 9%, respectively. Surwit diets, also used for this kind of study, contain hydrogenated coconut oil and corn-starch as fat and carbohydrate sources, respectively. Finally, the so-called cafeteria diets, containing elevated and undefined amounts of saturated fat and sugar have been also widely used to induce overweight/obesity. Hence, the complexity of all these diets makes it difficult to draw clear conclusions regarding the relative contribution of either fat or sugar to brain alterations. In this respect, exposure to sugar-sweetened beverages has been shown to have a negative impact on spatial memory (Reichelt et al., 2016) and neurogenesis (Reichelt et al., 2015), but systematic studies aimed at identifying the effect on the brain of either sugar- or fat-enriched diets are lacking.

As a result of all of the circumstances described above, comparison between different studies is very challenging and this might explain the discrepancies observed between such studies, despite them having been carried out under conditions that are apparently similar. Most studies reviewed in this article deal with the effect of van Heek-type diets.

UNHEALTHY DIET HAS AN IMPACT ON COGNITIVE PERFORMANCE: EPIDEMIOLOGICAL FEATURES

The relationship between diet composition and cognitive impairment has been the focus of observational studies showing that improving dietary habits positively influences cognition (Drover et al., 2009; Spencer, 2010), while reduction in diet quality is associated with declining psychological functioning over the follow-up period (Jacka et al., 2011).

A number of clinical studies highlight that hippocampus-dependent declarative memory is damaged in overweight/obese adults (Nilsson and Nilsson, 2009), as well as in children/adolescents (Cserjési et al., 2007; Afzal and Gortmaker, 2015). Accordingly, an inverse correlation between body mass index (BMI) and academic performance has been established (Yau et al., 2014).

Related studies have revealed slower learning rates in hippocampus-related tasks in humans consuming high-sugar diets (Attuquayefio et al., 2016). Another clinical study revealed that a 4-day HFD reduces the extent of hippocampus-dependent learning and memory as well as interoceptive sensitivity (Attuquayefio et al., 2017). Some meta-analyses have also provided evidence for the negative impact of obesity on neurocognitive functioning (Liang et al., 2014). In addition, although the mechanism linking overweight/obesity and cognitive dysfunction remains poorly characterized (Sellbom and Gunstad, 2012), cumulative evidence has identified obesity as a risk factor for cognitive impairment (for a review see Castanon et al., 2014), including Alzheimer disease (De Felice and Ferreira, 2014).

Many studies have reported that memory impairment triggered by diets is associated to specific insulin resistance (Banks et al., 2012; Kim and Feldman, 2015). In this regard, a study in humans indicated that glucose, but not insulin fasting levels, may have an impact on episodic memory in middle-aged women (Backeström et al., 2015). Thus, understanding the mechanisms connecting the consumption of unhealthy diets and cognition deficits is becoming both urgent and necessary in order to develop effective strategies aimed at preventing the expanding global burden of co-morbid obesity and dementia.

CONSUMPTION OF HIGH-FAT DIETS DURING THE JUVENILE PERIOD IMPAIRS HIPPOCAMPUS MORPHOLOGY AND FUNCTION

Neurogenesis and Cell Morphology

The negative impact of HFDs on hippocampal neurogenesis has been the focus of many studies (Hwang et al., 2008; Park et al., 2010), some of which have shown a differential effect on animals that started to consume a HFD after weaning compared to those that consumed the diet during adulthood (Boitard et al., 2012).

Evidence that HFDs can affect neuron development is provided by other studies showing that obese young mice consuming these diets for 8 weeks display an unexpected increase in hippocampal spine density, accompanied by an up-regulation of the neural cell adhesion molecule (NCAM) expression in CA1 pyramidal neurons (Valladolid-Acebes et al., 2013). Similarly, another study has recently reported a similar effect within the prefrontal cortex in young rats undergoing

prenatal exposure to HFD (Rincel et al., 2018). In contrast to these findings, Wang's group (Wang et al., 2016) identified a decrease in hippocampal spine density in obese juvenile rats exposed to HFDs. Interestingly, a study by Ferreira's group has reported that switching to a standard control diet reduces body weight (BW) and restores levels of hippocampal neurogenesis in these models (Boitard et al., 2016).

In addition to neuron morphology, astrocytes seem to be sensitive to HFD. This issue has mainly been investigated in hypothalamic areas (Chowen et al., 2016), but research specifically targeted within the hippocampus is less abundant. In this regard, a study carried out in HFD obese mice reported that consuming HFD from the time of weaning leads to longer and less abundant astrocyte prolongations (Cano et al., 2014) as well as to a reversible activation of hippocampal microglia (Hao et al., 2016). Obese adult rats undergoing a similar treatment displayed a lower number of GFAP-positive astrocytes (Gzielo et al., 2017), and a concomitant increase in the number of *Iba1* positive microglia cells was detected in non-obese mice subjected to a similar dietary intervention from weaning (Vinuesa et al., 2016).

Effect of High-Fat Diets on Hippocampal Neurotransmission

It has been reported that HFD impairs synaptic efficacy and blunts *N*-methyl-D-aspartate (NMDA)-induced long-term depression (LTD), but does not affect long-term potentiation (LTP; Valladolid-Acebes et al., 2012), in the hippocampus of obese mice exposed to these diets during the adolescent period. These findings differ from those of other authors. For instance, Mielke's group reported that HFD did not affect synaptic efficacy or LTP (Mielke et al., 2006), whereas other authors have demonstrated impairment of LTP after HFD treatment (Hao et al., 2016). The different periods of HFD treatment and the different protocols for synaptic plasticity processes may be responsible for these discrepancies. Moreover, Del Olmo's group has identified the impairment of LTD as the most relevant change in synaptic plasticity due to HFD consumed during the juvenile period (Valladolid-Acebes et al., 2012). Other authors have observed that, in spite of an identical basal synaptic transmission, obese HFD-treated adult male—but not female—mice display lower LTP and LTD values compared with their respective controls (Hwang et al., 2010)—a result that paves the way for research related to gender-specific effects of HFD on hippocampal function. In addition, a 6-month HFD treatment has been shown to decrease basal synaptic transmission and LTP in the *dentate gyrus* of the adult rat hippocampus (Karimi et al., 2013).

These apparent alterations of hippocampus plasticity strongly suggest an influence of HFD on glutamatergic neurotransmission. In fact, a study carried out in obese mice that consumed HFD during the adolescent period, has shown that HFD improves glutamate (GLU) up-take kinetics along with the up-regulation of glial GLU transporters (GLT-1 and GLAST) and a concomitant down-regulation of glutamine synthase. In addition, this treatment led to the down-regulation of the glucose transporter GLUT-1 (Valladolid-Acebes et al., 2012). All of these findings suggest that HFD can trigger a dramatic de-regulation of GLU turnover and provides further support for the above-mentioned changes in hippocampus synaptic transmission and plasticity elicited by HFD interventions during the juvenile period. This and other studies have reported that post-weaning HFD down-regulates the expression levels of both the NR2B subunit of the NMDA receptor and synaptophysin, concomitantly with a detrimental cognitive impairment in rats (Page et al., 2014) and mice (Valladolid-Acebes et al., 2012).

GLU is not the only neurotransmitter that is sensitive to HFD within the hippocampus; GABA levels appear to be decreased in the hippocampus as well as in the prefrontal cortex of adult rats, and it has been proposed that this change might contribute to the disruption of GABAergic inhibitory processes and be related to changes in GLU metabolism (Sandoval-Salazar et al., 2016), which are coherent with the above-mentioned worsening of synaptic plasticity.

Influence of High-Fat Diets During the Juvenile Period on Hippocampus-Dependent Spatial Memory

Research aimed at characterizing the effects of HFD consumption and/or HFD-induced obesity on learning and memory processes is abundant and has frequently been reviewed (Davidson et al., 2005; Cordner and Tamashiro, 2015; Noble and Kanoski, 2016). However, differences in the animal strain and age, the length of the dietary treatment and the experimental approach used all make it difficult to draw clear conclusions (Cordner and Tamashiro, 2015).

The effect of HFD on brain areas related to cognition, such as the amygdala, the prefrontal cortex, and especially the hippocampus, has received much attention in recent years. In the amygdala, Vega-Torres et al. (2018) recently reported that rats that consumed HFD exhibited attenuated fear learning associated to astrogliosis, which contrasts with the enhancement of emotional memory and amygdala plasticity reported by Boitard et al. (2015). Spencer et al. (2017) reported that HFD evokes neuroinflammation together with impaired amygdala-dependent memory. Janthakhin et al. (2017) also reported that maternal HFD impairs amygdala-dependent memory. Nevertheless, switching adolescent HFD to a control diet in adulthood reverses neurocognitive alterations (Boitard et al., 2016). With respect to the effect of HFD within the prefrontal cortex, a number of studies have implicated this brain area in cognitive deficits triggered by HFD (Reichelt et al., 2016), which may be related to morphological alterations evoked by these diets in this area (Dingess et al., 2017).

Although a study has reported that HFDs can have an influence in the ventral hippocampus of female mice (Krishna et al., 2015), the effect of HFD on memory seems to affect mainly dorsal hippocampus-dependent learning but spares other forms of learning (Stouffer et al., 2015). This may be due to the selective impairment of the dorsal hippocampus

caused by oxidative stress, inflammation and/or disrupted neurotransmission produced by consumption of these diets. In this sense, HFD intake results in a negative influence on the hippocampus in terms of both spatial learning and reference memory, and it has been proposed that HFD triggers obesity in part by interfering with the inhibition of hippocampal-dependent memory, which is critical to adjust energy intake to meet energy demands (Davidson et al., 2007; Kanoski and Davidson, 2011). Most of the studies agree that HFDs impair hippocampal function. Thus, impaired hippocampus-specific spatial memory, evaluated in the radial maze paradigm, was detected in adult rats after 3–5 days on a high-fat/high-sugar diet, while no effect was observed in the case of hippocampus-independent memory tasks (Kanoski and Davidson, 2011). The impact of HFD on hippocampus-dependent learning and memory during the particularly vulnerable juvenile period has been assessed in a number of studies showing that these diets deteriorate both relational and spatial memory (Valladolid-Acebes et al., 2011, 2013; Boitard et al., 2012; Kaczmarczyk et al., 2013; Del Rio et al., 2016). This behavioral impairment is accompanied by the inhibition of hippocampal neurogenesis in mice (Boitard et al., 2012), as well as by morphological alterations of dendritic spines (Valladolid-Acebes et al., 2013). Moreover, HFD has been shown to evoke hippocampal inflammation in rats (Boitard et al., 2014). These observations do not appear to be linked to obesity, as Vinuesa's group demonstrated that these processes are present without BW gain (Vinuesa et al., 2016).

Similarly, the effect of prenatal and perinatal exposure to HFD has also been shown to have a negative influence on the hippocampus in terms of spatial learning and reference memory (White et al., 2009; Lépinay et al., 2015; Wolfrum and Peleg-Raibstein, 2018). Related to this, a recent study carried out in humans demonstrated that 4 days of HFD reduces hippocampus-dependent learning and memory as well as interoceptive sensitivity (Attuquayefio et al., 2017). However, other studies carried out in adolescent mice have reported that short-term HFD fail to induce cognitive impairment in the novel object recognition paradigm (Del Rio et al., 2016).

The possibility that mood disorders triggered by obesity and/or HFD may have an impact on memory performance is a factor that has to be taken into account, since the increasing prevalence of childhood obesity has been accompanied by a parallel increase in comorbid psychological conditions such as depression and anxiety (Russell-Mayhew et al., 2012). Nevertheless, data in the literature do not allow the assessment of the comorbidity between memory and mood impairment. In this regard, some studies have reported anxiety and anhedonia after a 16-week HFD in adult rats (Dutheil et al., 2016) and after a 12-week treatment in mice (Sharma and Fulton, 2013), while other authors have reported a mood improvement together with a concomitant worsening of memory performance after 48 h HFD (Del Rio et al., 2016). The independence between mood and memory is stressed by the study by Kaczmarczyk et al. (2013), showing that learning/memory impairment evoked by HFD was not inhibited by the anti-depressants desipramine or reboxetine. For recent reviews on this topic, see Baker et al. (2017) and Reichelt and Rank (2017).

The influence of maternal obesity and high-fat feeding on synaptic plasticity, learning and memory has been also studied and recently reviewed by several authors (Contu and Hawkes, 2017; Edlow, 2017). Nevertheless, although there is compelling evidence of learning and memory deficits in the offspring on obese mothers (both in humans and in experimental models of diet-induced obesity), the mechanism that account for such an effect remains to be further investigated. As potential causes, a deficient production of BDNF within the hippocampus (Tozuka et al., 2010; Kim and Park, 2018), brain inflammation (Kang et al., 2014), and even a deficient leptin signaling (Dodds et al., 2011; Cordner and Tamashiro, 2015) may underlie deficient neurodevelopment and poor memory/learning performance observed in these models.

NUTRITIONAL VS. ENDOCRINE INPUTS IN MEMORY IMPAIRMENT TRIGGERED BY HIGH-FAT DIETS

Gut hormones and nutrients—which are pivotal for brain development and maturation—reach the brain after crossing the BBB and/or the choroid plexus by means of specific carriers as well as by unspecific diffusion mechanisms. These inputs can be altered by HFD as, in addition to an abundant supply of saturated FAs, these diets cause an imbalance of adipokines in white adipose tissue (WAT), characterized by the induction of leptin synthesis and the repression of adiponectin expression, which occur together with a rise in WAT-derived inflammatory cytokines, all of which are able to cross brain barriers (Tilg and Moschen, 2006; Spencer et al., 2017; **Figure 1**). In addition, HFDs have been shown to reduce the synthesis of bile acid receptor ligands, an event that may contribute to the impairment of neuroplasticity evoked by HFDs (Jena et al., 2018).

Systematic studies aimed at identifying the role of saturated fat as an independent risk factor for memory and mood impairment, specifically in the adolescent population, are lacking. A study by Baym et al. (2014) showed a negative correlation between hippocampus-dependent relational memory and the intake of saturated FAs in a child/adolescent population, supporting the concept that saturated fat impairs memory processes independently of metabolic factors. Nevertheless, the Baym study did not identify saturated fat intake as a BMI-independent risk factor for memory damage. Related to this, a study by Moon et al. (2014) demonstrated that acute administration of palmitic acid induces anxiety-like behavior in mice, independently of metabolic alterations.

The influence of HFDs on brain and particularly on hippocampus signaling, seems to involve many neurotransmitter (Hansen et al., 2018) and hormone-dependent pathways. In this context, insulin receptor signaling in hippocampal neurons is pivotal for spatial memory performance. The influence of HFD interventions on insulin responsiveness is difficult to

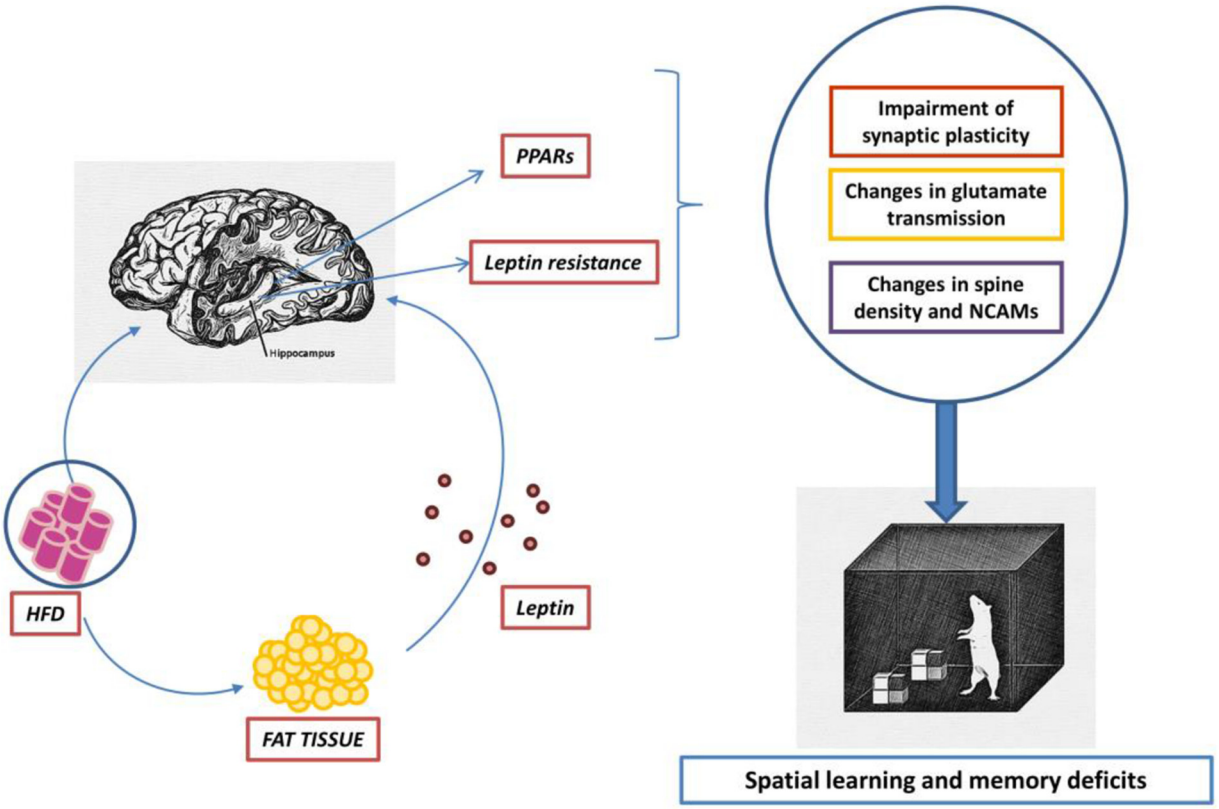

FIGURE 1 | Hypothetical dual effect of high-fat diets (HFDs) on the hippocampus. Fatty acids (FAs) contained in HFDs enter the brain by crossing brain barriers. Elevated concentrations within the hippocampus of saturated FAs, such as palmitic acid, lead to a deficient activation of PPARγ together with an overstimulation of PPARα. The consequence of such an imbalance may be inflammation accompanied by a decrease of synaptic plasticity. On the other hand, HFDs trigger adipose tissue inflammation together with an altered pattern of adipokine secretion, characterized by an increase of circulating leptin. Hyperleptinemia causes leptin resistance able to limit the activity of signaling pathways relevant in maintaining the structural and functional integrity of the hippocampus.

determine, as most studies do not go beyond the identification of insulin resistance indexes (HOMA-IR), which are not indicative of the responsiveness of insulin receptors within brain areas involved in learning/memory. In this regard, the study by Vinuesa et al. (2016) reported that memory impairment triggered by HFD in juvenile mice is associated to specific insulin resistance within the hippocampus, even in the absence of obesity, as already observed by Mielke et al. (2006). These findings are in line with previous studies, carried out in engineered rats, showing that lentiviral deletion of insulin receptors within the hippocampus has a negative influence on hippocampus-dependent learning/memory tasks (Grillo et al., 2011, 2015). This suggests that integral downstream signaling of insulin receptors is pivotal for learning/memory performance. Otherwise, brain insulin resistance evoked by HFDs has been shown to impair hippocampal synaptic plasticity and memory by increasing the palmitoylation of the AMPA GLU subunit GluA1 (Spinelli et al., 2017). Memory deficits have also been observed—both in mouse and rat models, after 1- (Molteni et al., 2002) and 8-month dietary treatments (Stranahan et al., 2008)—associated to marked obesity and insulin resistance. Nevertheless, other studies have reported that consumption of HFD for 4 or 8 weeks, triggering BW increase but failing to alter insulin resistance indexes, evokes hippocampus-dependent memory deficits specifically in adolescent mice (Valladolid-Acebes et al., 2011). In humans, one study indicates that glucose—but not insulin fasting levels—may have an impact on episodic memory in middle-aged women (Backeström et al., 2015). In the same vein, hippocampus-dependent memory deficits in obese adult mice, exposed to HFD during the juvenile period, were not reversed by further limited access to HFD (Valladolid-Acebes et al., 2013). Nevertheless, other studies have reported that cognitive function is improved by subsequently switching to a standard chow (Woo et al., 2013; Boitard et al., 2016), and this reversibility also concerns other aspects such as neuronal plasticity (White et al., 2009; Lépinay et al., 2015), morphological changes (Rincel et al., 2018) and memory (Boitard et al., 2016). Taken together, all of these findings support the concept that it is diet composition—rather than obesity or elevated caloric intake—that is pivotal for the long-term effect of HFD on learning/memory.

All of this research points to uncertainty regarding the relative contribution of nutritional inputs vs. the influence of endocrine-

metabolic impairment triggered by HFD. At this point, it should be highlighted that central inflammatory processes subsequent to HFD intake may be another key point to be considered. This topic has been the focus of recent reviews by Castanon et al. (2015), Morin et al. (2017), Spencer et al. (2017) and Layé et al. (2018).

THE ROLE OF SATURATED FATTY ACIDS IN MEMORY AND LEARNING DEFICITS: FACTS AND HYPOTHESIS

FAs are not only able to generate cell components or precursor metabolites, but also bind both intracellular and cell surface receptors, including peroxisome proliferator-activated receptors (PPARs) and G protein-coupled receptor 120 (GPR120).

PPARs mediate pleiotropic actions in the brain, including neurogenesis and synaptic plasticity (Roy et al., 2016; Zhou et al., 2016), as well as physiopathological responses, such as inflammation (Luna-Medina et al., 2005). Although the implication of PPARs in neural processes involved in learning and memory has been analyzed in a number of studies, research specifically devoted to assessing the relevance of FA receptors in neural damage triggered by HFD in the juvenile brain is lacking. Nonetheless, the importance of these receptors in neurogenesis and inflammation processes clearly points to their involvement in long-term memory deficits elicited by juvenile HFD. The up-regulation of NCAMs has been demonstrated in CA1 pyramidal neurons, occurring concomitantly with an increase in spine density in mice exposed to HFD only during the adolescence period (Valladolid-Acebes et al., 2013), showing the importance of these diets in neurogenesis. As NCAM play key roles in learning, memory and synaptic plasticity (Becker et al., 1996), it could be hypothesized that the up-regulation of NCAM expression induced by HFD is an integral compensatory reorganization of CA1 neurons aimed at improving synaptic connectivity (**Figure 1**).

Saturated FAs are less efficient PPARα and PPARγ agonists than unsaturated FAs (Kliewer et al., 1997; Varga et al., 2011). Therefore, a deficient activation of PPARγ and a subsequent limiting of PPARγ-mediated functions could be expected from the intake of diets containing elevated saturated/unsaturated FA ratios, as occurs with HFDs. In this regard, palmitic acid has been shown to impair amyloid processing both in neurons and astrocytes (Patil et al., 2006), whereas pioglitazone (PPARγ agonist) improves learning in Alzheimer disease models (Papadopoulos et al., 2013). Other studies have reported that palmitic acid induces lipotoxicity in cortical rat astrocytes (Wong et al., 2014), reduces hippocampal neurogenesis (Park et al., 2011) and promotes inflammatory responses, which were absent after oleic acid administration (Gupta et al., 2012). Concerning the effect of these diets on PPARα activation, a recent study by Huang et al. (2017) reported a down-regulation of the astrocyte GLU transporter, GLT-1, triggered by palmitic acid and other PPARα agonists. This result would be consistent with the presence of PPARα in rodent hippocampus (Roy et al., 2014; Rivera et al., 2014) as well as with the proposed role of PPARα in modulating synaptic plasticity in hippocampal neurons, and therefore in memory and learning (Roy et al., 2013).

Regarding the influence of HFD on GPR120 activity, there are no studies in the literature. Nevertheless, GPR120 seems to mediate the effects of Ω-3 PUFA and to improve both glucose metabolism and insulin sensitivity (Milligan et al., 2017). In addition, GPR120 seems to be relevant in the regulation of appetite and mood anxiety (Auguste et al., 2016). One speculative possibility is that GPR120 is poorly activated in animals subjected to experimental HFD, which might contribute to the metabolic and mood disorders triggered by these diets.

LEPTIN AS A KEY FACTOR FOR MEMORY CHANGES EVOKED BY HIGH-FAT DIETS IN JUVENILE ANIMALS

The rise in plasma leptin levels during HFD interventions has a dual effect, since leptin enhances CA1 LTP in rats (Oomura et al., 2006) and has been shown to be necessary for both brain maturation and learning/memory consolidation (Morrison, 2009; Guo and Rahmouni, 2011), whereas leptin resistance evoked by hyperleptinemia appears to be associated with deficits in hippocampal-dependent behaviors (Van Doorn et al., 2017).

Behavioral effects of leptin involve hippocampus leptin receptors (Harvey et al., 2006), which modulate JAK/STAT3, PI3K/Akt, MAPK and calcineurin signaling pathways (Morrison, 2009). The relevance of these pathways for hippocampal-dependent memory/learning has been investigated and there is compelling evidence that JAK/STAT3 modulates synaptic plasticity (Nicolas et al., 2012). Moreover, PI3K/Akt and MAPK, and MAPK and calcineurin regulate LTP and LTD, respectively (Harvey, 2013). In support of this, Farr et al. (2006) have reported that leptin therapy improves cognitive deficits in adult mice displaying a spontaneous overproduction of amyloid precursor protein. Some authors have shown that *db/db* mice, which have an inactivating mutation in the leptin receptor, display cognitive deficits (Dinel et al., 2011). Conversely, delivery of leptin within the ventral region of the hippocampus suppressed conditioned place-preference for food, increased the latency to run for food in an operant runway, and suppressed memory consolidation in a non-spatial appetitive response paradigm (Kanoski and Davidson, 2010).

On the other hand, hyperleptinemia triggers a rapid desensitization of leptin transport mechanisms located within the BBB and the choroid plexus—an effect that limits the effect of leptin. Although this process seems to be reversible in adult animals (Banks and Farrell, 2003), little is known about the influence that juvenile obesity can have on brain barrier permeability to leptin in the adult brain.

Hyperleptinemia also impairs leptin receptor signaling both in neurons and glial cells in brain areas relevant for learning/memory (Grillo et al., 2011). It has been demonstrated that leptin resistance selectively affected the functionality of the PI3K/Akt signaling pathway (Valladolid-Acebes et al., 2013).

Interestingly, Mainardi et al. (2017) have reported that HFDs causes a loss of leptin-induced modulation of hippocampal synaptic transmission in mice.

CONCLUDING REMARKS

Consumption of HFD during the juvenile/adolescent period has a negative impact on hippocampal memory and neural-related processes in the adult brain as revealed by a substantial amount of research carried out in murine models. Nevertheless, the variability of experimental conditions used to investigate this issue (dietary treatments of different duration, variety of diets, animals in different states of development, etc.) makes it difficult to draw reliable conclusions. Systematic studies carried out with diets of defined composition, which do not merge elevated amounts of saturated fat and sugar and that are preferably enriched in a particular FA, would be necessary to investigate in more depth the influence of fat on brain functions. In this regard, future research based on the use of experimental HFD manufactured with highly saturated or unsaturated oils, enriched in a particular FA, are needed to identify the specific contribution of the various types of dietary fat to memory processes.

Otherwise, the translational value of all of these findings remains unclear, as strong epidemiological studies are lacking. Therefore, it is necessary to carry out parallel clinical and basic research devoted to the identification of the molecular mechanism underlying memory deficits evoked by the regular consumption of HFD.

AUTHOR CONTRIBUTIONS

NDO and MR-G contributed to the conception and design of the review, as well as the drafting and revision of the manuscript. The authors have approved the final version of the manuscript.

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Combined Effect of Fatty Diet and Cognitive Decline on Brain Metabolism, Food Intake, Body Weight, and Counteraction by Intranasal Insulin Therapy in 3×Tg Mice

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Obesity and cognitive decline can occur in association. Brain dysmetabolism and insulin resistance might be common underlying traits. We aimed to examine the effect of high-fat diet (HFD) on cognitive decline, and of cognitive impairment on food intake and body-weight, and explore efficacy of chronic intranasal insulin (INI) therapy. We used control (C) and triple transgenic mice (3×Tg, a model of Alzheimer's pathology) to measure cerebral mass, glucose metabolism, and the metabolic response to acute INI administration (cerebral insulin sensitivity). Y-Maze, positron emission-computed tomography, and histology were employed in 8 and 14-month-old mice, receiving normal diet (ND) or HFD. Chronic INI therapy was tested in an additional 3×Tg-HFD group. The 3×Tg groups overate, and had lower body-weight, but similar BMI, than diet-matched controls. Cognitive decline was progressive from HFD to 3×Tg-ND to 3×Tg-HFD. At 8 months, brain fasting glucose uptake (GU) was increased by C-HFD, and this effect was blunted in 3×Tg-HFD mice, also showing brain insulin resistance. Brain mass was reduced in 3×Tg mice at 14 months. Dentate gyrus dimensions paralleled cognitive findings. Chronic INI preserved cognition, dentate gyrus and metabolism, reducing food intake, and body weight in 3×Tg-HFD mice. Peripherally, leptin was suppressed and PAI-1 elevated in 3×Tg mice, correlating inversely with cerebral GU. In conclusion, 3×Tg background and HFD exert additive (genes*lifestyle) detriment to the brain, and cognitive dysfunction is accompanied by increased food intake in 3×Tg mice. PAI-1 levels and leptin deficiency were identified as potential peripheral contributors. Chronic INI improved peripheral and central outcomes.

Keywords: positron emission tomography, high-fat diet, Alzheimer's disease, triple transgenic mice, cognitive disease, cerebral glucose uptake and insulin resistance, PAI-1, adipokines

Abbreviations: 3×Tg, Triple transgenic (mice); C, control (mice); CT, computed tomography; ^{18}FDG, ^{18}F-2-fluoro-2-deoxyglucose; FE, fractional glucose extraction; GU, glucose uptake; HFD, high-fat diet; ID, injected ^{18}FDG dose; INI, intranasal insulin therapy; ND, normal diet; PET, positron emission tomography.

INTRODUCTION

Obesity and neurodegenerative diseases are growing in prevalence. In humans, obesity was shown to predict cognitive impairment (Elias et al., 2005; Whitmer et al., 2005; Hassing et al., 2010), associating with cerebral matter losses (Driscoll et al., 2012), which were reversed by dieting (Haltia et al., 2007). In obese rodents, these abnormalities were related to, e.g., reduced hippocampal plasticity (Wu et al., 2003). In APP/PS1 double transgenic mice (with features of Alzheimer's disease), diet-induced obesity worsened amyloid burden and cognitive performance (Cao et al., 2007). In turn, there is also evidence that the damage to cognitive areas can lead to overeating (Davidson et al., 2009), and that the central nervous system exerts control on peripheral glucose homeostasis (Obici et al., 2002; Heni et al., 2017).

Among common underlying factors, cerebral insulin resistance has gained attention. Insulin promotes neuronal growth and differentiation, and amyloid and tau processing (Steen et al., 2005), protecting learning and memory (Zhao and Alkon, 2001), and defects in cerebral insulin receptors or action have been observed in patients with Alzheimer's disease (Zhao and Alkon, 2001; Talbot et al., 2012). In high-fat and/or high-fructose fed animals, defects in insulin action are accompanied by alterations in synaptic, dendritic, hippocampal integrity, and cognitive dysfunction (Winocur and Greenwood, 2005; Stranahan et al., 2008; Arnold et al., 2014; Calvo-Ochoa et al., 2014). On the other side, brain insulin signaling plays a critical role in the maintenance of energy balance, food intake, and weight gain (Stockhorst et al., 2004; Stranahan et al., 2008; Fronczek et al., 2012; Jauch-Chara et al., 2012; Madden et al., 2012; Talbot et al., 2012). INI treatment has shown promise as therapy to overcome cerebral insulin resistance in dementia and in obesity (Benedict et al., 2004; Craft et al., 2012; Jauch-Chara et al., 2012; Ott et al., 2012; Heni et al., 2017; Nedelcovych et al., 2018). However, there is much heterogeneity in the animal models and intervention protocols used, and in the level of efficacy attained. The current evidence suggests that short-term intra-cerebral insulin therapy results in cognitive improvements (Park et al., 2000; Marks et al., 2009; Vandal et al., 2014; Salameh et al., 2015), but efficacy of protracted insulin regimens has been less explored and not confirmed in most of the existing studies, in which phenomena of desensitization due to overdosing have been advocated (Kamal et al., 2012; Nazarians-Armavil et al., 2013; Bell and Fadool, 2017).

In this study, we tested the hypotheses that (a) high-fat feeding affects cognition, brain mass, cerebral metabolism, and cerebral insulin sensitivity, especially in genetically predisposed subjects, (b) regulation of food intake and body weight is compromised in subjects with impaired cognition, and (c) chronic INI prevents these effects. We used C and a 3×Tg mouse model of Alzheimer's type pathology fed high fat (HFD) or ND. An exploratory evaluation of efficacy of chronic INI to reverse the observed phenotype was carried out in the group showing more severe conditions, i.e., 3×Tg-HFD mice.

MATERIALS AND METHODS

Study Design

We studied 145 male mice, including n = 76 controls (B6129SF2/J, strain# 101045) and n = 69 3×Tg mice (B6;129-Psen1^{tm1Mpm}Tg(APPSwe,tauP301L)1Lfa/Mmjax; strain# 004807, The Jackson Laboratory, Bar Harbor, ME, United States). In a subset of 8 months old animals, microbiome-metabolome signatures associated with 3×Tg background, brain glucose extraction and HFD were recently published (Sanguinetti et al., 2018). The study design is summarized in **Figures 1A,B**. Animals were housed under 12-h light/12-h dark cycles and controlled room temperature (22°C), with *ad libitum* access to food and fresh water. Mice were divided in five groups: (1) ND (C-ND, B6129SF2J, n = 37, 11% kcals from fat, Mucedola, Milan, Italy); (2) high-fat diet (C-HFD, B6129SF2J, n = 39, 58% kcals from fat); (3) 3×Tg-ND (n = 22); (4) 3×Tg-HFD (n = 22); (5) 3×Tg-HFD and chronic INI (3×Tg-HFD + INI, n = 25). Diets and INI were started at 2 months of age. Body weight and food intake were monitored weekly, and random glycaemia every ∼7 weeks. At 8 and 14 ± 1 months of age, cognitive performance was measured by Y-maze test (Panlab, Harvard Apparatus, Barcelona, Spain), and positron emission and CT with ^{18}FDG (IRIS PET/CT, Inviscan SAS, Strasbourg, France) was performed in a subset of 85 mice. At the end of *in vivo* procedures, animals were euthanized, and the brain collected and weighted. The experimental protocol was conducted under the D.L.116/92 implementation of European Economic Community directive 609/86 regarding the protection of animals used for experimental and other scientific purposes.

Chronic Insulin Therapy

This explorative study was carried out in 3×Tg-HFD-INI mice. Weak sedation by 1–2% (v/v) isoflurane (IsoFlo®, Abbott Laboratories, Chicago, IL, United States) was used until correct positioning, and INI delivery was carried out in awake mice, under neck extension (Marks et al., 2009; Sanguinetti et al., 2018). INI was administered daily for 1 week, and weekly thereafter, to minimize desensitization or adverse effects (Kamal et al., 2012; Nazarians-Armavil et al., 2013; Anderson et al., 2017), accounting for the notion that each insulin dose has persisting effects over days (Meredith et al., 2015; Salameh et al., 2015). Each INI administration consisted of 0.87 UI in 24 µl vehicle solution (PBS, Sigma-Aldrich, St Louis, MO, United States), as delivered by pipette in four 6-µl drops, alternating nares every 1 min to ensure fluid inhalation (Marks et al., 2009).

Y-Maze Test

Cognitive performance and explorative behavior were measured by spontaneous alternation testing in a standard 3-arm Y-maze (Panlab, Harvard Apparatus, Barcelona, Spain) during an 8 min session. The test was performed at least 48 h after insulin administration in order to capture the effect of the chronic insulin therapy and to avoid any acute insulin effect. A visual automatic tracking system (Panlab, Harvard Apparatus, Barcelona, Spain) was used to measure: latency time (until first

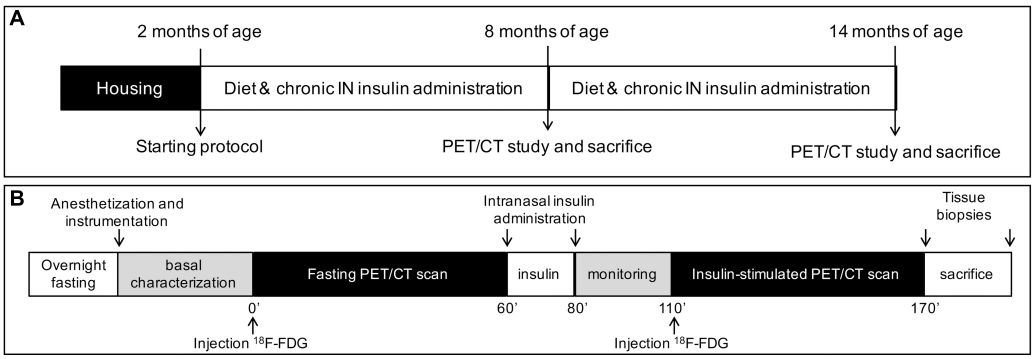

FIGURE 1 | The panels show the study design **(A)**, and PET-CT scanning session **(B)**. ND and HFD were tested in controls and 3×Tg mice, whereas chronic intranasal insulin therapy (INI) was tested in an additional group of 3×Tg-HFD mice.

arm choice), spontaneous alternation triplets (number of three consecutive entries in different arms), percentage of alternation triplets (against the maximum possible number), zone transition number, and total arm entries. Resting time, traveled distance, and speed were also measured.

PET-CT Scanning

The imaging session is shown in **Figure 1B**. Anesthesia was induced in fasted mice by 3–4% (v/v), and maintained with 1–2% (v/v) isoflurane. A rectal probe was positioned, and baseline temperature measured. Then, a heated pad was used to prevent the decline in body temperature due to anesthesia. Mice were positioned in a μPET-CT tomograph (IRIS PET/CT, Inviscan SAS, Strasbourg, France) and CT scans were acquired. Then, two 60-min PET scans were performed after i.p. ^{18}FDG injection (7.6 ± 0.1 and 7.9 ± 0.1 MBq, first and second scan), one in the fasted state, and one after 30-min of an acute INI dose (0.87 UI in 24 μl) (Marks et al., 2009) (**Figure 1B**). Glycemia was monitored in tail blood.

PET-CT Image Processing

Positron emission tomography data were corrected for dead time and radioactive decay, reconstructed by standard algorithms, and co-registered to CT images by AMIDE Medical Image Data Examiner 1.0.5. Volumes of interest were drawn in brain images corresponding to frontal, somatosensory and temporal cortices, dorsal striatum, globus pallidus, thalamus, hypothalamus, amygdala, hippocampus. Fractional tracer extraction (FE), reflecting the intrinsic ability of the brain to actively extract glucose from the circulation (Thie, 1995), was expressed as ratio of tissue activity to the ID per gram of body weight (% ID/g). GU, resulting from the combined effects of FE and glucose delivery from blood, was computed as product of % ID/g and glycaemia during imaging (Thie, 1995).

Circulating Markers

After the imaging session, animals were euthanized and blood was collected and centrifuged 10 min at 4000 rpm. Plasma concentrations of insulin, leptin, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), resistin, plasminogen activator inhibitor-1 (PAI-1), and monocyte chemoattractant protein-1 (MCP-1) were measured by Luminex® xMAP® technology (Mouse Adipokine Magnetic Bead Panel, Merck-Millipore Corp., MO, United States), according to manufacturer's instructions.

Brain Histology

Surface of the dentate gyrus, and thickness of the granule cell layer were measured in histological sections. After sacrifice, the brain was dissected, and one hemisphere was fixed in 10% neutral-buffered formalin (20–24 h), and processed for paraffin-embedding. Sections were cut on a coronal plane at a thickness of 8 μm on a rotary microtome and mounted on clean glass Polysine™ slides (Menzel-Gläser, Germany). Sections were stained with hematoxylin-eosin (Bio-Optica, Italy), according to standard protocol. Each section was documented at 5, 10, and 40× magnification using a Axioskop optical microscope connected with an AxioCam MRc5 color-camera and AxioVision analysis software (Carl Zeiss, Germany). By using the ImageJ software (version 2.0.0-rc-43/1.51k[1]), contour of the dentate gyrus was manually drawn in 10× images and its area recorded. Thickness of the granule cell layer of the suprapyramidal and of the infrapyramidal blades was based on the average of three measures obtained in proximity of the apex, the mid and the distal parts in each blade.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were performed by IBM® SPSS® Statistics for Mac OS X (version 24.0, Chicago, IL, United States). Data distribution was assessed by Shapiro-Wilk test. For normally distributed variables and variables that were normalized by logarithmic transformation (e.g., anthropometrics, cognitive parameters, and plasma levels of circulating markers), group comparisons were performed by ANOVA, with statistical significance localized by Fisher's least significant difference (LSD) *post hoc* analysis, or by two-tailed T-test. Mann-Whitney U test was used when normal

[1]https://imagej.nih.gov/ij

distribution was not achieved by logarithmic transformation (i.e., PET data). Analysis of intra-group changes in brain GU from fasting to acute insulin delivery was performed by Wilcoxon test. Two-way ANOVA was also used to confirm independent effects of diet and genotype, and their interaction, and brain metabolic responses to acute insulin administration (mixed design ANOVA). Statistical significance was set at p-value ≤ 0.05.

RESULTS

Table 1 provides sample sizes per group and time-point, **Tables 2**, **3** and **Supplementary Tables S1**, **S2** summarize two-way ANOVA and mixed-design ANOVA analyses.

C-HFD and 3×Tg-HFD mice consumed greater amounts of calories, gained more weight, and had greater glucose levels compared to C-ND and 3×Tg-ND mice. However, compared to diet-matched C, 3×Tg (either -ND or -HFD) mice ate larger amounts of calories, despite lower body weight, normal BMI and lower random glycaemia (**Figures 2A–F**, **3A–F**). Leptin levels were strikingly suppressed in 3×Tg models, with a small counteracting effect of HFD. PAI-1 levels were especially high in 3×Tg-HFD at 8 months (**Figure 4A**), but also elevated in 3×Tg-ND. MCP1 and IL6 were reduced in 3×Tg models at 8 and 14 months, and TNF-α and resistin levels also tended to be deficient in 3×Tg-ND (**Figures 4A,B**). In 3×Tg-HFD + INI, energy intake, body weight, and glycaemia were lower than in untreated 3×Tg-HFD mice, though the effect on glycemia was transient (**Figures 2D–F**). INI also modified inflammatory indices toward normal levels at 8 months, and progressively normalized PAI-1 levels (**Figures 4A,B**), paralleling the degree of preservation of cognitive function (**Figures 5A,B**).

Cognitive Function

In 8 months old mice (**Figure 5A**) a significant trend toward cognitive dysfunction was found across groups, from C-ND to C-HFD to 3×Tg-ND to 3×Tg-HFD. Spontaneous alternation triplets, both as total count and percentage, were significantly reduced in 3×Tg-ND and 3×Tg-HFD than control groups. These features were more pronounced at 14 months of age (**Figure 5B**), also involving total arm entries and zone transition number in 3×Tg-HFD (**Figure 5B**). Significant trends across the four groups, and group differences were seen in resting time, total distance, and speed at both 8 and 14 months (**Figures 5A,B**). In 14 months groups, reductions in traveled distance and speed and the increase in resting time were significant in 3×Tg-ND compared to ND controls, and severe in 3×Tg-HFD mice vs. all groups (**Figure 5B**). Interaction analyses (**Table 2**) confirmed a significant independent effect of genetic model (and a tendency of diet) on cognitive function.

In animals treated with chronic INI, cognitive performance was similar compared to control groups, and significantly higher than in age-matched untreated 3×Tg-HFD mice at 8 months (**Figure 5A**), and even more (3-folds) at 14 months of age (**Figure 5B**).

Brain Glucose Metabolism

Brain glucose metabolism showed a diffuse reduction in the whole brain (**Figures 6A,D**) and in all cerebral regions in fractional extraction of [^{18}F]FDG (**Supplementary Figures S1**, **S2**) in 3×Tg-ND and 3×Tg-HFD mice compared to age-matched controls. An independent effect of 3×Tg genotype to decrease glucose fractional extraction was confirmed in interaction analyses (**Table 2**). HFD consumption

TABLE 1 | Sample size referring to results shown in figures and tables.

	C-ND	C-HFD	3×Tg-ND	3×Tg-HFD	3×Tg-HFD + INI
8 months					
Body weight (20–24w)(g)	37	39	22	22	25
Random glycemia (21-22w) (mmol/l)	35	39	21	20	24
Caloric Intake (20-24w) (Kcal)	37	39	22	22	25
Circulating cytokines	9–11	10–12	8	6–7	10–11
Cognitive variables	39	39	21	20	23
Brain mass	5	5	4	4	7
Brain histology	3	5	3	3	5
Brain PET baseline	7	9	8	7	11
Brain PET + acute insulin	7	9	8	7	10
14 months					
Body weight (44w) (g)	14	16	11	11	8
Random glycemia (42–44w) (mmol/l)	14	16	11	11	8
Caloric Intake (Kcal)	14	16	11	11	8
Circulating cytokines	11–13	14–16	8–9	5–6	7
Cognitive variables	16	14	9	7	7
Brain mass	6	6	9	6	7
Brain histology	6	6	4	4	4
Brain PET baseline	10	12	8	6	7
Brain PET + acute insulin	10	11	8	5	7

per se did not aggravate, and INI did not alleviate this defect. Cerebral GU (extraction*glycemia) (**Figures 6B,C,E,F** and **Supplementary Figures S3**, **S4**) was greater in all brain regions in HFD compared to ND groups at 8 months, and this effect was blunted by 3×Tg background. Interaction analyses confirmed the independent effect of diet on cerebral GU, and a contrasting effect of 3×Tg background in selected brain regions (**Table 2** and **Supplementary Table S1**). At 8 months, acute INI lowered brain GU, suppressing peripheral glycemia in C-ND, C-HFD and 3×Tg-ND mice (**Figures 6B,C**, **Table 3**, and **Supplementary Table S2**), whereas no effect was observed in 3×Tg-HFD mice (indicative of brain insulin resistance in this group). Chronic INI in 3×Tg-HFD + INI mice was able to reduce the excess in brain GU seen in 3×Tg-HFD mice, and re-establish a normal acute response to insulin at 8 months of age (**Figures 6B,C**), although cerebral insulin resistance was present at 14 months (**Figures 6E,F**), consistent with raising blood glucose levels (**Figures 1D**, **6F**).

Among peripheral factors, PAI-1 levels were inversely related to brain GU in pooled age groups, and in 14 months old mice, during fasting ($r = -0.20$, $p = 0.058$, $r = -0.28$, $p = 0.033$) and acute insulin administration ($r = -0.21$, $p = 0.047$, $r = -0.22$, $p = 0.014$).

TABLE 2 | Two-way ANOVA for the effects of diet and genotype in group comparisons.

	Diet	Genotype	Diet* Genotype
8 months			
Body weight (g)	**<0.0005**	**<0.0005**	0.793
Body mass index (kg/m^2)	**<0.0005**	0.108	0.990
AUC body weight (g*time)	**0.015**	**<0.0005**	0.961
Random glycemia (mmol/l)(21-22w)	**0.001**	**<0.0005**	**0.035**
Insulin (pg/ml)	0.952	0.806	0.153
Leptin (pg/ml)	**0.039**	**<0.0005**	**0.036**
Resistin (pg/ml)	**0.045**	0.906	0.297
IL-6 (pg/ml)	0.937	0.187	0.724
TNF-alpha (pg/ml)	0.343	0.791	0.672
MCP-1 (pg/ml)	0.580	**0.004**	0.561
PAI-1 (pg/ml)	0.204	**0.001**	0.555
Alternation triplets (n)	0.125	**<0.005**	0.659
Alternation triplets (%)	0.143	**<0.005**	0.401
Resting time (s)	0.416	0.248	0.437
Total arm entries (n)	0.444	0.353	0.052
Total distance (cm)	0.133	0.106	0.259
Speed (cm/s)	0.141	0.106	0.252
Latency time (s)	0.825	0.293	0.946
Zone transition (n)	0.340	0.252	0.080
Brain mass (g)	0.333	0.239	0.710
Cerebral SUV (% ID/g) baseline	0.528	**<0.005**	0.849
Cerebral SUV (% ID/g) + insulin	0.434	**<0.005**	0.756
Cerebral GU (% ID/g * mmol/l) baseline	**<0.005**	0.067	0.351
Cerebral GU (% ID/g * mmol/l) + insulin	**<0.005**	0.593	0.477
14 months			
Body weight (g)	**0.004**	**0.001**	0.214
Body mass index (kg/m^2)	**0.011**	0.658	0.167
AUC body weight (g*time)	**0.011**	**<0.0005**	0.230
Random glycemia (mmol/l)	**0.003**	**<0.0005**	0.796
Insulin (pg/ml)	0.182	**0.004**	0.092
Leptin (pg/ml)	**0.026**	**<0.0005**	0.178
Resistin (pg/ml)	**0.022**	**0.025**	0.131
IL-6 (pg/ml)	0.543	**<0.0005**	0.063
TNF-alpha (pg/ml)	0.060	0.384	0.162
MCP-1 (pg/ml)	0.669	**0.005**	0.576
PAI-1 (pg/ml)	0.589	**0.029**	0.460
Alternation triplet (n)	0.120	**<0.0005**	0.495
Alternation triplet (%)	0.727	**0.001**	0.796
Resting time (s)	0.127	**<0.005**	0.154
Total arm entries (n)	**0.004**	0.085	0.079
Total distance (cm)	0.091	**<0.0005**	0.222
Speed (cm/s)	0.100	**<0.0005**	0.241
Latency time (s)	0.188	0.054	0.300
Zone transition (n)	**0.004**	0.077	0.083
Brain mass (g)	0.732	**<0.005**	0.270
Cerebral SUV (% ID/g) baseline	0.346	**0.001**	0.871
Cerebral SUV (% ID/g) + insulin	0.824	**0.001**	0.318
Cerebral GU (% ID/g * mmol/l) baseline	0.511	0.136	0.827
Cerebral GU (% ID/g * mmol/l) + insulin	0.268	0.607	0.644

In bold are indicated significant p-values.

TABLE 3 | Mixed design ANOVA for the effects of acute intranasal insulin stimulus.

	Acute insulin	Acute insulin * diet	Acute insulin * genotype	Acute insulin * diet * genotype
Cerebral fractional extraction				
8 months	0.359	0.927	0.794	0.878
14 months	0.329	**0.024**	0.814	0.102
Cerebral glucose uptake				
8 months	**<0.0005**	0.740	**0.001**	**0.011**
14 months	**<0.0005**	0.755	0.222	0.107

In bold are indicated significant p-values.

Brain Mass and Histology

Brain mass was significantly reduced in all 3×Tg models at 14 months (**Figures 7A–E**) and was not modified by chronic INI. The dimension of the dentate gyrus, and the thickness granule cell layer of both suprapyramidal and infrapyramidal blades showed not significant differences in 8 months old HFD or 3×Tg mice (**Figures 7B–D**). At 14 months (**Figures 7F–H**), HFD mice had lower infrapyramidal layer thickness, whereas the 3×Tg mice showed a reduction in dentate gyrus area and infrapyramidal layer thickness compared to C-ND mice. In 3×Tg-HFD mice, all measures (dentate gyrus area, suprapyramidal, and infrapyramidal layer thickness) were

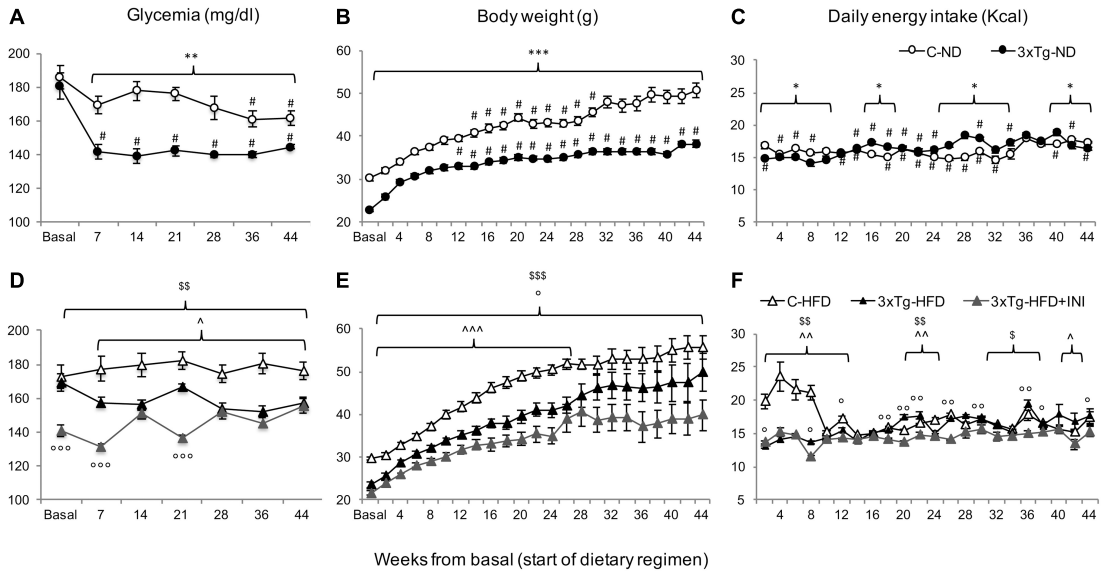

FIGURE 2 | The figure documents lower glycemia **(A,D)** and body weight **(B,E)**, and higher energy intake **(C,F)** in 3×Tg compared to diet-matched controls, with an effect of chronic INI to reduce all parameters **(D–F)**. Data are presented as mean ± SEM. Sample sizes are given in **Table 1**. Panels **A–F** $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ between groups (within-panel), $^{\#}p < 0.05$ ND **(A–C)** vs. HFD **(D–F)** within-strain (between panels), panels **D–F** $\hat{}p < 0.05$ or less ($\hat{}\hat{}$) C-HFD vs. 3×Tg-HFD, $^{\$}p < 0.01$ C-HFD vs. 3×Tg-HFD + INI, $^{\circ}p < 0.05$ or less ($^{\circ\circ}$,$^{\circ\circ\circ}$) 3×Tg-HFD vs. 3×Tg-HFD + INI.

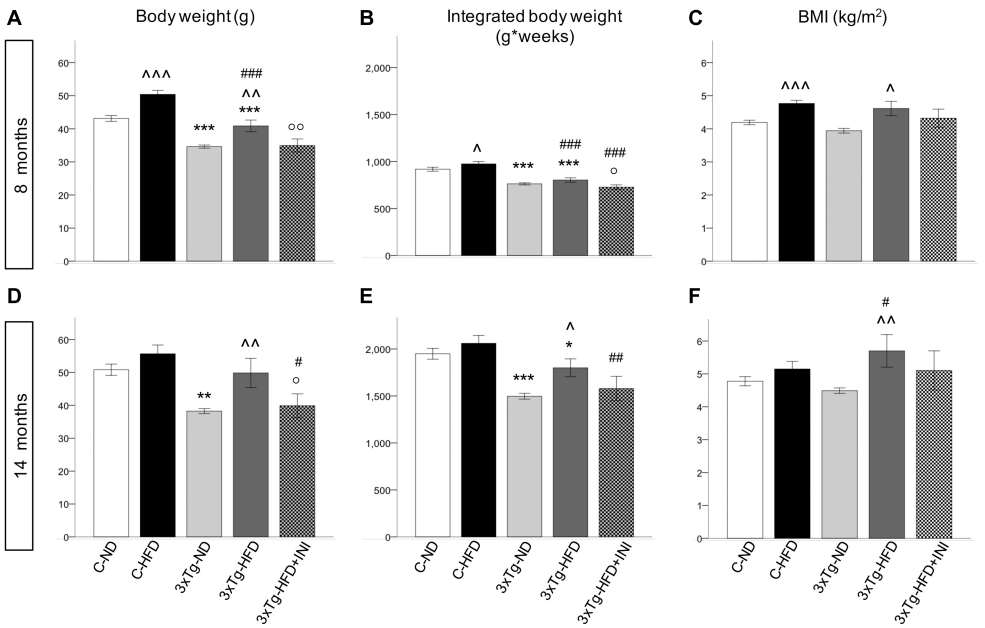

FIGURE 3 | Body weight, areas under the curves and BMI achieved by the study groups at final time-points of 8 **(A–C)** and 14 months of age **(D–F)**, showing that body weight was affected by diet (positively) and by 3×Tg background (negatively), whereas BMI was only dependent on diet, since 3×Tg mice were lighter, but also shorter (data not shown) than controls. Data are presented as mean ± SEM. Sample sizes are given in **Table 1**. $*p < 0.05$ or less ($**$,$***$) ND-ND and HFD-HFD (between strains), $\hat{}p < 0.05$ or less ($\hat{}\hat{}$,$\hat{}\hat{}\hat{}$) HFD vs. ND (within-strain), $^{\#}p < 0.05$ or less ($^{\#\#}$,$^{\#\#\#}$) vs. ND control group (reference group), $^{\circ}p < 0.05$ or less ($^{\circ\circ}$) for treated 3×Tg-HFD + INI vs. untreated 3×Tg-HFD.

defective. Chronic INI resulted in normal dimensions of dentate gyrus area, suprapyramidal, and infrapyramidal layer thickness, which were significantly larger in 3×Tg-HFD-INI than in 3×Tg-HFD mice, and comparable to C-ND mice.

In addition, at 14 months dentate gyrus and infrapyramidal layer dimensions were correlated with the percentage of alternation triplets, as assessed by Y maze ($R = 0.466$ and 0.467, $p = 0.029$). The dentate gyrus area was also significantly associated

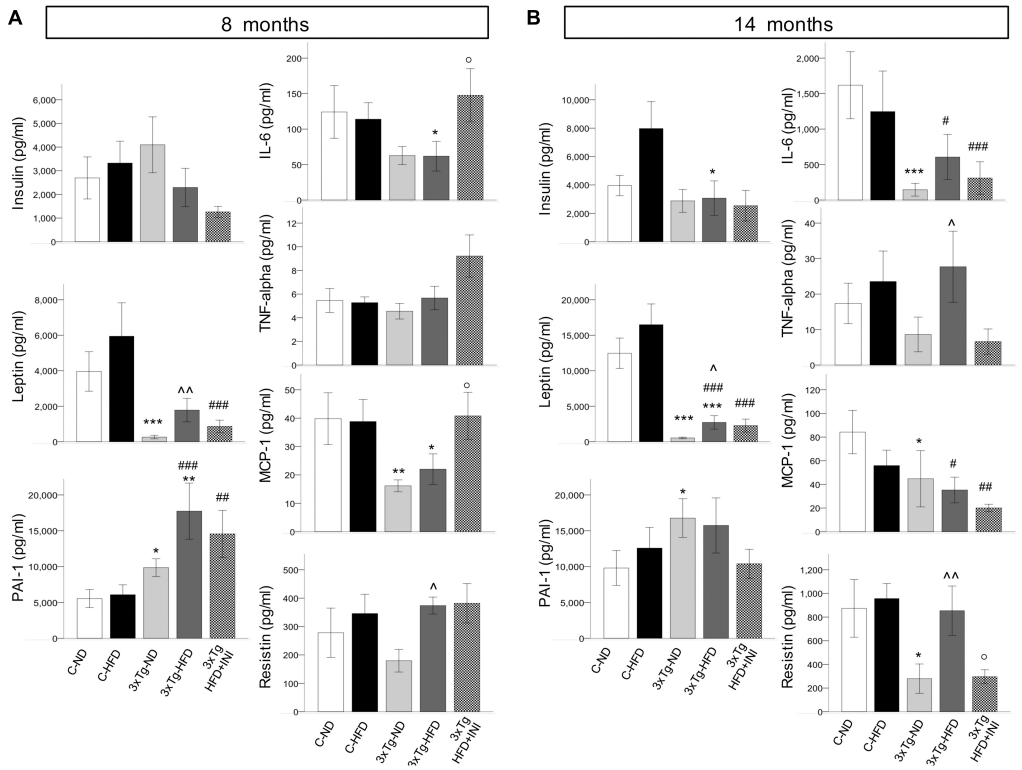

FIGURE 4 | Plasma levels of cytokines and adipokines measured in each study group at final time-points of 8 **(A)** and 14 months of age **(B)**. Data are presented as mean ± SEM (non-normally distributed data were normalized with logarithmic transformation, and parametric tests were used). Sample sizes are given in **Table 1**. $^{*}p < 0.05$ or less (**,***) ND-ND and HFD-HFD (between strains), $^{\wedge}p < 0.05$ or less ($^{\wedge\wedge}$) HFD vs. ND (within-strain), $^{\#}p < 0.05$ or less ($^{\#\#}$,$^{\#\#\#}$) vs. ND control group (reference group), $^{\circ}p < 0.05$ for treated 3×Tg-HFD + INI vs. untreated 3×Tg-HFD.

with resting time ($R = -0.466$, $p = 0.029$), total traveled distance ($R = 0.439$, $p = 0.041$), and speed ($R = 0.447$, $p = 0.037$).

DISCUSSION

This study documents a progressive reduction in cognitive function due to 3×Tg-background, HFD, and their combination. The former had stronger independent influence on alternation triplets, whereas the diet had more impact on the number of entries in Y-Maze arms. As a result, mice in the 3×Tg-HFD group presented worse profiles in all cognitive variables, especially at the age of 14 months. These results are consistent with previous evidence (Knight et al., 2014; Sah et al., 2017) pointing to additive mechanisms, i.e., amyloid-pathology (insoluble Aβ plaque area, tau neuropathology), restricted to 3×Tg mice, and an increase in soluble Aβ40-42 (Vandal et al., 2014), microglia activation (Knight et al., 2014), neuronal oxidative stress and apoptosis (Sah et al., 2017) also induced by HFD. Though it may be argued that the defects in speed and traveled distance seen in our 3×Tg mice can influence the interpretation of cognitive results, the decline in alternation triplets observed with Y-Maze testing was shown to reflect the outcomes of other tests (Morris water maze, smell recognition, novel object recognition tests) in non-Tg and 3×Tg mice fed ND or HFD (Knight et al., 2012; Sah et al., 2017). In fact, the authors of those studies concluded that regardless of the test used, HFD impairs both spatial and non-spatial memory (Knight et al., 2012). To further support our cognitive findings, we measured the surface of the dentate gyrus and thickness of the granule cell layer of the infra- and suprapyramidal blades in histological sections. These are the most affected hippocampal subfields during the development of Alzheimer's disease and show the greatest amyloid burden in the PDAPP (transgenic for the amyloid precursor protein) mouse model (Reilly et al., 2003). The reduction in dentate gyrus and granule cell layer dimensions did not achieve significance in 8 months old HFD and 3×Tg mice, showing only a tendency. They became significant in 14 months old HFD (infrapyramidal layer thickness) and 3×Tg-ND mice (dentate gyrus area and infrapyramidal layer thickness), and were most severely affected in 3×Tg-HFD mice (dentate gyrus area, suprapyramidal, and infrapyramidal layer thickness), correlating with cognitive findings. These timelines are consistent with the notion that the decline in dentate gyrus neurogenesis becomes measurable at 9 months, and is more evident at 12 months of age in 3×Tg compared to C (Rodriguez et al., 2008).

Considering that glucose is the main cerebral energy substrate, and given the growing implication of cerebral insulin resistance in the pathogenesis of Alzheimer's disease (Zhao and Alkon, 2001; Steen et al., 2005; Winocur and Greenwood, 2005;

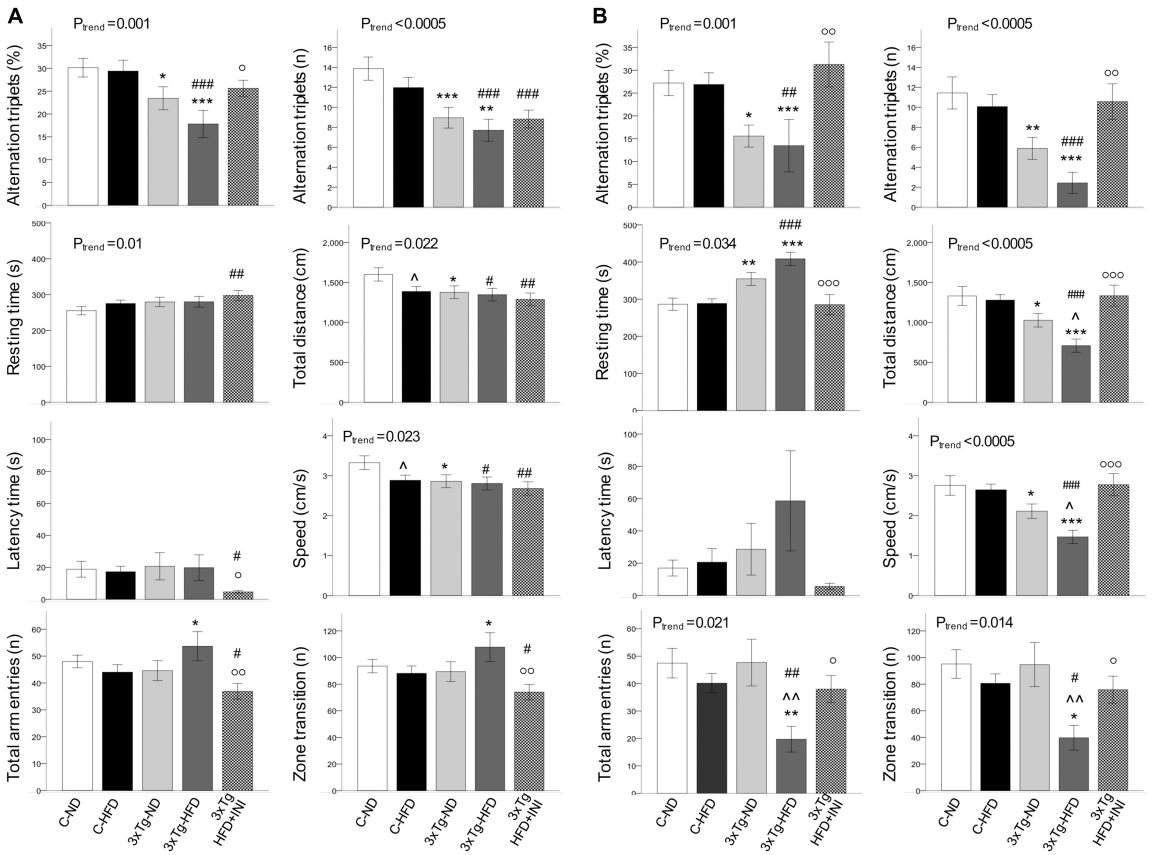

FIGURE 5 | Cognitive function parameters at the age of 8 **(A)** and 14 months **(B)**. Data are presented as mean ± SEM. Sample sizes are given in **Table 1**. P_{trend} refers to regression analyses across the first four groups. $^{*}p < 0.05$ or less (**,***) ND-ND and HFD-HFD (between strains), $^{\wedge}p < 0.05$ or less ($^{\wedge\wedge}$) HFD vs. ND (within-strain), $^{\#}p < 0.05$ or less ($^{\#\#}$,$^{\#\#\#}$) vs. ND control group (reference group), $^{\circ}p < 0.05$ or less ($^{\circ\circ}$,$^{\circ\circ\circ}$) for treated 3×Tg-HFD + INI vs. untreated 3×Tg-HFD.

Stranahan et al., 2008; Talbot et al., 2012; Arnold et al., 2014; Calvo-Ochoa et al., 2014), we examined for the first time the combined effects of diet and 3×Tg background on three processes underlying brain glucose metabolism *in vivo* (fractional extraction, FE, glucose uptake, GU during fasting, and their response to insulin), as determined by consecutive imaging sessions (fasting, acute INI delivery). First, we observed that the fractional extraction, i.e., the intrinsic capability of the brain to extract FDG from the circulation (Thie, 1995), was 30–40% deficient in our 3×Tg mice, regardless of diet. This defect is typically related to neuronal loss in patients with mild to severe cognitive dysfunction (Silverman et al., 2001; Ossenkoppele et al., 2012), and supports the translational potential of the current mouse model. Second, we found that cerebral GU, representing the actual glucose load entering the tissue (Ashraf et al., 2015) was in excess of 85% in HFD compared to ND groups, but this effect was reduced (especially in temporal cortex, globus pallidus, thalamus, somatosensory cortex) in 3×Tg-HFD mice. In human patients with mild cognitive impairment, cerebral hypermetabolism indicates compensatory neuronal recruitment during early disease stages of low amyloid deposition (Sperling et al., 2010; Mormino et al., 2011; Ashraf et al., 2015), but the overexposure of the brain to glucose might in turn promote amyloid deposition (Cohen et al., 2009), resulting in later neurodegeneration and hypometabolism. In line with this, Tg2576 mice feature brain glucose hypermetabolism at seven, but not at 19 months of age (Luo et al., 2012), and our 3×Tg mice showed lower metabolism during aging, together with a >15% reduction in brain mass. Third, our data document that brain responses to acute INI were blunted in 3×Tg-HFD mice, especially at 8 months. Summarizing these metabolic findings, middle-age 3×Tg-HFD mice were characterized by impaired cerebral glucose metabolism (compared to HFD mice) and cerebral insulin resistance (compared to ND, HFD, and 3×Tg-ND groups). Circulating PAI-1, i.e., a marker of metabolic syndrome and potential causal factor of Alzheimer's pathology in humans and rodents (Oh et al., 2014; Bi Oh et al., 2015), was markedly elevated in 3×Tg-HFD mice, and was the sole negative predictor of brain glucose metabolism.

Brain insulin treatment has been suggested to counteract cognitive deterioration in rodents and humans (Marks et al., 2009; Craft et al., 2012; Anderson et al., 2017). One or few insulin injections improved memory, learning, neurodegeneration and/or insulin signaling in different studies and in a variety of models, whereas protracted insulin regimens have been limitedly explored, and have generated unclear results. In Long-Evans

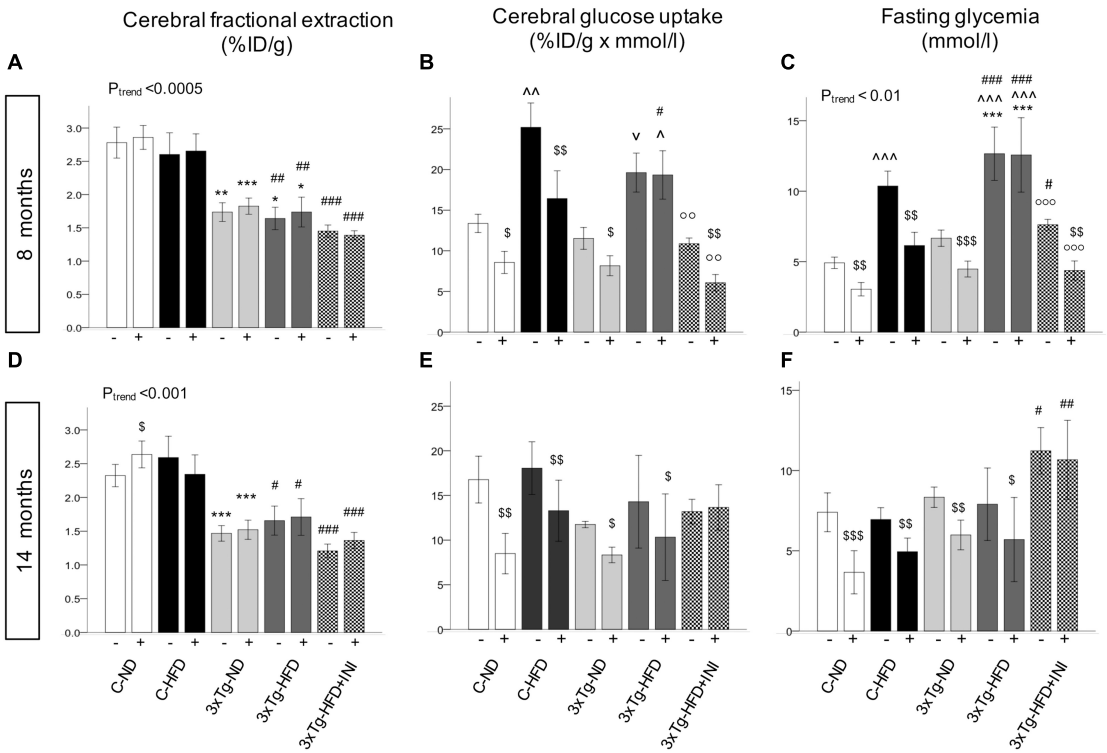

FIGURE 6 | Brain glucose metabolism in 8 (top) and 14 (bottom) months old mice. Fractional extraction **(A,D)** reflects the intrinsic ability of the brain to actively extract glucose from the circulation, and glucose uptake **(B,E)** takes into account the mass effect of circulating glucose, and was computed as product of fractional extraction **(A,D)** and glycaemia during imaging **(C,F)**. Data are presented as mean ± SEM. Sample sizes are given in **Table 1**. P_{trend} refers to regression analyses across the first four groups. $^{*}p < 0.05$ or less (**,***) ND-ND and HFD-HFD (between strains), $^{\wedge}p < 0.05$ or $^{\vee}p = 0.064$ HFD vs. ND (within-strain), $^{\#}p < 0.05$ or less ($^{\#\#}$,$^{\#\#\#}$) vs. ND control group (reference group), $^{\circ\circ}p < 0.01$ or less ($^{\circ\circ\circ}$) for treated 3×Tg-HFD + INI vs. untreated 3×Tg-HFD, $^{\$}p < 0.05$ acute intranasal insulin vs. baseline PET scans (within-group, paired tests).

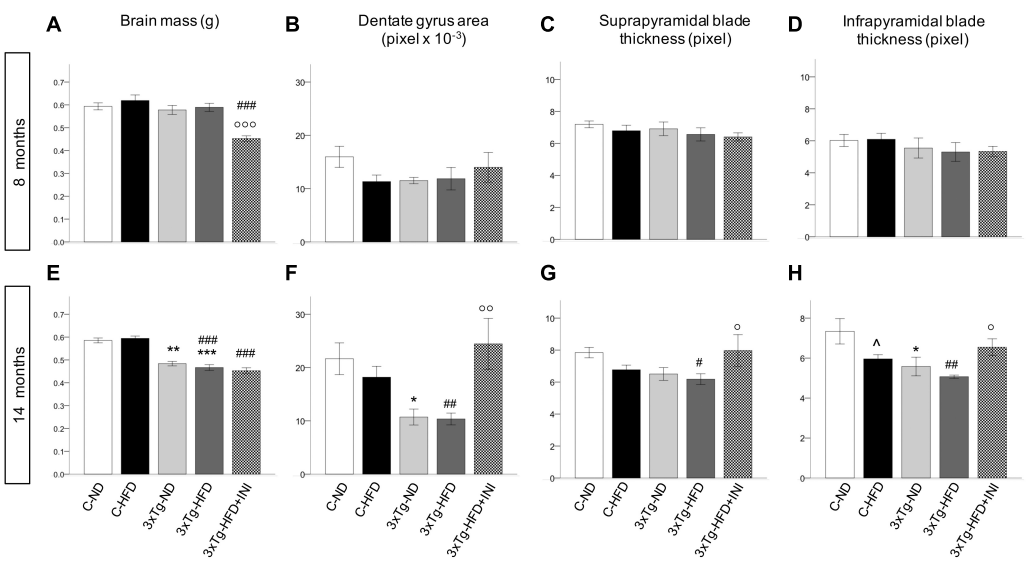

FIGURE 7 | Cerebral morphologicalcharacterization in 8 (top) and 14 (bottom) months old mice, including brain mass **(A,E)**, area of dentate gyrus **(B,F)**, and thickness of suprapyramidal **(C,G)** and infrapyramidal blades **(D,H)**, as measured in coronal brain sections stained with haematoxylin-eosin. Sample sizes are given in **Table 1**. $^{*}p < 0.05$ or less (**,***) ND-ND and HFD-HFD (between strains), $^{\wedge}p < 0.05$ HFD vs. ND (within-strain), $^{\#}p < 0.05$ or less ($^{\#\#}$,$^{\#\#\#}$) vs. ND control group (reference group), $^{\circ}p < 0.05$ or less ($^{\circ\circ}$, $^{\circ\circ\circ}$) for treated 3×Tg-HFD + INI vs. untreated 3×Tg-HFD.

rats, an intracerebroventricular injection of 4 mU of insulin increased memory function after 24 h (Park et al., 2000). In 12-months old SAMP8 mice, one-single or 14 daily injections of INI improved cognitive performance (Salameh et al., 2015). In a mouse HIV-associated neurodegenerative (HAND) model, nine daily injections of 2.4 IU enhanced the levels of glucose, ATP, ADP, phosphocreatine, and creatine in homogenized brain tissue (Nedelcovych et al., 2018). In F344 rats, insulin signaling was improved 2 h after a single INI injection (0.075 IU zinc-free insulin formulation), but no cognitive improvement was seen, even after prolonging treatment for 9-days (Anderson et al., 2017). In adult male C57BL6/J mice, short- and long-term memory, and odor discrimination were improved after acute (Marks et al., 2009) but not chronic INI treatment (twice daily for 30–60 days), and insulin signaling was blunted in the chronic arm (Bell and Fadool, 2017). Consistent with these negative outcomes, continuous (12-week) ICV insulin infusion impaired synaptic plasticity in Wistar rats (Kamal et al., 2012), and the prolonged exposure of neuronal cell cultures to insulin provoked neuronal insulin resistance (Nazarians-Armavil et al., 2013). We are aware of few reports addressing the effects of INI in 3×Tg mice or similar genetic models of AD pathology. Chen et al. (2014) showed that seven daily INI (1.75 IU) vs. vehicle injections restored brain insulin signaling, increased the levels of synaptic proteins, and reduced Aβ40 levels and microglia activation in an early disease stage, i.e., in 9 months 3×Tg mice. Still addressing early AD pathologies, Mao et al. (2016) found that 6 weeks of INI therapy (1.0 IU per day) improved cognitive deficits and insulin signaling, reduced Aβ production and amyloid plaque burden, and increased neurogenesis in young, i.e., 4.5-months old APPswe/PS1dE9 [amyloid precursor protein (APP)/PS1] mice. Finally, one report in 3×Tg-HFD mice showed that a single peripheral insulin injection reversed the deleterious effects of HFD on memory (intraperitoneal insulin injection) and soluble Aβ42 levels (intravenous insulin injection) (Vandal et al., 2014). In spite of a high degree of heterogeneity among studies, rodent models and intervention protocols, the above observations, together with the findings that chronic INI therapy is more effective at lower than higher dose regimens in humans with MCI or Alzheimer's disease (Craft et al., 2012), support the possibility that an excessive and continuous exposure of the brain to insulin may lead to a downregulation of insulin action. The novelty of our study is threefold. First, we used weekly administrations of INI to reduce continuity in brain overexposure to insulin; second, we tested the efficacy of chronic INI on cognition and hippocampal dimensions in 3×Tg-HFD mice, and examined different age groups, reflective of early and late AD pathology; third, we addressed the *in vivo* response of brain metabolism and cerebral insulin resistance to chronic INI in 3×Tg-HFD mice. An important finding in this study was that the efficacy of our weekly administration of INI was striking in magnitude and progressive over time, resulting in 3×Tg-HFD mice with entirely normal cognitive function. INI also normalized functional parameters of cerebral GU and insulin sensitivity, reducing peripheral glycemia and body weight. INI had a strong impact on hippocampal morphology, completely preventing the degeneration of the dentate gyrus and thinning of the granule cell layer of the infrapyramidal blade seen in untreated 3×Tg-ND and 3×Tg-HFD mice. The histological pattern was similar, and strongly associated to the number of alternation triplets observed in Y-Maze testing. The above finding is consistent with the neurogenesis shown in the dentate gyrus of 2×Tg (APP/PS1) mice (Mao et al., 2016) after 6 weeks of INI vs. vehicle treatment. Of note, the degree of preservation in cognition mirrored the decline in PAI-1, which was progressively normalized in INI treated mice. Instead, INI did not improve the deficit in glucose FE by the brain or the whole brain mass, which remained the earliest (FE) and latest (mass) non-modifiable hallmarks of the 3×Tg genetic background.

Our data confirm that 3×Tg mice overeat compared to controls (Adebakin et al., 2012; Knight et al., 2012), independent of diet type. Leptin deficiency in this model may partly explain this behavior. In Tg2576 mice, leptin deficiency was ascribed to reduced adiposity (Ishii et al., 2014), consistent with our observation of low adipokines in 3×Tg mice. Interestingly, leptin plays an important role in the pathogenesis of human dementia, and has been tested as therapy (Greco et al., 2010). However, leptin was not affected by chronic INI therapy, despite reduced feeding and improved cognition. One possibility is that leptin sensitivity in hypothalamic circuits was restored by INI (despite persistence of low circulating levels), considering that amyloid deposition can disturb arcuate NPY neuronal responses (Ishii et al., 2014). Alternatively, the 3×Tg model is characterized by high peripheral metabolism and thermogenesis (Knight et al., 2012, 2013) and by an impairment in gut-to-brain satiety regulation (Adebakin et al., 2012); both of these factors can stimulate food intake, but their response to chronic INI was not explored in our study. Finally, cognitive preservation may exert a direct effect to limit food intake due to improved memory, as lesions to cognitive areas cause hyperphagia (Cao et al., 2007; Davidson et al., 2009).

Study limitations include the use of anesthesia that may underestimate metabolism, but cannot be avoided during imaging, and the lack of molecular measures, since the study was meant to capture the phenotype in order to plan molecular evaluations. In addition, we did not administer INI to all groups, as this was an explorative study in the group with most severe conditions. We showed a complete normalization of cognition and hippocampal histology and metabolism in that group, but we cannot establish whether INI is more effective in 3×Tg-HFD vs. 3×Tg-ND or non-Tg mice.

CONCLUSION

In conclusion, this study confirms our hypotheses that the 3×Tg background and HFD exert additive (genes*lifestyle) detriment to the brain, and that cognitive dysfunction is accompanied by an increase in appetite in 3×Tg mice. PAI-1 levels and leptin deficiency were identified as potential peripheral contributors. Chronic INI preserved cognition, preventing hippocampus tissue loss and normalized PAI-1 levels, alleviating cerebral metabolic abnormalities, also reducing food intake, body weight, and glycemia in 3×Tg-HFD mice.

ETHICS STATEMENT

As stated in the manuscript, at the time this study was carried out, the law requested notification, without explicit approval, of the project to the Italian Ministry of Health, which serves as the official Ethical Authority for animal studies in Italy.

AUTHOR CONTRIBUTIONS

ES: data collection, contribution to data analysis and to manuscript drafting. MG: statistical analyses and results presentation, and contribution to manuscript drafting. VDS: histology data collection and analysis. DP, MT, MQ: PET-CT imaging and processing. PS: contribution to study design. SB: responsible for animal handling and wellbeing. PI: study design and coordination, data analysis, manuscript writing, and project funding. All the authors contributed to the revision of the manuscript and approved its submission for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2019.00188/full#supplementary-material

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Presymptomatic Treatment With Andrographolide Improves Brain Metabolic Markers and Cognitive Behavior in a Model of Early-Onset Alzheimer's Disease

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Alzheimer's disease (AD) is the most common type of dementia. The onset and progression of this pathology are correlated with several changes in the brain, including the formation of extracellular aggregates of amyloid-beta (Aβ) peptide and the intracellular accumulation of hyperphosphorylated tau protein. In addition, dysregulated neuronal plasticity, synapse loss, and a reduction in cellular energy metabolism have also been described. Canonical Wnt signaling has also been shown to be downregulated in AD. Remarkably, we showed previously that the *in vivo* inhibition of Wnt signaling accelerates the appearance of AD markers in transgenic (Tg) and wild-type (WT) mice. Additionally, we found that Wnt signaling stimulates energy metabolism, which is critical for the ability of Wnt to promote the recovery of cognitive function in AD. Therefore, we hypothesized that activation of canonical Wnt signaling in a presymptomatic transgenic animal model of AD would improve some symptoms. To explore the latter, we used a transgenic mouse model (J20 Tg) with mild AD phenotype expression (high levels of amyloid aggregates) and studied the effect of *andrographolide* (ANDRO), an activator of canonical Wnt signaling. We found that presymptomatic administration of ANDRO in J20 Tg mice prevented the reduction in cellular energy metabolism markers. Moreover, treated animals showed improvement in cognitive performance. At the synaptic level, J20 Tg animals showed severe deficiencies in presynaptic function as determined by electrophysiological parameters, all of which were completely restored to normal by ANDRO administration. Finally, an analysis of hippocampal synaptosomes by electron microscopy revealed that the length of synapses was restored with ANDRO treatment. Altogether, these data support the idea that the activation of canonical Wnt signaling during presymptomatic stages could represent an interesting pharmacological strategy to delay the onset of AD.

Keywords: andrographolide, Wnt signaling, glucose metabolism, Alzheimer's disease, neuroprotection

INTRODUCTION

Described over 100 years ago, Alzheimer's disease (AD) has become the most common age-related neurodegenerative disorder. AD is mainly characterized by progressive cognitive impairment, and current therapeutic approaches are limited by uncertainties about its precise etiology (Serrano-Pozo et al., 2011; Wang et al., 2017). This pathology is characterized by a slow loss of learning and memory cognitive functions, concomitant with the presence of senile plaques (Aβ aggregates) and neurofibrillary tangles in the hippocampus and cerebral cortex (Querfurth and LaFerla, 2010; Serrano-Pozo et al., 2011). Other molecular events also related to the progression of the disease include an increase in reactive oxygen species (ROS) production, mitochondrial dysfunction, inflammation, synaptic loss and a decrease in cerebral glucose uptake/utilization (Hamos et al., 1989; Kapogiannis and Mattson, 2011; Chen and Zhong, 2013; Cisternas and Inestrosa, 2017). Recently, the dysregulation of glucose metabolism has been postulated as one of the first events of AD pathology (Cisternas and Inestrosa, 2017). Early modulation of glucose metabolism has become an attractive target for AD patients in whom cognitive impairment has not yet become evident. Indeed, it has been documented that administration of hormones that stimulate glucose metabolism improves cognitive responses in humans diagnosed with AD, as well as in murine models of AD (Reger et al., 2006; Craft et al., 2012; Chapman et al., 2013; Freiherr et al., 2013). These findings suggest that the dysregulation of glucose in the brain could be critical for the onset and progression of AD and the development of other metabolic diseases, including diabetes.

Since the discovery of Wnt signaling three decades ago, this pathway has been implicated in several processes during the development and maintenance of the adult central nervous system (CNS). These processes include neurogenesis, neurite outgrowth, synapse establishment and maturation, and synaptic function (Ciani and Salinas, 2005; Varela-Nallar et al., 2010; Oliva et al., 2018). A strong relationship between loss of Wnt signaling function and neuronal dysfunction in AD has been documented (De Ferrari et al., 2003; Ciani and Salinas, 2005; Toledo and Inestrosa, 2010; Budnik and Salinas, 2011; Oliva et al., 2013). For example, in patients with AD, a decrease in the levels of canonical Wnt effectors such as β-catenin and an increase in the levels of Wnt inhibitors such as Dkk1 have been described (Purro et al., 2012, 2014). On the other hand, the presence of Aβ triggers a decrease in Wnt signaling in animal models of the disease (De Ferrari et al., 2003; Alvarez et al., 2004; Caricasole, 2004; Inestrosa et al., 2012). In a recent study, we showed that the inactivation of Wnt signaling in a transgenic mouse model of AD accelerates the progression of the disease. Specifically, we found increases in cognitive deficits, tau phosphorylation, and the $A\beta_{42}/A\beta_{40}$ ratio, as well as high levels of soluble Aβ species and changes in the number and size of senile plaques. Similarly, the inactivation of canonical Wnt signaling in WT mice triggers an increase in tau phosphorylation and hippocampal dysfunction, which correlates with the increase in $A\beta_{1-42}$ levels (Tapia-Rojas and Inestrosa, 2018). Altogether, the evidence suggests that Wnt signaling stability is required to guarantee a normal phenotype.

From previous data, we know that canonical and non-canonical Wnt signaling stimulate glucose metabolism in cultured hippocampal neurons by promoting glucose uptake and utilization (Cisternas et al., 2016a,b). Additionally, *in vivo* studies have demonstrated that symptomatic administration of canonical Wnt signaling activators (ANDRO and lithium) in a Tg AD mouse rescue the cognitive performance of the animals (Cisternas et al., 2018). This effect is mediated by an improvement of cellular glucose metabolism through a mechanism partially dependent on canonical Wnt signaling (Cisternas et al., 2018). Here, we provide evidence that the administration of the canonical Wnt signaling activator ANDRO, starting in the presymptomatic stage in J20 Tg mice, is able to rescue several cellular and physiological parameters. ANDRO improves cellular glucose metabolism and cognitive performance, both of which are accompanied by the recovery of neuronal aspects related to synaptic plasticity and morphology.

MATERIALS AND METHODS

Animals and Ethical Standards

High accumulation of amyloid aggregates, a condition described in humans with AD, was modeled in the PPSwInd Tg mouse line, which expresses a mutant form of human amyloid precursor protein (APP) bearing both the Swedish (K670N/M671L) and Indiana (V717F) mutations (APPSwInd). This mouse line is known as J20 and was obtained from The Jackson Laboratory (Stock no. 004462; Bar Harbor, ME, United States; RRID: MMRRC_034829-JAX). Three-month-old male (Hemizygous) mice were used as an asymptomatic AD model before starting the treatment (Mucke et al., 2000). Male C57BL/6 WT mice (3 months old) were used as a control.

The animals were maintained at the Animal Facility of the Pontifical Universidad Católica de Chile behind sanitary barriers in ventilated racks and in closed colonies. No sample-size calculation was performed. The experimental procedures were approved by the Bioethical and Biosafety Committee of the Faculty of Biological Sciences of the Pontifical Universidad Católica de Chile. Thirty mice were used and handled according to the National Institutes of Health guidelines (NIH, Baltimore, MD, United States). We used simple randomization to allocate mice to different cages. We divided the J20 animals into two groups (10 animals per group). In one group, we administered andrographolide (CAS Number 5508-58-7, cat: 365645, ANDRO, 2 mg/kg) three times per week for 16 weeks by intraperitoneal injection. The second group of J20 and the WT mice (10 animals each) were injected with saline solution as vehicle for 16 weeks. After the treatment, seven animals per group were used for the cognitive performance tests. After the cognitive test, the animals were housed for another 7 days and then used in the electrophysiology recording and other experiments. Only animals that completed the entire treatment and appeared healthy were used for the studies. For the inclusion/exclusion criterion, we evaluated the weight, and a visual inspection was performed. To prevent animal suffering, technical personnel checked the animals daily for evidence of distress (NIH

tables of supervision). All the cognitive tests (training and experiments) and the slice experiments were performed in a double-blind manner.

D-[1-^{14}C] Glucose Biodistribution

Upon completing the cognitive tests, three mice from each group were injected with D-[1-^{14}C] glucose (cat: NEC043X001 MC, PerkinElmer, United States) via the tail vein. Briefly, mice were anesthetized with isoflurane and injected intravenously via the tail with 50 μCi of tracer diluted to a final volume of 20 μL in isotonic saline. Following a 15 min uptake period, the animals were killed, and tissues were collected. Tissue radioactivity was quantified by liquid scintillation. D-[1-^{14}C] glucose levels were normalized to the weight of resected tissue and expressed as the percent of injected dose (Tsytsarev et al., 2012; Cox et al., 2015).

Glucose Uptake Analysis

Hippocampal slices were washed with buffer (15 mM HEPES (cat: H3375), 135 mM NaCl (cat: s3014), 5 mM KCl (cat: P5405), 1.8 mM $CaCl_2$ (cat: C1016), and 0.8 mM $MgCl_2$ (cat: 208337) supplemented with 0.5 mM glucose (Cisternas et al., 2014a). Then, the slices were incubated for 0–90 min with 1–1.2 μCi 2-[1,2-^{3}H(N)]-deoxy-D-glucose (cat: NET328250UC, PerkinElmer, United States) or ^{3}H-2-deoxyglucose (cat: NET328A250UC, PerkinElmer, United States) at a final specific activity of 1–3 disintegrations/min/pmol (∼1 mCi/mmol). Glucose uptake was arrested by washing the cells with ice-cold PBS supplemented with 1 mM $HgCl_2$ (cat: 203777, Sigma-Aldrich, United States). The incorporated radioactivity was quantified by liquid scintillation counting.

Determination of the Glycolytic Rate

Glycolytic rates were determined as previously described (Cisternas et al., 2014a). Briefly, slices were placed in tubes containing 5 mM glucose and then washed twice in Krebs–Henseleit solution (11 mM Na_2HPO_4, 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 1.3 mM $CaCl_2$, pH 7.4) containing the appropriate concentration of glucose. After equilibration in 0.5 mL of Hank's balanced salt solution/glucose (cat: 14025076, Thermo Fisher, United States) at 37°C for 30 min, 0.5 mL of Hank's balanced salt solution containing various concentrations of [3-^{3}H] glucose (cat: NET331C250UC, PerkinElmer, United States) was added, with a final specific activity of 1–3 disintegrations/min/pmol (∼1 mCi/mmol). Aliquots of 100 μL were then transferred to another tube, placed inside a capped scintillation vial containing 0.5 mL of water, and incubated at 45°C for 48 h. After this vapor-phase equilibration step, the tube was removed from the vial, the scintillation mixture was added, and the ^3H_2O content was determined by counting over a 5-min period.

Quantification of Hexokinase (HK) Activity

Brain tissue was washed with PBS, treated with trypsin/EDTA, and centrifuged at 500 × *g* for 5 min at 4°C. Then, the tissue was resuspended in isolation medium (250 mM sucrose (cat: S9378), 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA (cat: E6758), 1 mM DTT (cat: D0632), 2 mg/mL aprotinin (cat: A1153), 1 mg/mL pepstatin A (cat: 77170), and 2 mg/mL leupeptin (cat: L8511) at a 1:3 dilution, sonicated at 4°C, and then centrifuged at 1,500 × *g* for 5 min at 4°C. HK activity of the supernatant was quantified. For the assay, the purified fraction was mixed with the reaction medium (25 mM Tris-HCl (cat: T5941), 1 mM DTT, 0.5 mM NADP/Na^+ (cat: N8035), 2 mM $MgCl_2$, 1 mM ATP (cat: A1852), 2 U/mL G6PDH (cat: G6378), and 10 mM glucose (cat: G8270), and the mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of 10% trichloroacetic acid (cat: T6399, TCA), and the generation of NADPH was measured at 340 nm (Cisternas et al., 2016a). All reagents were purchased from Sigma-Aldrich, United States.

ATP Content

Brain tissues or hippocampal slices were treated with activators/inhibitors, and ATP levels were measured using an ATP determination kit (cat: A22066, Invitrogen/Molecular Probes, United States) (Calkins et al., 2011).

Activity of PFK and AMPK

For brain samples and hippocampal slices, AMPK activity was measured using an antibody specific to the phosphorylated T-172 (active) form of AMPK-α. Detection was performed by ELISA following the manufacturer's protocol. Experiments were conducted in triplicate and repeated at least three times (cat: KHO0651, Thermo Fisher Scientific Inc., United States) (Moreno-Navarrete et al., 2011; Stow et al., 2016). PFK activity was measured using the PFK Colorimetric Assay Kit (cat: K776, BioVision, United States) according to the manufacturer's instructions (Thurley et al., 2017).

Large Open-Field (LOF) Test

A 120 × 120 cm transparent Plexiglas cage with 35-cm-high transparent walls was used to study locomotor and stress behavior in our mouse model. The open field, which measured 40 × 40 cm, was defined as the "center" area of the field. Data were collected using an automatic tracking system (HVS Imagen, United Kingdom). Each mouse was placed alone in the center of the open field, and its behavior was tracked for 20 min. At the end of the session, the mouse was returned to its home cage. The parameters measured included the total time moving and the number of times the mouse crossed the center area of the platform (Walsh and Cummins, 1976; Cisternas et al., 2015).

Novel Object Recognition (NOR) and Novel Object Location (NOL)

The novel object recognition (NOR) and novel object location (NOL) tasks were performed as previously described (Bevins and Besheer, 2006; Vargas et al., 2014). Mice were habituated to the experimental room in the experimental cages for three consecutive days for 30 min per day (three consecutive days) and for 1 h on the testing day. The task was conducted on a 120 × 120 cm transparent Plexiglas platform with 35-cm-high transparent walls containing two identical objects placed at

specific locations. For object familiarization, mice were allowed to explore the platform for 10 min. The animals were subsequently returned to their home cages for 1 h, followed by a 5 min exposure to a novel localization of one of the familiar objects (NOL). The mice were again returned to their home cages for 1 h and were subsequently exposed to a novel object for 5 min. The mice had no observed baseline preference among the different objects. An object preference index was determined by calculating the time spent near the relocated/novel object divided by the cumulative time spent with both the familiar and relocated/novel objects. The cages were routinely cleaned with ethanol following mouse habituation and testing.

Memory Flexibility Test

This test was performed as previously described, and the conditions of the pool were the same as those of a Morris Water Maze (Chen et al., 2000; Salazar et al., 2017). Each animal was trained to one pseudorandom location of the platform per day for 5 days, with a new platform location each day. Training was conducted for up to 10 trials per day until the criterion of three successive trials with an escape latency of 20 s was achieved. When testing was completed, the mouse was removed from the maze, dried and returned to its cage. Animals were tested for the next location on the following day. Data were collected using a video tracking system (HVS Imagen).

ADP Content

ADP levels in whole-cell lysates of primary neurons and slices were measured using an ADP Assay Kit (cat: ab83359, Abcam, United Kingdom), according to the manufacturer's instructions (Chen et al., 2000).

Preparation of Mouse Brain Slices for Electrophysiology

Male mice at the age of 7 months were used to prepare acute coronal slices. Each animal was anesthetized with isoflurane and killed by decapitation, and the brain was removed rapidly. The brain was placed in a beaker with a cold sucrose-based solution composed of the following (in mM): 85 NaCl, 75 sucrose, 3 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 dextrose, 3.5 $MgSO_4$, 0.5 $CaCl_2$, 3 sodium pyruvate, 0.5 sodium L-ascorbate and 3 myo-inositol (305 mOsm, pH 7.4). Coronal sections (350 μm) were cut with a vibratome, and every slice was transferred to a beaker containing the same solution but at 36°C. An hour later, this solution was changed to recording solution, composed of (in mM): 126 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 dextrose, 1 $MgSO_4$, 2 $CaCl_2$, 3 sodium pyruvate, 0.5 sodium L-ascorbate and 3 myo-inositol (305 mOsm, pH 7.4) at room temperature (22°C). The slices were maintained there until used for recording. The recording was performed in the same solution, but its temperature was increased to 32–34°C for the full duration of the experiment. To prevent the γ-aminobutyric acid ($GABA_A$)-mediated inhibitory component from interfering with excitatory postsynaptic potentials (EPSPs), we applied 25 μM of picrotoxin. The experimental data were obtained from at least two brain slices from each animal. Multiple slices from a single animal were considered replicates. They were subsequently averaged representing a single animal. Hence, for WT $n = 8$ mice, two slices each; for J20 Tg: $n = 6$ mice, three slices each; and for J20+ANDRO: $n = 6$ mice, three slices each. Finally, all the n_s values in every experimental group (WT, J20 or J20+ANDRO) were also averaged and expressed as mean ± SEM.

Electrophysiological Recordings and Data Analysis

The slices were placed in a chamber under an upright infrared-differential interference contrast (IR-DIC) fluorescence microscope (Eclipse FNI, Nikon) equipped with a 40× water objective and a light-sensitive camera (TOPICA CCD Camera). We stimulated the Schaffer collaterals between CA3 and CA1 with a bipolar concentric electrode (World Precision Instruments, Sarasota, FL, United States) connected to an ISO-Flex stimulus generator (A.M.P.I., Jerusalem, Israel). To record the evoked field excitatory postsynaptic potentials (fEPSPs), we used a borosilicate glass electrode (World Precision Instruments, United States) ranging from 0.5 to 1 MΩ pulled on a P-97 Flaming/Brown Micropipette Puller (Sutter Instruments, United States). Each glass pipette was filled with the recording solution and placed in the *stratum radiatum* of the CA1 hippocampal region. The signal was collected using a MultiClamp 700B amplifier (Axon CNS, Molecular Devices LLC, United States) and digitally sampled at 30 kHz using a Digidata-1440A interface (Axon CNS, Molecular Devices). The data were acquired and analyzed offline using pClamp 10 (Molecular Devices LLC, United States). First, in each slice, we determined the relationship between the stimulus intensity of input and the magnitude of the evoked response. For this purpose, increasing levels of current were applied, and the slope of evoked fEPSP was determined. The intensity to evoke 60% of the maximum amplitude was used to perform the experiments. Second, to calculate the paired-pulse facilitation (PPF) index, we calculated the ratio of the second EPSP slope (R2) by the first EPSP slope (R1), evoked at different interstimulus intervals between 10 and 200 ms. Third, for long-term plasticity, every experiment consisted of the application of two pulses separated 50 ms each, every 15 s. The slope value of the first pulse (R1) was averaged during 15 to 20 min before TBS to obtain basal data, and then during 60 min after TBS to obtain the LTP induced data. We allowed enough time to obtain a stable basal signal and have at least 20 min of continuous data before applying theta burst stimulation (TBS). The necessary time period consisted of a series of 5 burst at 100 Hz, every 20 s. Subsequently, the same two pulses every 15 s were taken for 60 min more. To calculate the amount of long-term potentiation (LTP), we measured the slope (mV/ms) of the first fEPSP and averaged the 20 min pre-TBS and normalized pre- and post-TBS against this value (mean) at each time point. The normalized change was plotted as the relative value of fEPSP slope. To determine the degree of potentiation among groups, we compared. We also measured the fiber volley (FV) amplitude to show the correlation with fEPSP slope changes. Further analyses were performed using the same data. To determine the degree of facilitation, we measured the slope of the first evoked fEPSP

(R1) and the slope of the second evoked fEPSP (R2), and we established the (R2 − R1)/R1 or relative facilitation, which is a measure of facilitation, before and after TBS. These data were used to determine the PPF index, which is a measure of presynaptic Ca^{2+} and PPF attenuation, which correlates the PPF with LTP and postsynaptic mechanisms. All values of synaptic responses are presented as the mean ± SEM.

Synaptosome Preparation

Synaptosomes were prepared from hippocampus samples isolated from mouse brains. Hippocampi were homogenized in a weight:volume ratio of 1:10 using cold lysis buffer containing 0.3 M sucrose, 5 mM Hepes (pH 7.4) and protease and phosphatase inhibitors using an automatic Potter Elvehjem tissue homogenizer with Teflon Pestle (Pyrex) for 10 strokes at 3,000 rpm. The homogenate was centrifuged at 1,000 × *g* for 20 min, and then the supernatant was centrifuged at 16,000 × *g* for 20 min. The resultant pellet was resuspended in buffer 0.32 M sucrose, 5 mM Hepes pH 7.4 and passed six times through a 21-gauge syringe. The resuspended pellet was layered on top of a sucrose gradient of 0.85 M, 1.0 M, and 1.2 M and centrifuged at 100,000 × *g* for 2 h. The interface between 1.0 M and 1.2 M sucrose, corresponding to enriched synaptosomes, was fixed for electron microscopy. Synaptosomes were prepared from three animals in each group, and five to eight synapses were analyzed per group. Tukey's multiple comparison test was performed to compare the means.

Electron Microscopy

The synaptosome pellet was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.0 at room temperature overnight and was washed in three changes of cacodylate buffer for 2 h. The pellet was postfixed with aqueous 1% osmium tetroxide for 120 min, and after a rinse with bidistilled water, it was stained in block with 1% uranyl acetate for 90 min. The specimen was then dehydrated with graded concentrations of acetone (50, 70, 95, and 100%) for 30 min each, then pre-embedded with Epon: acetone 1:1 overnight and then included in pure Epon. The polymerization was carried out in an oven at 60°C for 48 h. Thin sections (80 nm) were cut with a Leica Ultracut R ultramicrotome, then stained with 1% uranyl acetate in methanol for 2 min and with Reynolds lead citrate for 5 min. The slices were observed with a Philips Tecnai 12 Biotwin microscope (Eindhoven, The Netherlands) at 80 kV.

Statistical Analysis

Unless specified, all experiments were performed three times, with triplicates for each condition in each experimental run (the graphs show the average of 9–15 points). The results are expressed as the means ± standard errors. The data were analyzed by one-way or two-way analysis of variance (ANOVA), followed by Bonferroni's *post hoc* test; $p \leq 0.05$ was considered the threshold for statistical significance with 95% confidence interval (CI). If other statistical analysis was used, it was specified accordingly. Statistical analyses were performed using Prism software (GraphPad, United States). To test normality, we used the numerical method in SPSS Statistics software (IBM, United States) using the numerical method.

RESULTS

In vivo Treatment With ANDRO Increases Brain Glucose Accumulation and Key Metabolic Markers

A reduction in cellular energy metabolism is an accurate marker of AD progression (Kapogiannis and Mattson, 2011; Cisternas and Inestrosa, 2017). Knowing that ANDRO increases glucose metabolism in symptomatic AD animals, we asked whether preventive administration of ANDRO was able to delay metabolic dysfunction. Mice were treated with ANDRO from postnatal week 13 for 16 weeks, and the treatment was finished when animals were 7 months old (**Figure 1A**). At this age, Tg animals show obvious cognitive deficits (Mucke et al., 2000; Saganich et al., 2006; Cheng et al., 2007; Tapia-Rojas and Inestrosa, 2018). First, we measured brain glucose accumulation by injecting radioactive glucose (D-[1-^{14}C] glucose) via the tail vein. After 15 min, we measured the amount of radioactivity in the whole brain and in the regions most affected in AD, i.e., hippocampus and cortex. Glucose accumulation in the whole brain of J20 Tg mice was 38% lower than that observed in the WT mice. The ANDRO treatment increased the glucose levels of the J20 Tg whole brain near the WT values (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, WT vs. J20 $^*p < 0.05$; J20 vs. J20+ANDRO $^*p < 0.05$) (**Figure 1B**). In the hippocampus, glucose accumulation was reduced ∼70% in J20 Tg mice relative to WT mice. The treatment of J20 Tg mice with ANDRO recovered glucose accumulation close to WT values (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, WT vs. J20 $^*p < 0.05$; J20 vs. J20+ANDRO $^*p < 0.05$) (**Figure 1C**). No significant differences in cortical glucose accumulation were observed in the three animal groups of this study (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, WT vs. J20 p = n.s.; J20 vs. J20+ANDRO p = n.s.) (**Figure 1D**). Hence, the accumulation of glucose is impaired in the hippocampus of J20 Tg mice, and it recovers to nearly normal values with presymptomatic ANDRO treatment.

We went a step further and examined the intracellular pathway of glucose utilization that is affected in the J20 Tg mice and targeted by ANDRO. After glucose is taken in by cells, it breaks down through glycolysis, a metabolic pathway that has several control points and includes the enzymes hexokinase (Hk) and phosphofructokinase (Pfk) and the brain metabolic sensor 5′ AMPK (Burkewitz et al., 2014). We measured the activity of Hk, Pfk, and AMPK and the activity of several metabolic markers in the hippocampus and cortex in all three groups in the present study. We did not find changes in Hk activity, the first control point for glucose utilization, in either the cortex or the hippocampus of WT, J20 Tg or J20 Tg+ANDRO mice (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, Cx WT vs. Cx J20 p = n.s.; Cx J20 vs. Cx J20+ANDRO p = n.s.; Hipp WT vs. Hipp J20 p = n.s.; Hipp J20 vs. Hipp

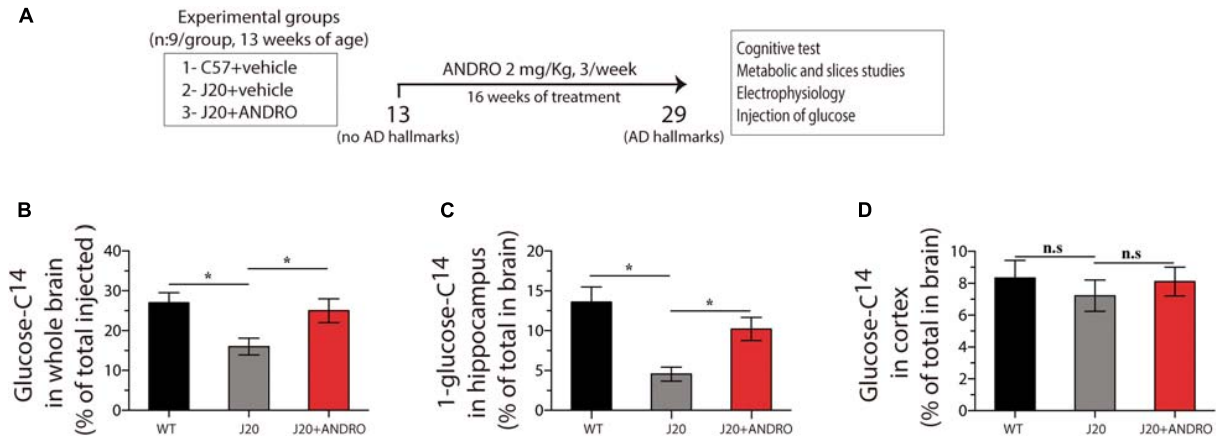

FIGURE 1 | Treatment with ANDRO increases the in vivo uptake of glucose. Schematic of the protocol for assaying the effect of ANDRO administration in a presymtomatic stage **(A)**. After the indicated treatments, radioactive glucose was injected in the tail vein, and 15 min later, glucose uptake in the whole brain **(B)**, hippocampus **(C)**, and cortex **(D)** was measured. The magnitude of the decrease in glucose uptake was mitigated in J20 mice. ANDRO treatment improved glucose uptake in the J20 animals to levels similar to those of WT mice. Data represent the mean ± SEM of $n = 6$ (number of animals for treatment), $p \leq 0.05$ (95% CI) was considered the threshold for statistical significance, Bonferroni test, p-values are indicated in the "Results" Section.

J20+ANDRO, p = n.s., **Figure 2A**). Interestingly, we observed a strong decrease in the activity of phosphofructokinase (Pfk) in both the hippocampus (2.56 ± 0.33) and the cortex (1.98 ± 0.21) of J20 Tg animals relative to control WT animals (3.88 ± 0.4 and 3.23 ± 0.34, respectively). The J20 Tg mice treated with ANDRO showed higher levels of Pfk activity in the cortex (4.35 ± 0.22) and hippocampus (4.97 ± 0.53), similar to the values observed in the WT mice (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, Cx WT vs. Cx J20 $^{*}p = 0.05$; Cx J20 vs. Cx J20+ANDRO $^{**}p = 0.01$; Hipp WT vs. Hipp J20 $^{*}p = 0.05$; Hipp J20 vs. Hipp J20+ANDRO, $^{*}p = 0.04$, **Figure 2B**). As mentioned above, one of the most important metabolic regulators of glucose metabolism is the enzyme AMPK. Thus, we measured the activity of this enzyme in the cortex and hippocampus. We detected a decrease in the activity of this enzyme in the hippocampus of J20 Tg mice compared with WT mice (4.55 ± 0.49), but not in the cortex (9.11 ± 0.92), and activity values similar to those of WT animals were found in the J20 Tg+ANDRO mice (8.53 ± 0.49 and 7.93 ± 0.83, respectively) (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, Cx WT vs. Cx J20 p = n.s.; Cx J20 vs. Cx J20+ANDRO p = n.s.; Hipp WT vs. Hipp J20 $^{*}p = 0.05$; Hipp J20 vs. Hipp J20+ANDRO, $^{*}p = 0.05$, **Figure 2C**). Taken together, the evidence shows that Pkf is the enzyme with the most severely affected activity in the cortex and hippocampus of J20 Tg mice and that ANDRO treatment is able to restore its activity to WT levels. Moreover, ANDRO mitigated the decrease in AMPK activity the hippocampus of J20 Tg mice.

We also measured the levels of ATP and the ATP/ADP ratio. We observed a decrease of ∼42% in the ATP levels in the cortex and hippocampus in the J20 mice, the treatment with ANDRO was able to recover in part the levels of ATP (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, Cx WT vs. Cx J20 $^{*}p = 0.05$; Cx J20 vs. Cx J20+ANDRO $^{**}p = 0.01$; Hipp WT vs. Hipp J20 $^{*}p = 0.05$; Hipp J20 vs. Hipp J20+ANDRO, $^{*}p = 0.05$, **Figure 2D**). The recovery in the levels of ATP was directly related with the increase in the ratio ATP/ADP, a direct form to describe an increase in the synthesis of ATP, the ratio value of the J20+ANDRO was similar to the WT values (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, Cx WT vs. Cx J20 $^{**}p = 0.01$; Cx J20 vs. Cx J20+ANDRO $^{*}p = 0.05$; Hipp WT vs. Hipp J20 $^{**}p = 0.01$; Hipp J20 vs. Hipp J20+ANDRO, $^{*}p = 0.05$, **Figure 2E**).

Glucose Metabolism in Slices From J20 Tg Animals Is Altered and Rescued by ANDRO Treatment

Next, to evaluate whether the augmented metabolic rate observed in the hippocampus of J20 Tg mice treated with ANDRO correlates with glucose cellular uptake, we measured the uptake of radioactive glucose by hippocampal slices at different times (**Figure 3Ai**). Hippocampal slices were recovered from the three groups of animals. At 15 min, the uptake of glucose was 3.21 ± 0.16 nmol/mg protein, 1.32 ± 0.13 nmol/mg protein and 1.71 ± 0.09 nmol/mg protein for WT, J20 Tg and J20 Tg+ANDRO, respectively. Significantly less glucose uptake was observed at 90 min in J20 Tg animals (5.10 ± 0.15 nmol/mg protein) relative to the control WT (7.83 ± 0.27 nmol/mg protein). Interestingly, J20+ANDRO slices showed a glucose uptake of 6.97 ± 0.12 nmol/mg (For the three curves, two-way repeated measures ANOVA: interaction: $F(18,60) = 14.46$, $p < 0.001$; animal group factor: $F(2,60) = 228.75$; time: $F(9,60) = 790.09$; **Figure 3A**. For the last point (90 min), one-way ANOVA: $p < 0.001$, followed by Bonferroni *post hoc* test, WT vs. J20 $^{***}p < 0.001$; J20 vs. J20+ANDRO $^{**}p < 0.01$; **Figure 3Aii**), close to WT animals, suggesting that the reduced glucose uptake in J20 Tg mice can be restored by ANDRO treatment.

Another approach to measure the cellular utilization of glucose is to determine the glycolytic rate by measuring the radioactive $^{3}H_2O$ molecules generated from enolase activity

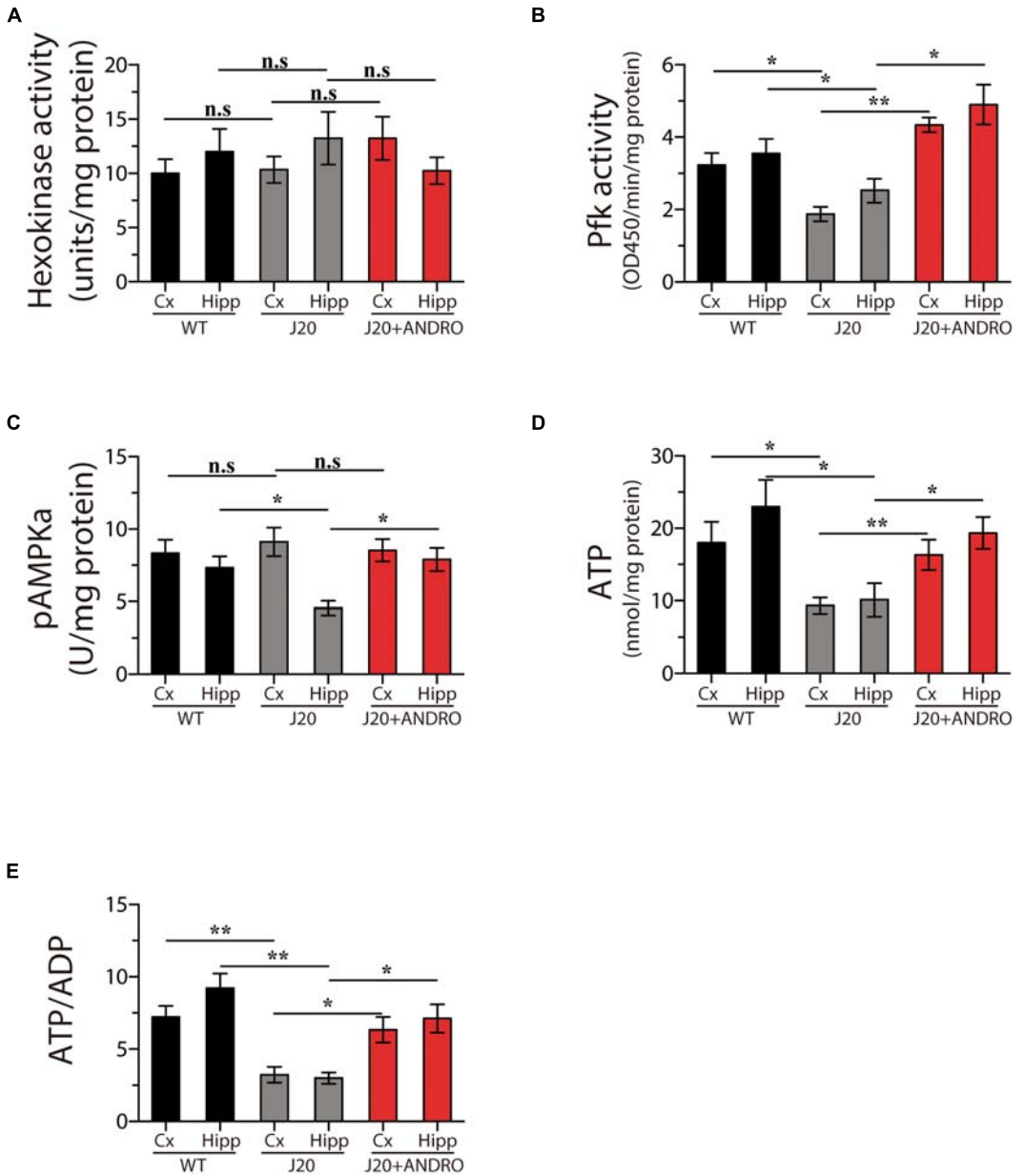

FIGURE 2 | ANDRO treatment induces recovery of general brain metabolism. Treatment with ANDRO stimulated the enzymatic activity of two checkpoint proteins of glycolysis: HK **(A)** and Pfk **(B)**. Effects of treatments on the levels of AMPK, an important metabolic sensor of glucose metabolism **(C)**. ATP **(D)** and the ATP/ADP ratio **(E)** were measured to correlate the previous parameters with the energy status of cells. ANDRO restored the levels of both parameters in J20 mice. Data represent the mean ± SEM of $n = 3$ (independent experiments), each performed in triplicate. $p \leq 0.05$ (95% CI) was considered the threshold for statistical significance, Bonferroni test, *p*-values are indicated in the "Results" Section.

(**Figure 3Bi**). A lower glycolytic rate was measured after 90 min in the J20 Tg mice (3.71 ± 0.20 pmol/mg protein) compared with the WT mice (8.57 ± 0.35 pmol/mg protein). Treatment with ANDRO partially rescued the level of activity after 90 min (5.52 ± 0.26 pmol/mg protein) [for the three curves, two-way repeated measures ANOVA: interaction: $F(8,30) = 41.49$, $p < 0.001$; animal group factor: $F(2,30) = 123.31$; time: $F(4,30) = 622.49$. For the last point (90 min), one-way ANOVA: $p < 0.001$, followed by Bonferroni *post hoc* test, WT vs. J20 $^{***}p < 0.001$; J20 vs. J20+ANDRO $^{*}p < 0.05$; **Figure 3Bii**]. Finally, to correlate the uptake of glucose and the glycolytic rate with ATP production, we measured ATP levels and ATP/ADP ratio (**Figures 3Ci,ii**, respectively). The brain slices obtained from J20 Tg animals showed a decrease of 50% in the levels of ATP and a similar decrease in the ATP/ADP ratio (5.23 ± 0.24) compared to what was observed in the WT mice (9.12 ± 0.93). The slices obtained from the J20 Tg+ANDRO group showed higher levels of ATP (one-way ANOVA, $p < 0.05$, followed by Bonferroni's

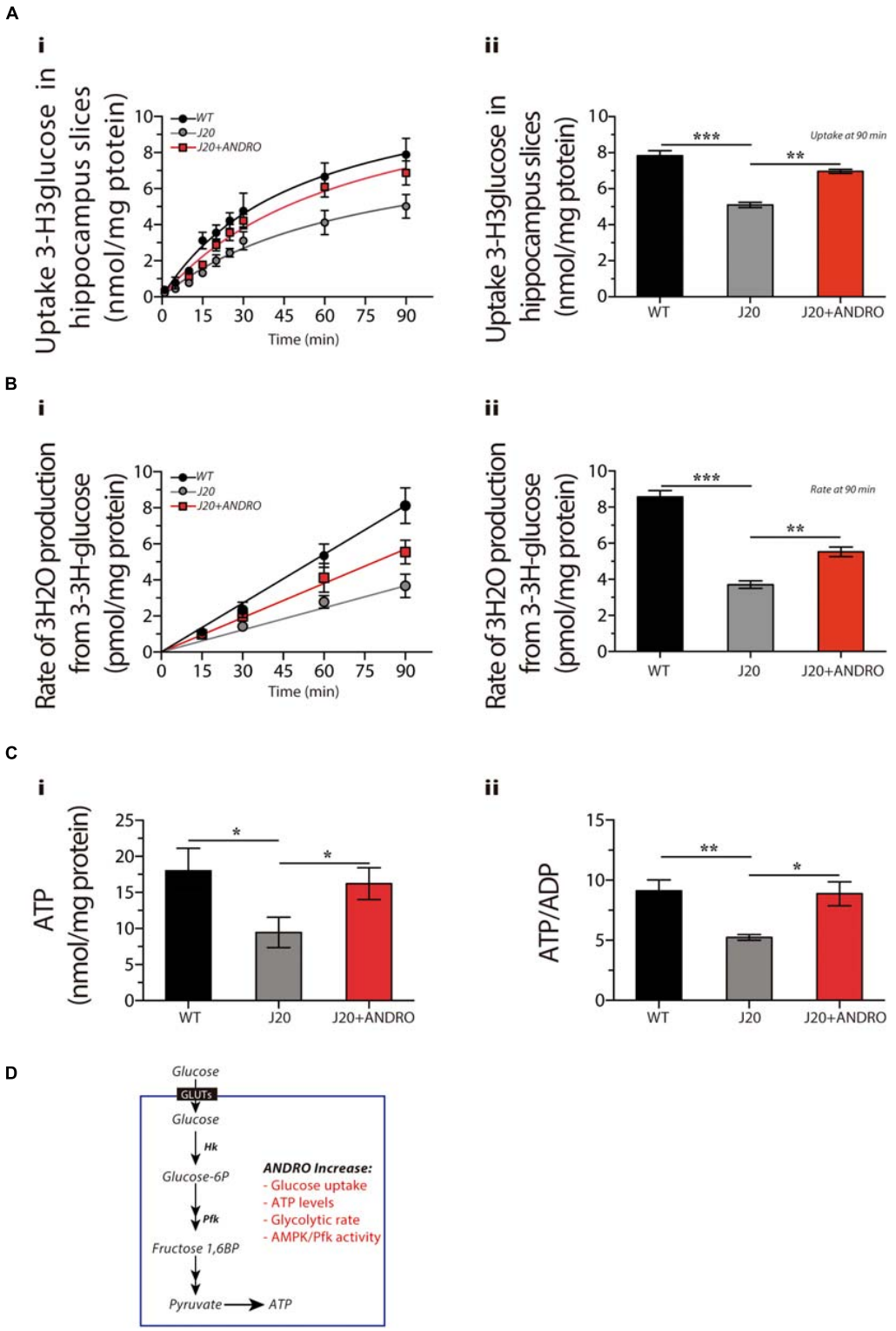

FIGURE 3 | ANDRO promotes the utilization of glucose and enhances the production of ATP in hippocampal slices. The uptake of radioactive glucose **(Ai)** in slices obtained from WT, J20, and J20+ANDRO. Treatment with ANDRO increased the uptake of glucose in J20 slices to levels similar to WT at 90 min **(Aii)**. Glycolytic rate after treatment **(Bi)**. The decreased glycolytic rate of J20 slices was rescued by treatment with ANDRO **(Bii)**. Both ATP and the ATP/ADP ratio were decreased in slices from J20 mice, and both were increased in the J20+ANDRO mice (**Ci,ii**, respectively). **(D)** Presymptomatic treatment with ANDRO induces recovery of several metabolic markers, including glucose uptake, glucose utilization through glycolysis and ATP recovery. The data represent the mean ± SEM of $n = 3$ (independent experiments), each performed in triplicate. $p \leq 0.05$ (95% CI) was considered the threshold for statistical significance, details in the "Results" Section.

post hoc test, WT vs. J20 $^{*}p = 0.05$; J20 vs. J20+ANDRO $^{*}p = 0.05$, **Figure 3Ci**) and ATP/ADP ratios (8.87 ± 0.99), similar to WT mice (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, WT vs. J20 $^{**}p = 0.01$; J20 vs. J20+ANDRO $^{*}p = 0.05$, **Figure 3Cii**). Our results indicate that preventive treatment with ANDRO was able to rescue several markers of energy metabolism (**Figure 3D**) that were deficient in the J20 Tg mice. To study whether these findings correlate with an improvement in neuronal activity, we analyzed and compared the cognitive performance of the Tg animals and the effect of ANDRO on neuronal plasticity.

Early Administration of ANDRO Rescues Cognitive Deterioration

We explored whether administration of ANDRO in the presymptomatic stage delays the cognitive impairment of J20 Tg mice. First, we first studied the activity of the mice using the open-field test. This test measures the time spent moving and the number of times that the animal crosses the center of the cage. Both parameters are used to rule out movement or stress problems. We observed no significant changes among groups, showing that the basal status does not affect the animal's performance (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, WT vs. J20 p = n.s.; J20 vs. J20+ANDRO p = n.s., **Figures 4Ai,ii**).

To evaluate memory, we performed NOR and NOL tests, both associated with hippocampal function. In the NOR test, we observed a significant decrease in the preference index in the J20 Tg mice treated with vehicle in comparison with the WT mice. However, in the J20 Tg animals treated with ANDRO, we observed preference indexes similar to those of WT mice (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, WT vs. J20 $^{**}p = 0.01$; J20 vs. J20+ANDRO $^{**}p = 0.01$, **Figure 4B**). In the NOL test, we observed that the treatment with ANDRO was able to recover the cognitive decline observed in the J20 Tg mice treated with vehicle (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, WT vs. J20 $^{*}p = 0.05$; J20 vs. J20+ANDRO $^{*}p = 0.05$, **Figure 4C**). The results suggest that ANDRO protects against memory loss in J20 Tg mice.

Next, we performed a highly sensitive memory flexibility test to study learning and memory performance (Chen et al., 2000). On the first trial day, the WT mice needed eight trials to reach criterion, while the J20 Tg mice treated with ANDRO or vehicle needed 13 and 14 trials, respectively (**Figure 4D**). However, after 4 days of testing, the WT mice needed six trials to reach criterion, whereas the J20 Tg mice needed the same 14 trials, showing a decrease in learning/memory performance (**Figure 4Di**). Interestingly, after 4 days, the J20 Tg+ANDRO mice needed only nine trials to reach criterion, showing that the treatment with ANDRO was able to delay the cognitive decline that affected memory/learning [**Figure 4D**: two-way repeated measures ANOVA: interaction $F(6,72) = 4.26$, $p < 0.01$; animal group factor: $F(2,72) = 222.54$; days: $F(3,72) = 12.65$. **Figure 4Dii**: we compared among groups the performance at the fourth day, observing significant differences in all groups. **Figure 4Dii**: one-way ANOVA, $p < 0.001$, followed by Bonferroni *post hoc* test, WT vs. J20 $^{***}p < 0.001$; J20 vs. J20+Andro $^{***}p < 0.001$].

Deficiencies in Basal Activity and Synaptic Plasticity Occurs in J20 Animals and Are Rescued by ANDRO

To determine whether cognitive disabilities and the restoration caused by ANDRO have a synaptic correlate, we studied the electrophysiological parameters involved in synaptic transmission and plasticity mechanisms. To do this, we studied synaptic transmission in the well-known synapse formed by Schaffer collaterals in the CA1 hippocampal region in 7-month-old WT, J20 and J20+ANDRO mice. We stimulated the collateral axons and recorded them at the level of the CA1. We applied increasing levels of current intensities that evoked increased fEPSP slopes and FV amplitudes, and we constructed an input–output relationship (**Figure 5A**). As shown, the plot of fEPSP slope against stimuli amplitude demonstrated that J20 animals have a deficiency in basal synaptic transmission compared to WT animals or to Tg animals treated with ANDRO [**Figure 5B**, measured at the maximum current intensity: WT = 0.38 ± 0.05 mV/ms; J20 = 0.18 ± 0.05 mV/ms; J20+ANDRO = 0.35 ± 0.04 mV/ms; two-way repeated measures ANOVA: interaction: $F(20,220) = 1.63$, $p > 0.05$; animal group factor: $F(2,220) = 29.35$, $p < 0.001$; stimulation intensity: $F(10,220) = 30.61$, $p < 0.001$, followed by Bonferroni *post hoc* test, $^{*}p < 0.05$, $^{**}p < 0.01$]. The input–output relationship based on the FV versus stimulus amplitude did not show a difference between animals, demonstrating that the number of axons recruited and their excitability did not differ between animal groups (measured at the maximum current intensity: WT = 0.8 ± 0.23 mV/ms; J20 = 0.53 ± 0.19 mV/ms; J20+ANDRO = 0.63 ± 0.17 mV/ms) (**Figure 5C**). We also plotted the correlation between the FV amplitude and the fEPSP slope (**Figure 5D**). Interestingly, the regression analysis resulted in a significant positive association between fEPSP slopes values and fiber volley amplitude for our three treatments [$WT_{slope} = 0.459 \pm 0.008$, R^2 0.99, $F(1,9) = 3498.13$, $p < 0.001$; $J20_{slope} = 0.3125 \pm 0.016$, R^2 0.98, $F(1,9) = 390.18$, $p < 0.001$; $J20{+}ANDRO_{slope} = 0.530 \pm 0.021$, R^2 0.99, $F(1,9) = 659.18$, $p < 0.001$; **Figure 5D**], demonstrating that the strength of synaptic transmission per activated axon is different among groups. Thus, J20 Tg mice that show a relatively small slope correlation also show an increase when treated with ANDRO. These data suggest that is not the number of axons but a synaptic mechanism that is altered in the J20 mice and that ANDRO pretreatment is able to revert.

We next investigated hippocampal synaptic plasticity in the form of LTP. Previously, our laboratory demonstrated that J20 Tg has several characteristics of the disease (Tapia-Rojas and Inestrosa, 2018). Then, we hypothesized that some of these defects would have consequences in the mechanisms that generate long-term synaptic plasticity. After obtaining a stable baseline of evoked fEPSPs, we applied TBS to induce LTP.

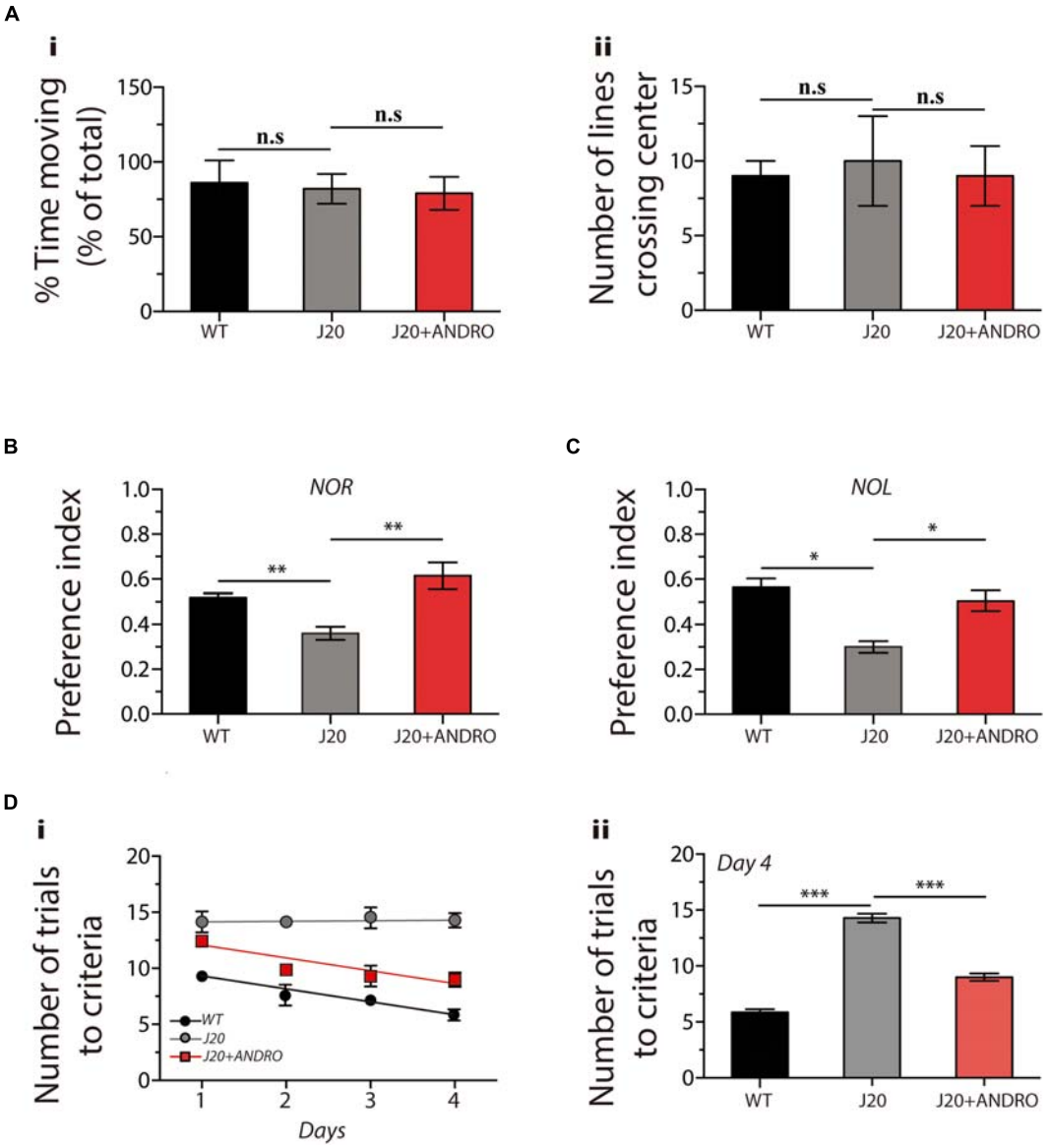

FIGURE 4 | The administration of ANDRO improves cognitive performance in a transgenic model of AD. After the indicates treatments, cognitve test related to hippocampal function were performed: large open field **(A)**, NOR **(B)**, NOL **(C)** and memory flexibility **(D)**. Data represent the mean ± SEM of $n = 9$ (number of animals), $p \leq 0.05$ (95% CI) was considered the threshold for statistical significance, details in the "Results" Section.

Two examples of fEPSPs evoked before and after TBS in WT, J20, and J20+ANDRO, are shown (**Figure 6A**). The analysis of the fEPSP slope after TBS relative to the slope before TBS was plotted against time and is shown in the graph (**Figure 6B**). The shape of LTP induction shows a slow time course until reaching a steady state in all three groups. This slow induction might be related to the differential equilibrium between long-term potentiation and depression (LTD), which shifts toward LTD in aged animals (Megill et al., 2015), or to age-dependent differences in the induction of LTP. Despite the differences in LTP amplitude observed in the plot, WT, J20 and J20+ANDRO showed significant potentiation above the basal amplitude (**Figure 6B**). The averaged fEPSP slope value of J20 Tg mice (60 ± 5% of baseline) after an hour of LTP induction is smaller than WT (150 ± 10% of baseline), while J20+ANDRO (110 ± 3% of baseline) shows better potentiation than J20 Tg (one-way ANOVA, $p < 0.001$, followed by Bonferroni *post hoc* test, WT vs. J20 $^{***}p < 0.001$; J20 vs. J20+ANDRO $^{***}p < 0.001$; WT vs. J20+ANDRO $^{**}p < 0.01$). This means that treatment with ANDRO increases the potentiation level in the transgenic mice almost twofold. The differences between animal groups under an LTP protocol should be attributable to postsynaptic mechanisms. To demonstrate that in our experiments, we first measured the range of FV amplitudes before and after LTP

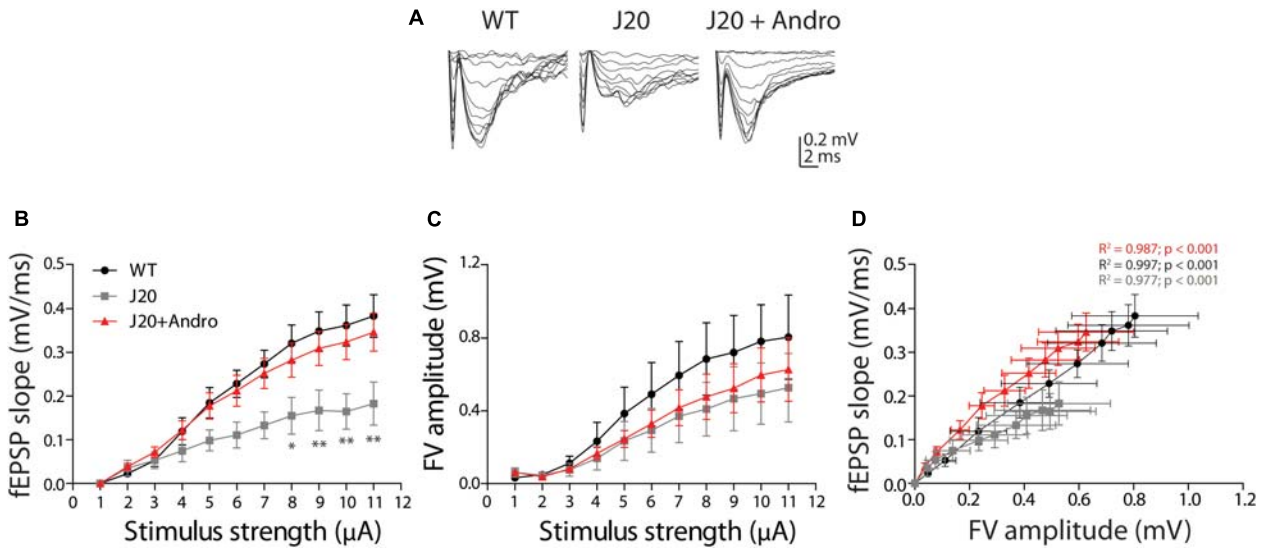

FIGURE 5 | Input–output curves showing reduced basal synaptic transmission in J20 Tg mice. Representative fEPSPs **(A)** evoked in response to successive increments of current intensity (from 1 to 11 μA), in WT (black dots), J20 (gray squares) and J20+ANDRO (red triangles). Input–output curves of fEPSP slope **(B)** and FV amplitude **(C)**, both against stimulus intensity, demonstrating deficient synaptic transmission. Linear regression plot of fEPSP slopes dependence on FV amplitude **(D)**, evidenced that Tg animals cannot sustain responses with high stimulation. In B, WT vs. J20+ANDRO are significantly different from J20 [two-way repeated measures ANOVA: interaction: $F(20,220) = 1.63$, $p > 0.05$; animal group factor: $F(2,220) = 29.35$, $p < 0.001$; stimulation intensity: $F(10,220) = 30.61$, $p < 0.001$, followed by Bonferroni *post hoc* test: WT vs. J20 at 8 μA $^*p < 0.05$, 9 to 11 μA $^{**}p < 0.01$; J20 vs. J20+Andro at 8 μA $^*p < 0.05$, 9 to 11 μA $^{**}p < 0.01$; WT vs. J20+Andro p = n.s.]. In panel **(D)**, the data were fitted with a linear regression: $R^2 = 0.99$, 0.98, and 0.99 correspondingly; and slopes for WT = 0.459 ± 0.008; J20 = 0.3125 ± 0.016; J20+ANDRO = 0.530 ± 0.021. ANCOVA analysis between WT and J20: $F(1,19) = 12.51$, $p = 0.002$; WT and J2+ANDRO: $F(1,19) = 49.87$, $p < 0.001$; and J20 and J20+ANDRO = $F(1,19) = 37.64$, $p < 0.001$, gave significant differences among all linear regressions.

induction (WT before: 0.47 ± 0.006 mV, after: 0.46 ± 0.001 mV, p = ns; J20 before: 0.41 ± 0.005 mV, after: 0.39 ± 0.002, p = ns; J20+ANDRO before: 0.29 ± 0.003 mV, after: 0.28 ± 0.001 mV, p = ns, by two-way ANOVA and Bonferroni *post hoc* test). Then, we plotted the input–output function of these experiments. We averaged the FV amplitudes of all experiments and plotted them against the averaged fEPSP slope of all experiments, both during basal stimulation (open symbols) and after LTP induction (solid symbols) (**Figure 6C**). Therefore, every point represents the averaged FV value and the averaged slope of the evoked response. We analyzed the correlation between these two variables to quantify the degree to which they are related, and whether they differ among the three groups. By Pearson's correlation we found the correlation coefficient 'r' of each group during pre-TBS condition (open symbols) (by Pearson's correlation WT: $r = 0.5290$, J20: $r = 0.0152$, J20+ANDRO: $r = 0.3244$). Using Fisher 'r' to 'z' transformation, we used z-scores to compute the significance of the difference when we compare two correlation coefficients (WT vs. J20: z = 2.11, two-tailed $p = 0.032$; J20 vs. J20+ANDRO: $z = 0.93$, two-tailed $p = 0.352$; WT vs. J20+ANDRO: $z = -1.18$, two-tailed $p = 0.238$). When z is positive means that the 'r' of that group (e.g., WT) is greater than the one which is compared to (e.g., J20); if z its negative, the 'r' of the first group (e.g., J20) is smaller than the one which is compared to (e.g., J20+ANDRO). The first case is the only one significant: WT correlation coefficient is greater than the 'r' of J20. Then, we found the correlation coefficient 'r' of each group during post-TBS condition (closed symbols) (by Pearson's correlation WT: $r = -0.1430$, J20: $r = 0.3657$, J20+ANDRO: $r = 0.07478$). As above, z-scores were computed to assess the significance of the difference between two correlation coefficients (WT vs. J20: $z = -5.78$, two-tailed $p = 0$; J20 vs. J20+ANDRO: $z = 3.39$, two-tailed $p = 0.0007$; WT vs. J20+ANDRO: $z = -2.4$, two-tailed $p = 0.016$). We found significant differences in all comparisons: WT and J20+ANDRO are significantly less correlated than J20, what is explained by their increment in slope after TBS. Interestingly, the response to ANDRO pretreatment shows that the system appears to be optimized to generate a broad range of responses or synaptic transmission with reduced axonal recruitment activity. Assuming that the number of axons is consistent among the different animal groups (**Figure 5C**), we propose that ANDRO acts at the presynaptic region and modulates the postsynaptic machinery to optimize the strength of the response.

To decipher the mechanisms targeted by ANDRO, we analyzed the PPF, which is a presynaptic form of plasticity. This protocol measures the probability of release of presynaptic terminals. We evoked two presynaptic spikes separated at different times (10–200 ms) and recorded the postsynaptic responses (fEPSP) as R1 and R2. The resultant PPF index is shown in **Figure 7B**. The three groups showed facilitation (ratio above 1) at a range of interstimulus intervals (10–90 ms). At this time, WT is undistinguishable from J20 Tg+ANDRO, and J20 shows a significantly lower ratio level [WT: 2.77 ± 0.36; J20: 2.02 ± 0.24; J20+ANDRO: 2.98 ± 0.16; two-way repeated

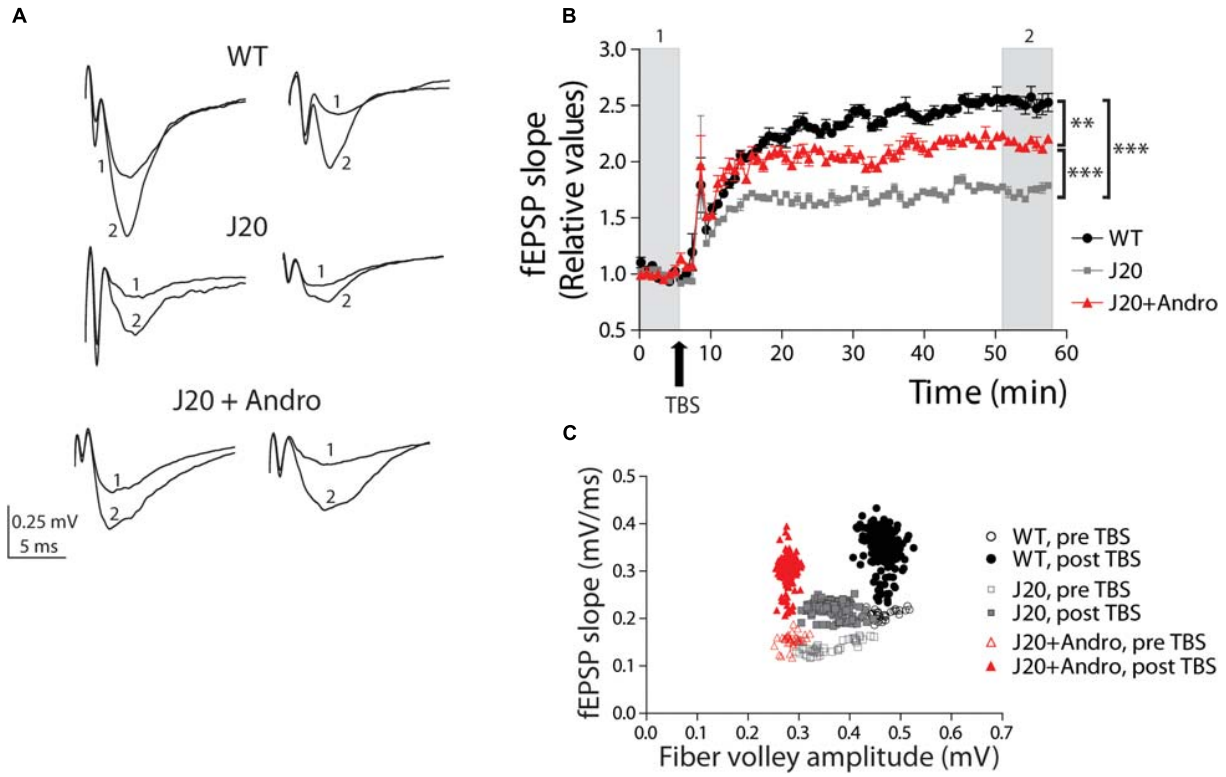

FIGURE 6 | Pretreatment with ANDRO increases the level of LTP. Two representative traces of field EPSPs **(A)** recorded before (1) and after LTP induction (2) in each animal group. WT (black dots), J20 (gray squares), and J20+ANDRO (red triangles). Twenty minutes of basal activity was recorded **(B)** before LTP was induced by theta burst stimulation (TBS, arrow), as explained in the "Results" Section. The fEPSPs slope measurements after LTP induction were normalized to the slope measurements during basal time. Treatment with ANDRO increased the amount of LTP compared to J20, but to a lesser extent than WT. After TBS, comparing the averages of the last 10 min among groups, all three curves become significantly different (one-way ANOVA, $p < 0.001$, followed by Bonferroni *post hoc* test WT vs. J20, $^{***}p < 0.001$; WT vs. J20+ANDRO, $^{**}p < 0.01$; J20 vs. J20+ANDRO, $^{***}p < 0.001$). **(C)** Correlation plot between FV amplitude and fEPSP slope before and after LTP induction. WT: pre-TBS (open black circles), post-TBS (solid black circles); J20: pre-TBS (open gray squares), post-TBS (solid gray squares); J20+ANDRO: pre-TBS (open red triangles), post-TBS (solid red triangles). The correlation between FV amplitude and fEPSP slope was found using Pearson's correlation. Using Fisher '*r*' to '*z*' transformation, we used *z*-scores to compute the significance of the difference when we compare two correlation coefficients before TBS (open symbols). The same analysis was performed to compare correlation coefficients after TBS (closed symbols). After LTP induction, we found significant differences in all group comparisons (for details, see main text). Data represents the mean ± SEM from 16 slices of 8 (WT) mice; 18 slices of 6 (J20) mice; 18 slices of 6 (J20+Andro) mice. Further details, see Section "Materials and Methods."

measures ANOVA: interaction: $F(12,105) = 1.09$, $p > 0.05$; animal group factor: $F(2,105) = 19.87$, $p < 0.001$; interstimulus intervals: $F(6,105) = 18.58$, $p < 0.001$, followed by Bonferroni *post hoc* test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$]. We also measured the PPF between pulses before and after TBS (**Figures 7A,C,D**). The application of two pulses (R1 and R2) separated by 50 ms during the experiment allowed us to determine the degree of facilitation and how this value changed with the induction of LTP. **Figure 7A** shows two examples of WT, J20 and J20+ANDRO, with the two pre-TBS evoked responses (R1 and R2) and its corresponding (R1' and R2') 40 min after application of TBS. We plotted the relative facilitation [(R2 − R1)/R1] before and after TBS [(R2' − R1')/R1']. We showed that even during basal transmission, the facilitation index differs significantly between WT and J20 and between J20 and J20+ANDRO (WT: 2.3 ± 0.5; J20: 0.7 ± 0.2; J20+ANDRO: 1.9 ± 0.4, by one-way ANOVA and *post hoc* Bonferroni test, $^{**}p < 0.01$, $^{*}p < 0.05$) (**Figure 7C**). This finding confirms that the facilitation index determined by PPF is severely defective in J20, while ANDRO is able to revert this condition, showing values close to WT (**Figure 7C**). Strikingly, the facilitation index after TBS shows that facilitation decreases in WT and J20+ANDRO, which is expected if the first pulse increases more in relation to the increment in the second pulse (WT: 1.9 ± 0.4; J20+ANDRO: 1.4 ± 0.3; J20: 0.6 ± 0.2, by one-way ANOVA and *post hoc* Bonferroni test, $^{*}p < 0.05$). A summary of both sets of graph data, showing the comparison within groups, is shown below (**Figure 7D**). These data indicate another characteristic of the plasticity process, that is, the dynamics of PPF during LTP. TBS induces LTP but also attenuation of PPF responses. This suggests that the expression of LTP can induce changes in the presynaptic component (Schulz et al., 1994). The plot of R1 and R2 shows the dynamic change in synaptic transmission after TBS (WT: R1 = 232 ± 29%, R2 = 210 ± 27%; J20: R1 = 166 ± 31%, R2 = 153 ± 27%; J20+ANDRO: R1 = 217 ± 23%, R2 = 163 ± 15% all relative to baseline, $p < 0.01$) and on

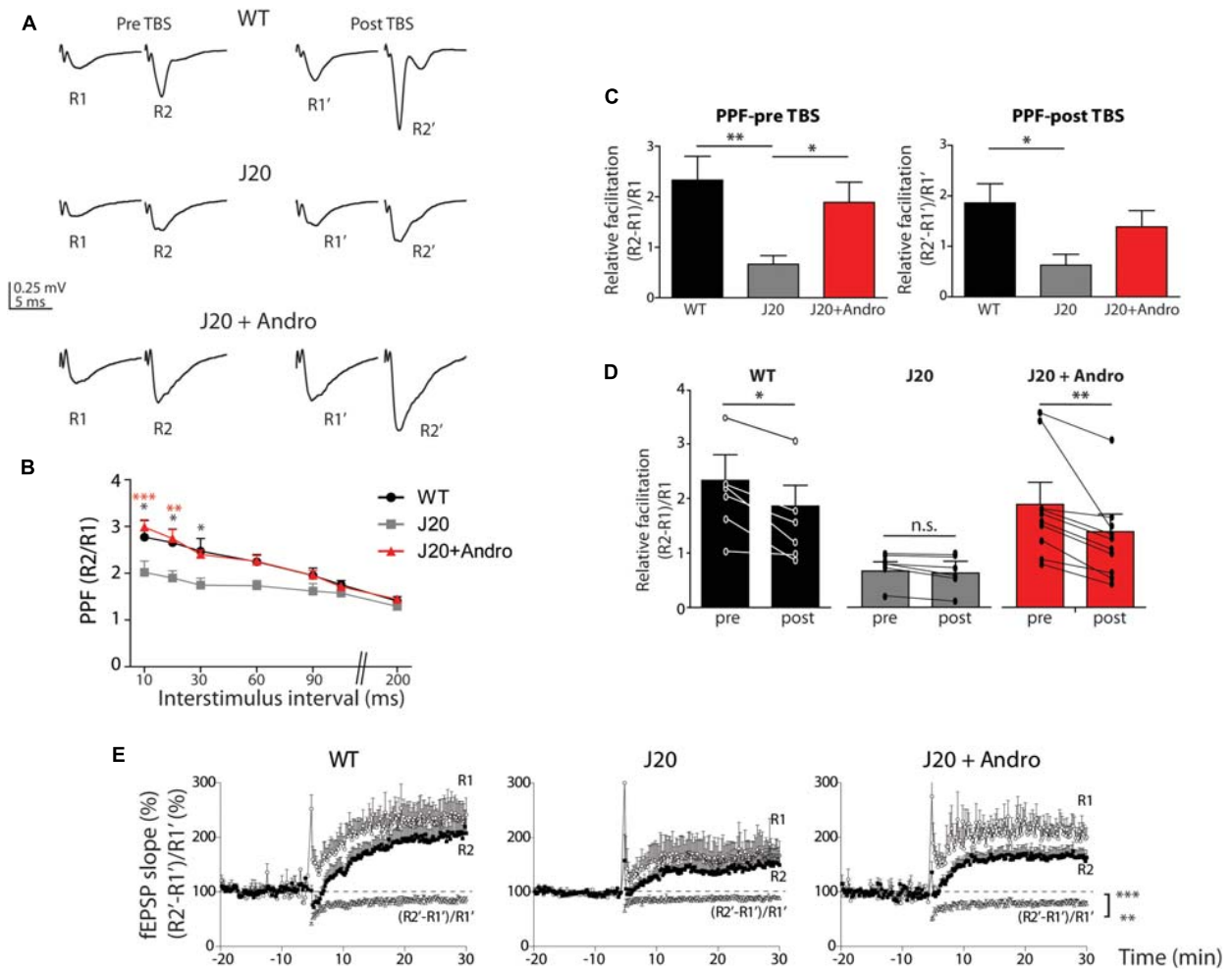

FIGURE 7 | Short-term plasticity is rescued by pretreatment with ANDRO. Representative traces **(A)** of two evoked fEPSPs separated by 50 ms before (pre-TBS) and after LTP induction (post-TBS) are shown. The graph **(B)** shows the ratio of the second fEPSP slope to the first fEPSP slope (R2/R1), recorded at different interval times: 10, 20, 30, 50, 60, 80, 100, 150, and 200 ms. WT (black dots), J20 (gray squares) and J20+ANDRO (red triangles). The values are expressed as mean ± SEM [two-way repeated measures ANOVA: interaction: $F(12,105) = 1.09$, $p > 0.05$; animal group factor: $F(2,105) = 19.87$, $p < 0.001$; ISI: $F(6,105) = 18.58$, $p < 0.001$, followed by Bonferroni *post hoc* test: WT vs. J20 at 10, 20 and 30 ms $^{*}p < 0.05$, beyond p = n.s.; J20 vs. J20+Andro at 10 ms $^{***}p < 0.001$, at 20 ms $^{**}p < 0.01$, beyond p = n.s.; WT vs. J20+Andro p = n.s.]. For the same stimulus point, the three curves were compared **(C)** show the relative facilitation during the baseline period [(R2 – R1)/R1], pre-TBS, left plot (WT: 2.3 ± 0.5; J20: 0.7 ± 0.2; J20+ANDRO: 1.9 ± 0.4; one-way ANOVA, $p < 0.05$, followed by Bonferroni *post hoc* test, $^{*}p < 0.05$, $^{**}p < 0.01$) and after LTP induction [(R2' – R1')/R1'], post-TBS, right plot (WT: 1.9 ± 0.4; J20+ANDRO: 1.4 ± 0.3; J20: 0.6 ± 0.2, one-way ANOVA, $p < 0.05$, followed by Bonferroni *post hoc* test, $^{*}p < 0.05$). The average of each bar was calculated over all the points during the baseline period and the last 20 min recorded during LTP. Also shown **(D)** are the same data as in **(C)**, but plotted by animal group. Comparing facilitation within each group per separated, we showed that facilitation pre-TBS is significantly different than facilitation post-TBS in WT [two-way repeated measures ANOVA: interaction: $F(2,14) = 2.68$, $p > 0.05$; pre and post TBS condition: $F(1,14) = 18.33$, $p < 0.001$; animal group factor: $F(2,14) = 8.43$, $p < 0.01$, followed by Bonferroni *post hoc* test, WT pre vs. post TBS $^{*}p < 0.05$, J20 pre vs. post TBS $p > 0.05$, J20+Andro pre vs. post $^{**}p < 0.01$]. Plot of R1' and R2' (%) and the percentage of attenuation [(R2' – R1')/R1'] **(E)**, revealing a reduced degree of response attenuation in J20 during the first 30 min after LTP induction (one-way ANOVA, $p < 0.001$ followed by Bonferroni *post hoc* test, WT vs. J20 $^{***}p < 0.001$, J20 vs. J20+Andro $^{***}p < 0.001$, WT vs. J20+Andro $^{**}p < 0.01$). Data represents the mean ± SEM from 16 slices of 8 (WT) mice; 18 slices of 6 (J20) mice; 18 slices of 6 (J20+Andro) mice.

the PPF, showing attenuation (mean value relative to baseline: WT = 82 ± 0.4%, J20 = 92 ± 0.24%, J20+ANDRO = 77 ± 0.3%, by one-way ANOVA and *post hoc* Bonferroni test, $^{***}p < 0.001$, $^{**}p < 0.01$) (**Figure 7E**). This previously reported phenomenon (Schulz et al., 1994; Wang and Kelly, 1997), suggests that high frequency activity causes plasticity changes both at the level of LTP and PPF. Although we have not explored the mechanism associated with this effect, whether presynaptic or postsynaptic, it is interesting that the amount of attenuation is lesser in J20 and greater with ANDRO.

ANDRO Treatment Prevents Increases in the Size of Hippocampal Synapses

It has been reported from postmortem AD brains that the number of synapses in the hippocampus is dramatically

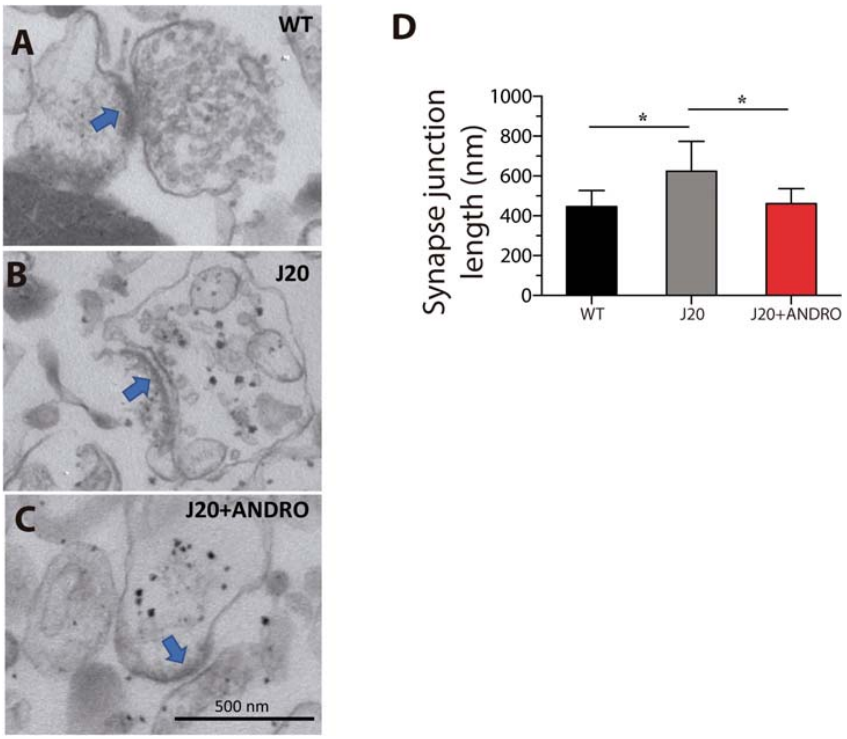

FIGURE 8 | Effect of ANDRO on synapse length. Electron micrographs of synaptosomes from wild-type **(A)**, J20 Tg **(B)** and J20 Tg+ANDRO mice **(C)**. The graph **(D)** shows a significant increase in the synapse length in the J20 Tg mice in comparison with WT mice and J20 Tg+ANDRO. Images are representative of three different experiments. The data represent the mean ± SEM of $n = 3$ (independent experiments, one-way ANOVA and post Bonferroni Multiple Comparison Test was performed, $^*p < 0.05$ and 95% CI).

decreased, but the remaining synapses are large in sides, which led to the idea that there are compensatory mechanisms working to overcome the synapse loss (Dekosky and Scheff, 1990; Scheff et al., 1990). Here, we biochemically prepared synaptosome-enriched fractions from the hippocampi of the three groups of animals in the study. **Figure 8** shows electron micrographs of synaptic junctions of WT, J20 Tg and J20 Tg+ANDRO mice. The right panel shows a quantitative analysis of the synaptic junction length. J20 Tg showed a larger synaptic junction (624 ± 149 nm) in comparison with the control (469 ± 52 nm). ANDRO treatment brought the length of the synaptic junction for the J20 Tg close to the control value (462 ± 75 nm) (one-way ANOVA, $p < 0.05$, followed by Bonferroni's *post hoc* test, WT vs. J20 $p = 0.05$; J20 vs. J20+ANDRO $p = 0.05$, **Figure 8**).

DISCUSSION

Although first described over 100 years ago, the etiology of AD is not well understood, which limits the ability to treat the disease or slow its progression (Querfurth and LaFerla, 2010; Serrano-Pozo et al., 2011). AD is the most common form of dementia, affecting approximately 10% of individuals over the age of 65 and approximately 50% of people 85 years and older (Association, 2013). Most cases are sporadic, yet approximately 1–2% are genetically linked and can be distinguished by early-onset dementia (Bettens et al., 2013). Increasing longevity in humans, combined with the high incidence of AD in older adults, will only exacerbate the global public health cost (Association, 2013; Chen and Zhong, 2013). In this context, the prevention of AD has emerged as a real strategy against the AD burden. However, no correlation has been described between presymptomatic modulation of any specific signaling pathway and AD marker expression in AD mouse models, including aspects related to metabolic dysfunction and impairment of synaptic function. In the present work, we studied whether the preventive administration of ANDRO, a well-described agonist of canonical Wnt signaling, is able to delay the specified markers of AD.

During the onset and progression of AD, a decrease in the utilization of glucose by brain cells has been described, leading to low ATP levels, which could be a critical step that partially explains the beginning of the synaptic failure described in AD patients and in AD animal models. The brain is characterized by a high rate of glucose consumption, and defective glucose utilization has been described in various diseases related to dementia, including AD (Chen and Zhong, 2013; Cisternas and Inestrosa, 2017). The dysregulation of glucose metabolism in AD has gained importance as a therapeutic target because the administration of hormones that stimulate glucose metabolism, such as insulin and glucagon-like peptide 1 (GLP-1), improves

cognitive responses in humans diagnosed with AD, as well as in mouse models of AD (Craft et al., 2012; Chapman et al., 2013; Freiherr et al., 2013), suggesting that the dysregulation of glucose in the brain could be critical in the onset and progression of AD as well as the progression of other metabolic diseases including diabetes. However, the effects of the hormones insulin and GLP-1 on downstream pathways are not well understood and represent an interesting field of research; in fact, several reports implicate Wnt signaling in the regulation of glucose metabolism (Cisternas and Inestrosa, 2017).

Several pathways have been postulated as molecular links between glucose metabolism and brain function, including Wnt signaling. Dysregulation of canonical Wnt signaling through a loss or gain of function has been linked to the progression of various diseases, including diabetes mellitus type II and AD (Inestrosa and Toledo, 2008; Farias et al., 2010; Toledo and Inestrosa, 2010; Inestrosa et al., 2012; Oliva et al., 2013; Cisternas et al., 2014b; Inestrosa and Varela-Nallar, 2014; Rios et al., 2014). The involvement of the Wnt pathway in the regulation of glucose metabolism has regained prominence in recent years, as studies in humans have suggested different components of the Wnt pathway as risk factors for the development of diseases such as DMTII and age-related dementia. However, the final effect depends on whether canonical or non-canonical Wnt signaling is affected (Lyssenko, 2008; Schinner, 2009; Shao et al., 2013). Despite all these efforts, the relationship between this signaling pathway and glucose metabolism in the brain has not been clearly established (Inestrosa and Arenas, 2010; Inestrosa and Varela-Nallar, 2014; Rios et al., 2014).

Recently, we have shown that acute treatment with the ligand Wnt3a induces a large increase in glucose uptake in neurons without changing the expression or localization of GLUT3, a glucose transporter expressed mostly in neurons (Cisternas et al., 2016a). We also observed that Wnt3a treatment increased the activation of the metabolic sensor Akt. Moreover, the increases in HK activity and glycolytic rates depended on activation of the Akt pathway. Furthermore, we did not observe changes in the activity of G6PDH or in the PPP. The effect of Wnt3a did not depend on the transcription of Wnt target genes or on synaptic changes (Cisternas et al., 2016a). Moreover, a recent study showed that the activation of canonical Wnt signaling by the *in vivo* administration of Wnt agonists, such as ANDRO and lithium, was able to induce a significant metabolic improvement and rescue cognitive performance in a transgenic model of advanced AD (Cisternas et al., 2018). Therefore, Wnt signaling stimulates the use of glucose in cortical neurons through glycolysis to satisfy the high energy demand of these cells, a critical effect that would explain the improvements in cognitive performance and synaptic plasticity observed in the presence of Wnt ligands. However, the final effect on neuronal metabolism depends on the specific Wnt pathway activated.

In AD, one of the most severely affected brain regions is the hippocampus, a region critical in the generation of new memories and other cognitive functions (Eichenbaum, 2014, 2017). The neural network of the hippocampus implies a high level of neuronal activity that is accompanied by a high energy demand; in fact, in WT animals, a significant proportion of the injected glucose was detected in the hippocampus. Interestingly, in the presymptomatic J20 mice, a significant decrease in the accumulation of glucose was observed in the hippocampus; therefore, although the animals presented normal cognitive performance, an early decrease in the utilization of glucose was detected. The latter supports the idea that metabolic dysfunction is one of the first molecular alterations in AD (Kapogiannis and Mattson, 2011). Given that both ATP and glucose uptake decreased in J20 mice, our data suggest that the metabolic dysfunction occurred mainly in neurons, since it is known that these cells use glucose mainly through glycolysis to generate ATP. The recovery in both metabolic parameters could be explained by a dual effect of ANDRO. First, ANDRO could modulate the activity of key metabolic enzymes such as Pfk, and consequently the increase in the activity of this enzyme could trigger an increase in the generation of pyruvate which is the main substrate for the mitochondrial machinery to produce ATP. But we cannot rule out a direct effect on the activity of the mitochondria, what would be subject for another study. Another action of ANDRO could be the activation of some metabolic sensors, such as AMPK, promoting cells to initiate an adaptive response to recover the general metabolic state (Ríos et al., 2018).

The improvement in the utilization of glucose and the generation of ATP both *in vivo* and in hippocampal slices was correlated with significant recovery of learning and memory function to levels similar to those of WT mice. Since this observation suggests a synaptic effect of ANDRO, we used electrophysiological approaches to explore synaptic function in J20 Tg mice with and without ANDRO treatment. We found severe deficiencies in synaptic transmission in the J20 Tg mice. The input–output curve revealed a deficiency in the level of response evoked by a given stimulus strength. However, it is unlikely that the mutant mice had fewer available axons or less excitable axons than the WT mice, since the FV plot did not show any difference among groups. The FV component and the evoked fEPSP showed a linear correlation in all groups. However, the same stimulation intensity activates fewer axons in J20 Tg mice than in the other two groups. These results suggest that the intrinsic activation of the axon and not the number of axons is altered in J20 Tg mice. On the other hand, the number of release sites or the efficiency of neurotransmitter release could be affected in the J20 Tg mice. Interestingly, treatment with ANDRO prevents the phenotypic effect of the transgene. These observations could, to some extent, be explained by observations made in humans wherein postmortem brains of AD patients have a significant decrease in the number of synapses and an increase in synaptic size (Dekosky and Scheff, 1990; Scheff et al., 1990), suggesting that a compensatory mechanism might strengthen the remaining synapses. Here, we explore one feature of the synapses in a biochemical preparation of synaptosomes. We found an increase in the length of the synaptic junction in the J20 Tg mice, mimicking the observation in human patients. Preventive treatment with ANDRO avoided that phenotype. Overall, this early cognitive and metabolic protection are the first such effect reported for the diterpene ANDRO since a previous report showed the ability of ANDRO to decrease neuropathology in AD mice; however, in that study, ANDRO treatment of AD mice

started at 7 and 12 months of age, when the cognitive deficits of the disease were already evident (Serrano et al., 2014; Tapia-Rojas and Inestrosa, 2018).

We also studied classical long-term plasticity in the form of LTP. We found that all three groups of animals showed LTP, with J20 Tg mice showing the lowest LTP. Treatment with ANDRO significantly increased the LTP level in J20 Tg mice, which reached an intermediate level between those of the mutant and the WT mice. These data can explain, to some extent, the observation in the memory flexibility test, where ANDRO partially reestablished the normal phenotype. A very striking observation is that ANDRO rescues the deficit in synaptic transmission. In other words, the correlation between FV size and fEPSPs is improved and similar to that of WT synapses. After LTP induction, lower FV amplitudes cause larger evoked responses. Notably, after LTP induction, there is a clear reduction in the basal FV amplitude of WT. Because the J20 mice were treated months before we performed the experiments, we hypothesized that ANDRO altered some of the presynaptic mechanisms with the consequent increase in the efficiency of the synapse.

Additional electrophysiological studies provided some clues regarding the mechanisms involved (**Figure 7**). The PPF protocol shows the evoked response to successive pulses separated by different time intervals (**Figure 7B**). PPF is a form of short-term plasticity and reflects the probability of release from presynaptic sites (Debanne et al., 1996; Jackman and Regehr, 2017). We demonstrated that J20 Tg mice have deficient PPF compared to WT and J20+ANDRO mice over a wide range of intervals. Moreover, the PPF index, comparing the basal PPF (pre-TBS) and after LTP induction (post-TBS), was also deficient during LTP studies. Thus, if there is a presynaptic component during the expression of LTP, both pre- and postsynaptic mechanisms should interfere (Schulz et al., 1994). We measured this interference (**Figure 7E**), which has been called attenuation (Wang and Kelly, 1997). A reduced degree of attenuation was found in J20 Tg mice, suggesting reduced participation of the presynaptic component during LTP. Instead, ANDRO-treated animals showed similar levels to WT mice. These data have several implications. One implication is that LTP appears to be less affected in this mutant than in other Tg mouse models of AD (Gengler et al., 2010; Serrano et al., 2014). We can report that, at the time we performed these experiments, LTP, which is regulated postsynaptically (Glanzman, 1994; Malenka and Bear, 2004), is significantly lesser in J20 mice compared to WT. The amount of attenuation is significantly different from those in the other two groups, which implies that the influence of presynaptic terminals is stronger in WT and ANDRO-treated mice than in J20 Tg mice. Therefore, we could infer that presynaptic failure was one of the first signs to be manifested in the J20 Tg model and might account for early markers of the disease, at least at the time we performed the experiments (7 months). We can predict that those failures will reach postsynaptic components later in life causing more severe deficiencies in LTP, or even its complete suppression, as occurs in the more severe double Tg model mice (Gengler et al., 2010; Serrano et al., 2014). The origin of this mechanism has been controversial, but there is strong evidence attributing it to postsynaptic activity dependent on Ca^{2+} and Ca^{2+}-calmodulin (CaM) signaling and to inhibition of calcineurin (CaN) activity (Wang and Kelly, 1997). We did not explore these dependencies in our experiments.

In summary, we present evidence that ANDRO restores several aspects of neurophysiology including glucose metabolism, cognitive performance and synaptic transmission. ANDRO normalized the synaptic mechanisms acting on the presynaptic side to avoid failures in the output signals. An important step forward from here would be to demonstrate that presymptomatic treatment with ANDRO is able to delay the principal symptoms of AD, including those that concern glucose utilization, synaptic failure and the cognitive task. These findings suggest that a preventive treatment against AD could be feasible.

In future research, we would like to go a step further and test the preventive effect of ANDRO in more complex models of AD and, ultimately, to test this treatment in patients with AD or even in patients with early clinical manifestations of AD. Overall, we propose ANDRO as a feasible therapeutic drug to delay the early- and late-onset manifestations of neurodegenerative diseases such as AD.

AUTHOR CONTRIBUTIONS

PC and NI conceived and designed the experiments. PC, CO, DB, and VT performed the experiments. PC, CO, and VT analyzed the data. NI contributed to the reagents, materials, and analysis tools. PC, CO, VT, and NI wrote the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Regulation of Hippocampal Synaptic Function by the Metabolic Hormone, Leptin: Implications for Health and Neurodegenerative Disease

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The role of the endocrine hormone leptin in controlling energy homeostasis in the hypothalamus are well documented. However the CNS targets for leptin are not restricted to the hypothalamus as a high density of leptin receptors are also expressed in several parts of the brain involved in higher cognitive functions including the hippocampus. Numerous studies have identified that in the hippocampus, leptin has cognitive enhancing actions as exogenous application of this hormone facilitates hippocampal-dependent learning and memory, whereas lack or insensitivity to leptin results in significant memory deficits. Leptin also markedly influences some of the main cellular changes that are involved in learning and memory including NMDA-receptor dependent synaptic plasticity and glutamate receptor trafficking. Like other metabolic hormones, there is a significant decline in neuronal sensitivity to leptin during the ageing process. Indeed, the capacity of leptin to modulate the functioning of hippocampal synapses is substantially reduced in aged compared to adult tissue. Clinical studies have also identified an association between circulating leptin levels and the risk of certain neurodegenerative disorders such as Alzheimer's disease (AD). In view of this, targeting leptin and/or its receptor/signaling mechanisms may be an innovative approach for developing therapies to treat AD. In support of this, accumulating evidence indicates that leptin has cognitive enhancing and neuroprotective actions in various models of AD. Here we assess recent evidence that supports an important regulatory role for leptin at hippocampal CA1 synapses, and we discuss how age-related alterations in this hormonal system influences neurodegenerative disease.

Keywords: leptin, hippocampus, synaptic plasticity, Alzheimer's disease, amyloid

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LEPTIN AND LEPTIN RECEPTORS

Leptin, an endocrine hormone, is the functional product of the obese (*ob*) gene. It is principally made in white adipose tissue and once secreted, the circulating levels of leptin correlate directly to body fat content (Maffei et al., 1995; Considine et al., 1996). The fasting leptin levels that circulate in the plasma generally range from 1 ng/ml to 100 ng/ml (Boden et al., 1996). Peripherally-derived leptin readily accesses the brain via a transport mechanism that is saturable and highly sensitive to triglycerides and adrenaline (Banks et al., 2004). Hypothalamic neural circuits are a key site for the central actions of leptin with the arcuate nucleus in particular being crucial for the energy regulating functions of this hormone. However, the neuronal actions of leptin extend

beyond the hypothalamus, with many areas of the brain including the hippocampus and cerebral cortex expressing leptin receptors at a high density.

Leptin receptors are produced by the diabetes (*db*) gene and six isoforms of the leptin receptor, ObRa-f, exist. All the isoforms, with the exception of ObRe, are expressed at the plasma membrane, have analogous extracellular domains, but differ in the length of their intracellular domain. The intracellular domain of the short isoforms (ObRa, c, d, f) range from 30 to 40 amino acid residues in length. Contrastingly, the long isoform, ObRb incorporates an extended (302 residues) intracellular domain, which enables the full complement of leptin receptor signaling pathways to be activated by this isoform. In contrast, the short isoforms have limited capacity to signal, with only some of the Ob-Rb-driven signaling molecules being activated by the short isoforms. ObRe is a novel leptin receptor isoform as it does not have a membrane spanning region, but it readily binds leptin. Consequently, leptin binding to ObRe is thought to aid its transport in the plasma.

Leptin receptors are highly homologous to other class I cytokine receptors; a superfamily of receptors that includes interferon receptors. Leptin binding to ObR activates janus tyrosine kinase 2 (JAK2), which promotes JAK2 phosphorylation. This in turn allows particular tyrosine residues located intracellularly to be phosphorylated. This sequence of events enables a number of signaling pathways to be activated by ObRs. The principle signaling molecules that are stimulated by neuronal ObRs include the signal transduction and activator of transcription (STAT) transcription factors, phosphoinositide-3 kinase (PI 3-kinase) and mitogen-activated protein kinase (MAPK; Farooqi and O'Rahilly, 2014).

LEPTIN REGULATION OF HIPPOCAMPAL SYNAPTIC FUNCTION

Anatomical evaluation of ObR expression in the brain has detected a high density of this receptor in different regions of the hippocampus and specifically at hippocampal synapses (Hâkansson et al., 1998; Shanley et al., 2002). In line with this expression pattern, leptin treatment potently regulates excitatory synaptic transmission evoked at hippocampal Schaffer-collateral (SC)-CA1 synapses (Shanley et al., 2001; Oomura et al., 2006; Moult et al., 2010; Moult and Harvey, 2011). Moreover, studies in obese rodents that are leptin-insensitive have also identified significant impairments in two key forms of hippocampal synaptic plasticity, namely long-term potentiation (LTP) and long-term depression (LTD). Additionally, marked deficits in behavioral assessment of hippocampal-dependent memory have also been reported in these obese rodents (Li et al., 2002; Winocur et al., 2005). Together these findings support the notion that leptin has potential cognitive enhancing actions.

More recent studies indicate that application of leptin also influences the magnitude of excitatory synaptic transmission at another synaptic input to CA1 neurons. This input, which arises in the entorhinal cortex (EC), forms part of the temporoammonic (TA) pathway (Luo et al., 2015; McGregor et al., 2018; **Figure 1**). Indeed, application of leptin to juvenile (11–18) hippocampal slices induces LTP at this synaptic connection (Luo et al., 2015), which directly contrasts with the leptin-induced depression of synaptic transmission observed at SC-CA1 synapses at this age (Moult and Harvey, 2011). Interestingly, although leptin has divergent actions at the two synaptic inputs to pyramidal neurons, activation of NMDA receptors is essential for both forms of leptin-dependent synaptic plasticity (Moult and Harvey, 2011; Luo et al., 2015). The involvement of NMDA receptors parallels the dependance on NMDA receptors for other key modulatory effects of leptin in the hippocampal formation. For instance, the ability of leptin to rapidly increase the density of hippocampal dendritic filopodia is dependent on NMDA receptor activation as the morphological changes induced by leptin are diminished in the presence of the NMDA receptor antagonist, D-AP5 (O'Malley et al., 2007). Similarly, a requirement for NMDA receptors has been demonstrated for the leptin-driven reversal of LTP (known as depotentiation) as this process is blocked following antagonism of NMDA receptors. In the thalamic-lateral amygdala pathway, NMDA receptors are also required for the depotentiation of amygdala LTP induced by leptin (Wang et al., 2015). Interestingly recent studies indicate that NMDA receptors, and specifically GluN2B-containing NMDA receptors in hypothalamic AgRP neurons, are critically involved in leptin-dependent homeostatic control of body weight (Üner et al., 2015).

LEPTIN REGULATION OF AMPA RECEPTOR TRAFFICKING

Synaptic insertion and removal of AMPA receptors is known to be important for driving long term alterations in excitatory synaptic efficacy at hippocampal synapses (Collingridge et al., 2004; Herring and Nicoll, 2016). Transient changes in the molecular identity of synaptic AMPA receptors following LTP induction has been detected in some studies (Plant et al., 2006; Morita et al., 2014), but there are some exceptions (Tang et al., 1999; Clayton et al., 2002). In a similar manner, leptin promotes insertion of AMPA receptors into hippocampal synapses, and this is pivotal for leptin driven changes in synaptic efficacy (e.g., LTP) at adult hippocampal SC-CA1 synapses (Moult et al., 2010). Thus, the rectification index of synaptic AMPA receptors is enhanced by leptin, and addition of philanthotoxin, to selectively inhibit GluA2-lacking AMPA receptors, results in reversal of LTP induced by leptin. Together this suggests that incorporation of GluA2-lacking AMPA receptors into synapses is a key mechanism underlying leptin-induced LTP (Moult et al., 2010). In agreement with this, studies using a combination of immunocytochemical and biotinylation techniques have demonstrated that exposure to leptin increases the plasma membrane expression of the AMPA receptor GluA1 subunit in primary cultures of hippocampal neurons and in hippocampal slices (Moult et al., 2010).

Trafficking of AMPA receptors is also essential for the reported effects of leptin at TA-CA1 synapses (**Figure 1**).

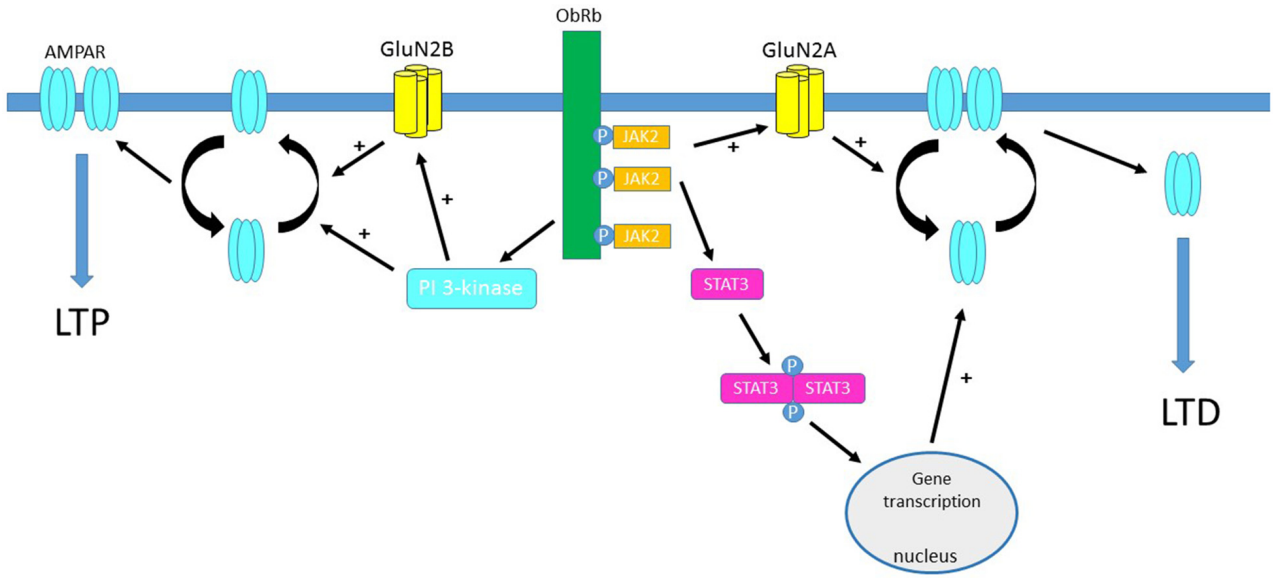

FIGURE 1 | Activation of leptin receptors influences hippocampal synaptic efficacy at temporoammonic (TA)-CA1 synapses. Schematic representation of the predominant signaling pathways that are activated by leptin receptors located at hippocampal TA-CA1 synapses. Leptin binding to ObRb results in phosphorylation of janus tyrosine kinase 2 (JAK2) which in turn promotes phosphorylation and dimerization of signal transducer and activator of transcription 3 (STAT3), leading to gene transcriptional changes in the nucleus. Activation of JAK2-STAT3 signaling mediates leptin-induced AMPA receptor internalization and induction of long-term depression (LTD) at adult TA-CA1 synapses. The ability of leptin to induce LTD at adult TA-CA1 synapses also requires the activation of GluN2A-containing NMDA receptors, which also promotes the removal of AMPA receptors from hippocampal synapses. In contrast, at juvenile TA-CA1 synapses, leptin-driven stimulation of the phosphoinositide-3 kinase (PI 3-kinase) cascade, culminates in the synaptic insertion of AMPA receptors and the induction of long-term potentiation (LTP). Activation of GluN2B NMDA receptor subunits is pivotal for leptin-induced LTP and the synaptic insertion of GluA2-lacking AMPA receptors at juvenile TA-CA1 synapses.

Indeed, application of philanthotoxin also reverses the persistent increase in synaptic transmission induced by leptin at juvenile TA-CA1 synapses, suggesting that GluA2-lacking AMPA receptor are inserted into synapses by leptin and that this process is required for leptin-induced LTP (Luo et al., 2015). Conversely, the synaptic removal of GluA2-lacking AMPA receptors is implicated in leptin-induced LTD as leptin fails to induce LTD in the presence of selective inhibitors of clathrin-mediated endocytosis (McGregor et al., 2018). In a manner similar to SC-CA1 synapses, application of leptin is not only capable of inducing LTD but this also results in a significant reduction in GluA1 surface expression in hippocampal slices (McGregor et al., 2018).

Significant evidence indicates that activity-dependent LTP and LTD is likely to be key cellular events that underlie learning and memory processes that are hippocampus-dependent (Bliss and Collingridge, 1993). Moreover, occlusion studies have demonstrated that leptin-induced changes in excitatory synaptic strength evoked at SC-CA1 and TA-CA1 synapses occlude activity-dependent synaptic plasticity, indicating similar expression mechanisms (Moult and Harvey, 2011; Luo et al., 2015; McGregor et al., 2018). Thus, as leptin induces novel forms of synaptic plasticity, and regulates AMPA receptor trafficking processes, which mirrors the cellular processes that are implicated in hippocampal-dependent learning and memory, it suggests that leptin has cognitive enhancing properties.

PROTECTIVE ACTIONS OF LEPTIN IN THE CNS

A possible protective role for leptin in the CNS was first suggested in comparative studies that identified significant brain changes in leptin deficient *ob/ob* mice compared to their wildtype littermates (Ahima et al., 1999). In these studies, the brain weight of *ob/ob* mice was markedly lower than wildtype mice suggesting that lack of leptin reduces neuronal viability (Ahima et al., 1999). In support of this, treatment of *ob/ob* mice with leptin for up to 4 weeks completely reversed the observed brain abnormalities (Ahima et al., 1999). Numerous subsequent studies support the notion that leptin has protective actions centrally. Thus studies performed in central and peripheral neurons indicate that exposure to leptin not only enhances the rate of neuronal survival, but also prevents neuronal cell death induced by a variety of apoptotic stimuli (Doherty et al., 2008; Guo et al., 2008; Davis et al., 2014).

Protective effects of leptin have been shown in several CNS-driven disease models that are associated with neuronal apoptosis. In a mouse model of cerebral ischemia, treatment with leptin decreased the extent of brain injury as the overall infarct volume was attenuated by leptin (Zhang et al., 2013). Similarly, in an oxygen/glucose deprivation model of ischemia, leptin protects against ischemic damage (Zhang and Chen, 2008). In various models of Parkinson's disease (PD), the viability

of dopaminergic neurons treated with either 6-OH dopamine or MPTP is significantly enhanced after treatment with leptin (Weng et al., 2008; Doherty et al., 2008). Moreover, prior exposure of human SH-SY5Y neuroblastoma cells with leptin also prevented neuronal cell death induced by the neurotoxin 1-methyl-4-pyridinium (MPP^+; Lu et al., 2006).

It is interesting to note that the neuroprotective properties of metabolic hormones is not restricted to leptin. Thus, ghrelin, which is produced in the stomach and regulates body weight by promoting food intake, also markedly influences neuronal viability (de Candia and Matarese, 2018). In hypothalamic cells, treatment with ghrelin protects against oxygen-glucose deprivation and subsequent apoptosis by inhibiting mitochondrial formation of reactive oxygen species (Chung et al., 2007). The toxic apoptotic actions of Aβ are also attenuated in hypothalamic cells and hippocampal neurons following treatment with ghrelin (Moon et al., 2011; Gomes et al., 2014). Moreover, in a mouse model of traumatic brain injury, ghrelin limits the degree of neuronal degeneration by reducing apoptosis (Lopez et al., 2014). Recent evidence indicates that like leptin, age-related alterations occur in ghrelin function which are thought to lead to an increased risk of neurodegenerative disease (de Candia and Matarese, 2018). As both these metabolic hormones have comparable neuroprotective actions in the hippocampus, and given their closely related hypothalamic functions, it is feasible that there is some cross-talk between the hormones in terms of neuroprotective mechanisms. Although evidence of this is limited, leptin and ghrelin are both reported to prevent Aβ-induced cell death via inhibition of GSK-3β (Martins et al., 2013). However, further studies are required to examine potential interactions between these metabolic hormones and in turn how this impacts on CNS health.

LEPTIN AND AGING

It is known that as individuals get older, the functioning of metabolic hormones deteriorates and this has implications for normal CNS function as metabolic dysfunction has been linked to faster ageing and an increased likelihood of developing neurodegenerative conditions such as Alzheimer's disease (AD; Stranahan and Mattson, 2011; Kim and Feldman, 2015). Several lines of evidence support the notion that age-related changes in the effectiveness of the leptin system occurs in the CNS. Indeed, hypothalamic neurons are less responsive to leptin with age as the satiety effects of leptin are markedly reduced in aged (30 month) compared to adult (6 month) rats (Shek and Scarpace, 2000). At the cellular level, reductions in leptin-driven activation of STAT3 as well as phosphorylated-STAT3 binding activity have been observed in aged rats (Shek and Scarpace, 2000; Scarpace et al., 2001). Attenuated uptake of leptin into the hypothalamus, as well as reductions in the capacity of ObRs to signal, due to elevations in suppressor of cytokine signaling-3 (SOCS-3), have also been reported in aged animals (Wang et al., 2001; Peralta et al., 2002).

Age-dependent changes in response to leptin have also been detected at hippocampal synapses. At juvenile (P14–21) SC-CA1 synapses, treatment of hippocampal slices with leptin markedly depresses excitatory synaptic transmission, however this effect is transient as it is readily reversed on leptin washout (Shanley et al., 2001; Xu et al., 2008; Moult and Harvey, 2011). In contrast, leptin has directly opposing actions in hippocampal slices obtained from adult (3–4 months) rats as exposure to leptin culminates in LTP induction at SC-CA1 synapses. Moreover the magnitude of leptin-induced LTP at SC-CA1 synapses is significantly altered with age, as leptin-induced LTP is attenuated by around 50% in aged (12–14 months) relative to adult tissue (Moult and Harvey, 2011). Thus, not only does leptin have bi-directional age-dependent effects on synaptic efficacy at excitatory SC-CA1 synapses, but there is also a marked reduction in the sensitivity to leptin with age.

Recent evidence indicates that the modulatory actions of leptin at excitatory TA-CA1 synapses is also highly dependent on age (McGregor et al., 2018; **Figure 1**; **Table 1**). In juvenile tissue, application of leptin leads to TA-CA1 LTP (Luo et al., 2015), whereas a persistent depression (LTD) is observed after leptin addition to adult hippocampal slices (McGregor et al., 2018). Moreover like SC-CA1 synapses, the leptin-sensitivity of TA-CA1 synapses markedly decreases in aged tissue, as application of leptin fails to induce LTD at aged TA-CA1 synapses (McGregor et al., 2018). It is unclear why leptin has no effect at aged excitatory TA-CA1 synapses. However the lack of leptin responsiveness is unlikely to be due to age-related changes in NMDA receptor expression as TA-CA1 LTP is readily induced by a high frequency stimulation protocol in slices from aged animals, and like leptin-induced LTD, the cellular mechanisms underlying TA-CA1 LTP are dependent on GluN2A-containing NMDA receptors (McGregor et al., 2018).

Studies examining the potential cellular mechanisms contributing to the age-dependent actions of leptin have evaluated the role of NMDA receptors, as hippocampal NMDA receptors comprising different NMDA receptor subunits are implicated in divergent types of synaptic plasticity (Liu et al., 2004; Bartlett et al., 2007), and NMDA receptor expression and molecular identity varies at different stages of development and ageing (Monyer et al., 1994). Using pharmacological tools to selectively block different GluN2 subunits, it has been shown that distinct NMDA receptor subunits contribute to the bi-directional and age-related actions of leptin at hippocampal synapses. Thus, NMDA receptors comprised of GluN2B subunits mediate the leptin-driven synaptic depression at juvenile SC-CA1 synapses. By contrast, in adult hippocampus, activation of NMDA receptors that contain GluN2A subunits is key for the induction of LTP by leptin at SC-CA1 synapses. (Moult and Harvey, 2011). At TA-CA1 synapses, NMDA receptors with distinct molecular composition are also implicated in the opposing age-dependent effects of leptin. Thus, activation of GluN2B subunits is required for leptin-induced LTP in juvenile tissue (Luo et al., 2015) whereas GluN2A subunits underlie leptin-induced LTD in adult hippocampus (McGregor et al., 2018; **Table 1**). The signaling pathways that link leptin receptor

TABLE 1 | Summary of the opposing actions of leptin at hippocampal synapses with age.

SC-CA1 synapse	TA-CA1 synapse
Juvenile (P11–18) hippocampal slices	
Leptin-induced synaptic depression	**Leptin-induced LTP**
GluN2B-dependent ERK signaling Removel of GluA2-lacking AMPARs	GluN2B-dependent PI 3-Kinase signaling Insertion of GluA2-lacking AMPARs
Adult (3–6 months) hippocampal slices	
Leptin-induced LTP	**Leptin-induced LTD**
GluN2A-dependent PI 3-Kinase signaling Insertion of GluA2-lacking AMPARs	GluN2A-dependent JAK2-STAT3 signaling Removel of GluA2-lacking AMPARs
Aged (12–15 months) hippocampal slices	
Leptin-induced LTP	**Leptin fails to induce LTD**
Magnitude of leptin-induced LTP is reduced.	LFS also fails to induce LTD

Summary table illustrating the bi-directional effects of the hormone leptin at hippocampal Schaffer-collateral (SC)-CA1 and temporoammonic (TA)-CA1 synapses. At juvenile SC-CA1 synapses, application of leptin results in a transient depression of excitatory synaptic transmission that is not only NMDA receptor dependent but involves selective activation of GluN2B-containing NMDA receptors. In addition, activation of an ERK-dependent process and subsequent removal of GluA2-lacking AMPA receptors underlies this effect of leptin. Conversely at adult (3–6 months) SC-CA1 synapses, a novel form of long-term potentiation (LTP) is evoked by leptin. This process is GluN2A-dependent, requires activation of phosphoinositide-3 kinase (PI 3-kinase) and the synaptic insertion of GluA2-lacking AMPA receptors. The ability of leptin to regulate SC-CA1 synapses markedly declines with age as the magnitude of leptin-induced LTP at SC-CA1 synapses is significantly attenuated in aged hippocampus. At all stages of development and ageing, leptin has opposing actions on synaptic efficacy at the anatomically distinct TA input to CA1 neurons. Thus in contrast to its actions at SC-CA1 synapses, leptin induces a novel form of NMDA-dependent LTP at juvenile TA-CA1 synapses. Leptin-induced LTP involves selective activation of GluN2B subunits and PI 3-kinase-driven trafficking of GluA2-lacking AMPA receptors to synapses. In contrast, application of leptin to adult hippocampal slices leads to the induction of a novel form of NMDA receptor-dependent long-term depression (LTD) at TA-CA1 synapses. Leptin-induced LTD is GluN2A-dependent and involves activation of canonical janus tyrosine kinase 2 (JAK2)-signal transducer and activator of transcription 3 (STAT3) signaling and internalization of GluA2-lacking AMPA receptors. Like SC-CA1 synapses, there is a marked reduction in the sensitivity of TA-CA1 synapses with age, as leptin fails to induce LTD at aged TA-CA1 synapses.

activation to modulation of synaptic efficacy also differ depending on age and synaptic connection. Thus, at adult SC-CA1 synapses, PI3-kinase activation is crucial for leptin-induced LTP, but stimulation of ERK-dependent signaling is essential for the synaptic depression induced by leptin at juvenile slices. Interestingly, divergent signaling pathways also mediate the age-related modulatory actions of leptin at TA-CA1 synapses as LTP induce by leptin at juvenile synapses requires GluN2B subunits and PI 3-kinase-driven signaling (Luo et al., 2015; **Figure 1**). However in adult, leptin-induced TA-CA1 LTD is unique as JAK2-STAT3 signaling which in turn drives gene transcriptional changes are fundamental for this form of synaptic plasticity (McGregor et al., 2018; **Figure 1**). Overall these data suggest that the age-dependent effects of leptin on excitatory synaptic transmission at the two distinct inputs to CA1 pyramidal neurons are dependent on the molecular composition of NMDA receptors.

LEPTIN AND ALZHEIMER'S DISEASE

Age significantly increases the risk of developing AD, and as a consequence the prevalence of this disease is steadily increasing as people live longer. Other prominent factors that influence AD risk, include diet and lifestyle. Indeed, mid-life obesity significantly alters the risk of AD later in life, when compared to individuals with normal body weight. Increased body fat content results in higher circulating levels of leptin and subsequent development of leptin resistance in the obese state (Friedman, 2014). Thus AD risk may also be significantly altered in individuals with resistance to leptin and/or altered responsiveness to leptin. In support of this notion, attenuated levels of leptin in the plasma are documented in AD patients (Power et al., 2001), and prospective studies have found that low leptin levels is linked to an elevated risk of AD with age (Lieb et al., 2009). The fact that very high leptin levels (and subsequent development of leptin resistance) as well as low leptin levels are both associated with an increased risk of AD suggests that divergent metabolic states can influence the risk of AD. Although these clinical findings appear to be contradictory, there are parallels to the regulatory actions of leptin in the hypothalamus. Thus, the ability of leptin to regulate food intake and body weight occurs within a tightly regulated concentration range, such that too little circulating leptin fails to influence energy homeostasis, whereas highly elevated leptin levels that occur in the obese state not only results in leptin resistance but also loss of leptin's capacity to regulate food intake (Friedman, 2014). Overall this suggests that failure to maintain normal body weight and thus keep leptin levels within the physiological range, results in an increased risk of developing AD.

In a manner similar to AD patients, alterations in leptin function have also been observed in several rodent models of AD. Indeed transgenic models that have the same mutations that occur in familial AD, also exhibit reduced circulating leptin levels, suggesting compromised leptin function (Fewlass et al., 2004). However some clinical studies have found no apparent link between plasma leptin levels and AD. One possible reason for this discrepancy is that the plasma levels of leptin may not be a good indicator of leptin levels in the CNS. Indeed, altered blood-brain transport of leptin has been documented in AD (Dietrich et al., 2008), suggesting that the CNS levels of leptin are reduced in AD compared to normal. However, studies that have directly measured the brain levels of leptin have reported increased or unaltered leptin levels in AD patients (Bonda et al., 2014; Maioli et al., 2015). However, attenuated expression of leptin receptor mRNA and immunoreactivity is evident in post-mortem AD tissue, suggesting possible development of resistance to leptin (Bonda et al., 2014; Maioli et al., 2015). Reductions in some key signaling

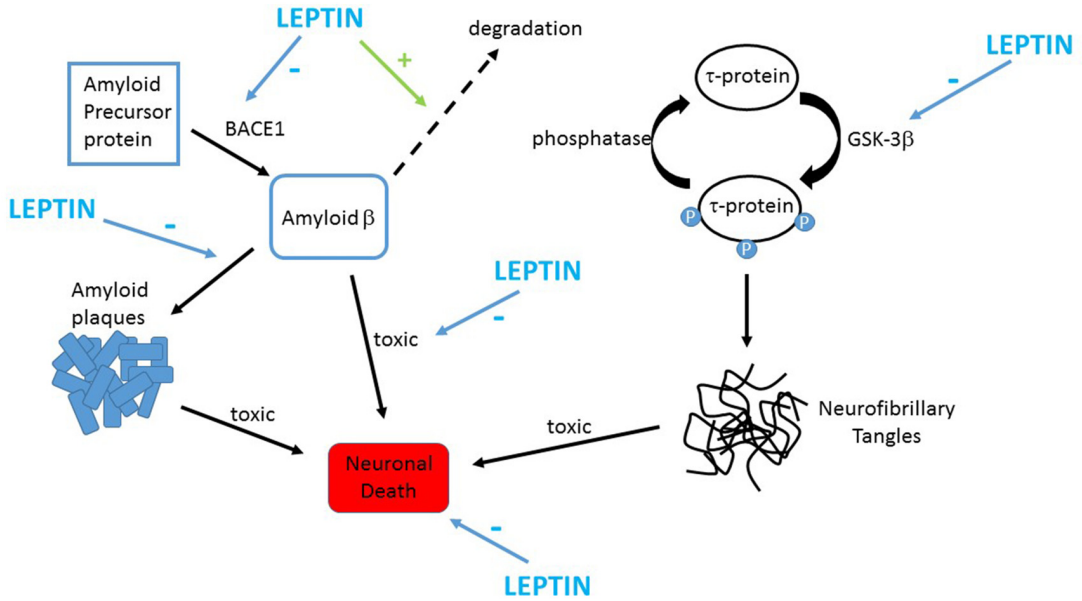

FIGURE 2 | Leptin prevents the key pathological changes in Alzheimer's disease (AD). Schematic representation of the protective actions of leptin in AD. Toxic forms of Aβ are formed by the proteolytic processing of APP by the enzyme BACE1. Leptin limits production of Aβ in neurons via inhibiting BACE1 activity. Leptin also attenuates extracellular Aβ levels by promoting the clearance and degradation of Aβ. Accumulation of Aβ promotes assembly of fibrillary forms of Aβ that leads to formation amyloid plaques. Leptin reduces plaque formation by reducing expression of GM1 gangliosides thereby limiting assembly of fibrillary Aβ. Leptin also counteracts the harmful effects of Aβ as it protects against the toxic actions of Aβ via stimulation of pro-survival signaling pathways. Hyper-phosphorylation of tau protein occurs in AD which results in formation of neurofibrillary tangles. GSK-3β drives phosphorylation of tau, and leptin limits this via inhibiting the activity of GSK-3β.

pathways that are activated downstream of leptin receptors have been observed in AD tissue which also supports the possibility that central resistance to leptin develops in AD (Maioli et al., 2015).

PROTECTIVE ACTIONS OF LEPTIN IN AD MODELS

Increasing evidence indicates that leptin has protective actions in a variety of cellular systems that model the neuronal degeneration that occurs in AD. Several studies have revealed that the levels of toxic Aβ are diminished after treatment with leptin (**Figure 2**). Indeed, the activity of β secretase, a key enzyme involved in the production of $A\beta_{1-42}$, is reduced by leptin resulting in decreased levels of $A\beta_{1-42}$ (Fewlass et al., 2004). In H4 neuroglioma cells, transcription of presenilin 1 is also down-regulated by leptin which in turn leads to attenuated $A\beta_{1-42}$ levels (Niedowicz et al., 2013). Uptake of Aβ is also modulated by leptin as LRP1-mediated uptake of Aβ is elevated after treatment of human SHSY-5Y neuroblastoma cells with leptin (Fewlass et al., 2004). Furthermore, leptin enhances the degradation of Aβ by increasing the levels of insulin degrading enzyme in organotypic brain slices (Marwarha et al., 2010).

In neurotoxicity assays, exposure of hippocampal or cortical neurons to leptin protects against the harmful actions of oligomeric Aβ on neuronal viability (Doherty et al., 2013; Martins et al., 2013; **Figure 2**). Leptin also prevents the increased aggregation of Aβ that occurs after treatment of cortical neurons with Cu^{2+} ions (Doherty et al., 2013). In addition, leptin is reported to have neuroprotective actions in hypothalamic cells, as the ability of oligomeric Aβ to increase superoxide production and elevate intracellular calcium levels are significantly attenuated by leptin in mHypoE-N42 cells (Gomes et al., 2014).

It is known that increased expression of the presynaptic protein, endophilin 1 occurs in those suffering from AD and in transgenic AD models (Ren et al., 2008). As endophilin 1 inhibits the synaptic release of glutamate (Weston et al., 2011), AD-linked increases in endophilin 1 are likely to have a direct impact on excitatory synaptic transmission. Recent evidence has demonstrated that exposure to leptin also alters expression of endophilin1, as leptin inhibits the increase in endophilin 1 induced by Aβ (Doherty et al., 2013). Moreover, the cortical levels of endophilin 1 are up-regulated in Zucker *fa/fa* rats which indicates that insensitivity to leptin elevates the brain levels of endophilin 1 *in vivo* (Doherty et al., 2013). Consequently it is possible, that leptin, by regulating endophilin 1 expression, indirectly influences excitatory synaptic transmission at hippocampal synapses.

In addition to preventing the chronic actions of Aβ, leptin is also capable of directly influencing the impact of acute Aβ on hippocampal synaptic function. It is well established that short-term treatment with Aβ not only results in inhibition of activity-dependent LTP, but it also enhances LTD in hippocampal slices (Shankar et al., 2008; Li et al.,

2009). Moreover exposure to leptin is reported to reverse Aβ-driven inhibition of hippocampal LTP and it prevents facilitation of LTD induced by Aβ (Doherty et al., 2013; Malekizadeh et al., 2017). Direct effects of Aβ on AMPA receptor trafficking processes have also been observed, such that treatment with Aβ promotes internalization and synaptic removal of GluA1 subunits in hippocampal cultures (Li et al., 2009; Doherty et al., 2013). Prior treatment with leptin also counteracts this detrimental effect of Aβ as the ability of Aβ to internalize GluA1 subunits is blocked by leptin (Doherty et al., 2013; Malekizadeh et al., 2017). PI 3-kinase activation is required for the protective effects of leptin as pharmacological inhibition of this signaling cascade blocked the ability of leptin to prevent the acute actions of Aβ on hippocampal synapses (Doherty et al., 2013).

It has been proposed that oligomeric Aβ facilitates the induction of LTD via preventing uptake of glutamate at hippocampal synapses (Li et al., 2009). This process is likely to enhance glutamate levels within the synaptic cleft leading to activation and subsequent desensitization of synaptic NMDA receptors (Li et al., 2009). One of the proposed functions of LTD is to enhance the flexibility of networks by providing a way of re-setting potentiated synapses, thereby preventing saturation of hippocampal synapses which would limit capacity for learning. However this normal function of LTD is likely to be compromised in AD, as hippocampal synapses exposed to Aβ are unable to be potentiated/saturated due to the block of LTP induction by Aβ. Consequently in AD, hippocampal excitatory synapses are likely to remain in a depressed state; an effect that would be reinforced by sustained removal of AMPA receptors from synapses by Aβ. As treatment with leptin prevents the aberrant effects of Aβ on hippocampal LTP and LTD, as well as AMPA receptor trafficking, it is likely that restoration of normal hippocampal synaptic function would occur after treatment with leptin.

However, as leptin also has direct effects on excitatory synaptic efficacy, including its ability to induced novel forms of LTP and LTD at hippocampal synapses, do the protective actions of leptin have a bearing on its potential cognitive enhancing properties? What is clear is that the ability of leptin to prevent the acute synapto-toxic actions of Aβ occurs at low concentrations of leptin that have little or no effect of basal excitatory synaptic transmission (Doherty et al., 2013). In contrast, however, the marked effects of leptin on hippocampal synaptic plasticity that are likely to enhance cognition, occur at much higher leptin concentrations. This suggests that the protective and cognitive enhancing actions of leptin are distinct, as they have differing pharmacological profiles.

LEPTIN INFLUENCES TAU-RELATED PATHOLOGY IN AD

Neurofibrillary tangles are another major feature of AD pathology and hyper-phosphorylated tau is a key element of these tangles. Increasing evidence suggests that leptin limits accumulation of tau within the brain. Indeed, significantly lower phosphorylated tau (p-tau) levels are detected in TgCRND8 mice, that overexpress mutant human APP, after treatment with leptin (Greco et al., 2010). Tau phosphorylation status is also directly reduced by leptin as the activity of GSK3β, a key enzyme that drives tau phosphorylation, is inhibited by this hormone (Greco et al., 2009, 2010; **Figure 2**). Cortical neurons that are chronically treated with Aβ also exhibit elevated levels of p-tau and this process is markedly reduced by leptin (Doherty et al., 2013). In addition, impaired leptin function is associated with alterations in cortical p-tau levels *in vivo* as markedly elevated levels of p-tau have been detected in leptin-insensitive Zucker *fa/fa* rats (Doherty et al., 2013). Moreover, development of leptin resistance has recently been associated with enhanced tau pathology in mouse models of AD (Platt et al., 2016). Thus it is clear that leptin not only limits phosphorylation of tau, but also that lack of leptin receptor-driven signaling increases p-tau levels which lends support to the hypothesis that AD risk is influenced by impairments in the leptin system (Power et al., 2001; Lieb et al., 2009).

LEPTIN IMPROVES MEMORY IN AD MODELS

Significant evidence indicates that activity-dependent LTP and LTD are likely to be key cellular events that underlie learning and memory processes that are hippocampus-dependent (Bliss and Collingridge, 1993). Moreover, occlusion studies have demonstrated that leptin-driven changes in excitatory synaptic strength evoked at SC-CA1 and TA-CA1 synapses occlude activity-dependent synaptic plasticity, indicating similar expression mechanisms (Moult and Harvey, 2011; Luo et al., 2015; McGregor et al., 2018). Thus, as leptin induces novel forms of synaptic plasticity, and it potently regulates AMPA receptor trafficking processes, both of which mirror the cellular processes that are implicated in hippocampal-dependent learning and memory, it suggests that leptin has cognitive enhancing properties. Indeed, this is backed up in numerous behavioral studies which demonstrate that leptin has cognitive enhancing effects in rodents. Indeed intravenous administration of leptin facilitates performance in hippocampal spatial memory tests assessed using the Morris water-maze (Oomura et al., 2006). Conversely, deficits in hippocampal-dependent spatial memory are observed in leptin-insensitive rodents, suggesting that insensitivity to leptin markedly influences hippocampal-dependent memory (Li et al., 2002; Winocur et al., 2005). Diet-induced leptin resistance also interferes with memory consolidation in rats, such that marked deficits are observed in object recognition tests in diet-induced obese rats compared to wild type mice with normal body weight (Zanini et al., 2017).

Application of leptin also enhances performance in many hippocampal-dependent memory tasks in rodent models of AD. Thus, in SAMP8 mice which have elevated levels of Aβ and hippocampal memory deficits, administration of leptin improves performance in a T-maze task (Farr et al., 2006). In

TgCRND8 mice, a transgenic line derived from an APP Swedish mutation that develop amyloid plaques in the hippocampus and cortex from 9 weeks of age, treatment with leptin enhances the ability to perform behavioral tests of novel object recognition and fear conditioning (Greco et al., 2010). Hippocampal memory deficits in APP/PS1 mice have also been reversed by intracerebroventricular (ICV) administration of a lentiviral vector expressing leptin (Pérez-González et al., 2014). Recent *in vivo* studies also support the notion that leptin counteracts the harmful actions of Aβ on hippocampal-dependent memory, as the spatial memory deficits induced by ICV application of Aβ(1–42) are alleviated in rats exposed to leptin (Tong et al., 2015). Thus, there is clear evidence that treatment with leptin enhances cognition in both healthy animals and in models of AD.

THE THERAPEUTIC POTENTIAL OF THE LEPTIN SYSTEM IN AD

Although leptin has cognitive enhancing and neuroprotective actions in rodents, it is key for future development of leptin as an AD therapy, that its central actions in humans are well defined. Several studies have observed cognitive enhancing actions of leptin in human clinical studies. Thus, significant increases in gray matter volume have been detected in adults with congenital leptin deficiencies following treatment with physiological levels of leptin (Matochik et al., 2005). Leptin replacement therapy has also led to a significant improvement in cognitive function in a 5 year old with leptin deficiency due to a rare *ob* gene mutation (Paz-Filho et al., 2008). Although chronic leptin replacement therapy is safe and well tolerated in humans (Paz-Filho et al., 2015), there have been no clinical trials carried out to examine whether leptin is beneficial in AD patients.

However, several key factors need to be taken into account when using leptin in future clinical studies. Thus identifying which individuals are most likely to respond to leptin, and when in the disease process a leptin-based therapy will be beneficial is key. For instance, identifying individuals who have developed resistance to leptin, as a consequence of midlife obesity, may be pertinent as these individuals may be unresponsive to leptin-based therapies. In contrast, leptin treatment may be highly beneficial in AD patients with low baseline leptin levels. As leptin receptors are expressed throughout the brain, it is also vital that steps are taken to limit potential CNS side effects associated with a leptin-based therapy. In this respect, modification of the leptin molecule may enable specific targeting of the key brain regions affected in AD and thus enhance therapeutic efficacy. In support of this approach, a recombinant methionyl human form of leptin (metreleptin) has recently been developed, and gained FDA approval for use in treating lipodystrophy (Paz-Filho et al., 2015). Several studies have found that the whole leptin molecule is not required as leptin fragments have reported biological activity and can replicate the hypothalamic actions of leptin (Grasso et al., 1997; Rozhavskaya-Arena et al., 2000). Consequently, another approach may be to develop small leptin-like molecules. It would have to be established that such leptin mimetics replicate the full spectrum of cognitive enhancing and protective actions of leptin. Moreover, retention of key properties of the whole leptin molecule such as brain penetrability will be fundamental for the future success of a leptin-based mimetic. However recent studies in rodents have produced extremely promising results as one leptin fragment, namely $leptin_{116–130}$ was found to replicate leptin action as it displayed powerful protective effects on hippocampal synaptic function (Malekizadeh et al., 2017). Indeed, $leptin_{116–130}$ prevented the ability of Aβ to interfere with hippocampal synaptic plasticity and promote neuronal toxicity. Moreover, peripheral administration of $leptin_{116–130}$ replicated leptin's cognitive enhancing properties as it improved the ability of mice to performance specific episodic-like memory behavioral tasks (Malekizadeh et al., 2017). This indicates not only that the $leptin_{116–130}$ fragment readily enters the brain but that it also reaches the hippocampus where it markedly influences synaptic physiology and function.

A ROLE FOR LEPTIN IN OTHER NEURODEGENERATIVE DISORDERS?

In a manner similar to AD, increasing evidence indicates that metabolic dysfunction is a prominent feature in several other neurodegenerative diseases. Indeed, a link has been identified between mid-life obesity and the risk and progression of PD (Abbott et al., 2002; Procaccini et al., 2016). Alterations in metabolic function related to mid-life obesity are also associated with an increased likelihood of developing vascular dementia (Kivipelto et al., 2005) and Huntington's disease (HD; Gaba et al., 2005; Procaccini et al., 2016). Clinical studies have revealed almost a two fold increase in the incidence of multiple sclerosis in obese (BMI >30) children compared to those of normal body weight (Munger et al., 2009). Several studies have observed alterations in the circulating levels of leptin in both patients and in models of these CNS disorders. Indeed, α-synuclein A53T transgenic mice, that model familial PD, exhibit metabolic abnormalities and hypoleptinemia (Rothman et al., 2014). In addition attenuated plasma levels of leptin have been detected in HD (Pratley et al., 2000; Popovic et al., 2004), and PD patients (Evidente et al., 2001).

Several studies have found that an obese phenotype exacerbates neurodegeneration in various disease models. For instance, leptin deficient *ob/ob* mice treated with either meth-amphetamine or kainic acid display significantly greater neurotoxic damage and mortality rates than lean control mice (Sriram et al., 2002). Moreover in a PD model, exposure to the neurotoxin MPTP triggers far greater degeneration of dopaminergic neurons in overfed obese mice than lean littermates fed a normal diet (Choi et al., 2005). Degeneration of dopamine neurons induced by central infusion of 6-OHDA is also significantly elevated in diet-induced obese rodents relative to lean littermates (Morris et al., 2010). Together this suggests not only that leptin dysfunction contributes to disease pathology, but also that the leptin system may be a novel target for treatment of other neurodegenerative diseases (Evidente et al., 2001; Aziz et al., 2007; Procaccini et al.,

2016). However further work is required to evaluate fully the potential use of the leptin system in treating these CNS diseases.

CONCLUSION

It is well documented that the hippocampus is a key CNS target for the hormone leptin and that the potential cognitive enhancing effects of leptin are due to its actions at hippocampal synapses. An established role for leptin is in its ability to regulate synaptic efficacy at SC-CA1 synapses. However, recent evidence indicates that the TA input to hippocampal CA1 neurons is also an important functional target for this hormone. The regulatory actions of leptin at excitatory TA-CA1 synapses is important as the TA pathway is an early site for degeneration in AD, and clinical studies demonstrate a link between leptin and an increased risk of AD. In addition, treatment with leptin and specific fragments of the whole leptin peptide have advantageous effects in a range of systems that model AD, suggesting that the leptin system is novel therapeutic target in AD. Although there may be limitations to the therapeutic use of leptin, due to development of leptin resistance in some individuals, it is clear that clinical studies are needed to enable thorough assessment of the therapeutic potential of the leptin system in AD patients. As the risk of developing several other neurodegenerative disorders is also linked to altered leptin function, it is feasible that targeting the leptin system may also offer possible therapeutic benefit in these CNS diseases.

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JH and GM both contributed to writing the review.

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The Link Between Tau and Insulin Signaling: Implications for Alzheimer's Disease and Other Tauopathies

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The microtubule-associated protein tau (MAPT) is mainly identified as a tubulin binding protein essential for microtubule dynamics and assembly and for neurite outgrowth. However, several other possible functions for Tau remains to be investigated. Insulin signaling is important for synaptic plasticity and memory formation and therefore is essential for proper brain function. Tau has recently been characterized as an important regulator of insulin signaling, with evidence linking Tau to brain and peripheral insulin resistance and beta cell dysfunction. In line with this notion, the hypothesis of Tau pathology as a key trigger of impaired insulin sensitivity and secretion has emerged. Conversely, insulin resistance can also favor Tau dysfunction, resulting in a vicious cycle of these events. In this review article, we discuss recent evidence linking Tau pathology, insulin resistance and insulin deficiency. We further highlight the deleterious consequences of Tau pathology-induced insulin resistance to the brain and/or peripheral tissues, suggesting that these are key events mediating cognitive decline in Alzheimer's disease (AD) and other tauopathies.

Keywords: insulin resistance, tau protein, MAPT, Alzheimer's disease, tauopathy, cognitive decline, diabetes

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INTRODUCTION

Tau protein was first isolated from the porcine brain as a factor essential for microtubule assembly (Weingarten et al., 1975). Eleven years after its first characterization, Tau was shown to be a component of paired helical filaments (PHFs), the main constituents of Neurofibrillary tangles (NFTs) in the Alzheimer's disease (AD) brain (Grundke-Iqbal et al., 1986). The need to understand the role of Tau in AD and other tauopathies resulted in years of studies focusing on the pathological role of this protein and therefore, currently there exists a gap in what we know about the physiological functions of Tau. Bridging this gap will be instrumental to better comprehend the contribution of this protein to disease states.

Abbreviations: Aβ, Amyloid β; AD, Alzheimer's disease; CSF, Cerebrospinal fluid; FTD, Frontotemporal dementia; GSK3β, Glycogen synthase kinase 3 β; NFTs, Neurofibrillary tangles; PHFs, Paired helical filaments; PTEN, Phosphatase and tensin homolog protein; PTMs, Post-translational modifications; AkT, Protein kinase B; SH3, Src homology 3; T2D, Type 2 diabetes.

Tau is a natively soluble and unfolded protein that undergoes a variety of post-translational modifications (PTMs), which directly or indirectly modulates its physiological and pathological functions. Phosphorylation is the most common PTM described for Tau, and Tau has 85 different phospho-sites (Guo et al., 2017). A proper balance in the number of phosphorylated and dephosphorylated residues of Tau favors its classical physiological role in neurite outgrowth, axonal transport and microtubule dynamics. In addition, due to its scaffolding property, Tau can bind to a variety of proteins, therefore impacting multiple physiological functions (Meier et al., 2015; Suzuki and Kimura, 2017; Zhou et al., 2017; Stefanoska et al., 2018). Many of these interactions still remain to be uncovered. Under pathological conditions, an imbalance between the activities of kinases and phosphatases originates high levels of abnormally phosphorylated Tau, which have reduced affinity to microtubules and results in cytoskeleton destabilization. In addition, Tau hyperphosphorylation induces its missorting from axons into somatodendritic compartments, which is linked to synaptic dysfunction and cell death (Guo et al., 2017). Highly phosphorylated Tau self-assembles into PHFs and then NFTs, insoluble dense intracellular lesions considered a key hallmark of important human neurodegenerative disorders, such as AD, frontotemporal dementia (FTD), and other Tauopathies (Goedert and Spillantini, 2011).

In this review article, we discuss recent findings suggesting a novel physiological function for Tau protein as a regulator of insulin signaling in the brain and in peripheral tissues, and we further discuss the possible implications of Tau pathology-induced insulin resistance to cognition. A thorough understanding of the mechanisms linking Tau and insulin signaling becomes important when elucidating the increased risk of dementia associated with diabetes.

Tau PROTEIN AND INSULIN SIGNALING IN THE BRAIN

Insulin, the main regulator of glucose homeostasis and metabolism, is also crucial for a variety of different brain functions such as synaptic plasticity, learning and memory (Duarte et al., 2012; Fernandez and Torres-Alemán, 2012; De Felice, 2013; Kleinridders et al., 2014). In addition to its classical role as a microtubule-stabilizer, Tau acts as a scaffolding protein and interacts with components of the insulin signaling pathway in the brain. The N-terminal portion of Tau can bind to Src homology 3 (SH3) domains of Src family tyrosine kinases, which include domains from the p85alpha subunit of phosphatidylinositol 3-kinase (PI3K), a key protein in the insulin signaling pathway (Reynolds et al., 2008). Co-immunoprecipitation studies using wild-type (WT) mouse brain tissue and N1E115 cells transfected with WT human Tau reported that Tau binds to phosphatase and tensin homolog protein (PTEN), a negative regulator of insulin transduction that catalyzes the dephosphorylation of phosphatidylinositol (3,4,5)-triphosphate (PIP3) resulting in the formation of phosphatidylinositol (4,5)-diphosphate (PIP2). Therefore, by interacting with PTEN, Tau reduces its activity and favors insulin signaling (Marciniak et al., 2017). These studies raise the possibility that the ability of insulin to maintain proper brain activity depends on Tau and, conversely, pathological forms of Tau could be deleterious due to a loss of proper protein function. This idea was corroborated by a study showing that Tau deletion impaired hippocampal insulin sensitivity (Marciniak et al., 2017). Tau ablation in mice further resulted in deficits in long-term potentiation and contextual fear conditioning responses (Ahmed et al., 2014). In another study, the knockout (KO) of Tau in a mouse of B6129PF1/J background strain induced age-dependent defects in short-term memory and in synaptic plasticity (Biundo et al., 2018).

Impaired brain insulin signaling has been consistently associated with cognitive decline in animal models and in humans (Moloney et al., 2010; Bomfim et al., 2012; Craft et al., 2012, 2017; Lourenco et al., 2015; Benedict and Grillo, 2018). Under pathological conditions, loss of physiological function when Tau is hyperphosphorylated may trigger impairments in insulin signaling that will negatively impact the brain. The ability of Tau to interact with SH3 domains inversely correlates with its phosphorylated levels (Reynolds et al., 2008), suggesting that the scaffolding properties of Tau are regulated by its phosphorylation status. Moreover, increased Tau phosphorylation and decreased insulin signaling was observed in the hippocampus of middle-aged (17 months) rats, and it was linked to deficits in spatial learning (Kuga et al., 2018). In another study, hyperphosphorylated Tau-containing neurons from insoluble fractions of post-mortem AD and other tauopathies were shown to contain insulin accumulated as oligomers (Rodriguez-Rodriguez et al., 2017). In this study, the authors showed that the intraneuronal accumulation of insulin was associated with impairments in insulin signaling, characterized by reduced neuronal insulin receptor expression, as well as decreased downstream components of the insulin signaling pathway, such as phosphorylated protein kinase B (AkT). Studies using animal models that develop Tau pathology are warranted to determine whether impaired synaptic function and insulin signaling are a result of Tau loss of function due to Tau aggregation.

The loss of function hypothesis for Tau toxicity also extends to the findings in clinics. Reduced brain levels of soluble Tau are characteristic of the most common pathological variant of FTD, known as dementia lacking distinctive histopathology (DLDH). In these cases, reduced soluble Tau and the absence of insoluble or fibrillary Tau inclusions are associated with disease severity (Zhukareva et al., 2003). Proteomic analysis has found a high correlation between reduced Tau protein levels, synaptic impairment and reactive gliosis in FTD cases (Papegaey et al., 2016).

Although the influence of Tau pathology on insulin signaling is not completely understood, it is known that insulin resistance can induce Tau hyperphosphorylation and cognitive decline in human and in animal models (Deng et al., 2009; El Khoury et al., 2014; Yarchoan et al., 2014; Benedict and Grillo, 2018). Systemic insulin resistance was linked to poorer performance on cognitive tests and higher levels of cerebrospinal fluid (CSF) phosphorylated and total Tau in cognitively normal individuals

(Laws et al., 2017). Conversely, systemic insulin resistance was only associated with higher CSF levels of phosphorylated and Total Tau when the presence of the APOE e4 allele was considered (Starks et al., 2015). One of the mechanisms underlying this event involves the glycogen synthase kinase 3 β (GSK3β), a Tau kinase regulated by insulin *via* the AkT pathway (Welsh and Proud, 1993). Impaired brain insulin signaling, either as a result of insulin resistance due to the chronic exposure of neurons to high levels of insulin (Kim et al., 2011), or as a result of eventual decrease in brain insulin levels, triggers a reduction in AkT phosphorylation that leads to an increase in GSK3β activity and ultimately Tau phosphorylation (Zhang et al., 2018). Insulin can also affect phospho-Tau levels by decreasing the activity of Tau phosphatases (Gratuze et al., 2017). Protein phosphatase 2 (PP2A) is the primary Tau phosphatase implicated in AD, the activity of which is suppressed by insulin administration in humans and animals (Gong et al., 1995; Kins et al., 2001; Vogelsberg-Ragaglia et al., 2001). It remains to be investigated if defective insulin signaling, or tau pathology comes first in the disease pathogenesis. Our hypothesis is that it is individual specific. Regardless, in both cases, dysfunctional Tau protein and insulin signaling would aggravate each other and prompt or exacerbate cognitive decline in AD and other tauopathies.

In addition to insulin, insulin-like growth factors (IGFs) are important for the regulation of physiological functions such as glucose and energy metabolism, and they play crucial metabolic and neurotrophic roles in the brain (Gasparini and Xu, 2003; Sharma et al., 2016). The administration of IGFII in rats enhanced memory retention and prevented memory impairment (Chen et al., 2011). Serum levels of IGF1 reduces with aging and correlates with cognitive performance of individuals in the Mini Mental State Examination (MMSE) and in other neuropsychological assessments (Rollero et al., 1998; Kalmijn et al., 2000; Dik et al., 2003; Landi et al., 2007; Al-Delaimy et al., 2009; Angelini et al., 2009). Moreover, lower levels of IGF1 are both a risk factor and a feature of AD (Mustafa et al., 1999; Steen et al., 2005; Talbot et al., 2012; Westwood et al., 2014). Intriguingly, IGF proteins have been shown to regulate Tau phosphorylation *in vivo* (Schubert et al., 2003) and *in vitro* (Hong and Lee, 1997; Lesort et al., 1999; Lesort and Johnson, 2000), with the brain IGFI null mouse having increased hyperphosphorylated Tau when compared to WT animals (Cheng et al., 2005). Therefore, considering our hypothesis of Tau loss of function when this protein is hyperphosphorylated and aggregated in pathological conditions, it is likely that dysfunctional Tau is also a consequence of impaired IGF signaling in AD and other tauopathies. Moreover, because insulin and IGF signaling pathways share common intracellular components, it is possible that in disease, Tau loss of function affects IGF signaling. However, the association between the IGF system and Tau loss of function still needs to be investigated.

Apart from intracellular Tau, the existence of extracellular forms of Tau protein has been reported. The secretion of Tau *via* direct translocation across the plasma membrane was observed in neuroblastoma and in Chinese hamster ovary (CHO) cells overexpressing Tau constructs. In another study, Tau protein was released *via* exosomes by primary neuronal cultures and N2a cells overexpressing Tau constructs (Wang et al., 2017). While our current knowledge on the physiological functions of intracellular Tau is expanding, less is known about the role of extracellular Tau in physiology. A recent study has argued that the secretion and transfer of Tau between neurons is a physiological process rather than a disease-specific event (Evans et al., 2018). However, Tau secretion was shown to increase with Tau phosphorylation (Katsinelos et al., 2018), leading to the hypothesis that pathological Tau is secreted as a consequence of its reduced affinity to MTs when abnormally phosphorylated. Indeed, the exposure of neuroblastoma cells (SH-SY5Y) to conditioned medium from activated human microglia, caused an increase in the production and secretion of Tau (Lee et al., 2015), reinforcing the idea that neuroinflammation may impact Tau pathogenesis (Laurent et al., 2018). Therefore, with inflammation as a feature of the metabolic syndrome, a detrimental cycle might arise escalating both neurodegeneration and metabolic dysfunction in Tauopathies.

Neuronal-derived exosomes of total plasma from AD subjects presented increased levels of pIRS-1Ser312, a marker of insulin resistance, detected as early as 10 years before clinical onset of AD (Kapogiannis et al., 2015). In other studies, neuronal-derived exosomes in plasma of AD patients had higher levels of phosphorylated Tau (Abner et al., 2016), and the ratio of oligomeric to total Tau was higher in the CSF of AD patients (Sengupta et al., 2017). Despite the detection of markers of both Tau pathology and insulin resistance in exosomes derived from AD patients, it still remains to be elucidated how these species interact with each other regarding cause and consequence, their potential to act as trans-synaptic transmitter of pathology across neurons and their implication on brain function.

Tau PROTEIN AND INSULIN SIGNALING IN PERIPHERAL TISSUES

A recent publication suggested that Tau in the brain can efflux into the blood and can be cleared in the periphery, and therefore, a chronic increase of peripheral Tau clearance can reduce pathological Tau accumulation, neurodegeneration, and neuroinflammation in the brain (Wang et al., 2018). Tau is cleared in kidney and liver under physiological conditions in both human and mice. Therefore, compromised kidney and liver function might impede Tau clearance from the brain and thereby contribute to neurodegeneration. Insulin resistance has been suggested to contribute to kidney dysfunction and may also lead to non-alcoholic fatty liver disease during type 2 diabetes (T2D; De Cosmo et al., 2013; Mohamed et al., 2016), providing an additional mechanism by which T2D may contribute to cognitive dysfunction.

While Tau has played a primary role in the pathogenesis of some neurodegenerative diseases and has been extensively studied in the context of the brain, Tau has only recently been proposed to play a role in peripheral metabolic regulation. Recent work by our group and others has shown that Tau is highly expressed in pancreatic islets, in insulin-secreting beta cells (Bharadwaj et al., 2017; Wijesekara et al., 2018). Interestingly,

mice with a global KO of Tau show an increase in body weight and defects in glucose-stimulated insulin secretion and impaired glucose tolerance at a very young age (Wijesekara et al., 2018). A recent study also showed central insulin resistance in Tau KO animals, proposing that impaired insulin signaling in the hypothalamus, the main regulator of body weight, is a possibility. In accordance with this last hypothesis, Tau ablation inhibited the anorexigenic effects of insulin when delivered icv in mice (Marciniak et al., 2017). Mice with genetic deletion of insulin receptors in the brain [i.e., neuron-specific IR KO (NIRKO)] exhibit diet-sensitive obesity, increased food intake, and insulin resistance (Brüning et al., 2000). Moreover, brain insulin resistance typically results in increased hypothermia, augmented hepatic glucose output and impaired response to hypoglycemia (Kleinridders et al., 2014), all characteristics mirrored in the Tau KO animals, clearly suggesting the presence of reduced brain insulin signaling in these animals. While the effects on beta cell function could be a consequence of this, modulated by alterations in leptin levels, it was also evident that Tau plays a direct role in insulin production and secretion in these cells (Maj et al., 2016; Wijesekara et al., 2018). As a microtubule binding protein, Tau may be important for insulin granule movement to or sequestration at the plasma membrane mediating insulin secretion, and movement of proinsulin from the endoplasmic reticulum to the Golgi and subsequently to the insulin granule mediating insulin synthesis. In addition, one previous study suggested that Tau may also regulate insulin gene transcription (Maj et al., 2016).

Interestingly, elevated levels of Tau phosphorylation have been reported in the pancreas of patients with T2D (Miklossy et al., 2010), and recently, we showed significant Tau hyperphosphorylation in pancreatic islets from a transgenic mouse model of AD and T2D (Wijesekara et al., 2017). Whether this contributes to diabetes pathogenesis or is a consequence of diabetes itself remains to be understood. During diabetes, PI3 kinase pathway is typically down-regulated and therefore, GSK3 activity may be up-regulated, which could drive phosphorylation of Tau in diabetes (Hooper et al., 2008). Indeed, in pancreatic beta cell-derived rodent cell line, RIN-5F, inhibition of P13K was shown to increase Tau phosphorylation (Maj et al., 2016). Furthermore, it has been suggested that hypothermia, a consequence of insulin resistance could also drive Tau phosphorylation (El Khoury et al., 2016). Conversely, since hyperphosphorylated Tau may contribute to microtubule disassembly, changes in mitochondrial dynamics and inflammation (Beharry et al., 2014; Laurent et al., 2018), pathological Tau likely further contribute to beta cell dysfunction and insulin resistance. Regardless, the end result is an eventual reduction in insulin secretion, therefore, lower brain insulin levels leading to further aggravation of synaptic and cognitive impairments.

Although we were unable to observe appreciable expression of Tau in both mouse skeletal muscle and fat tissue (Wijesekara et al., 2018), its expression was shown in rat skeletal muscle in a previous study (Gu et al., 1996). An important feature of T2D is impaired glucose transport into skeletal muscle and adipose tissue, which is facilitated by GLUT4 glucose transporter translocation to the plasma membrane. While some have suggested that an intact microtubule system is important for the insulin-induced actin remodeling prior to the transporter translocation, some studies using agents that inhibit microtubule polymerization has suggested against this (Fletcher et al., 2000; Olson et al., 2001; Liu et al., 2013). Nonetheless, it has been shown that GLUT4 storage vesicles travel along microtubules *via* kinesins and actin filaments, from the perinuclear region, bringing them into close proximity with the plasma membrane SNARE proteins (Tunduguru and Thurmond, 2017). Newly formed GLUT4 vesicles also are transported from the plasma membrane to the cell interior by the microtubule-based motor protein dynein. However, it has been shown that heterologous overexpression of Tau protein, despite being localized to microtubules in 3T3-L1 adipocytes, delays the initial appearance of GLUT4 at the cell membrane following insulin stimulation (Emoto et al., 2001). This is in accordance with the view that excess Tau blocks axonal transport due to interference with motor proteins (Mandelkow et al., 2003). If Tau is indeed a critical regulator of GLUT4 movement in insulin-sensitive tissues, reduced glucose uptake into these tissues may have contributed to the early development of hyperglycemia in Tau KO mice and remains to be explored in future studies.

Current data suggests that there is clearly a role of Tau in peripheral tissue, particularly in regulating glucose homeostasis. However, the mechanisms linking central insulin resistance and peripheral Tau hyperphosphorylation and the metabolic consequences remain largely unknown. There are many factors that require consideration such as the impact of brain insulin resistance, particularly the hypothalamus on peripheral

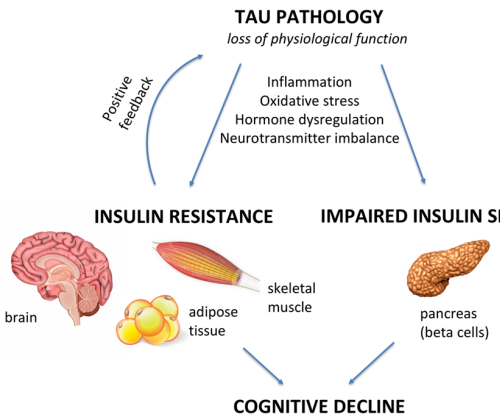

FIGURE 1 | Tau pathology as a mediator of insulin resistance and cognitive and metabolic alterations. Under pathological conditions, Tau loss of function can negatively impact the brain and peripheral tissues, such as skeletal muscle, adipose tissue and pancreas. In this review article, we explore the hypothesis of Tau pathology being a key inductor of insulin resistance and beta cell dysfunction in Alzheimer's disease (AD) and other tauopathies. When defective insulin signaling is present in the brain, it triggers neurodegeneration and cognitive decline. On the other hand, when it is present in peripheral tissues, it leads to a diabetes-like phenotype. Inflammation, oxidative stress, hormonal dysregulation and neurotransmitter imbalance are mechanisms that can lead to/aggravate Tau pathology-induced alterations in insulin signaling/secretion in the brain and in the periphery, which are further associated with cognitive defects.

metabolic regulation, especially in the context of feeding behavior, body temperature regulation, energy expenditure; influence of pathological Tau on hormone regulation such as leptin and ghrelin and their impact on metabolism; direct changes to the microtubule system in individual tissues; and impact of gluco- and lipo-toxicity and peripheral insulin resistance and inflammation. Regardless, even with limited data, it is becoming quite clear that we cannot fully comprehend neurodegeneration without fully understanding the physiological roles of its key players such as Tau within the periphery.

CONCLUSION

Tau pathology has emerged as a trigger of insulin resistance and insulin deficiency in the brain and peripheral tissues, and it is suggested to be an early event in the pathogenesis of AD and other tauopathies, representing a promising therapeutic target capable of interfering with disease progression. Impaired insulin signaling can also trigger Tau pathology, sustaining a vicious cycle, with cognitive decline being the end result as illustrated in **Figure 1**. However, it remains to be elucidated whether impaired insulin signaling, or Tau pathology comes first in AD pathogenesis and other tauopathies.

The molecular mechanisms of how Tau impairs insulin signaling and insulin secretion also remains to be better investigated and might involve loss of Tau function or indirect mechanisms such as inflammation, oxidative stress or changes in hormone/neurotransmitter release. Regardless, it is becoming apparent that Tau may be an important link between neurodegeneration and diabetes and while Tau KO mice provide a good foundation for these studies, future experiments need to be geared towards understanding these phenomena at a whole-body level using transgenic Tau hyperphosphorylation models and tissue-specific Tau KO animals.

AUTHOR CONTRIBUTIONS

RG and FDF defined the topics to be covered and drafted the main part of the manuscript. NW participated in the conception and design of the figure, with contributions from RG. RG, NW, FDF and PF participated in the development and draft of the article, critical revision of the article and final approval of the version to be published.

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Disorders of Body Weight, Sleep and Circadian Rhythm as Manifestations of Hypothalamic Dysfunction in Alzheimer's Disease

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While cognitive decline and memory loss are the major clinical manifestations of Alzheimer's disease (AD), they are now recognized as late features of the disease. Recent failures in clinical drug trials highlight the importance of evaluating and treating patients with AD as early as possible and the difficulties in developing effective therapies once the disease progresses. Since the pathological hallmarks of AD including the abnormal aggregation of amyloid-beta (Aβ) and tau can occur decades before any significant cognitive decline in the preclinical stage of AD, it is important to identify the earliest clinical manifestations of AD and elucidate their underlying cellular and molecular mechanisms. Importantly, metabolic and non-cognitive manifestations of AD such as weight loss and alterations of peripheral metabolic signals can occur before the onset of cognitive symptoms and worsen with disease progression. Accumulating evidence suggests that the major culprit behind these early metabolic and non-cognitive manifestations of AD is AD pathology causing dysfunction of the hypothalamus, a brain region critical for integrating peripheral signals with essential homeostatic physiological functions. Here, we aim to highlight recent developments that address the role of AD pathology in the development of hypothalamic dysfunction associated with metabolic and non-cognitive manifestations seen in AD. Understanding the mechanisms underlying hypothalamic dysfunction in AD could give key new insights into the development of novel biomarkers and therapeutic targets.

Keywords: hypothalamus, obesity, diabetes, sleep, circadian rhythm, dementia, amyloid-beta, tau

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INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia in the elderly and remains an incurable and devastating neurodegenerative disease (Alzheimer's Association, 2018). Though the exact pathogenesis of AD remains unclear, abnormal accumulation of amyloid-beta (Aβ) peptides and the microtubule-associated protein tau over time leads to neuronal and synaptic dysfunction and the neuropathological hallmarks of extracellular Aβ plaques and neurofibrillary tau tangles (Sala Frigerio and De Strooper, 2016). While cognitive symptoms are the most prominent feature of AD, they are now recognized as a late manifestation. Aβ and tau pathology can be detected by cerebrospinal fluid (CSF) analysis or positron emission tomography (PET)

imaging in the preclinical stage of AD, decades prior to the cognitive impairment seen in mild cognitive impairment (MCI) or dementia (Dubois et al., 2016). Notably, various metabolic and non-cognitive manifestations of AD including weight loss and sleep and circadian rhythm disorders can precede the cognitive decline (Ishii and Iadecola, 2015). The hypothalamus, which serves as the brain's integrator of peripheral metabolic signals and houses the central circadian pacemaker, the suprachiasmatic nucleus (SCN), is uniquely positioned to mediate many of these non-cognitive changes. Importantly, both Aβ and tau pathology have been found in the hypothalamus of AD brains (**Table 1**). Moreover, non-cognitive symptoms can worsen with disease progression and are associated with increased morbidity and mortality in AD, highlighting the importance of hypothalamic dysfunction in AD. In this review article, we discuss recent developments in understanding the relationship between AD pathobiology and select metabolic and non-cognitive manifestations that are putatively linked to hypothalamic dysfunction.

BODY WEIGHT AND SYSTEMIC METABOLISM

Late-Life Weight Loss: An Early Manifestation of AD

Weight loss has long been recognized as a clinical manifestation of AD and was considered a criteria consistent with the diagnosis of probable AD in the 1984 NINCDS-ADRDA work group report (McKhann et al., 1984). Importantly, weight loss in AD patients correlated with increased morbidity and mortality (White et al., 1998; Jang et al., 2015) and cortical Aβ load (Blautzik et al., 2018). Furthermore, MCI subjects who are underweight or lose weight have an increased risk for progressing to AD (Sobów et al., 2014; Joo et al., 2018). These studies collectively suggest that weight loss is an intrinsic feature of AD pathobiology.

While weight loss once dementia manifests could be attributed to impairments in appetite and eating behavior, epidemiological studies have found consistently that late-life weight loss can precede the cognitive decline in AD (Barrett-Connor et al., 1996; Buchman et al., 2005; Stewart et al., 2005; Johnson et al., 2006; Gao et al., 2011; Emmerzaal et al., 2015; Jimenez et al., 2017). Additionally, in a large community cohort study, late-life weight loss increased the risk for developing MCI regardless of mid-life body weight, suggesting that late-life weight loss is a clinical manifestation of early stages of AD regardless of mid-life metabolic risk factors (Alhurani et al., 2016). Furthermore, a recent study from the Dominantly Inherited Alzheimer Network (DIAN) found that asymptomatic carriers of gene mutations for autosomal dominant AD had significantly lower body mass index (BMI) compared to nonmutation carriers with weight loss starting more than a decade before onset of cognitive symptoms (Müller et al., 2017). Importantly, lower BMI was found to be associated with higher brain Aβ burden and lower scores on a delayed memory recall test in the asymptomatic AD mutation carriers.

Mounting evidence suggests that the adipocyte-derived hormone (adipokine) leptin is affected in AD. Leptin is produced in proportion to adiposity and serves as a critical negative afferent signal to the brain and in particular the hypothalamus to regulate body weight and systemic metabolism (Friedman, 2014). Low circulating leptin levels have been consistently found in AD subjects (Lieb et al., 2009; Bigalke et al., 2011; Khemka et al., 2014; Ma et al., 2016; Yu et al., 2018). While the underlying mechanisms for the early weight loss and low circulating leptin levels remain to be fully elucidated, dysfunction of the hypothalamus is likely to be a major driver. Compared to wild-type littermates, young transgenic mice with Aβ pathology (Tg2576 mice) prior to plaque formation or significant cognitive impairment exhibited low body weight/adiposity and low plasma leptin levels, which was associated with Aβ-mediated dysfunction of select hypothalamic neurons important for the regulation of body weight (Ishii et al., 2014). Taken together, these findings from animal and human studies raise the intriguing possibility that Aβ could interfere with hypothalamic sensors of peripheral metabolic signals such as leptin, preventing the brain from responding to signals of low body weight/adiposity resulting in pathologically low circulating leptin levels and unintentional weight loss.

TABLE 1 | Hypothalamus and Alzheimer's disease (AD): select pathological and neuroimaging studies.

Study type	Hypothalamic findings
Histopathology (amyloid)	• Amyloid deposition was observed in all hypothalamic nuclei by Braak Stage C (Braak and Braak, 1991) • Specifically, amyloid plaques have been reported in many hypothalamic regions including the mammillary bodies, SCN, TMN, LTN, VMN and fornix (Stief, 1927; Rudelli et al., 1984; McDuff and Sumi, 1985; Stopa et al., 1999)
Histopathology (tau)	• Isolated neurofibrillary tangles (NFT) was observed first in TMN at Braak Stage III and by Stage VI are widespread in TMN and LTN (Braak and Braak, 1991) • NFT and tau staining have been reported in the mammillary bodies, SCN, DMN, VMN, arcuate (infundibular) nucleus and fornix (Stief, 1927; Rudelli et al., 1984; McDuff and Sumi, 1985; Saper and German, 1987; van de Nes et al., 1998; Schultz et al., 1999; Stopa et al., 1999)
MRI (volume)	• Decrease in hypothalamic volume was observed by moderate AD (Callen et al., 2001) • Hypothalamic atrophy was more pronounced in males than in females (Callen et al., 2004) and positively correlated with BMD loss in mild AD (Loskutova et al., 2010)
^{18}FDG-PET (glucose metabolism)	• Reduced glucose metabolism in the hypothalamus was observed in MCI and AD patients (Nestor et al., 2003; Cross et al., 2013)

Abbreviations: BMD, bone mineral density; DMN, dorsomedial nucleus; FDG, fluorodeoxyglucose; MCI, mild cognitive impairment; MRI, magnetic resonance imaging; NFT, neurofibrillary tangles; LTN, lateral tuberal nucleus; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; TMN, tuberomammillary nucleus; VMN, ventromedial nucleus.

Additionally, leptin is important for maintaining hippocampal structure and function (McGregor and Harvey, 2017) and exerting neuroprotective effects under a variety of neurotoxic conditions including Aβ (McGuire and Ishii, 2016). In humans, low circulating leptin levels have been associated with cognitive decline in the elderly (Holden et al., 2009) and decreased hippocampal gray matter volume (Narita et al., 2009). Similarly, studies using various transgenic mouse models of Aβ pathology have consistently found that leptin levels correlate positively with cognitive function and negatively with Aβ burden (Greco et al., 2010; Takeda et al., 2010; Pérez-González et al., 2014). Therefore, alterations in leptin signaling associated with unintentional weight loss may serve not only as a marker of early AD but may contribute to AD pathogenesis. While leptin has been the most extensively investigated adipokine in AD, a significant role for other adipokines and peripheral metabolic signals cannot be excluded (Kiliaan et al., 2014).

Mid-Life Obesity and Diabetes: Risk Factors for Developing AD

In contrast to late-life weight loss, mid-life obesity and related comorbid conditions including insulin resistance and type 2 diabetes mellitus (T2DM) have been found in several epidemiological studies to be risk factors for cognitive decline and AD (Arvanitakis et al., 2004; Kivipelto et al., 2005; Biessels et al., 2014; McGuire and Ishii, 2016). In contrast, a large population study in the UK found that mid-life obesity decreased risk for dementia (Qizilbash et al., 2015); however, this study may have potential confounding factors including reverse causation bias and ill-defined range of mid-life (Gustafson, 2015; Kivimäki et al., 2015). While additional studies are clearly needed, the current evidence suggests that age is an important factor when considering body weight and adiposity changes in AD with mid-life obesity being a risk factor and late-life weight loss being an early manifestation of AD.

In light of the association between mid-life obesity and T2DM and AD, it has been noted that these metabolic disorders cause damage to the hypothalamus by similar mechanisms to those seen in AD (Clarke et al., 2018). Physiological consequences of obesity and T2DM, including chronic hyperinsulinemia and high circulating levels of free fatty acids have been shown to lead to hypothalamic insulin and leptin resistance (Thon et al., 2016), ER stress (Zhang et al., 2008; Mayer and Belsham, 2010) and pro-inflammatory intracellular cascades (Milanski et al., 2009) in hypothalamic neurons. Similarly, Aβ oligomers induced TNF-alpha mediated inflammation and ER stress in cultured hypothalamic neurons and the hypothalamus of mice and macaques (Clarke et al., 2015). Furthermore, an NMR-based metabolomics study of the transgenic amyloid precursor protein/presenilin 1 (APP/PS1) mouse model of Aβ pathology found that these mice had significant hypothalamic metabolic abnormalities prior to memory impairment (Zheng et al., 2018). These studies provide further support that hypothalamus dysfunction can occur early in the development of AD and is likely mediated by mid-life metabolic risk factors of obesity and T2DM.

There is also substantial evidence to suggest that obesity and T2DM related pathologies could directly promote early AD pathology. Studies in mouse models and humans have shown that hyperinsulinemia and insulin resistance can increase Aβ load by interfering with clearance mechanisms and increasing production of Aβ (Stanley et al., 2016; Ramos-Rodríguez et al., 2017; Benedict and Grillo, 2018). Additionally, obesity and T2DM lead to increased deposition of human islet APP (hIAPP or amylin) in not only pancreatic islets but in the brain parenchyma and cerebrovascular system, which may exacerbate AD pathology by causing neurotoxicity and decreased Aβ clearance (Jackson et al., 2013; Wijesekara et al., 2017). The cross-seeding of misfolded hIAPP and Aβ peptides has been hypothesized as a mechanism for shared disease pathogenesis between AD and T2DM (Moreno-Gonzalez et al., 2017). However, not all studies show that obesity and T2DM worsens AD pathology. For example, a mouse model of human tau pathology given a high-fat, high-sugar and high-cholesterol diet had no significant changes in hippocampal and cortical tau pathology (Gratuze et al., 2016).

SLEEP AND CIRCADIAN RHYTHM DISORDERS

Sleep Disorders

Sleep disorders affect 25%–66% of AD patients and are a leading cause for institutionalization (Bianchetti et al., 1995; Moran et al., 2005; Guarnieri et al., 2012). Importantly, sleep quality in AD declines early in the disease and worsens with disease progression (Vitiello et al., 1990; Liguori et al., 2014). Furthermore, cognitively normal subjects with Aβ deposition by CSF measurements had worse sleep quality compared to those without Aβ deposition (Ju et al., 2013). This association between AD pathology and poor sleep quality has been recapitulated in multiple mouse models with increased Aβ deposition (Wisor et al., 2005; Roh et al., 2012; Sethi et al., 2015). Additionally, a single intracerebroventricular (ICV) infusion of Aβ oligomers disrupted sleep patterns in mice (Kincheski et al., 2017). Taken together, these findings provide evidence that AD pathology impacts sleep early in AD and may occur prior to the onset of cognitive symptoms.

Accumulating evidence suggests that hypothalamic dysfunction is responsible for the sleep dysfunction in AD. A recent study found reduced hypothalamic glucose uptake, as measured by ^{18}F-flurodeoxyglucose PET, in AD subjects compared to non-demented control subjects, which was associated with sleep impairment and CSF AD biomarkers (Liguori et al., 2017). Evidence also exists for the involvement of specific hypothalamic nuclei in the sleep dysfunction in AD. The intermediate nucleus of the hypothalamus, the putative analog to the ventrolateral preoptic nucleus (VLPN) in rodents, contains neurons that are active in both rapid eye movement (REM) and non-REM (NREM) sleep (Chung et al., 2017; Saper and Fuller, 2017). A decrease of galanin-positive neurons in

the intermediate nucleus was reported in postmortem AD brains (Lim et al., 2014). Because these neurons are active during sleep and inhibit wake-promoting neurons, loss of VLPN galanin neurons presents a potential mechanism for decreased NREM sleep and increased awakenings in AD (Saper and Fuller, 2017).

Another important hypothalamic nucleus in the regulation of sleep is the lateral hypothalamic area (LH), which contains neurons that synthesize the neuropeptide orexin (hypocretin). Orexin is critical for the maintenance of sleep-wake architecture by promoting arousal with orexin deficiency resulting in narcolepsy (Tsujino and Sakurai, 2013). In human studies, there are conflicting reports regarding orexin levels in AD with multiple studies reporting unchanged or decreased CSF and hypothalamic levels (Fronczek et al., 2012; Schmidt et al., 2013; Liguori et al., 2014). In contrast, more recent studies suggest that accumulating AD pathology is associated with increased CSF orexin levels and sleep disruption. In AD biomarker-defined MCI subjects, increased CSF orexin levels were associated with REM sleep disruption and sleep fragmentation (Liguori et al., 2016). In another study, higher CSF orexin levels were found in biomarker-defined AD subjects compared to MCI and control groups (Gabelle et al., 2017).

Substantial support also exists for sleep dysfunction worsening AD pathology and increasing the risk for developing dementia (Mander et al., 2016). A recent meta-analysis found that sleep disorders such as insomnia and sleep-disordered

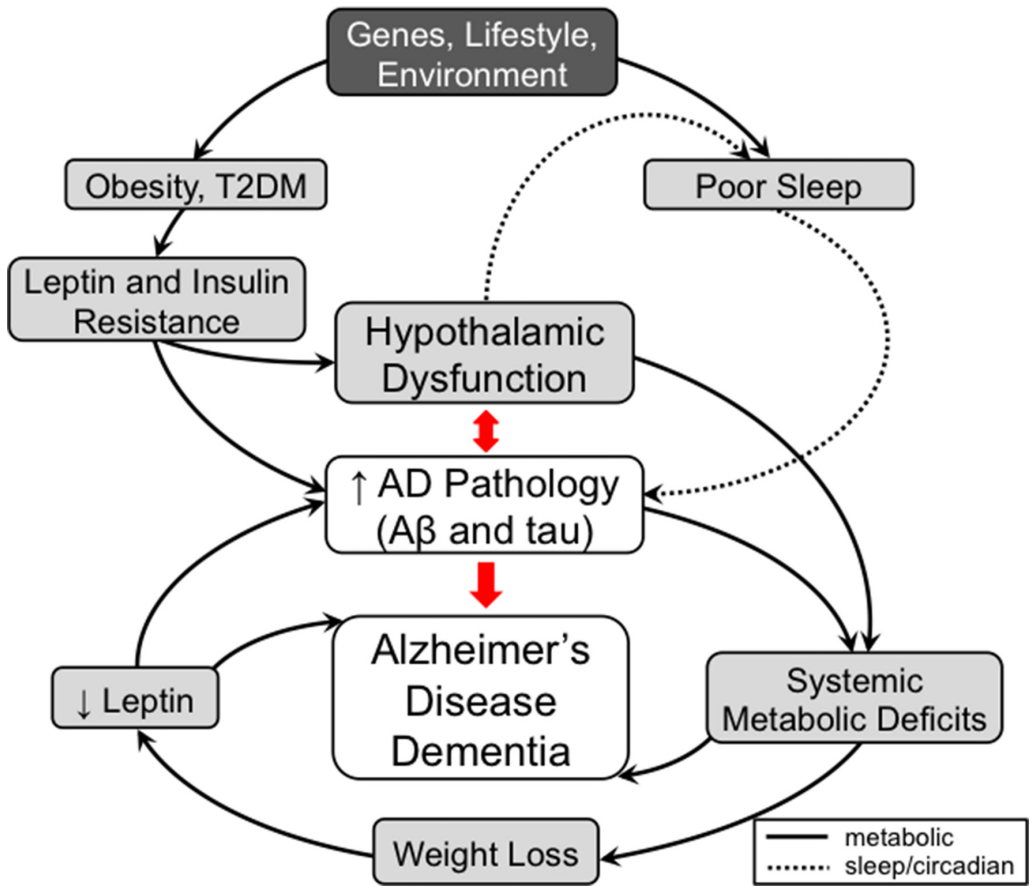

FIGURE 1 | A model for the contribution of metabolic and non-cognitive factors (e.g., sleep/circadian rhythm) in the pathogenesis of Alzheimer's disease (AD). In mid-life, obesity and type 2 diabetes mellitus (T2DM) are risk factors for AD. These conditions are associated with high circulating insulin and leptin levels leading to the development of hypothalamic dysfunction, including insulin and leptin resistance, as well as to worsening AD pathology directly. Development of hypothalamic insensitivity to peripheral metabolic signals in mid-life sets the stage for exacerbation of metabolic dysregulation in late-life AD, when accumulation of AD pathology can lead to further neuronal injury. A possible model to explain the correlation between late-life weight loss and AD posits that AD pathology-mediated neuronal injury in the hypothalamus leads to a hypermetabolic (catabolic) state, which results in weight loss and a pathologically low leptin state. As leptin has possible roles as a neuroprotective factor and a regulator of hippocampal structure and function, deficiency in leptin signaling could also contribute to cognitive impairment independent of hypothalamic signaling. Similar to metabolic dysfunction, sleep disorders play a role in AD pathogenesis. Poor sleep quality in mid-life has been associated with increased AD pathology. In late-life, hypothalamic dysfunction caused by AD pathology contributes to the sleep dysfunctions seen in AD. The worsening sleep disorders would then feed forward into the development of further AD pathology and eventually dementia. Therefore, disorders of metabolism and non-cognitive (e.g., sleep/circadian rhythm) factors mediated by hypothalamic dysfunction are both early risk factors and manifestations of AD that can contribute in a feed-forward manner that ultimately results in AD dementia. Solid lines represent metabolic pathways and dashed lines represent pathways related to sleep and circadian rhythms.

breathing increased the risk for developing AD (Shi et al., 2018). Prolonged sleep duration in older adults was also associated with increased development of dementia (Westwood et al., 2017). Therefore, abnormal sleep, regardless of the duration, is associated with increased dementia risk. Additionally, human and animal studies have found that poor sleep quality including deprivation can worsen AD pathology. In healthy human adults, a single night of lost sleep was associated with an increased Aβ load as measured by CSF and brain PET studies (Ooms et al., 2014; Shokri-Kojori et al., 2018). Furthermore, several studies have found various measures of poor sleep quality were associated with increased brain Aβ load in cognitively normal individuals (Spira et al., 2013; Branger et al., 2016) Consistent with these human studies, sleep deprivation or increased wakefulness in a *Drosophila* or transgenic mouse model of Aβ pathology increased Aβ burden (Kang et al., 2009; Roh et al., 2014; Tabuchi et al., 2015). The underlying mechanism behind the association between sleep and Aβ pathology has been hypothesized to be due to increased clearance of Aβ during sleep (Xie et al., 2013) or neuronal activity-dependent increases in Aβ secretion during wakefulness (Cirrito et al., 2005; Tabuchi et al., 2015). Despite some conflicting studies, the current evidence supports a bidirectional relationship where AD pathology can cause increased orexin levels and disruption of sleep, while disruption of sleep can lead to increased AD pathology.

Circadian Rhythm Disorders and Sundowning

Closely related to sleep disorders, circadian rhythm abnormalities including disrupted day-night activity patterns are common in AD patients (Musiek et al., 2015). In particular, aggressive behaviors in AD are often temporally dependent, worsening in the afternoon and evening, in a pattern that is clinically termed Sundown Syndrome or "sundowning" (Khachiyants et al., 2011). Importantly, agitation such as seen with sundowning in AD patients can precede significant adverse outcomes including institutionalization, accelerated cognitive decline and increased caregiver burden (Canevelli et al., 2016). Yet, current strategies for managing aggressive symptoms rely on pharmacological interventions including anti-psychotics that may not target the underlying pathways affected and can have significant adverse effects (Ballard and Corbett, 2013). Therefore, understanding the underlying mechanisms of sundowning would be critical for improving the clinical care of AD patients.

The hypothalamus has long been recognized as a major regulator of both circadian rhythm and aggressive behaviors, suggesting a potential role in sundowning. Dysfunction in the hypothalamic SCN, the central pacemaker, is a likely mediator of circadian rhythm disorders in AD (Van Erum et al., 2018). In AD patients, the SCN shows increased aging-related atrophy and neurodegeneration with evidence for neurofibrillary tangle accumulation (Swaab et al., 1985; Stopa et al., 1999). Additionally, in postmortem AD brains, blunted fluctuations in circadian motor activity and increased SCN amyloid plaque burden are reported to be correlated with reduction of two central circadian neurotransmitters, vasopressin and neurotensin (Stopa et al., 1999; Harper et al., 2008; Hu et al., 2013), although one study reported no change in SCN vasopressin levels in AD (Wang et al., 2015). Similarly, the hypothalamus has been long implicated in the role of aggressive behaviors. In the early 20th century, electrical stimulation of specific regions of the hypothalamus including the LH and the VMH promoted aggression in cats (Hess and Akert, 1955). These areas of the hypothalamus have been classically identified as "attack areas," and their stimulation in a variety of animal species has been linked with distinct aggressive behaviors (Haller, 2013). A recent study identified a hypothalamic circuit involving projections from the SCN to the VMH that regulated the daily rhythm in aggression propensity of male mice (Todd et al., 2018), suggesting that disruption of this hypothalamic circuit could lead to sundowing in AD.

Molecular and genetic studies in animal models further support the hypothalamus and in particular the SCN playing a central role in circadian rhythm disorders associated with AD pathology. A mouse model of Aβ pathology was found to have dampened SCN excitability rhythms, concurrent with circadian-associated behavioral disturbances and reduced daytime A-type potassium currents (Paul et al., 2018). In contrast, several Aβ mouse and *Drosophila* models exhibit circadian behavioral abnormalities despite normal central clock function, suggesting that Aβ-related circadian abnormalities may also stem from a "central clock output failure" in which the SCN fails to entrain brain-resident and peripheral clocks (Chauhan et al., 2017).

CONCLUSIONS

We have briefly reviewed select recent findings on the metabolic and non-cognitive manifestations of AD that can occur before the cognitive decline and focused specifically on disorders of body weight, sleep and circadian rhythm. We provide evidence that these metabolic and non-cognitive manifestations of AD are due to hypothalamic dysfunction caused by AD pathology and can be bidirectional and feed-forward in nature (**Figure 1**). Furthermore, while body weight and sleep/circadian rhythm may appear to act independently from each other, they often share common neurotransmitters (e.g., orexin, galanin) and brain regions (e.g., VMH, LH) in the hypothalamus, which can be modulated by peripheral circulating factors such as leptin and glucose (Fang et al., 2012; Tsujino and Sakurai, 2013; McGuire and Ishii, 2016). Therefore, seemingly disparate clinical manifestations of AD may be due to alterations of common hypothalamic pathways affected early in AD.

Despite recent advances, there are significant gaps in our knowledge. The hypothalamus is a complex brain region comprised of numerous distinct molecular cell types with each potentially a part of multiple different pathways. While several studies using a candidate-based approach have identified select individual cell types affected by AD pathology (Ishii et al., 2014; Clarke et al., 2015), the exact cell types affected in the hypothalamus are not known. Therefore, large unbiased molecular screens such as with Drop-Seq and similar approaches will likely be needed (Campbell et al., 2017). Additionally, once the cell types affected by AD pathology are identified,

the exact cellular mechanisms leading to the dysfunction of those neurons and whether they are similar to those seen in more extensively studied brain regions such as the hippocampus need to be elucidated. Finally, any mechanistic studies in cellular or animal models needs to be validated and verified in carefully conducted AD biomarker-defined human studies.

While cognitive manifestations have deservedly received the bulk of the attention in AD research, non-cognitive manifestations are often correlated with disease progression, increased morbidity including institutionalization, and increased mortality. These non-cognitive signs and symptoms could be developed as inexpensive and readily accessible markers of AD progression in a clinical setting. Moreover, elucidating the underlying molecular mechanisms for these early clinical manifestations of AD may yield important insights into novel pathways affected in AD, which could lead to the development of important new therapeutic targets.

AUTHOR CONTRIBUTIONS

All authors participated in the study design, drafted/revised the manuscript, approved the final version and agreed to be accountable for all the aspects of the work.

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The Strategic Location of Glycogen and Lactate: From Body Energy Reserve to Brain Plasticity

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Brain energy metabolism has been the object of intense research in recent years. Pioneering work has identified the different cell types involved in energy production and use. Recent evidence has demonstrated a key role of L-Lactate in brain energy metabolism, producing a paradigm-shift in our understanding of the neuronal energy metabolism. At the center of this shift, is the identification of a central role of astrocytes in neuroenergetics. Thanks to their morphological characteristics, they are poised to take up glucose from the circulation and deliver energy substrates to neurons. Astrocyte neuron lactate shuttle (ANLS) model, has shown that the main energy substrate that astrocytes deliver to neurons is L-Lactate, to sustain neuronal oxidative metabolism. L-Lactate can also be produced from glycogen, the storage form of glucose, which is exclusively localized in astrocytes. Inhibition of glycogen metabolism and the ensuing inhibition of L-Lactate production leads to cognitive dysfunction. Experimental evidence indicates that the role of lactate in cognitive function relates not only to its role as a metabolic substrate for neurons but also as a signaling molecule for synaptic plasticity. Interestingly, a similar metabolic uncoupling appears to exist in peripheral tissues plasma, whereby glucose provides L-Lactate as the substrate for cellular oxidative metabolism. In this perspective article, we review the known information on the distribution of glycogen and lactate within brain cells, and how this distribution relates to the energy regime of glial vs. neuronal cells.

Keywords: glycogen, lactate, astrocyte, ANLS, synaptic plasticity

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INTRODUCTION

L-Lactate was isolated in the 18th century and found to be released by muscle cells upon exertion, its physiological role been reduced, for a long time, to a simple waste product of anerobic metabolism. Interesting work in the '80s started to unveil the metabolic properties of L-Lactate in skeletal muscles (Brooks, 1985). In contrast, our understanding of the energy metabolism in the central nervous system (CNS) was delayed because of the technical challenges in studying the brain compared to the peripheral organs. However, in the '90s, it was proposed that astrocytes release L-Lactate as a result of aerobic glycolysis, i.e., the processing of glucose to lactate in the presence of physiological concentrations of oxygen, upon synaptic stimulation to support neuronal function, providing the first evidence of a lactate shuttle in the CNS (Pellerin and Magistretti, 1994; Magistretti and Allaman, 2018). This metabolic profile of astrocytes and

their role in brain energy metabolism was initially received with skepticism, as mammalian cells are known to generate their main energy source molecule, ATP, within mitochondria, starting from glucose. Indeed, since glucose is almost fully oxidized by the brain, this implied that a transfer of L-Lactate from astrocytes to neurons should exist. ATP is mainly produced by oxidative phosphorylation, fueled by the tricarboxylic acid (TCA) cycle. Pyruvate originating from the glycolysis is transformed in a sequence of reactions to produce substrates supporting the TCA activity. Neurons are no different and express similar transporters for glucose (GLUT) at their membrane. Consistent with their high energetic demands neurons are mainly oxidative (80%–90% of their metabolism; Magistretti and Allaman, 2015). Questions then arise, as to why should neurons behave differently, and somehow rely on astrocyte-derived lactate to support their energy needs? Is this metabolic profile due to the specific expression of metabolic enzymes or because of the inability of neurons to store energy in the form of glycogen (Magistretti and Allaman, 2018)? Also, how do astrocytes sustain neuronal metabolism? In this short perspective article, we will briefly analyze these three points, and provide a review of recent evidence that address these questions.

THE ASTROCYTE NEURON LACTATE SHUTTLE (ANLS)

According to the neuro-metabolism view up to the early '90s, cells in the CNS simply need to consume glucose, whose constant supply needs to be provided by the vascular system, in order to sustain the brain homeostasis. Indeed, as already demonstrated by Sherrington at the end of the 19th century, blood flow is coupled to neuronal activity through a mechanism known as neurovascular coupling (Roy and Sherrington, 1890) mediated by a variety of vasoactive molecules (Magistretti and Allaman, 2015). This activity-dependent increase in local blood flow was considered sufficient to provide the necessary amount of glucose for the direct use by neurons. However, particularly upon intense neuronal activity, such as during long term potentiation (LTP), when synaptic plasticity requires additional energy support, glucose does not seem to be the preferred substrate to maintain neuronal activity (Suzuki et al., 2011). Experiments that investigated learning and memory formation in the context of the energy metabolism have shown that lactate, rather than glucose, was effective in reversing the amnestic effect caused by the inhibition of monocarboxylate transporters (MCTs) and of pharmacological inhibition of glycogenolysis, one of the mechanisms responsible for lactate production (Suzuki et al., 2011; Boury-Jamot et al., 2016; Gao et al., 2016). Supporting this view is the "Astrocyte-Neuron Lactate Shuttle (ANLS)," a neuroenergetic model first proposed in the '90s, according to which glutamate uptake into astrocytes as a result of synaptic activity triggers an intracellular signaling cascade within astrocytes that results in the production of L-Lactate through aerobic glycolysis (Pellerin and Magistretti, 1994).

The ANLS model reconciled, the morphological-based hypotheses of Camillo Golgi related to astrocytic metabolic support of neurons, with experimental evidence. *In vivo* experiments have demonstrated that indeed astrocytes are the predominant site of glucose uptake during synaptic activity (**Figure 1**). Thus, downregulating the expression of glutamate transporters on astrocytes drastically reduces the activity-dependent uptake of glucose into the brain parenchyma (Cholet et al., 2001; Voutsinos-Porche et al., 2003). A mirror experiment in which an increase in glutamate transporters in astrocytes was induced pharmacologically, resulted in an increase in glucose uptake into the brain parenchyma as determined by *in vivo* 2-deoxyglucose PET (Zimmer et al., 2017).

Additional *in vivo* experiments have shown that a gradient exists between the concentration of L-Lactate in neurons and astrocytes, favoring its efflux from astrocytes and its influx in neurons (Mächler et al., 2016), a phenomenon that has been also validated using computational models (Jolivet et al., 2015; Coggan et al., 2018).

Lactate is not only an energy substrate for neurons. Indeed, recent evidence, triggered by the observation that lactate transfer from astrocytes to neurons is necessary for LTP expression, synaptic plasticity and memory consolidation (Suzuki et al., 2011) has shown that lactate is also a signaling molecule for synaptic plasticity. Indeed lactate modulates the expression of at least 20 genes related to synaptic plasticity and neuroprotection (Yang et al., 2014; Margineanu et al., 2018). This signaling action of lactate is due to a positive modulation of N-Methyl-D-aspartate (NMDA) receptor signaling (Yang et al., 2014).

GLYCOGEN

Recent findings about the specific role of lactate derived from glycogen, rather than direct glycolysis of glucose, represents another modality through which the ANLS operates. Glycogen has a well-known structure, formed by linear chains of glucose that accumulates around a core protein called glycogenin, forming round granules of various size, between 20 and 80 nm in diameter in astrocytic processes (Calì et al., 2016). Glycogen was first discovered in peripheral tissues, and its concentration in the brain, compared to muscle and liver, is considerably lower, in a concentration ratio of 1:10:100, respectively (Magistretti and Allaman, 2013). Interestingly, glycogen granules are specifically located in astrocytes, although under pathological conditions they can accumulate in neurons, eventually to cause neurodegeneration, like in the Lafora disease (Magistretti and Allaman, 2007; Vilchez et al., 2007).

As glycogen is the storage form of glucose, it is safe to speculate about its physiological role as energy storage, which implies that astrocytes can be considered energy reservoirs. A pioneering work in the '80s demonstrated how, in the cortex, two neuromodulators, vasoactive intestinal peptide (VIP) and noradrenaline (NA; Magistretti et al., 1981; Magistretti and Morrison, 1988), are potent glycogenolytic signals, a phenomenon resulting in local increase of phosphate-bound energy sources (ATP) within the stimulated networks

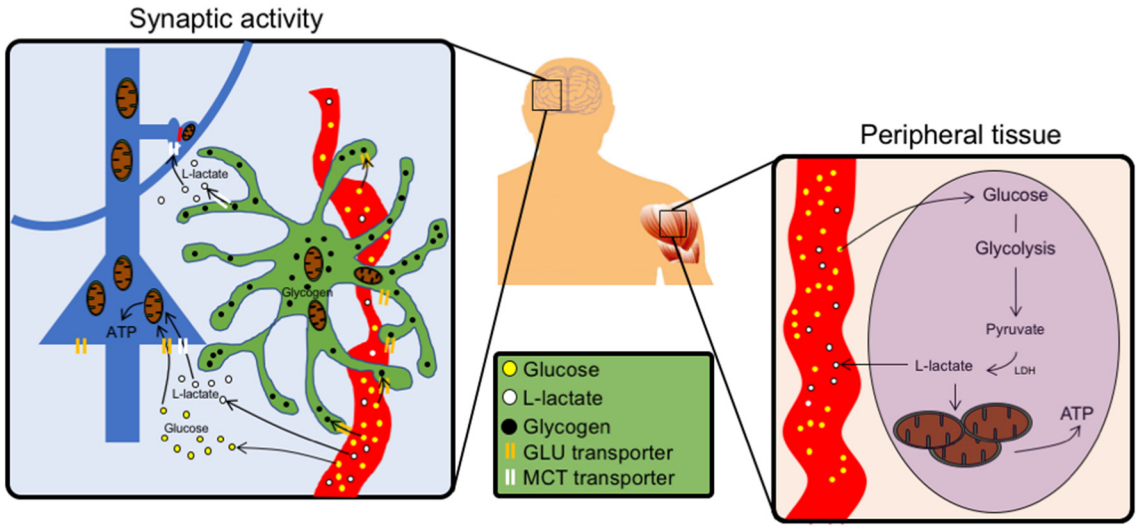

FIGURE 1 | Schematics of the peripheral vs. central action of L-Lactate. **Left**, In the brain, neurons (blue) can uptake glucose using glucose transporters (GLUTs) and use it as a source of energy at the level of their soma. Around synapses, astrocytes (green) take up glucose from the blood vessels (red) and store it as glycogen granules (black). Upon synaptic activity, astrocytes produce L-Lactate in proximity of synapses, which express monocarboxylate transporter (MCT) transporters to import lactate as local source of energy. Lactate can also be formed from glycogen, the storage form of glucose. **Right**, In peripheral tissues glucose fuels tricarboxylic acid (TCA) cycle *via* circulating lactate through glycolysis.

(Magistretti and Schorderet, 1984). Recent evidence confirms these findings, expanding our understanding of the role of NA in particular, whose network activation is mobilized during attentional states necessary for cognitive functions such as learning and memory (Gao et al., 2016; Alberini et al., 2017). From a molecular point of view, NA binds to β2 adrenergic receptors in astrocytes, whose activation triggers glycogenolysis (Magistretti and Morrison, 1988; Sorg and Magistretti, 1992) and the subsequent rise of extracellular lactate levels that are needed for LTP and memory formation (Suzuki et al., 2011; Gao et al., 2016).

Therefore, given the role of lactate derived from astrocytic glycogen in synaptic plasticity, a functional relationship between astrocytic processes filled with glycogen and synaptic profiles is likely to exist (Calì et al., 2016, 2017; Agus et al., 2018). Indeed, recent reports using 3D electron microscopy have shown the preferential location of glycogen granules in astrocytic processes around synapses, rather than being randomly distributed in the astrocytic cytosol, both in the hippocampus and in the cortex (Calì et al., 2016; Mohammed et al., 2018). From an ultrastructural point of view, such distribution suggests that when high firing rate results in phenomena like LTP, that are translated into higher functions such as learning and memory stabilization, lactate, derived from glycogen stored within astrocytic granules close to synapses may exert its dual role of both energy substrate and signaling molecule for plasticity (**Figure 1**).

Sustained neuronal activity, like the one leading to LTP, does not merely induce a metabolic response in astrocytes, whose effect would be measurable after hours, but is also known to trigger an immediate calcium elevation (Araque et al., 2014; Bazargani and Attwell, 2016; Santello et al., 2019). Astrocytic calcium waves are diverse and complex (Di Castro et al., 2011; Agarwal et al., 2017; Bindocci et al., 2017), and their exact nature is still under debate, although evidence has shown their role in triggering glutamate release both *in vitro* and *in situ* (Bezzi et al., 1998). One potential mechanism involves exocytosis of synaptic-like microvesicles (Calì et al., 2008, 2009, 2014; Marchaland et al., 2008) upon activation of astrocytic GPCRs (Bezzi et al., 2004). It is worth mentioning that in a recent report, astrocytes have been shown to modulate levels of another monamine, dopamine, in the prefrontal cortex (Petrelli et al., 2018). These astrocytes express channels and enzymes that regulate homeostasis of dopamine, which could potentially modulate glycogen phosphorylase (GP) activity *via* cAMP (Smith et al., 2004). Furthermore, dopamine activation of D1-like receptors increases intracellular calcium levels, a mechanism likely to take place in astrocytes. Despite the link between LTP and calcium waves in astrocytes, a similar effect on metabolic substrates like lactate or glycogen is not known. A direct link has been reported between the activation of the store-activated calcium channels (SOCE) in astrocytes and glycogenolysis. This process serves as a glycolytic source of ATP to fuel SERCA pumps, to maintain adequate calcium levels in ER stores (Müller et al., 2014). Calcium, in particular, is an indirect signal for GP activation; therefore, one could speculate about the role of calcium in mobilizing energy stores in close proximity of microdomains. Conversely, calcium signaling in astrocytes might be affected by their metabolic turnover, as they depend on NAD+/NADH redox state, which is highly influenced by lactate fluxes (Requardt et al., 2012; Wilhelm and Hirrlinger, 2012).

PERIPHERAL LACTATE UTILIZATION

The role of L-Lactate is not limited to the CNS. Metabolism in peripheral tissues, and the action of lactate have also been extensively investigated in skeletal muscle, heart and in tumoral tissues. Several groups identified the presence of lactate dehydrogenase (LDH) on the mitochondria of sperm cells (Hochachka, 1980) and then in kidney, liver and muscle cells (Kline et al., 1986; Brandt et al., 1987). Brooks (1985) first named the cell-to-cell lactate shuttle in muscle, in 1985. Interestingly, the lactate shuttle is not limited to cytoplasm-mitochondria communication but also to cytosol-peroxisome where it supports the β-oxidation (McClelland et al., 2003). Lactate is produced continuously under aerobic conditions in skeletal muscle and oxidative muscle cells have the capacity to oxidize lactate present in the plasma or released by glycolytic muscle cells. It was also shown that rodent and human muscles cells possess the mitochondrial lactate oxidation complex (mLOC; Dubouchaud et al., 2000) that includes the presence of LDH isoforms, MCTs and cytochrome C oxidase, in their mitochondria. Furthermore, lactate can also be oxidized by mitochondria isolated from liver, heart and skeletal muscle cells.

During exercise, the oxidation of L-Lactate released by muscle cells increases up to 75%–80% of the basal values in the blood stream (Mazzeo et al., 1986) and it is now demonstrated that lactate can stimulate mitochondria biogenesis through activation of PGC1α (Hashimoto et al., 2007) which in turn influences the transcription of LDH isoforms to increase the ratio of LDHA/LDHB. This change promotes the formation of Lactate over pyruvate (Summermatter et al., 2013). In the heart, during exercise, it is also believed that lactate becomes the predominant source of energy compared to other metabolic sources (Gertz et al., 1988) and longitudinal studies have demonstrated that trained animals have reduced lactate blood levels, most likely due to an enhanced capacity to use it as a substrate by different organs (Bergman et al., 1999). Interestingly, it appears that during endurance exercise, significant amounts of brain derived neurotrophic factor (BDNF) are released in the bloodstream correlating with the release of L-Lactate (Schiffer et al., 2011). BDNF is an important trophic factor in the brain. L-Lactate has been demonstrated to increase BDNF expression in different neural cell systems (Coco et al., 2013; Yang et al., 2014). Interestingly, recent work has shown that cortical astrocytes can recycle BDNF and ultimately promote TrkB phosphorylation, to sustain LTP (Vignoli et al., 2016).

Besides its role in brain and muscle physiology, lactate has an important role in cancer cells. Tumors have high glycolytic metabolism even under normal O_2 levels, a phenomenon known as the Warburg effect. This environment supports cancer cell survival and leads to accumulation of L-Lactate. This buildup has been reported to inhibit the migration of CD8+, CD4+ T-cells (Haas et al., 2015). Moreover, tumors producing high level of LDHA (favoring the conversion of Pyruvate to Lactate) have less positive outcomes (Brand et al., 2016). Blood lactate concentration observed around a tumorigenic environment can vary massively, raising from 1.5 to 3 mM in physiological conditions, up to 30–40 mM in cancerous tissues (Hirschhaeuser et al., 2011; Colegio et al., 2014; Haas et al., 2015). Moreover, the inhibition of the immune system by L-Lactate is not limited to a disturbance of immune cells metabolism, but also through an increase of pro-survival factor such as HIF-1α or angiogenic factors (Shi et al., 2011; Magistretti and Allaman, 2018).

Overall, lactate has been shown to be involved in multiple processes besides its metabolic support to muscle cells. L-Lactate ensures the survival of tumoral tissues by both promoting an environment favorable for their growth and reducing the reactivity of the adaptive immune system.

LACTATE AS A METABOLIC BUFFER BETWEEN GLUCOSE AND OXIDATIVE METABOLISM IN PERIPHERAL TISSUES

Recently a mechanism reminiscent of the ANLS has been shown to operate at the whole-body level (**Figure 1**). Indeed using *in vivo* Magnetic Resonance Spectroscopy to trace the fate of various metabolites in fed and starving mice, it was shown that ^{13}C-lactate was extensively labeling TCA intermediates in peripheral organs (Hui et al., 2017). By measuring glucose metabolites in all organs, the authors found a considerably higher amount of circulating lactate compared to other metabolites, concluding that L-Lactate can act as a reservoir molecule whose turnover can be glycolytically fine-tuned on demand, rather than directly using glucose. This mechanism is reminiscent of what is observed in tumorigenic environment, where circulating lactate represents a more efficient way to use local energy reserves and uncouple it from glucose availability, which can be influenced by multiple factors. Interestingly, the only exception was the brain, where glucose was surpassing the amount of circulating lactate. As shown by the ANLS, a lactate gradient between astrocytes and neurons (Mächler et al., 2016) allows its exchange *via* the MCTs. Such a metabolic flow relies on astrocytic glycolysis, which is necessarily coupled to glucose utilization, triggered by synaptic signaling (Pellerin and Magistretti, 1994; Magistretti and Allaman, 2015). An even more tightly regulated way of energy delivery on demand occurs *via* lactate derived from glycogen (Suzuki et al., 2011; Gao et al., 2016). In this case, energy stores, under the form of glycogen granules, around synapses, can serve as metabolic reservoirs of lactate for energy delivery and plasticity signals for synapses. Consistent with the fact that astrocytes are locally synthesizing on demand lactate from glucose and glycogen, the amount of peripheral lactate accessing the brain is minimal (Hui et al., 2017).

From the above considerations, one should consider a dual action of lactate; one, as a source of energy, based on the uncoupling of glucose metabolism from the TCA cycle in astrocytes and the delivery of lactate to neurons. The second one, *via* the glycogen, as a signaling molecule for plasticity.

ANY ISSUES?

At its time of publication in 1994 (Pellerin and Magistretti, 1994), the ANLS model has been challenged, although the concept of the lactate shuttle, at least in the skeletal muscle,

was not a novelty (Brooks, 1985). It is worth mentioning that most controversies around the ANLS raised from calculations inferred from theoretical models or metabolic stoichiometry rather than experimental data, opposing to the ANLS a model hypothesizing lactate flow from neurons to astrocytes, for disposal into the blood stream. (Dienel, 2012, 2017) To summarize, the few opponents to the ANLS argue that considering the rapid release of lactate in the bloodstream upon brain activity and the small concentration of lactate in the brain its oxidation in the neurons cannot support their synaptic activity. However, a compelling number of *in vivo* investigations have demonstrated that synaptic activity, and glutamate release trigger upstream intracellular cascades in astrocytes promoting glucose utilization mainly by astrocytes (Chuquet et al., 2010; Jakoby et al., 2014). Moreover, experimental work has also shown that upon glutamate activity, the glycolytic activity in the astrocytes is enhanced, compared to neurons (Mongeon et al., 2016) and that the loss of astrocytic glutamate transporters reduced the glucose consumption in activated brain areas (Cholet et al., 2001). Finally, some questions arose about the neuronal type that can support the model. For example, since GABA uptake by the astrocytes does not trigger aerobic glycolysis (Peng et al., 1994; Chatton et al., 2003) it is clear that energy delivery to GABA neurons operates through other mechanisms. However, since GABA neurons are mostly interneurons that are activated by glutamatergic inputs, it is conceivable that the glutamate-stimulated ANLS may provide energy to GABAergic neurons. Overall, converging evidence from several laboratories indicates that the ANLS provides an operational model for the coupling between neurons and astrocytes (Barros and Weber, 2018).

AUTHOR CONTRIBUTIONS

CC, AT and PM wrote the manuscript.

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