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**SEDE SANTIAGO.**

# GALECTIN-8 AS A NOVEL REGULATOR OF PRIMARY CILIUM AND ENERGY BALANCE

Thesis presented to obtain the academic degree of PhD in Cell Biology and Biomedicine

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## EVALUACIÓN

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*A Nelly, por todo su tiempo y dedicación.*

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**Table of content.**

## Figure Index.

## 1.0- Abstract.

Primary cilium (PC) is cell surface antenna-like organelle that contribute to elaborate and integrate signaling pathways triggered by a variety of extracellular stimuli. In neurons of the hypothalamic region, the PC plays a crucial role in metabolism regulation by signals such as leptin and insulin. Loss of cilia in these neurons leads to leptin and insulin resistance and obesity, which are main public health problems. Therefore, exploring brain-dependent factors and conditions that regulate PC function in hypothalamic neurons is necessary to improve our understanding of metabolic physiology and its disorders.

The leptin-melanocortin circuit in the hypothalamus depends on PC function to control energy balance and food intake by enhancing anorexigenic signals and energy expenditure in response to leptin. Two antagonistic populations of neurons are involved in this circuit: AgRP neurons that express neuropeptide-Y/agouti-related peptide and POMC neurons that express pro-opiomelanocortin. During fasting, AgRP neurons inhibit POMC neurons through AgRP and GABA neurotransmitters, thus generating appetite and low energy expenditure signals. After food intake, leptin secreted into the bloodstream inhibits AgRP neurons and activates POMC neurons, which now trigger satiety and energy expenditure signals.

Galectin-8 (Gal-8) is a beta-galactosidase binding protein that modulates various cellular processes by interacting with different glycoproteins, both intracellularly and extracellularly. In the central nervous system, Gal-8 is expressed at different levels in several regions, including the hypothalamus. It is highly expressed in the choroid plexus and is found in the cerebrospinal fluid (CSF), which permeates the entire brain. In the brain, Gal-8 has been shown to play neuroprotective and immunosuppressive roles. Proinflammatory conditions, such as those observed in obesity and diabetes, upregulate Gal-8 expression. Studies in platelets suggest that Gal-8 can induce calcium influx in certain cellular systems, although the underlying

mechanism remains unknown. Gal-8 binds and activates β1 integrins, which are major receptors for this lectin and have been shown to induce calcium influx through L-type calcium channels. In this study, we investigate the role of Gal-8 on primary cilia biogenesis, leptin signaling, and calcium homeostasis related to the metabolic function of hypothalamic neurons.

**Hypothesis**: Galectin-8 induces disassembly of primary cilia in Clu-177 hypothalamic cells, affecting leptin signaling and involving calcium influx through L-type calcium channels.

The results demonstrate that Gal-8 induces loss of primary cilia in the hypothalamic Clu-177 cell line, in a time- and concentration-dependent manner. Treatment with 30nM Gal-8 reduced the number of ciliated cells by ~20% and the length of primary cilia by 22.5% involving the AurkA/HDAC6 axis. Pulldown assays showed that Gal-8 binds α3β1 and α5β1 integrins. Accordingly, Gal-8 treatment induced activation of β1-integrin downstream elements such as *src* and FAK kinases. Inhibition of these kinases prevented cilia loss. Gal-8 treatment also induced calcium influx through L-type calcium channels. Blocking these channels, as well as chelating extracellular calcium, prevented calcium influx and loss of primary cilia. Gal-8 treatment reduced leptin signaling in hypothalamic Clu-177 cells. *In vivo* experiments show that Gal-8 knockout (Gal-8-KO) mice exhibit lower weight and food intake, as well as higher locomotor activity and respiratory exchange ratio (RER), compared to wild-type (WT) mice. Moreover, intranasal administration of Gal-8 in Gal-8-KO animals restored the RER to the WT phenotype.

All these results are consistent with a potential role of Gal-8 in brain contributing to control energy balance through the PC signaling function in the hypothalamus. The Gal-8-dependent signaling pathway that includes integrin/FAK/Src activation, calcium influx through L-type calcium channel $Ca_v1.3$ and downstream AurKA/HDAC6 axis, impacting upon PC biogenesis and structure in hypothalamic neurons, may provide new elements amenable to metabolic alterations and therapeutic opportunities.

Key word: Galectin-8, Primary cilium, Energy balance, L-Type Calcium Channel.

# 2.0- Introduction.

## 2.1.0.- Problem statement.

The primary cilium (PC) is an antenna-like organelle that extends from the cell surface and is present in nearly all mammalian cells. It serves as a platform for signal transduction of various receptors and plays crucial roles in numerous biological processes (Besschetnova et al., 2010; Senatore et al., 2022; Veland et al., 2009), including embryogenesis, cell proliferation, differentiation, and migration (Ma et al., 2022; Senatore et al., 2022; Veland et al., 2009). In neurons, PC function is essential for dendritic development and neuroendocrine circuits (Senatore et al., 2022; Veland et al., 2009). Disruption or loss of the PC, as well as alterations in its structure and function contribute to pathological conditions called ciliopathies (Besschetnova et al., 2010; Czajka, 2020; Veland et al., 2009). Therefore, the mechanisms that regulate PC maintenance and their potential dysfunctions entail significant biomedical interest.

In neuronal cells of the arcuate nucleus of the hypothalamus, the PC is a crucial component of the mechanisms that regulate appetite (food intake) and energy expenditure (Han et al., 2014; C. H. Lee et al., 2020; Seo et al., 2009; Varela & Horvath, 2012). Here, AgRP neurons express Y/agouti-related peptide whereas POMC neurons express pro-opiomelanocortin, neuropeptides with opposing functions (Baldini & Phelan, 2019; Oh et al., 2015; Toda et al., 2017; Varela & Horvath, 2012). During fasting, AgRP neurons are activated and release signals that inhibit POMC neurons, leading to increased appetite and reduced energy expenditure. Conversely, food intake deactivates AgRP neurons and activates POMC neurons through the action of leptin released from adipocytes (Varela & Horvath, 2012; D. J. Yang et al., 2022). The function of leptin receptors on POMC neurons is regulated by PC (Berbari et al., 2013; Guo et al., 2016; Seo et al., 2009). The absence of PC results in a reduction of leptin receptors on the cell surface and an impaired leptin-mediated signaling (Berbari et al., 2013; Guo et al., 2016). Mouse

models of ciliopathies, characterized by PC loss or alterations in morphology, exhibit leptin resistance, increased food intake, obesity, impaired thermoregulation, and reduced oxygen consumption (Berbari et al., 2013; Jacobs et al., 2016; Seo et al., 2009). However, in contrast with the largely studied mechanisms that regulate PC dynamic biogenesis during the cell cycle in proliferating cells (Izawa et al., 2015), whether extracellular signals modulate the biogenesis of PC in hypothalamic cells remain largely unexplored.

Galectins constitute a family of 16 carbohydrate-binding proteins implicated in a wide range of cellular processes (Nabi et al., 2015). The carbohydrate recognition domain (CRD) of galectins is highly conserved and interacts with β-galactosides, particularly N-acetyllactosamines (Galβ1-3GlcNAc or Galβ1-4GlcNAc), which are present in the glycosylation patterns of cell surface proteins (Nabi et al., 2015). Variations in the affinity for these glycoconjugates are observed among different galectins due to variations in the amino acid residues within their CRDs (Kaltner & Gabius, 2012; Nabi et al., 2015). As a result, galectins play redundant or complementary roles in various cellular processes (Barake et al., 2020).

The role of galectins on PC function has been little studied. Potential connections have been described for Galectin-3 (Gal-3) and Galectin-7 (Gal-7) (Chiu et al., 2006; Hafsia et al., 2020; Rondanino et al., 2011; Torkko et al., 2008). Gal-3 has been found in the centrosome and PC axoneme (Chiu et al., 2006), and its silencing in polarized epithelial MDCK cells resulted in longer cilia, with 70% displaying morphological abnormalities (Chiu et al., 2006; Torkko et al., 2008). Studies in Gal-3-KO mice show that chondrocytes exhibit slightly longer cilia with curved or stunted morphology, cells with two cilia, and a 15% reduction in the number of ciliated cells compared to WT animals (Hafsia et al., 2020). On the other hand, Gal-7 has been shown to stabilize the PC and promote cilium elongation in MDCK and mpkCCD$_{c14}$ cells (Rondanino et al., 2011). Whether galectins have the potential to regulate ciliogenesis in hypothalamic cells remain unknown.

Gal-8 is one of the most widely expressed galectins in various tissues and displays a unique preference for certain carbohydrates (Hadari et al., 1995; Nabi et al., 2015).

Gal-8 belongs to the tandem-repeat type of galectins characterized by an N-terminal and a C-terminal carbohydrate recognition domain (CRD) separated by a linker peptide of variable length (Hadari et al., 1997). In the brain, Gal-8 is expressed in different regions, including the choroid plexus, basal ganglia, thalamus, and hypothalamus (John & Mishra, 2016; Pardo et al., 2017, 2019). Previous studies conducted in our laboratory demonstrated that Gal-8 exhibits neuroprotective and immunosuppressive functions in the brain (Pardo et al., 2017, 2019). Other studies have shown that Gal-8 induces an increase in intracellular calcium levels in platelets (Romaniuk et al., 2010). The mechanism of this effect and whether this also occurs in other cells is unknown.

Studies focused on cell proliferation have shown that intracellular calcium activates the Aurora kinase A (AurkA)/HDAC6 axis leading to PC loss (Mirvis et al., 2019; Plotnikova et al., 2010, 2011, 2012). In this thesis, we asked whether Gal-8 has the capability to modulate the PC biogenesis involving intracellular calcium changes associated with the AurkA/HDAC6 axis in hypothalamic neurons. Answering this question may contribute to our understanding of the mechanisms that regulate appetite and energy balance.

## 2.2.0.- Literature review.

### 2.2.1- Primary cilia.

The structure of PC has three main components: the basal body, the transition zone, and the axoneme (Kiesel et al., 2020; Veland et al., 2009). The basal body anchors the cilium to the mother centriole and is involved in the regulation of PC assembly and disassembly during the cell cycle. The transition zone acts as a gatekeeper, controlling the traffic of proteins to and from the cilia. The axoneme is a tubulin-based structure that protrudes from the cell surface surrounded by the cell membrane (Kiesel et al., 2020; Veland et al., 2009). Unlike motile cilia, which have a 9+2 arrangement of microtubules (nine peripheral microtubule doublets in a ring conformation and a central pair of singlet microtubules), the PC has a 9+0 microtubule arrangement lacking the central pair and also the inner and outer dynein

arms responsible for movement in other cilia (Kiesel et al., 2020; Veland et al., 2009) (Figure 1A).

Although the PC was first described by Zimmermann in 1898 (Zimmermann, 1898), its function was not defined until the early 2000s. Studies conducted during that time revealed that the loss of PC is associated with the development of polycystic kidney disease (PKD) (Lin et al., 2003; Pazour et al., 2000). Subsequent research demonstrated that the PC serves as a mechanosensing organelle that responds to fluid flow influencing gene expression (Delling et al., 2013; Praetorius & Spring, 2001). PC is now considered a signaling platform where various proteins, including integrins, tyrosine kinase receptors, ion channels, and other membrane receptors, are enriched. These proteins, along with their associated transduction machinery, participate in signal transduction processes within the PC (Senatore et al., 2022; Teves et al., 2019; Veland et al., 2009; Wann et al., 2012).

Complete absence of PC characterizes of a group of ciliopathies, including Bardet-Biedl syndrome, Senior-Loken syndrome, Jourbet syndrome, polycystic kidney disease, retinitis pigmentosa, nephronophthisis and situs inversus (Czajka, 2020; Nishimura et al., 2018; Senatore et al., 2022). Additionally, various abnormalities in the primary cilium structure have been associated with other pathogenic conditions, such as hydrocephalus, atherosclerosis, hyperphagia, obesity, and diabetes (Czajka, 2020; Nishimura et al., 2018; Senatore et al., 2022; Veland et al., 2009). These associations highlight the importance of understanding the mechanisms underlying PC maintenance and function.

### 2.2.2.- Primary cilia assembling (Ciliogenesis).

In proliferating cells, PC assembly, also known as ciliogenesis, occurs after cell division, during the G1 phase of the cell cycle, influenced by extracellular and intracellular conditions (Nishimura et al., 2018; Pedersen et al., 2008) (Figure 1B). In the extracellular pathway, PC assembly begins with the docking of the mother centriole to the cell membrane through a process facilitated by the protein CEP83 (Tanos et al., 2013). This process involves loss of the CP110 protein that acts as a "cap" on the centriole preventing microtubule and axoneme extension

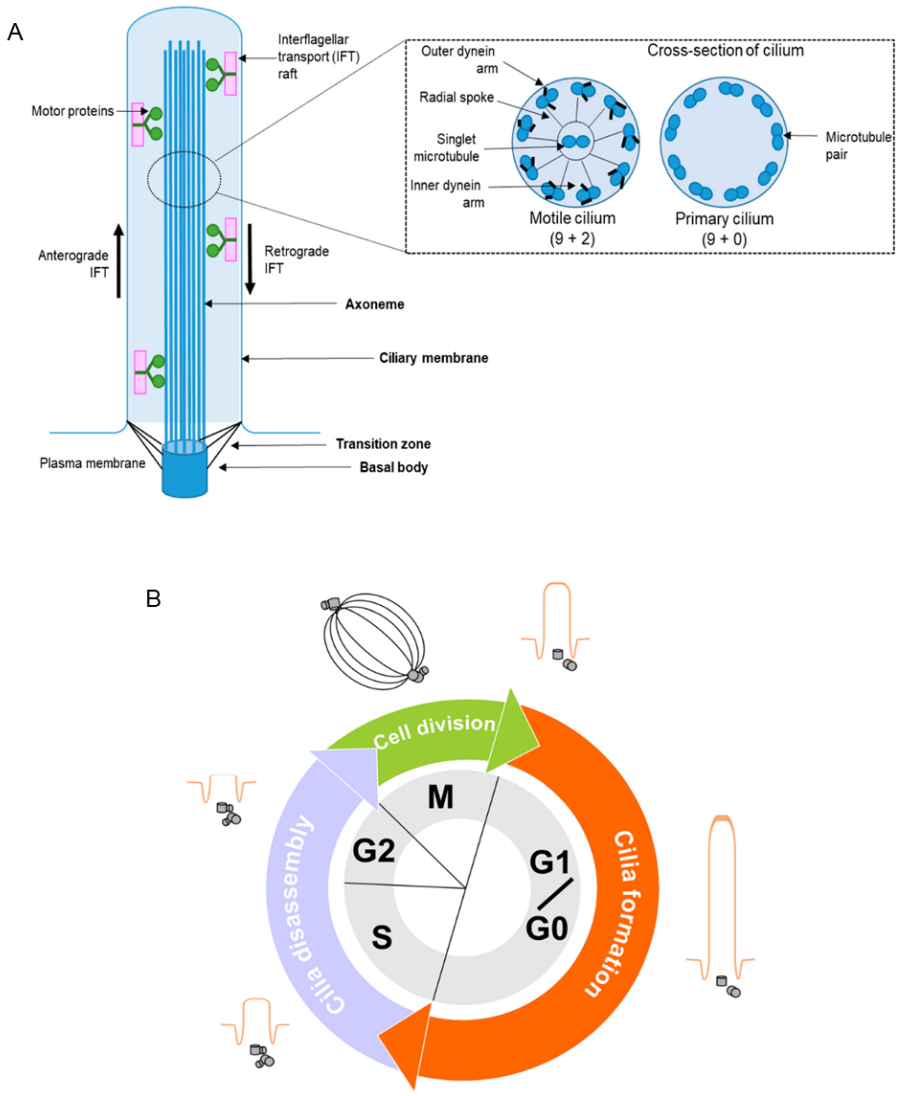


**Figure 1.- Structure and stages of primary cilium.**

A) The primary cilium is divided into three components: basal body, transition zone, and axoneme. The axoneme of the primary cilium have a 9+0 microtubules conformation, in contrast to the 9+2 conformation of motile cilia; B) Changes in the PC during the cell cycle. Ciliogenesis occurs after each cell division and therefore cilium can be detected in G0/G1 cells, while it starts to be disassembled at the S phase start through a process that is finished at mitosis.

(Kobayashi et al., 2011; Prosser & Morrison, 2015; Tanos et al., 2013). Once CP110 is removed, axonemal microtubules are nucleated and PC assembly starts (Kobayashi et al., 2011; Prosser & Morrison, 2015; Tanos et al., 2013). The intraflagellar transport (IFT) complex is responsible for transporting proteins along the axoneme during PC assembly and elongation (Follit et al., 2006; Pedersen et al., 2008; Sedmak & Wolfrum, 2010; W. Wang et al., 2021).

In addition to microtubule assembly in the extracellular pathway, the intracellular pathway of PC ciliogenesis involves vesicles originating from both the Golgi apparatus and recycling endosomes (Pedersen et al., 2008; W. Wang et al., 2021). After cell division, the mature mother centriole becomes associated with various proteins called distal appendage proteins, which include CEP83, CEP89, SCLT1, CEP164, and FBF1 (L. Wang & Dynlacht, 2018; T. T. Yang et al., 2018). These proteins are crucial for docking preciliary vesicles (PCV) to the mother centriole. PCVs are loaded with ciliary proteins and transported to the mother centriole via microtubules and actin networks, utilizing motor proteins such as KIFC-1 and Myosin-VA (S. Lee et al., 2018; C.-T. Wu et al., 2018).

Intracellular vesicle trafficking involved in ciliogenesis is sensitive to factors present in the serum. Serum starvation induces ciliogenesis by promoting the transport of Rabin8, a guanine-nucleotide exchange factor, to the mother centriole via Rab11 vesicle-mediated transport (Lu et al., 2015; Westlake et al., 2011). Rabin8 activates Rab8a, a small GTPase that facilitates the coupling of PCVs to the mother centriole (Lu et al., 2015; Westlake et al., 2011). Conversely, serum inhibits ciliogenesis through lysophosphatidic acid (LPA) (Walia et al., 2019), which activates its receptor LPAR1 followed by AKT-mediated stabilization of Rab11a/WDR44 complex (Walia et al., 2019). This stabilized Rab11a/WDR44 complex blocks transport of Rabin8 to the mother centriole, thereby inhibiting ciliogenesis (Walia et al., 2019).

### 2.2.3.- Primary cilia disassembling.

Loss of the PC is considered a prerequisite for cells to enter mitosis (Izawa et al., 2015). The PC is present in G1/G0 cells, starts to be lost during the S phase, and is completely absent during the G2 phase, thus allowing the duplication of the

centrosome that is required for bipolar mitotic spindle formation (Izawa et al., 2015) (Figure 1B).

During mitosis, the activation of Aurora Kinase A (AurkA) plays a central role in PC disassembly. AurkA phosphorylates and activates HDAC6, a cytosolic histone deacetylase that deacetylates and destabilizes α-tubulin in the axoneme of the PC, leading to its loss (Hubbert et al., 2002; Plotnikova et al., 2012; Pugacheva et al., 2007).

PC loss can also occur through mitosis-independent mechanisms (K. H. Lee et al., 2012; Plotnikova et al., 2010). For example, non-canonical Wnt5a signaling can activate the AurkA/HDAC6 pathway leading to cilia resorption (K. H. Lee et al., 2012). Increases in intracellular calcium can promote Calmodulin (CaM) binding to and activation of AurkA, as observed in kidney HEK293 and HK2 cell lines stimulated with vasopressin (Plotnikova et al., 2010). Calcium influx and CaM/AurkA binding and activation have also been implicated in PC disassembly in both mitotic and non-mitotic hTERT-RPE1 cells (Plotnikova et al., 2012).

Therefore, regulation of PC dynamics is not only crucial for cell cycle progression but also for the proper functioning of this organelle as a signaling platform. Extracellular factors that regulate the balance between PC assembly and disassembly are expected to impact upon PC signaling functions.

### 2.2.4.- Primary cilia and signal transduction.

PC is involved in several signaling pathways that are crucial for cellular functions and development (Ma et al., 2022; Senatore et al., 2022; Veland et al., 2009). These pathways include receptor tyrosine kinase signaling, Wnt signaling, Hedgehog signaling, and TRP (Transient Receptor Potential) channel signaling (Nishimura et al., 2018; Veland et al., 2009). Thus, PC serves as a signaling hub where various receptors and their downstream signaling components are localized and can be coordinately integrated and regulated (Nishimura et al., 2018; Veland et al., 2009).

In neurons, the PC has been involved in the development and maintenance of dendrites, participating in the formation of synapses (Kumamoto et al., 2012; Sheu

et al., 2022). PC has been implicated in serotonergic transmission through axon-cilium synapses, playing a role in physiological processes like mood regulation (Dupuy et al., 2023; Sheu et al., 2022).

Dysfunctions of the PC in hypothalamic neurons, including POMC neurons involved in regulating food intake and energy balance, have been associated with hyperphagia (excessive eating) and obesity (Ávalos et al., 2022; Berbari et al., 2013; Guo et al., 2016; Hernández-Cáceres et al., 2019; Jacobs et al., 2016; C. H. Lee et al., 2020; Oh et al., 2015; Seo et al., 2009).

#### 2.2.4.1.- Primary cilium and energy balance in hypothalamus.

The arcuate nucleus in the hypothalamus plays a crucial role in regulating food intake and energy expenditure through the leptin-melanocortin system (Baldini & Phelan, 2019). This system consists of two populations of neurons with opposing functions: AgRP (Agouti-Related Peptide) neurons and POMC (Pro-Opiomelanocortin gene expression) neurons, which are connected to secondary neurons that express the Melanocortin Receptor 4 (MC4R) and integrate signals from POMC or AgRP neurons producing appetite or satiety (Baldini & Phelan, 2019; Sohn, 2015). AgRP neurons are activated during fasting and inhibit the activity of POMC and MC4R neurons in a Gaba-dependent manner, leading to increased appetite and decreased energy expenditure (Baldini & Phelan, 2019; Sohn, 2015; Varela & Horvath, 2012). POMC neurons are activated by leptin and insulin after food intake and release alpha-melanocyte stimulating hormone (α-MSH), which activates MC4R neurons, promoting satiety and increasing energy expenditure (Baldini & Phelan, 2019; Sohn, 2015; Varela & Horvath, 2012). In addition, leptin and insulin released into the bloodstream after food intake, also inhibits the activity of AgRP neurons (Baldini & Phelan, 2019; Sohn, 2015; Varela & Horvath, 2012) (Figure 2). Leptin binds to its receptor, Leptin Receptor-B (LepR-B), and activates the JAK/STAT3 pathway. This results in the phosphorylation of the transcription factor STAT3 in its tyrosine 1138 residue by JAK kinase, and the

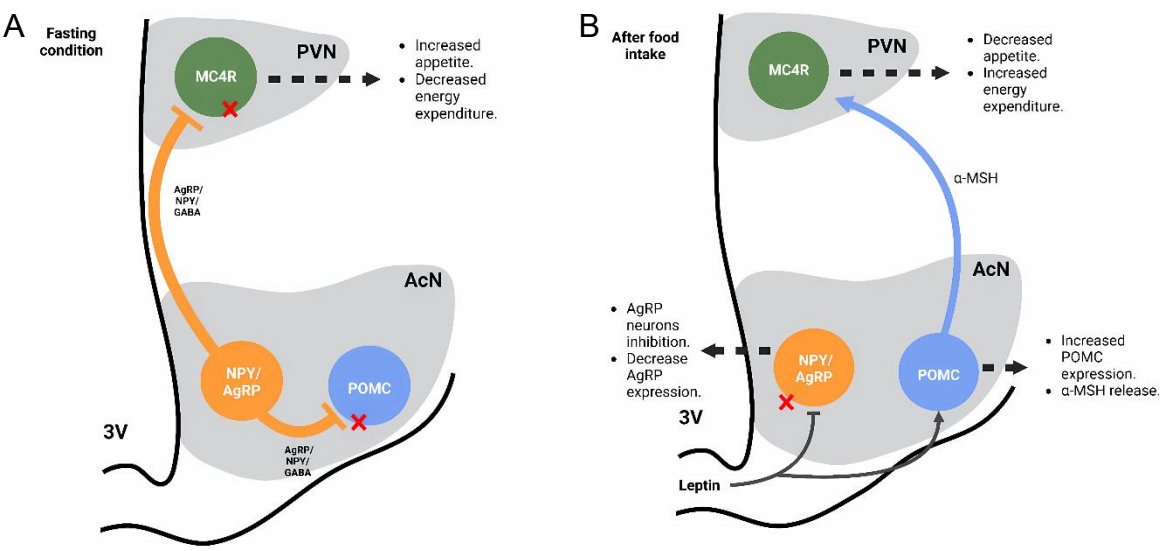


**Figure 2.- Hypothalamic leptin-melanocortin circuit.**

Energy expenditure and appetite are controlled in the hypothalamus by the leptin-melanocortin system conformed by POMC and AgRP neurons within the Arcuate Nucleus (can) and MC4R neurons in the Paraventricular Nucleus (PVN). A. Fasting conditions are characterized by the inhibition that AgRP neurons exert upon POMC and MC4R neurons underlying appetite and decrease energy expenditure. B. After food intake, the leptin released from adipocytes reaches the hypothalamus where it inhibits AgRP neurons and actives POMC neurons. Activated POMC neurons release $\alpha$-MSH neuropeptide that activates MC4R neurons, thus triggering satiety and increasing energy expenditure signals.

translocation of pSTAT3 into the nucleus, with the subsequent activation of POMC transcription (Baldini & Phelan, 2019; Oh et al., 2015; Sohn, 2015; Varela & Horvath, 2012).

POMC and AgRP neurons are also implicated in the peripheric glucose homeostasis. Chemogenetic activation of AgRP neurons in mice determines insulin resistance and impaired glucose homeostasis involving gene expression changes in the brown adipocyte tissue resulting in decreased insulin-stimulated glucose uptake (Steculorum et al., 2016). In contrast, POMC activation improves glucose tolerance and insulin sensibility in mice models (Alsina et al., 2018; Burke et al., 2017; Zhou et al., 2007). Specific deletion of POMC gene in arcuate neurons diminishes the tolerance of glucose in non-obese mice (Alsina et al., 2018). In diet induced obesity and *ob/ob* mice models an increased POMC expression improves glucose tolerance and insulin sensibility (Burke et al., 2017; Zhou et al., 2007).

The PC is involved in the regulation of LepR-B presence at the plasma membrane and its ability to sense leptin. Mouse models of ciliopathies characterized by alterations in PC-related proteins, such as BBS1, IFT88, and IFT139, show that the loss of PC in hypothalamic neurons associates with leptin resistance, increased food intake, obesity, decreased thermoregulation, and reduced oxygen consumption (Berbari et al., 2013; Jacobs et al., 2016; Seo et al., 2009). Disruption of PC using short hairpin RNA (shRNA) against BBS1 and BBS2 leads to 80% reduction of LepR-B cell surface levels (Guo et al., 2016; Seo et al., 2009). Similarly, the cell surface expression of the serotonin receptor 5-HT2cR, another receptor important for energy homeostasis, is also reduced under BBS1 silencing conditions (Guo et al., 2016, 2019).

Other circuits within the central nervous system are involved in behaviors related to food intake independently of metabolic needs (Matafome & Seiça, 2017). One of these circuits involves the mesolimbic system, which influences hedonic behavior in a dopamine-dependent manner by activating the reward system through signals associated with ghrelin and endocannabinoids (Petrovich, 2021; Sárvári et al., 2014). Also, stress and conditioned fear signals can regulate eating behavior

independently of energy homeostasis (Z. F. Li et al., 2021; Petrovich et al., 2009). Amygdala activity has been implicated in the development of food aversion, anorexia nervosa, or even compulsive eating (Z. F. Li et al., 2021; Petrovich et al., 2009). In this thesis we focus on the effects of Gal-8 in PC and the Leptin-melanocortin circuit.

### 2.2.5.- Galectins.

Galectins are a family of proteins consisting of 16 members that share a highly conserved carbohydrate-recognition domain (CRD). This domain has a strong affinity for β-galactosides, particularly N-acetyllactosamines (Galβ1-3GlcNAc or Galβ1-4GlcNAc) present in the glycosylation patterns of cell surface proteins and lipids (Kaltner & Gabius, 2012; Nabi et al., 2015). Galectins are classified into three subtypes: prototypic, chimeric, and tandem-repeat galectins. The prototypic galectin subgroup includes galectins 1, 2, 5, 7, 10, 11, 13, 14, 15, and 16. These galectins possess a single CRD domain and can form homodimers through non-covalent interactions. Galectin-3 (Gal-3) is the only member of the chimeric galectin subgroup and is characterized by a single CRD domain coupled to a collagen-like oligomerization domain through which this galectin can form aggregates, particularly pentamers. The tandem-repeat galectin subgroup consists of Galectins 4, 6, 8, 9, and 12, which contain two CRD domains connected by a linker peptide (Kaltner & Gabius, 2012; Nabi et al., 2015).

Galectins can be found in the cytosol and secreted into the extracellular space through an unconventional secretion mechanism (Hughes, 1999; Kaltner & Gabius, 2012; Nabi et al., 2015). At least for Gal-3 the secretion mechanism involves exosomes (Bänfer et al., 2018). In the cytosol, certain galectins, including Gal-8, Gal-3, and Gal-9, have been involved in the detection, removal, and repair of damaged endosomes and endolysosomes by interacting with the exposed glycans of their ruptured membranes (Chauhan et al., 2016; Falcon et al., 2018; Jia et al., 2018, 2019; Liu & Rabinovich, 2005). Gal-1 and Gal-3 also participate in mRNA maturation processes (Coppin et al., 2017; Fritsch et al., 2016; Patterson et al., 2014; Vyakarnam et al., 1997). Once secreted to the extracellular environment, galectins bind to the glycosylation patterns on the cell surface and extracellular matrix proteins

and promote a variety of cellular processes, including proliferation, differentiation, migration, and apoptosis (Hughes, 1999; Kaltner & Gabius, 2012; Metz et al., 2016; Nabi et al., 2015; Norambuena et al., 2009; Oyanadel et al., 2018).

Overall, galectins exhibit diverse functions by acting both inside and outside cells through interactions with glycosylated proteins, contributing in this way to physiological and pathological processes. Elucidating their functions may provide new clues for therapeutic interventions in many diseases.

### 2.2.6.- Galectins and primary cilia.

Hereto, galectins that have been associated with PC ciliogenesis and function are restricted to Gal-3 and Gal-7 (Chiu et al., 2006; Clare et al., 2014; Rondanino et al., 2011; Torkko et al., 2008). Gal-3 has been found to colocalize with γ-tubulin in the centrosome and is also present in the PC axoneme (Chiu et al., 2006). Studies conducted on MDCK cells, a canine kidney epithelial cell line, have shown that reducing Gal-3 levels with siRNA leads to longer cilia, with more than 70% displaying morphological alterations (Torkko et al., 2008). Slightly longer cilia have been observed in chondrocytes derived from Gal-3 knockout (Gal-3-KO) mice compared with cells from wild-type (WT) mice (Hafsia et al., 2020). The cilia in Gal-3-KO cells exhibit different alterations, being mostly curved, stunted, or duplicated, though in some cells is also absent (Hafsia et al., 2020). These findings indicate a role of Gal-3 in ciliogenesis and maintenance of cilia morphology. Other studies proposed a role of Gal-7 in stabilizing and promoting elongation of PC (Rondanino et al., 2011). Cilia length becomes is reduced by 27% in mpkCCDc14 cells transduced with inducible Gal-7 shRNA (Rondanino et al., 2011). Gal-7 binds to the PC extracellularly suggesting autocrine or paracrine effects on cilia elongation (Rondanino et al., 2011).

### 2.2.7.- Galectin-8.

Gal-8 is a tandem-repeat galectin first identified in prostate cancer and then found to be expressed in various tissues, including lung, kidney, gastrointestinal tract, brain, and others (Hadari et al., 1995; Nabi et al., 2015). The N-terminal CRD of Gal-8 exhibits a high affinity for glycans bearing terminal α-2,3-sialic acid, a property that

is not seen in other galectins and is provided by the residues Arginine 45, Glutamine 47, and Arginine 59 in Gal-8 (Hadari et al., 1995, 1997; Ideo et al., 2003, 2011). In the cytosol, Gal-8 plays a role recognizing damaged endomembranes and targeting them to autophagy degradation by recruiting the autophagy receptor NDP52 (Thurston et al., 2012). This clearance of damaged endomembranes mechanism constitutes a protection system against the infection of certain bacteria and the spread of pathogenic protein aggregates (Falcon et al., 2018; Fraser et al., 2019; Jia et al., 2019; Thurston et al., 2012).

Extracellularly, Gal-8 has diverse functions in a variety of cell types and tissues, including the brain (Barake et al., 2020). Gal-8 promotes the maturation of B cells into plasma cells (Tsai et al., 2011), regulates lymphocyte T formation (Norambuena et al., 2009; Sampson et al., 2016), has immunosuppressor functions in models of autoimmune encephalomyelitis and protects neurons against a variety of damaging conditions (Pardo et al., 2017, 2019). In human platelets, Gal-8 promotes spreading, fibrinogen binding and elevations of intracellular calcium levels (Romaniuk et al., 2010).

In various cell types, Gal-8 exerts its effects by activating integrins and their downstream signaling pathways (Levy et al., 2001; Zick, 2022). Outcomes of Gal-8-integrin interactions include neuroprotection, EMT, cell proliferation and cytoskeleton rearrangements (Cárcamo et al., 2005; Diskin et al., 2012; Levy et al., 2001; Oyanadel et al., 2018; Pardo et al., 2019; Tribulatti et al., 2009). Signaling pathways mediated by kinases such as SRC, FAK, MAPKs, and PI3K have been involved in these effects (Cárcamo et al., 2005; Diskin et al., 2012; Levy et al., 2001; Oyanadel et al., 2018; Pardo et al., 2019; Tribulatti et al., 2009). The specific cellular responses triggered by Gal-8 binding to integrins depend on the cell type and context. For instance, in non-tumoral MDCK cells, Gal-8 has been shown to bind to α5β1 integrin and activate a FAK/EGFR/proteasome pathway leading to partial EMT and invasive tumorigenic capabilities (Oyanadel et al., 2018).

In the brain, Gal-8 has been detected in various regions, including the choroid plexus, basal ganglia, thalamus, and hypothalamus (John & Mishra, 2016; Pardo et

al., 2017, 2019). Studies in our laboratory have revealed neuroprotective roles of Gal-8 against oxidative stress, excitotoxicity, nutritional deprivation, and the formation of amyloid-beta (Aβ) oligomers (Pardo et al., 2019). These effects are mediated by the interaction between Gal-8 and β1 integrin leading to activation of the ERK 1/2 and PI3K/AKT signaling pathways (Pardo et al., 2019).

Our laboratory has also described function-blocking autoantibodies in patients with lupus (Massardo et al., 2009; Pardo et al., 2006) or with multiple sclerosis (Pardo et al., 2017). These antibodies block the interactions of Gal-8 with cell surface glycans (Cárcamo et al., 2005) and have been associated with lymphopenia in lupus (Massardo et al., 2009) and with worse evolution of the disease in patients with multiple sclerosis (Pardo et al., 2017).

Overall, Gal-8 exhibits a wide range of functions both intracellularly and extracellularly, including roles in autophagy, immune regulation, cell adhesion, platelet function, and neuroprotection. The interaction with specific glycans and cell surface proteins, among which integrins stand out, underlies Gal-8 functions in various tissues and biological processes.

### 2.2.8.- The role of Integrins, Src and Fak on L-type calcium channels.

Integrins are transmembrane proteins that link the extracellular matrix to microfilaments in the cytosol and are therefore crucial elements in cell adhesion and migration (Horton et al., 2016). Integrins are composed by α and β subunits, which form heterodimeric complexes with distinct binding specificities to matrix substrates (Horton et al., 2016). Activation of integrins triggers the activation of focal adhesion proteins, such as FAK, Src, Paxillin, Talin, and Vinculin, leading to cellular responses such as cell migration, proliferation, differentiation and EMT depending on the kind of cell and cellular context (Harburger & Calderwood, 2009; Horton et al., 2016; Widmaier et al., 2012). Interestingly, integrins have been associated with the regulation of L-type calcium channels (LTCC) and subsequent calcium influx (Chao et al., 2008; Gui et al., 2006; X. Wu et al., 2001; Yan Yang et al., 2010).

L-type calcium channels belong to the voltage-gated calcium channel (VGCC) family, with four subtypes: CaV1.1 (CACNA1S), CaV1.2 (CACNA1C), CaV1.3

(CACNA1D), and CaV1.4 (CACNA1F) (Catterall, 2011; Dolphin, 2016). While all LTCC subtypes are found in lymphocytes, their expression in other tissues varies. CaV1.1 is predominantly found in skeletal muscle, CaV1.2 and CaV1.3 are present in cardiovascular cells and neurons, CaV1.3 is also found in the pancreas, kidney, and cochlea, and CaV1.4 is primarily found in the retina (Catterall, 2011; Dolphin, 2016).

Previous studies have demonstrated that integrins such as α5β1 and α4β1 can induce calcium influx by promoting the opening of LTCC (Chao et al., 2008; Gui et al., 2006; X. Wu et al., 2001; Yan Yang et al., 2010). Activation of these integrins leads to phosphorylation of the C-terminal intracellular loop of LTCC in a FAK and Src-dependent manner, which triggers the opening of the channels and subsequent calcium influx (Chao et al., 2008; Gui et al., 2006; X. Wu et al., 2001; Yan Yang et al., 2010). So far, integrin-mediated opening of LTCC and calcium influx have been involved in vascular contraction (Gui et al., 2010; Waitkus-Edwards et al., 2002), regulation of hippocampal neuron gene expression (Gall et al., 2003) and cell migration (Jacquemet et al., 2016; Ouyang et al., 2022).

### 2.2.9.- Galectin-8 as a potential regulator of the primary cilium.

In both mitotic and non-mitotic cells, disassembly of primary cilia can occur through a pathway initiated by an increase of intracellular calcium levels, which then activates AurkA in a CaM-dependent manner resulting in HDAC6 activation and the subsequent microtubule depolymerization (Plotnikova et al., 2010, 2012). As mentioned, Gal-8 has been shown to induce an elevation of cytosolic calcium levels in platelets (Romaniuk et al., 2010) and integrins that are primary counter-receptors of Gal-8 (Barake, 2021; Cárcamo et al., 2005; Nabi et al., 2015; Oyanadel et al., 2018; Pardo et al., 2019), can induce calcium influx by regulating the opening of L-type calcium channels through FAK and Src activity (Chao et al., 2008; Gui et al., 2006; X. Wu et al., 2001; Y. Yang et al., 2010). Therefore, we hypothesize that Gal-8 can induce the loss of PC in hypothalamic cells through the activation of the AurkA/HDAC pathway downstream of an Integrin-FAK/Src-LTCC-dependent influx

of calcium. We propose that this Gal-8-induced loss of PC results in reduced leptin signaling and alterations in energy balance.

## 2.3.- Hypothesis.

Galectin-8 induces primary cilium disassembling, decreased leptin signaling and a positive energy balance through calcium influx by L-Type calcium channels.

## 2.4.- General Objective.

To determine the role of Galectin-8 and L-type calcium channels activity on ciliogenesis and leptin signaling.

### 2.4.1.- Specifics Objectives.

**1.- To determine the effects of Gal-8 on the primary cilium of hypothalamic Clu-177 cells.**

1.1.- To analyze the effect of Gal-8 on the primary cilium of Clu-177 cells in a time- and concentration-dependent manner.

1.2.- To determine the carbohydrate binding dependence of the effect of Gal-8 on the primary cilium in Clu-177 cells.

**2.- To define the mechanism of Gal-8-induced changes in primary cilum in Clu-177 cells.**

2.1.- To define whether Gal-8 treatment leads to primary cilium shearing and/or reabsorption.

2.2.- To define the role of integrins and focal adhesion proteins in the effects of Gal-8 treatment.

2.3.- To define the participation of calcium influx and the role of L-type calcium channels.

**3.- To determine the role of Gal-8 in leptin signaling and mice energy balance.**

3.1.- To determine the effect of Gal-8 in leptin signaling.

3.2.- To compare metabolic parameters related with primary cilium function in WT and Gal-8 KO mice.

# 3.0- Materials and Methods.

## 3.1.0.- Materials.

### 3.1.1.- Animals.

Mice were housed at the Faculty of Biological Sciences of Pontificia Universidad Católica de Chile. All protocols were approved by the Institutional ethics committee on the care and use of animals in research of the Universidad San Sebastián. Mice were maintained under conditions of strict confinement, including automatic control of temperature (21°C) and photoperiod (12 h light / 12 h dark), with water and food ad libitum. Lgals8/Lac-Z knock-in (here called Gal-8 KO) mice were generated from C57BL/6NTac mice engineered in Regeneron Pharmaceuticals Inc., New York, using Velocigene technology for replacing the entire coding region of the mouse Lgals8 gene (18,427 bp) with LacZ lox-Ub1-EM7-Neo-lox Cassette containing the LacZ gene that encodes β-galactosidase (Pardo et al., 2019; Pardo et al., 2017).

### 3.1.2.- Reagents and materials.

Thrombin from human plasma (T1063), protease inhibitor cocktail powder for bacterial (P8465), Src inhibitor PP2 (P0042) (Sigma), Nifedipine powder (SC-3589, Santa Cruz Biotech), glutathione-sepharose 4B (#17075601, cytiva), bradford solution (BM1470, Winkler), cover glasses (Marienfeld, #0111520), protease and phosphatase inhibitor mini tablets (A32959, Pierce).

### 3.1.3.- Antibodies and dyes.

Mouse anti α-tubulin (B-5-1-2) (1:1000), mouse anti Cav α1C (D6) (1:1000), mouse anti Cav α1D (E3), mouse anti alpha -3 (A-3), -5 (H-104) and beta1 (4B7R) integrins, and mouse anti FAK (H-1), pFAK (2D11), Src (B-12) and pSrc (9A6) were obtained from Santa Cruz Biotech. Rabbit anti acetylated-α-tubulin K40 (D2063) (1:1000), rabbit anti HDAC6 (D21B10) (1:1000), rabbit anti STAT3 (D3Z26) (1:1000) and mouse anti pY705-STAT3 (M9C6) (1:1000) were purchased from Cell Signaling

Technology. Rabbit anti pS22-HDAC6 (Ab61058) (1:1000) was purchased from Abcam.

Primary antibodies were recognized with horseradish peroxidase (HRP) conjugated antibodies (Rockland) for western blots (1:5000 dilution) or with Alexa conjugated antibodies (Molecular Probes) for immunofluorescence (1:500 dilution).

For microtubules live cell imaging, SiR-Tubulin (Cytoskeleton, Cat# CY-SC002) was used. Ratiometric calcium imaging was performed using Fura-Red AM from Invitrogen (Cat # F3020).

## 3.2.0.- Methods.

### 3.2.1.- Cell culture.

Clu-177 cells were maintained with high glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100 ug/mL penicillin and 0,1 mg/mL of streptomycin (P/S) in a cell incubator with a constant flow of 95% O2 y 5% CO2 at 37 °C. Cells were tested again mycoplasma contamination every two weeks. Eighteen hours prior the experiments the cells were washed with PBS 1x and maintained with DMEM-P/S media without FBS to induce ciliogenesis.

Jurkat cells were maintained with Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS and 100 ug/mL penicillin and 0,1 mg/mL of streptomycin in a cell incubator with a constant flow of 95% O2 y 5% CO2 at 37 °C.

### 3.2.2.- Recombinant protein production.

Bacteria transformed with pGEX-4T-3 (Pharmacia Biotech) plasmid bearing Gal-8-GST or GST were grown in a pre-inoculum in LB supplemented with ampicillin (100 µg/ml) (LB/Amp) at 37°C and 200 RPM of agitation. Then, the bacteria were transferred to 1 Lt of LB/Amp and grown for 2 hours until an absorbance of 0.7-0.9A. IPTG was added at 0.1 mM final concentration and the bacteria were maintained at 37°C and 200 RPM for recombinant protein production. Then, bacteria were centrifuged in 250 mL aliquots at 5000 RPM for 10 minutes in a Sorvall RC5C plus refrigerated centrifuge at 4°C with a GSA fixed angle rotor. The supernatants were discarded, and the bacterial pellets were frozen at -80°C until further use.

### 3.2.3.- Recombinant protein purification.

Pellets were resuspended in 1 ml of PBS and 250 ul of bacterial antiproteases cocktail (stock concentration 43 mg/ml) for 30 minutes. The lysozyme was added at 100 µg/ml and the mix was kept at 4°C in rotation. Triton X-100 was added at final 1% concentration, the resuspension was sonicated with an ultrasonic processor Sonics Vibra-Cell VC50 and then the mix was incubated for 30 min at 4°C in rotation. Lysates were centrifuged at 14,000 RPM for 10 min and the supernatant was keep on ice. Glutathione-Sepharose beads were washed once with 30 volumes of water and then two times with 30 volumes of PBS to balance it. The bacteria lysate supernatant was incubated with glutathione-sepharose beads for 3 h at 4ºC in rotation (500 ul of beads for 1 liter of initial bacterial culture). Afterwards, the beads were washed at least 3 times with 30 volumens of PBS 1x and centrifuged at 500g in a Sorvall Biofuge Stratos with a Heraeus #3047 Swinging Bucket Rotor at 4°C. For Gal-8 elution, Gal-8-GST bound to glutathione-sepharose beads were incubated with 10 U of Thrombin per 500 µl of beads in a final volume of 1000 µl at room temperature (RT) for 4 h under agitation. The beads were centrifuged at 500 g for 5 min at 4°C and the protein in the supernatant was measured with Bradford.

### 3.2.4.- Galectin-8 activity test.

Glass coverslips in a 24 wells plate were coated with Gal-8 (20 ug/ml) or PBS 1x for 1 hour at 37°C. Then the liquid was subtracted from the wells and the covers were dried for 30 min at 37°C. Jurkat cells are seeded at a concentration of 50,000 cells per well in the absence of FBS and incubated for 45 min. At least 95% of Jurkat cells seeded on Gal-8 coated coverslips must be attached and spread on the substrate to considerate a correct Gal-8 activity (Fig 3).

### 3.2.5.- Inmunofluorescence.

Clu-177 cells, seeded at 25,000 cells per ml in glass coverslips for 24 hours, were washed with PBS, incubate 18 hours with DMEM-P/S medium without FBS, fixed

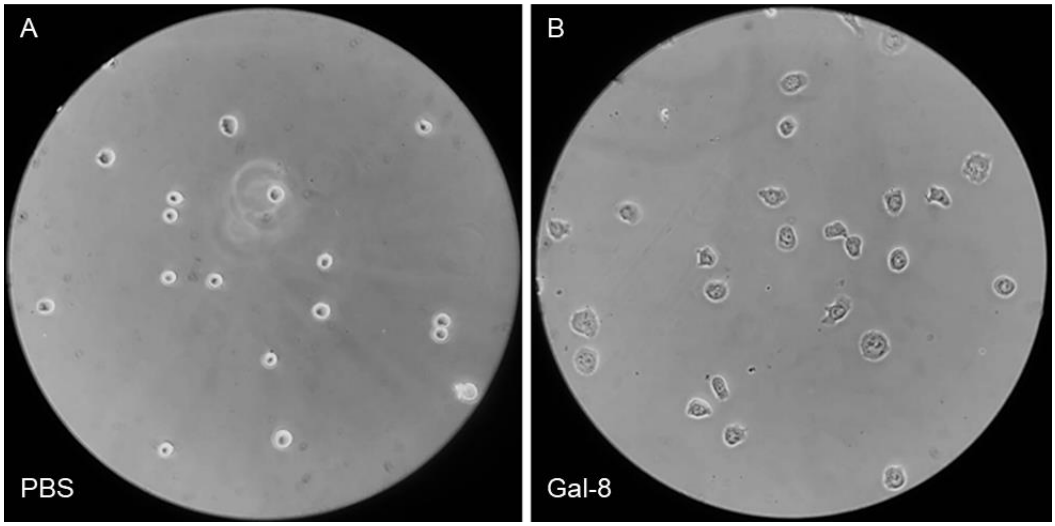


**Figure 3.- Galectin-8 activity testing by cell adhesion and spreading.**

Jurkat cells seeded in glass coverslips coated with PBS (left) or Gal-8 (right) show different morphology due to the cell spreading that active Gal-8 induces when used as supportive matrix.

with ice-cold PFA 4%/Sucrose 4% for 10 minutes, incubated with blocking buffer (TBS/Saponin 0.05%/BSA 1%) for 30 minutes at RT and then overnight with primary antibody diluted in blocking buffer at 4°C. After three washes with TBS, cells were incubated with secondary fluorescent antibodies and Hoechst nucleus tinction for 30 minutes at 37°C, washed three times TBS and mounted with Fluoromount-G.

### 3.2.6.- EdU proliferation assay.

Clu-177 cells (25,000 cells/ml) seeded in glass coverslips for 24 hours were washed with PBS 1x and incubated 18 hours with DMEM-P/S. The cells were co-treated with Gal-8 (30 nM) and 2X EdU (ThermoFisher Scientific, #C10340) complete medium solution for a final concentration of 10 µM for 2 hours. Then, cells were fixed with PFA 4% for 15 minutes, and washed twice with a PBS 1x/BSA 3%. Cells were permeabilized with a PBS 1X 0.5% Triton X-100 solution for 20 minutes, and then washed twice with PBS 1x/BSA 3%. The Click-iT reaction was developed according to the manufacturer's instructions. Cells were washed with PBS 1x and incubated for 30 minutes with Hoechst 33342 at room temperature protected with light. Coverslips were then washed twice with PBS 1x and mounted into microscope slides and posteriorly analyzed with confocal microscopy.

### 3.2.7.- Western-blot.

Cells with the different treatments were lysed with RIPA lysis buffer (Tris 50mM, NaCl 150 mM, EDTA 5 mM, NP40 1%, Sodium Deoxycholate 0.5%, SDS 0.1%, pH 8.0) for 30 minutes at 4 °C, supplemented with protease and phosphatase inhibitor. Protein samples were resolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at different percentages depending on the protein of interest. Loading buffer contained 100 mM DTT as a reducing agent. After electrophoresis, samples were transferred into a PVDF membrane in Bio-Rad Trans-Blot Turbo transfer system. After transferring, membranes were blocked with TBST/BSA 1% buffer for 45 minutes. Primary antibodies were incubated ON at 4ºC in blocking buffer. Then, membranes were washed three times with TBST buffer for 5 minutes at RT. Secondary antibodies conjugated with horseradish peroxidase (HRP) were incubated 1 hour in blocking buffer then washed. Membranes were

incubated 1 minute with Cyanagen Westar Ultra 2.0. Chemiluminescent images were acquired in Syngene G:Box detection system with the corresponding filter. Densitometric band analysis was performed with FIJI software.

### 3.2.8.- Pull-down assay.

Protein extracts were incubated with GST-Glutation Sepharose column, for 1 h at 4ºC. The beads were centrifuged and then the supernatant was incubated for 2 h at 4ºC with Gal-8-GST-Glutation-Sepharose previously incubated for 1 h with the indicated inhibitor or vehicle as described in (Pardo et al., 2019). Beads were then washed 3 times with lysis buffer at 4ºC then 3 times at RT for 5 min and eluted with loading buffer for western blot.

### 3.2.9.- Conditioned media analysis.

Clu-177 cells were seeded in 100mm petri dishes at a 25,000 cell per ml in 10 ml of DMEM/FBS 10%. Twenty-four hours later the cells were washed with PBS 1x and the medium was replaced with DMEM without FBS for 18 hours. Then, cells were treated with Gal-8 or vehicule for 2 hour or with Sharing cilia buffer (112 mM NaCl, 3.4 mM KCl, 10 mM CaCl2, 2.4 mM NaHCO3,2mM HEPES, pH 7.0) for 10 min. The conditioned media was centrifuged at 1,000g for 10 min to avoid cell detritus contamination (Mirvis et al., 2019; Raychowdhury et al., 2005). The supernatant was incubated with trichloroacetic acid (TCA) at 20% ON at 4°C and agitation. Then, the samples were centrifuged at maximum speed for 10 min, washed three times with cold acetone 80% and added 5ul of NaOH 0.01M. Finally, the samples were prepared to western-blot analysis.

### 3.2.10.- Primary cilium live cell imaging.

Clu-177 cells seeded in live cell imaging plates were incubated with SiR-Tubulin 100 nM ON. The cells were then washed twice with 1mL of DMEM-Hepes. The treatments were diluted in DMEM-Hepes at the times indicated in the experiments. Live cell imaging was performed with a Leica SP8 confocal microscope, using a 63x oil immersion objective, following a single field of view per experiment with a 180 seconds per frame time resolution.

### 3.2.11.- Ratiometric calcium imaging.

Clu-177 cells were incubated with Fura-red AM (5uM) for 30 minutes The treatments were diluted in DMEM-Hepes at the times indicated in the experiments. Live cell imaging was performed with a Leica SP8 confocal microscope, using a 63x oil immersion objective, following a single field of view per experiment with a 5 seconds per frame time resolution.

### 3.2.12.- Locomotor activity.

Metabolic cage was used to measure the mice locomotor activity. Seven hours before the measurement, the mice were placed in the metabolic cage. Then, during the night phase, locomotor activity was measured using the metabolic cage (UGO BASILE, model 41801) for 12 hours. During the measurement period the mice were kept with food and water *ad libidum.*

### 3.2.13.- Respiratory exchange ratio.

Measurement of CO2 production and O2 consumption was performed using the iWorx GA200 measurement system. The mice were placed in the metabolic cage at a maximum concentration of 0.24% CO2. Gas measurement was performed until a CO2 concentration of 3% was reached. The data were analyzed with the LabScribe 2.0 software according to the manufacturer's instructions.

### 3.2.14. Glucose tolerance test.

Basal glycemia of mice was measured after 6 hours of fasting. Subsequently, a glucose solution (2g glucose per kg body weight) was injected and subsequent glycemia measurements were performed every 30 minutes for 2 hours. During the measurement time the mice were deprived of food to avoid interference with the measurement.

### 3.2.15. Intranasal Gal-8 administration.

Intranasal administration of Gal-8 was performed in 3-month-old WT and Gal-8-KO mice partially anesthetized with isoflurane. After anesthesia, in prone position 3uL of Galectin-8 50ug/ml was administered to the treated group and 3ul of PBS to the control group, in each of their nostrils. The procedure was repeated once a day for 2 days.

### 3.2.16.- Statistical analysis.

All analysis were carried out using GraphPad Prism 8.0 software. Student’s t test was used for two-group comparison, and one-way or two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used for comparisons of more than two groups. P-values equal or minor than 0.05 ($P \leq 0.05$) was considered statistically significant, and higher p-values were considered non-significant (ns). The P-value graphic style is indicated according to APA format. Data points and error bars in the figures represent mean and standard error of the mean (SEM).

# 4.0.- Results.

## 4.1.- Effects of Gal-8 on the primary cilium of hypothalamic cells.

### 4.1.1- Galectin-8 induces loss and length reduction of primary cilium in Clu-177 hypothalamic cells.

Gal-8 treatment at a concentration of 600nM for 4 hours decreased the percentage of ciliated cells from 80% in control cells to 35% in Gal-8 treated cells (Fig. 4). Gal-8 concentrations of 30nM, 300nM, and 600nM showed similar effects at different time points. The percentage of ciliated cells decreased to 14-17% at 0.5 hours, 18% at 1 hour, 20-22% at 2 hours, and 15-29% at 4 hours of treatment (Fig. 5). We did not observe these effects under conditions that denature proteins but not lipopolysaccharide (LPS) (Fig. 6 A-B). Therefore, Gal-8 itself and not possible traces of LPS contamination caused cilia loss.

Gal-8 treatment also impacted primary cilium morphology, decreasing cilia length and volume while the shape complexity index remained unaffected (Fig. 7A). Control and cells treated with denatured Gal-8 (dnGal-8) displayed an average primary cilium size of 3.64 µm and 3.4 µm, respectively, whereas Gal-8-treated cells exhibited an average size of 2.82 µm (Fig. 7B). Moreover, Gal-8-treated Clu-177 cells showed significantly lower proportion (4%) of cilia larger than 4 µm compared to control cells (40%) (Fig. 7C). Ciliary volume decreased by 33.5% in Gal-8-treated cells compared to control cells (Fig. 7D).

These results show that Gal-8 treatment reduces the percentage of ciliated cells and affects cilia morphology in cells that maintain the primary cilium.

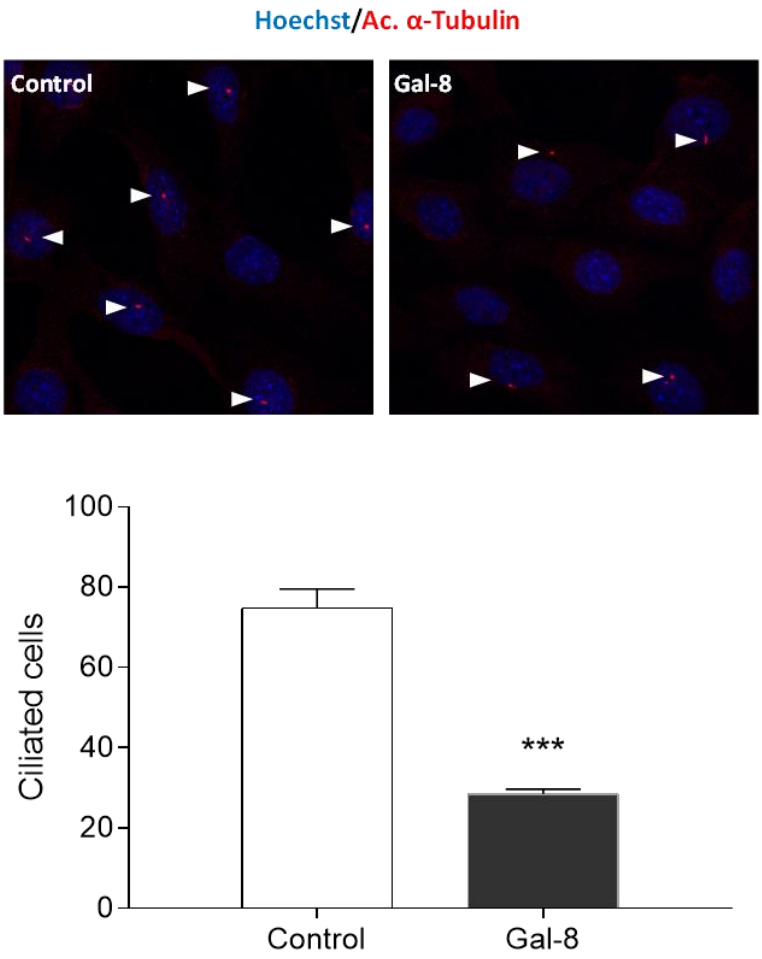


**Figure 4.- Galectin-8 induces primary cilium loss in Clu-177 cells.**

Percentage of ciliated cells after Gal-8 treatment. Hypothalamic Clu-177 cells were treated with Gal-8 at 600 nM for 4 hours and the cilia were visualized by immunofluorescence against acetylated $\alpha$-tubulin. Gal-8 treatment induces a 45% reduction in the ciliated cells. (t-test, P<.001).

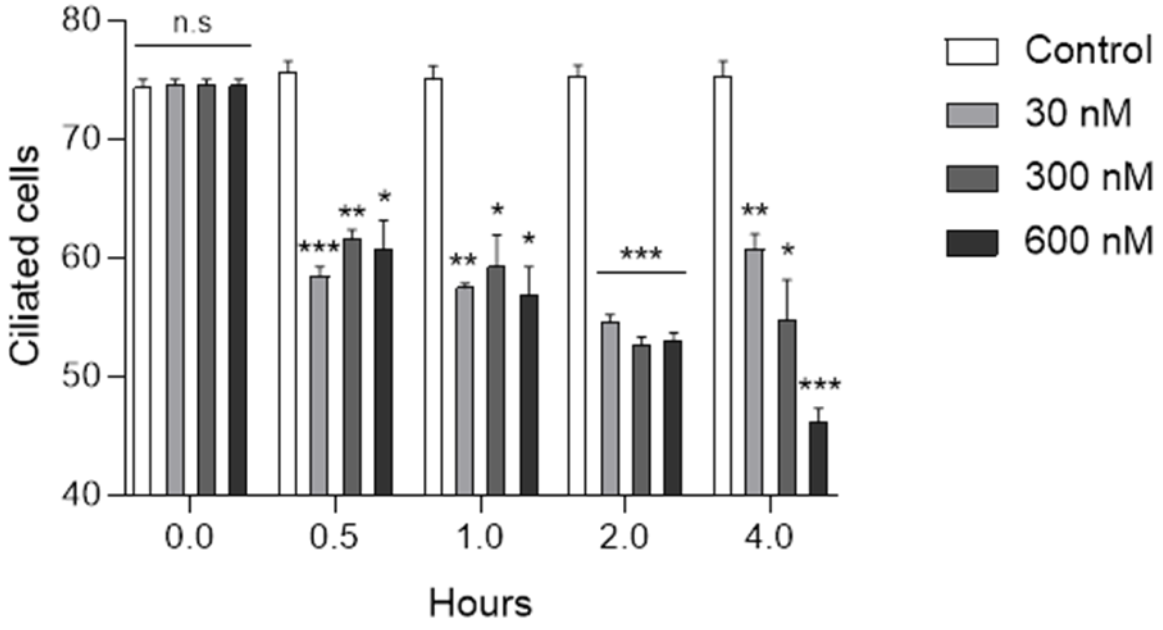


**Figure 5.- Gal-8 treatment induces PC loss in Clu-177 cells.**

Percentage of ciliated cells after Gal-8 treatment. Hypothalamic Clu-177 cells were treated with Gal-8 at 30, 300 and 600 nM for 0.5, 1, 2 and 4 hours and the cilia were visualized by immunofluorescence against acetylated $\alpha$-tubulin and then analyzed with FIJI. Data shows that cells loss PC in a concentration and time dependent manner. (Two-way Anova. *P≤ .05;**P< .001 or ***P <.001).

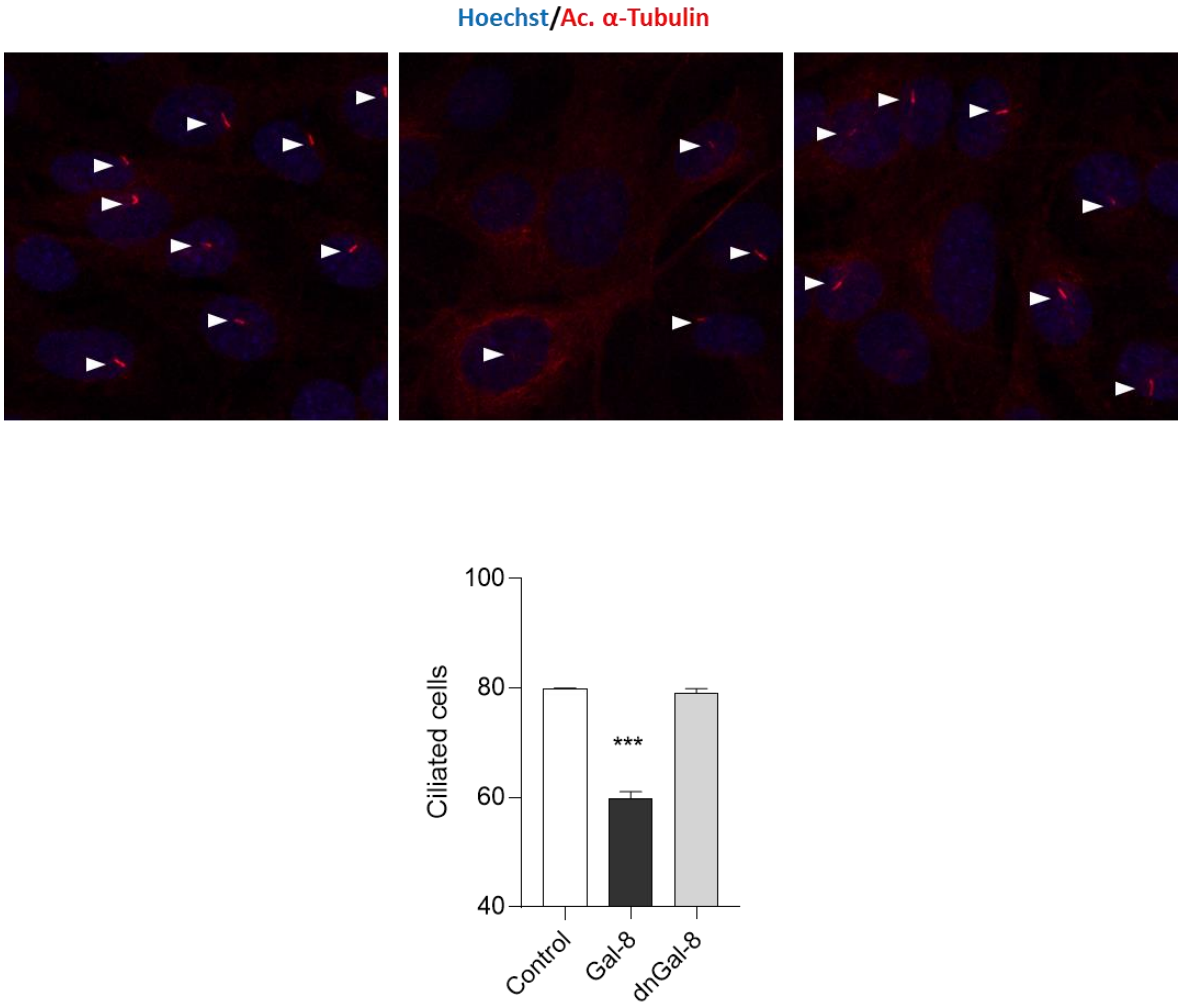


**Figure 6.- Galectin-8 induction of PC loss is independent of LPS contamination.**

Cells were treated with 30nM of Gal-8 or denaturated (dnGal-8) for 2 hours. A) Indirect immunofluorescence against acetylated α-tubulin showing the PC in CLU177 cells; B) Graph of the percentage of ciliated cells shows that Gal-8 but not dnGal-8 reduces the number of ciliated cells from 79.9% to 59.8% (One-way Anova $P<0.001$).

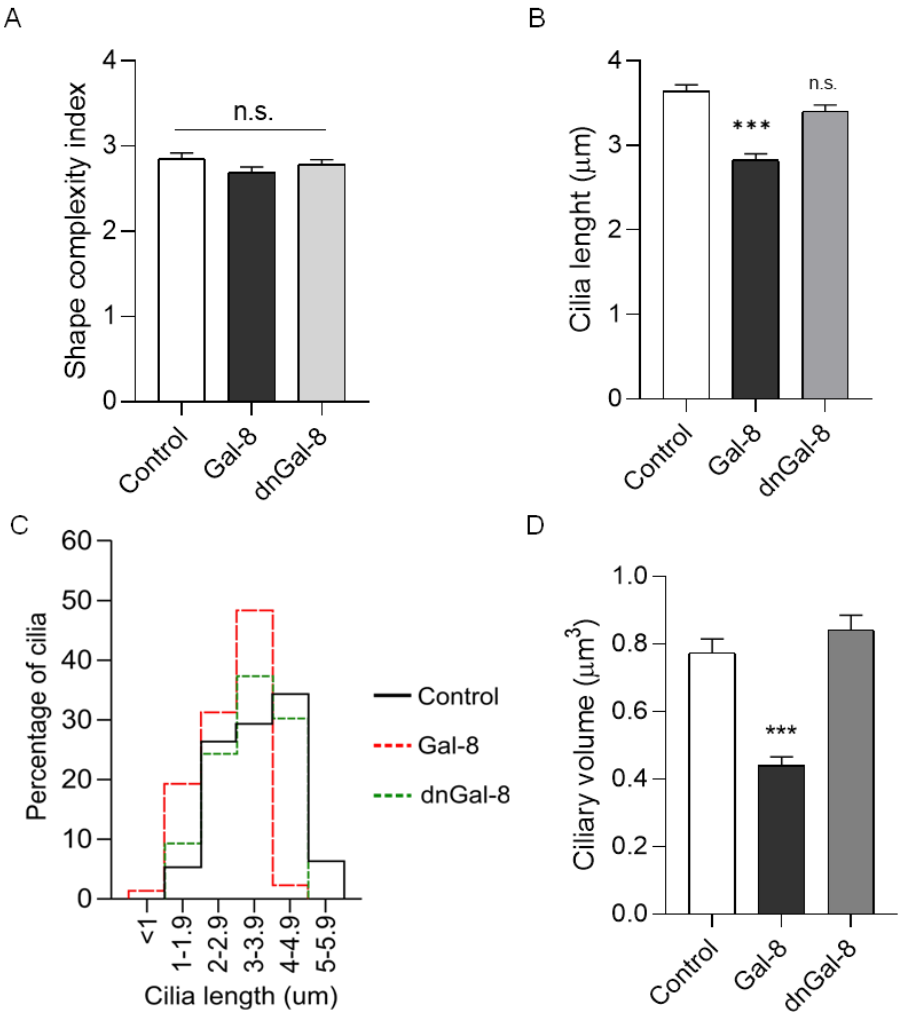


**Figure 7.- Gal-8 reduces PC length and volume but not shape complexity in Clu-177 cells.**

Clu-177 cells treated with 30 nM Gal-8 or dnGal-8 for 2 h were analyzed for morphology parameters using CiliaQ software. Shape complexity of PC remains unaltered by Gal-8 and ndGal-8treatment (A); Gal-8 but not dnGal-8 treatment reduces PC length (B and C) and ciliary body volume (D). (One-way Anova P<.001).

### 4.1.2.- Gal-8 induced effects on primary cilium involve interactions with cell surface carbohydrates.

To elucidate the mechanism by which Gal-8 exerts its effects on primary cilium, we first investigated its interactions with cell surface glycosylated molecules employing conditions known to inhibit Gal-8 binding to glycans interactions (Ideo et al., 2011; Oyanadel et al., 2018). We preincubated Gal-8 with either β-lactose or α-2,3-sialyllactose (2,3SL) to block glycan binding through its two carbohydrate recognition domains (CRDs) or only its N-terminal CRD, respectively.

Preincubation with 2,3SL reduced Gal-8 effect on cilia loss from 19% to 9.7% whereas β-lactose completely prevented the Gal-8 effects on cilium (Figure 8). Neither sucrose nor 2,6SL, which are used as an osmolarity control and does not bind to the CRD of Gal-8, showed any blocking effect (Figure 8). These findings indicate that Gal-8 interactions with specific cell surface carbohydrates mediate its effects on primary cilium.

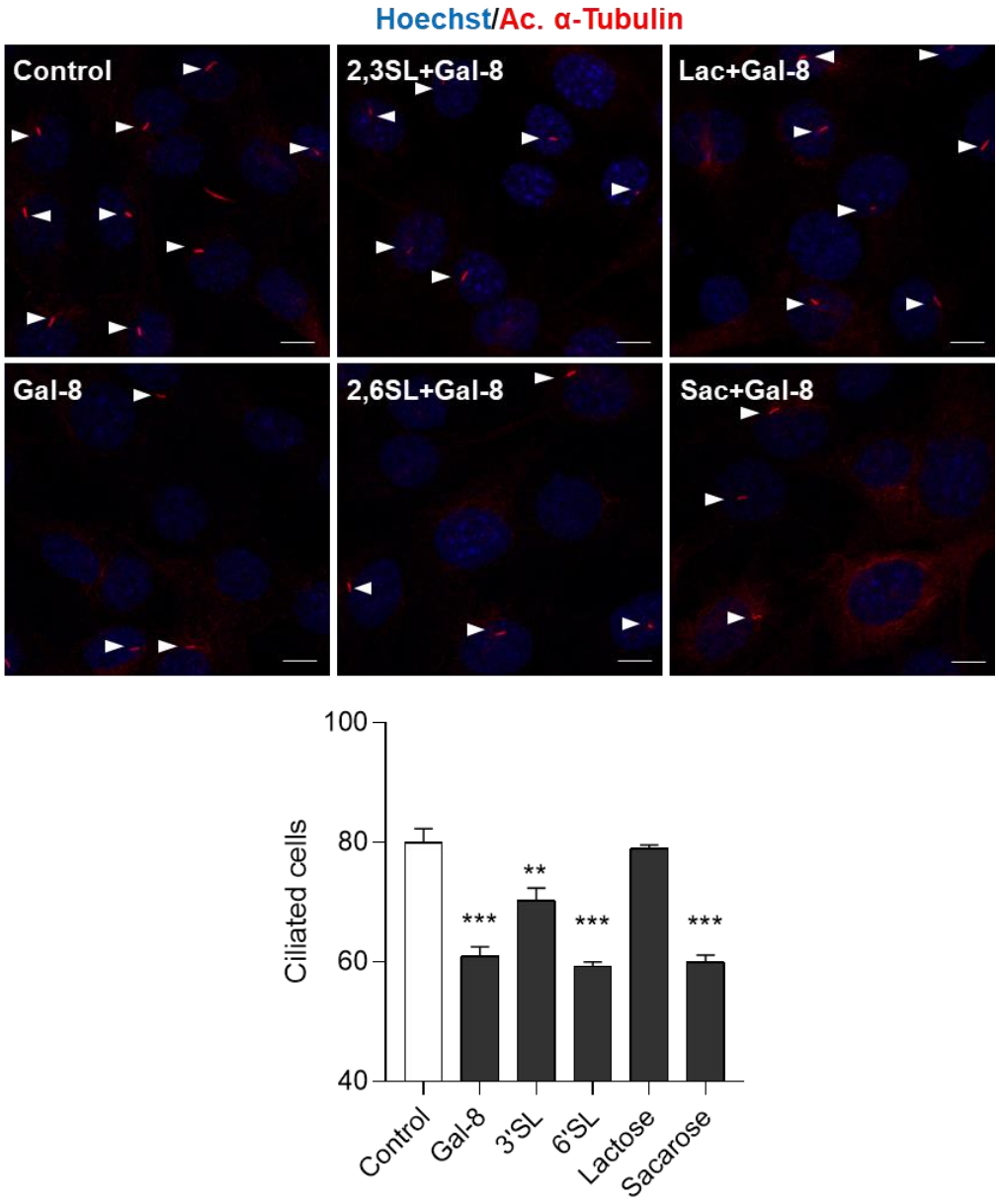


**Figure 8.- Loss of primary cilia by Gal-8 requires carbohydrate binding.**

Cells were treated or cotreated with Gal-8 (30 nM) and α-2,3-syalillactose (2,3SL, a Gal-8 N-domain blocker, 10 mM), α-2,6-syalllactose (2,6SL, homeostasis control), β-lactose (lactose, a blocker of both Gal-8 domains, 10 mM), or sacarose (homeostasis control). Co-treatment with the Gal-8 blockers α-2,3-syalillactose and lactose, but not with α-2,6-syalillactose or sacarose, prevents Gal-8-induced cilia loss. (One-way Anova **P<.01 or ***P<.001).

### 4.1.3.- Galectin-8 treatment under conditions that affect primary cilium does not increase cell proliferation.

As cilia loss can be secondary to stimulation of cell proliferation, we examined the incorporation of Edu as a maker of DNA synthesis. Cells treated with 30nM Gal-8 for 2 hours showed no significant difference in EdU incorporation compared to control cells (Figure 9A), indicating that DNA replication does not increase under the conditions that induce cilia loss. Gal-8 treatment for 24 hours did not alter the overall cell number compared to the control condition (Figure 9B). These findings indicate primary cilium loss induced by Gal-8 treatment is not due to stimulation of cell proliferation.

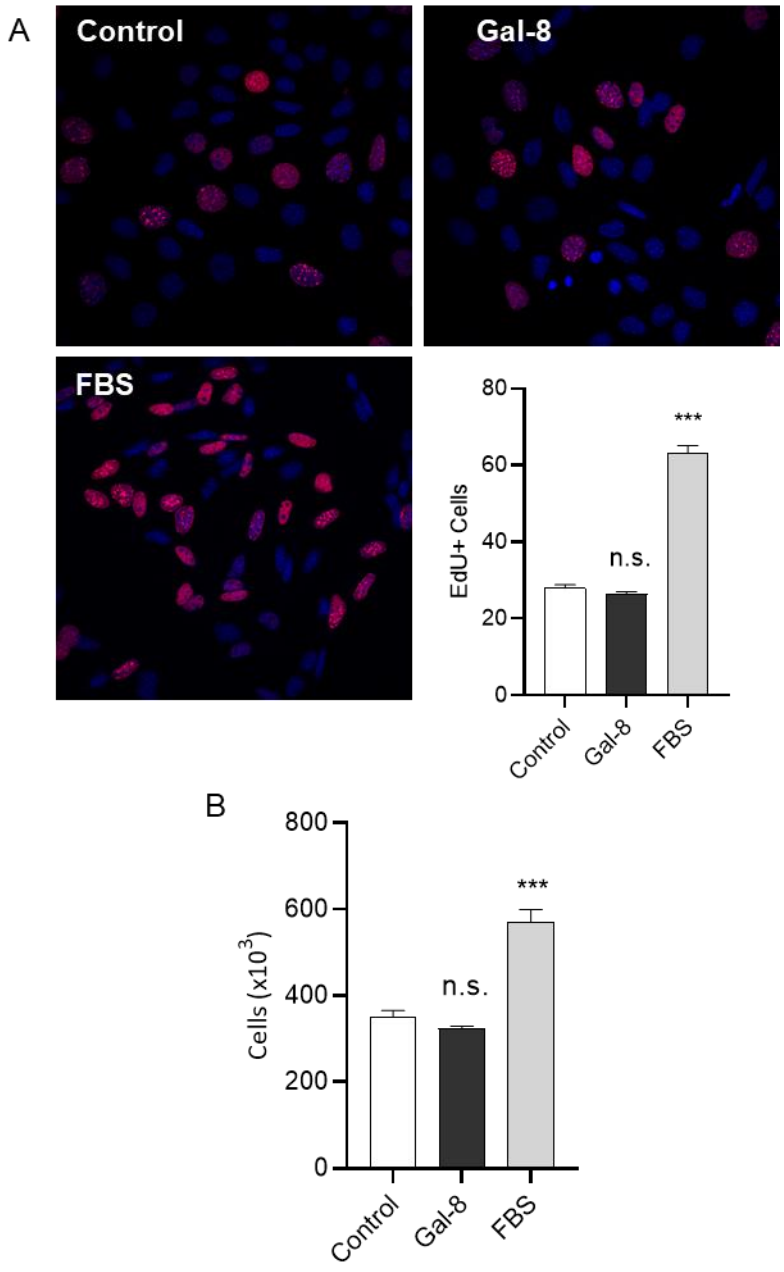


**Figure 9.- Gal-8 does not increase cell proliferation in Clu-177 cells.**

Clu-177 cells were treated with Gal-8 at 30 nM for 2 or 24 hours. A) Gal-8 treatment for 2 h did not increase DNA synthesis measured by the thymidine analog EdU, contrasting with the 40% increase induced by 10% FBS; B) 24 hours of Gal-8 treatment did not change cell number, whereas 10% FBS increased 62.9% the cell number. (One-way Anova P<.001).

### 4.1.4. Conclusions.

All these results demonstrate that Gal-8 treatment reduces the percentage of ciliated cells and affects the morphology of primary cilium in Clu-177 hypothalamic cells. These effects are mediated by Gal-8 interactions with cell surface carbohydrates, as blocking its glycan binding attenuates the loss of ciliated cells. In addition, Gal-8 induced cilia loss do not involve cell proliferation, indicating a distinct mechanism of action.

## 4.2.- Galectin-8 induces cilium resorption involving HDAC6 activity downstream an integrin/Src/FAK/LTCC pathway

### 4.2.1- Galectin-8 Induces Resorption of Primary Cilium.

Loss of primary cilia (PC) can occur through three different mechanisms: 1) gradual PC resorption or disassembly leading to a progressive shortening until complete disassembly; 2) cleavage and release of PC into the extracellular medium as ectosomes; and 3) combined mechanism involving gradual shortening of the PC followed by cleavage and release as ectosomes after reaching a critical point (Mirvis et al., 2019). The gradual resorption of PC relies on the activation of the AurkA/HDAC6 axis, while PC cleavage has been associated with the levels and activation of Katanin (p60) (Hubbert et al., 2002; Izawa et al., 2015; K.H. Lee et al., 2012; Mirvis et al., 2019; Plotnikova et al., 2012; Pugacheva et al., 2007).

To investigate the dynamics of primary cilia loss we conducted live cell microscopy experiments in cells previously incubated with SiR-Tubulin probe (100 nM) in medium without fetal bovine serum (FBS) for 18 hours. Treatment with Gal-8 resulted in gradual resorption of PC, starting 30 minutes after treatment, whereas control cells showed no changes in their cilia (Fig. 10A).

We also analyzed conditioned media attempting to detect acetylated alpha-tubulin as ectosome release indicator, using cilium shedding buffer (CSB) as positive control (Mirvis et al., 2019; Raychowdhury et al., 2005). Treatment with 30 nM Gal-8 for 0.5, 1, or 2 hours did not increase the levels of acetylated alpha-tubulin and total tubulin in the conditioned media, whereas treatment with CSB led to a 240% increase in the ratio of acetylated alpha-tubulin to total tubulin (Fig. 10B). Only at high concentrations of 600 nM Gal-8 we observed acetylated alpha-tubulin in the conditioned media indicative of PC release. Therefore, at the 30 nM concentration used in previous experiments, Gal-8 induces PC disassembly and reabsorption, practically ruling out the cleavage mechanism of PC loss.

Then, we examined the activation of the AurkA/HDAC6 axis, which has been implicated in PC assembly-disassembly processes associated with the cell cycle (Izawa et al., 2015). Treatment with Gal-8 resulted in a 45.3% increase in

phosphorylated HDAC6 (pHDAC6) levels and induced PC loss, whereas both effects were prevented by pretreatment with the AurkA inhibitor VX-680 (Fig. 11).

These findings suggest that Gal-8, at a concentration of 30 nM, induces PC loss in Clu-177 cells through a reabsorption mechanism involving activation of the AurkA/HDAC6 axis.

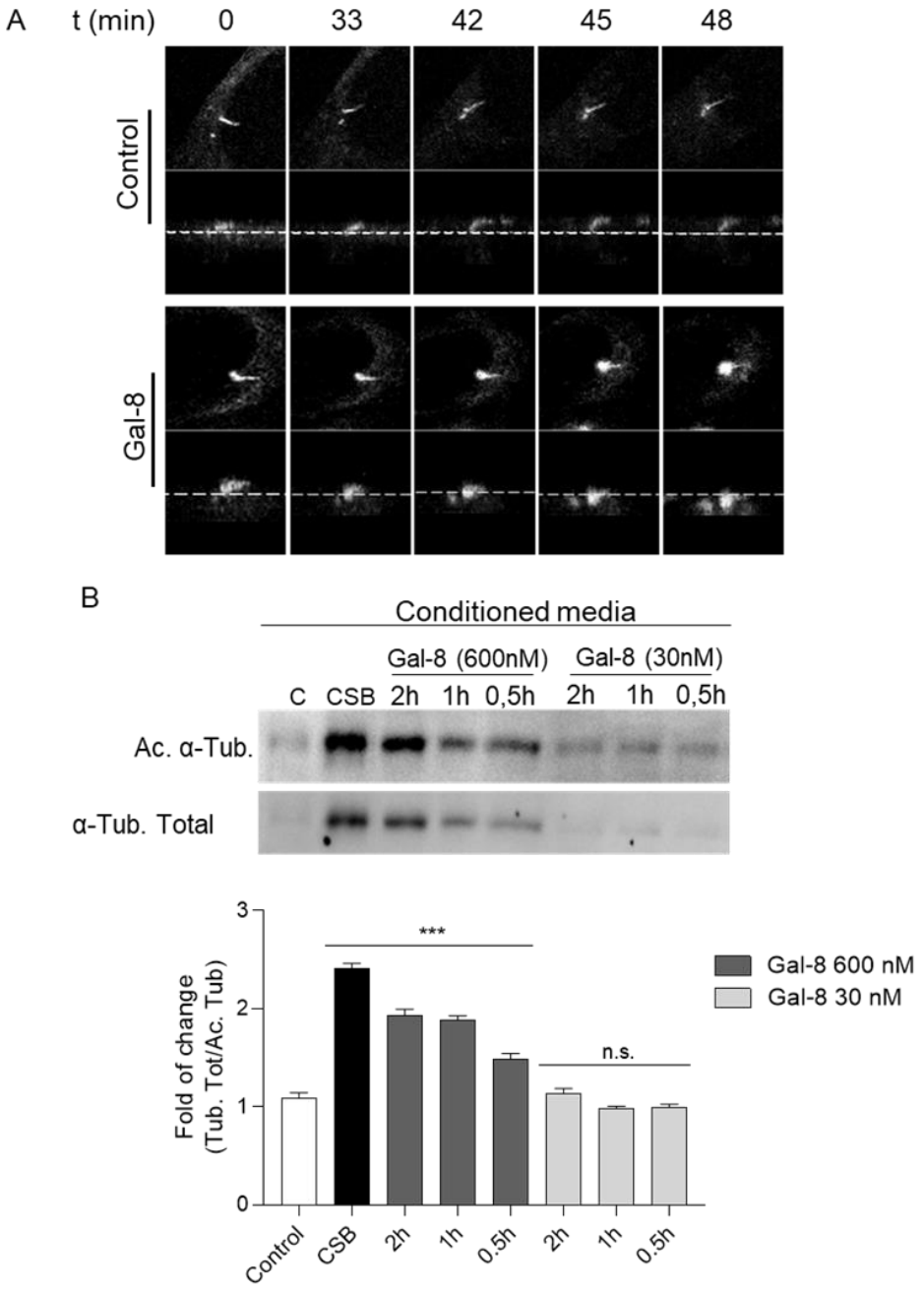


**Figure 10.- Gal-8 can induce PC resorption or shedding depending on its concentration.**

A) Live cell imaging of Clu-177 cells stained with SiR-Tubulin shows cilia resorption to cytosol in cells treated with 30 nM Gal-8; B) Conditioned media of cells treated with 600 nM but not 30 nM Gal-8 showed the presence of acetylated α-tubulin indicating PC shedding. (One-way Anova P<.001).

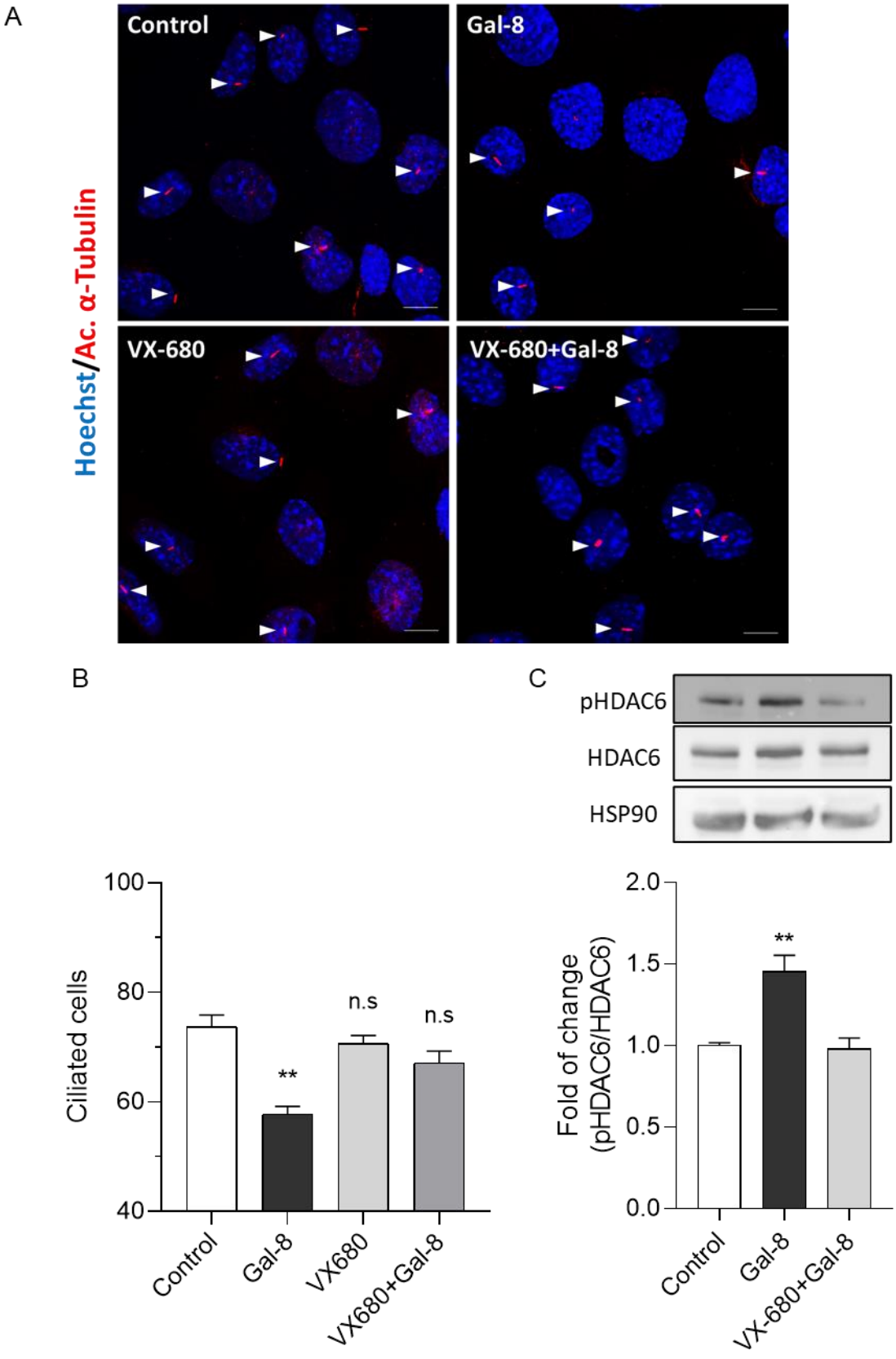
A
Control
Gal-8
VX-680
VX-680+Gal-8
Hoechst/Ac. α-Tubulin
B
Ciliated cells
100
80
60
40
**
n.s
n.s
Control
Gal-8
VX680
VX680+Gal-8
C
pHDAC6
HDAC6
HSP90
Fold of change (pHDAC6/HDAC6)
2.0
1.5
1.0
0.5
0.0
**
Control
Gal-8
VX-680+Gal-8

**Figure 11.- PC loss induced by Gal-8 treatment requires AurkA activity.**

Clu-177 cells pretreated with AurkA inhibitor VX-680 or vehicle were incubated with Gal-8 (30nM) for 2 h and then number of ciliated cells was evaluated. A) Indirect immunofluorescence against acetylated $\alpha$-tubulin shows loss of PC after Gal-8 treatment and the avoidance of this effect when cells are pretreated with AurkA inhibitor VX-680; B. Graphs shows the quantification of these effects; C) Western blot shows that Gal-8 treatment increases HDAC6 phosphorylation except when cells are pretreated with VX-680. (One-way anova $P<.01$).

### 4.2.2- Galectin-8 binding to integrins and activation of focal adhesion proteins mediate cilium loss.

One of the major receptors for Gal-8 is the integrin family of proteins (Nabi et al., 2015). Previous studies from our laboratory and others have demonstrated that the binding of Gal-8 to β1 integrins is involved in various cellular processes, including cytoskeletal rearrangements, cell adhesion, neuronal protection, and epithelial-mesenchymal transition (Cárcamo et al., 2005; Diskin et al., 2012; Levy et al., 2001; Oyanadel et al., 2018; Pardo et al., 2017, 2019).

To investigate the interaction between Gal-8 and integrins in Clu-177 cells, a pulldown assay was performed using Gal-8-GST bound to glutathione-sepharose beads, as previously described (Oyanadel et al., 2018). The Gal-8-GST beads showed interaction with alpha-3, alpha-5, and beta-1 integrins, but not with alpha-1, alpha-2, and beta-3 integrins (Fig. 12A).

Activation of Src kinase has been shown to inhibit ciliogenesis and reduce ciliary size in mouse dermal and embryonic fibroblast cells, as well as 10T1/2 cells (Bershteyn et al., 2010; Drummond et al., 2018). Src and FAK are components of the focal adhesion complex activated by integrins (Horton et al., 2016; Mitra & Schlaepfer, 2006; Widmaier et al., 2012) We found that treatment with Gal-8 increased by 21% and 25% Src and FAK phosphorylation, respectively (Fig. 12B). Furthermore, Src and FAK inhibitors counteracted the effect of Gal-8 on primary cilia (Fig. 13).

We can conclude that the binding of Gal-8 to α-3, α-5, and β-1 integrins, and subsequent activation of focal adhesion proteins such as FAK and Src are involved in the effects of Gal-8 on primary cilia.

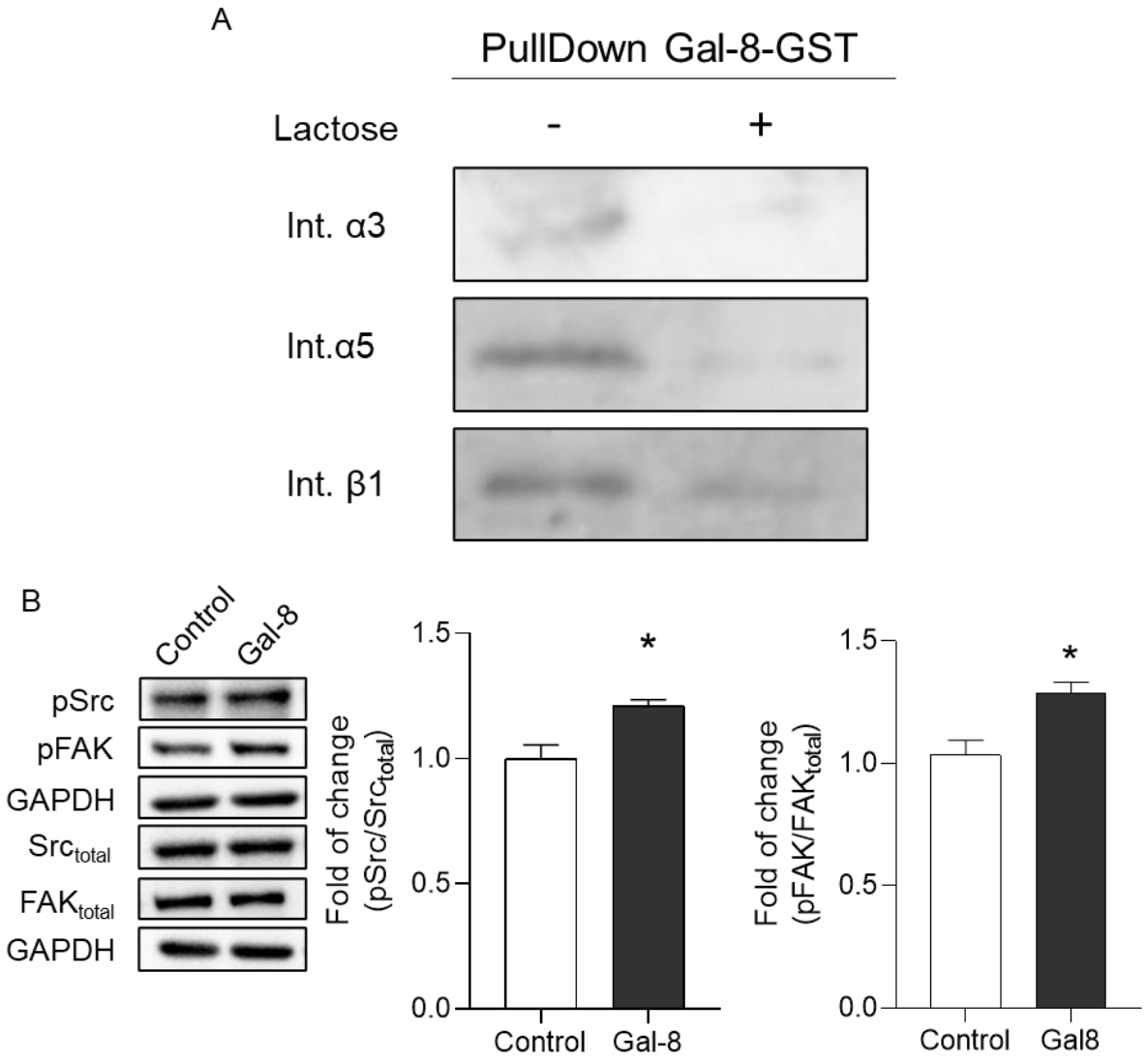


**Figure 12.- Gal-8 interacts with integrins and induces Src and FAK phosphorylation.**

A) Pulldown with Gal-8-GST indicates interaction of Gal-8 with $\alpha$3 and $\alpha$5 integrin, which also pulled down the $\beta$1 subunit; B and C) Gal-8 treatment increases Src and FAK phosphorylation in Clu-177 cells. (t-test P<.05).

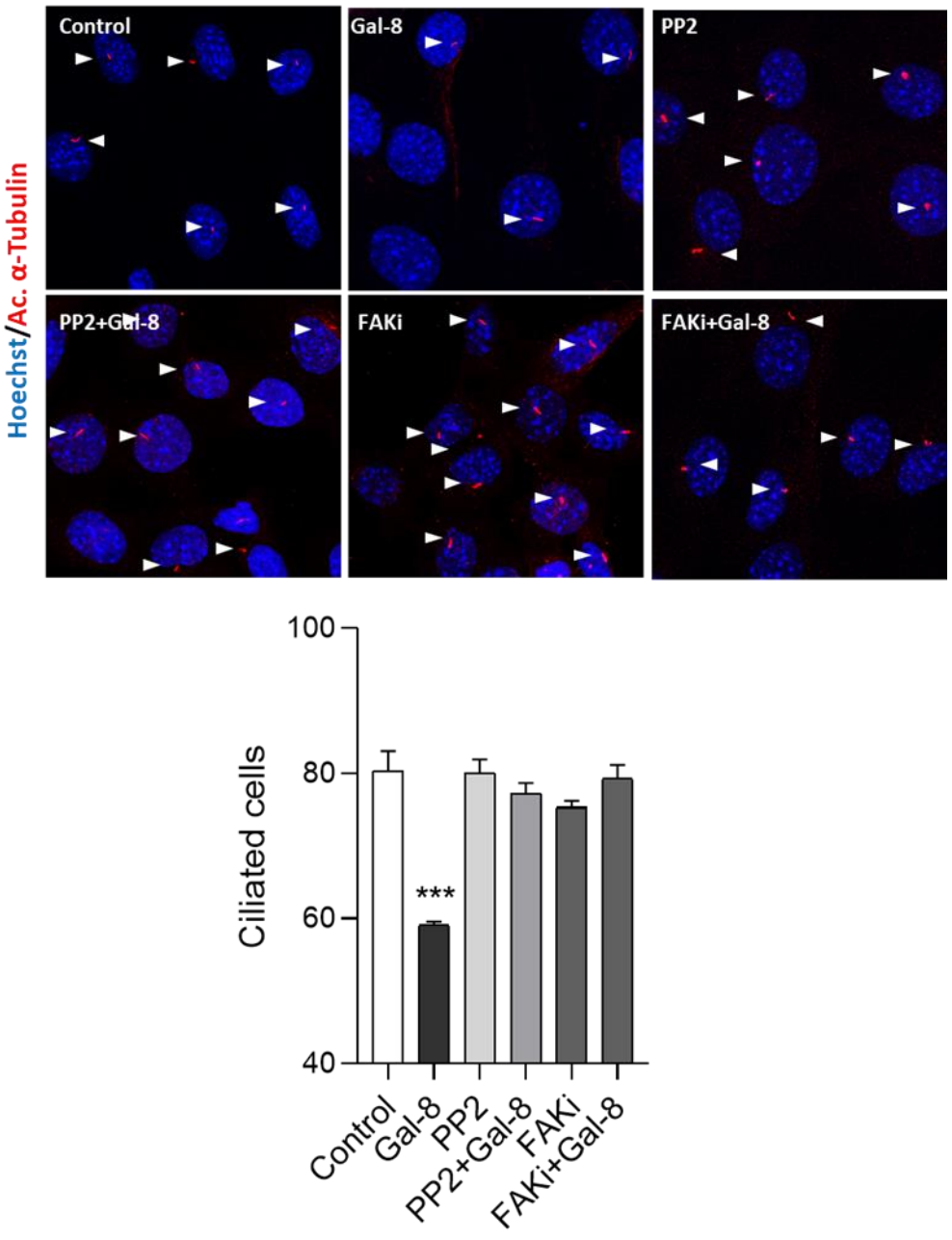


**Figure 13.- PC loss induced by Gal-8 requires FAK and *Src* activity.**

Gal-8 (30nM) treatment reduced by 21.2% the number of ciliated Clu-177 cells while this effect is avoided in cells pretreated with the Src and FAK inhibitors PP2 and FAKi, respectively. (One-way Anova P<.001).

### 4.2.3- Galectin-8 induces cilium loss through calcium influx.

The involvement of intracellular calcium in both reabsorption and shear mechanisms of PC loss has previously been describe in mitotic and non-mitotic cells (Mirvis et al., 2019; Plotnikova et al., 2010, 2012). Increases in cytosolic calcium levels can activate the AurkA/HDAC6 axis through CaM-mediated signaling and this results in depolymerization of alpha-tubulin and reabsorption of the PC (Plotnikova et al., 2010, 2012). Gal-8 treatment has also been shown to increase intracellular calcium concentration in platelets (Romaniuk et al., 2010). Therefore, we asked whether Gal-8 might induce calcium influx associated with PC loss in hypothalamic cells.

Using ratiometric calcium imaging with Fura-Red AM, we found that Gal-8 treatment induced an increase in cytosolic calcium concentration that reached a peak within 10-15 seconds in Clu-177 cells. The fluorescence intensity during the calcium peak was 75% higher than basal conditions (Fig. 14A). At a concentration of 90 nM Gal-8, the calcium peak reached 120% over basal fluorescence, while at a lower concentration of 3 nM, we observed a sustained increment in calcium levels over time, reaching 10% above basal fluorescence in 4 minutes (Fig. 14A). This increase in cytosolic calcium was completely abolished in the presence of EGTA, indicating calcium influx from the extracellular medium (Fig. 14A). Furthermore, EGTA completely abolished the effect of Gal-8-induced primary cilia loss. As previously shown, Gal-8 treatment decreased by 27.1% the number of ciliated cells, while no significant differences were observed in cells co-incubated with EGTA (Fig. 14B).

These findings suggest that Gal-8-induced loss and shortening of the primary cilium in Clu-177 cells involve calcium influx, which subsequently activates the AurkA/HDAC6 axis.

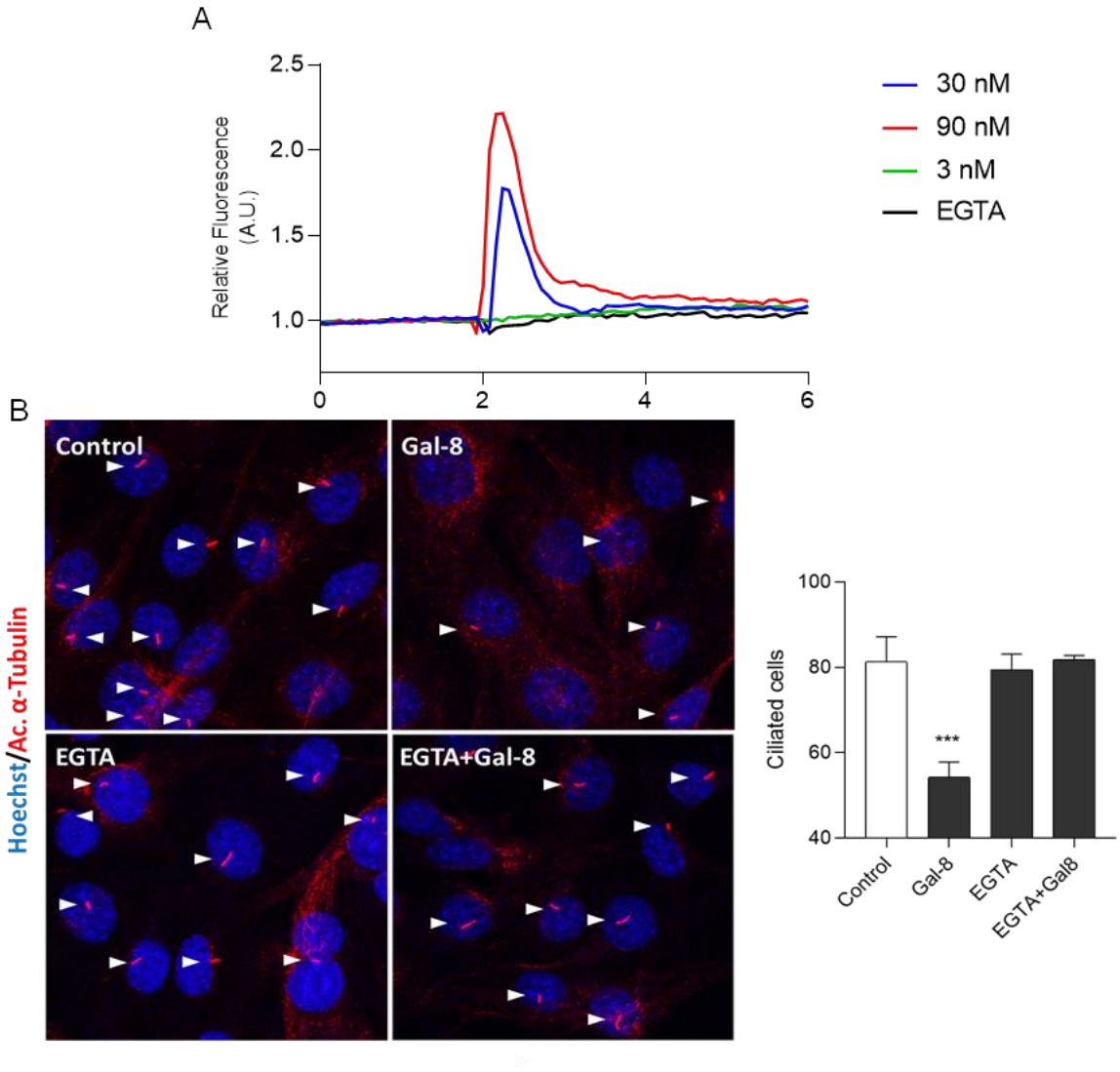


**Figure 14.- Gal-8 induces a calcium influx involved in PC loss.**

A) Cytosolic calcium levels measured by Fura-Red AM calcium imaging are increased under Gal-8 treatment and EGTA avoids this effect indicating calcium influx; B) EGTA co-treatment avoids the loss of PC induced by Gal-8. (One-way Anova P<.001).

### 4.2.4- Gal-8 induces calcium influx through Src and FAK-mediated opening of L-Type calcium channel CaV 1.3

L-type calcium channels are divided into 4 subtypes depending on its regulatory and pore α-subunit. The $Ca_v$ 1.1, 1.2, 1.3, and 1.4 channels are associated with α-subunits S, C, D, and F, respectively (Dolphin, 2016). $Ca_v$ 1.1 is found in muscle, $Ca_v$ 1.4 with vision, and Cav 1.2 and 1.3 channels regulate calcium in neurons (Catterall, 2011; Dolphin, 2016).

As integrins, Src, and FAK, which can be activated by Gal-8, have been shown to regulate L-type calcium channels (Chao et al., 2008; Gui et al., 2006; Waitkus-Edwards et al., 2002; X. Wu et al., 2001), we assessed this possibility in Clu-177 cells. Detection of the α1D regulatory subunit indicated that Clu-177 cells express the Cav 1.3 calcium channel subtype (Fig. 15A). Pretreatment with the L-type calcium channel blocker nifedipine abrogated the cytosolic calcium increase induced by Gal-8 treatment (Fig. 15B). PP2 and FAKi inhibitors of Src and FAK activity, respectively, also prevented the Gal-8-induced increase in cytosolic calcium (Fig. 15B).

These findings suggest that Gal-8 induces calcium influx through the Src and FAK-mediated opening of the L-type calcium channel CaV 1.3, leading to the subsequent loss of the primary cilium in Clu-177 cells.

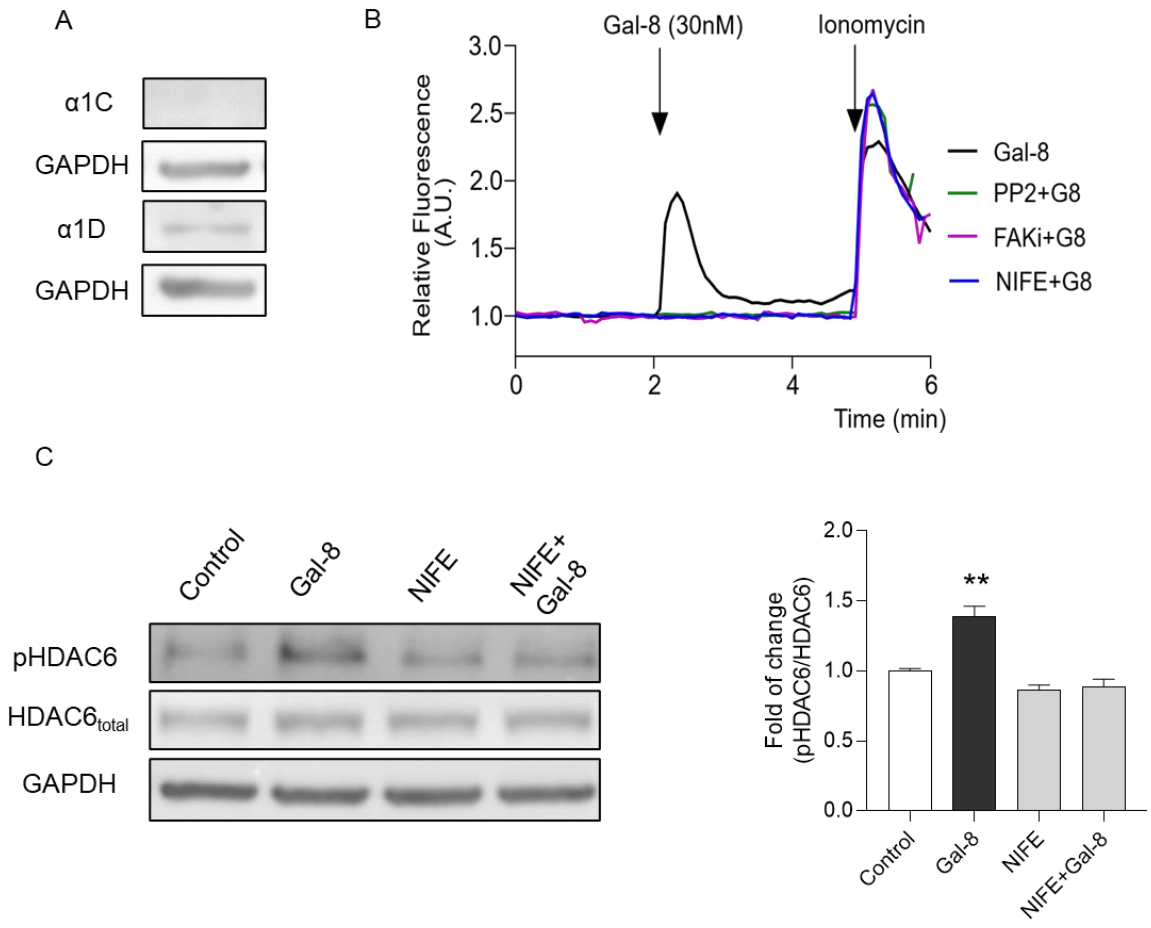


**Figure 15.- Gal-8-induced calcium influx is abrogated by an inhibitor of calcium channel $Ca_v$ 1.3.**

A) Clu-177 cells express the L-type calcium channel $Ca_v$ 1.3 at levels detectable by Western blot, as shown by the band corresponding to the regulatory subunit α1D, while the $Ca_v$ 1.2 regulatory subunit α1C was undetectable; B) L-type calcium channel blocker Nifedipine (NIFE) or Src and FAK inhibitor (PP2 and FAKi, respectively), precluded the calcium influx induced by Gal-8 treatment when added 30 min before; C) Nifedipine pretreatment avoids HDAC6 phosphorylation induced by Gal-8 (30nM). (One Way ANOVA P<.01).

### 4.2.5. Conclusions

These results reveal that Gal-8 induces gradual resorption of the primary cilium through the activation of the AurkA/HDAC6 axis. This process is mediated by the binding of Gal-8 to integrins leading to the activation of focal adhesion proteins Src and FAK, which in turn triggers calcium influx through the L-type calcium channel CaV 1.3.

## 4.3.- Functional consequences of Gal-8 induced primary cilia loss.

### 4.3.1.- Gal-8 avoids leptin signaling in hypothalamic cells.

We examined the effect of Galectin-8 (Gal-8)-induced primary cilium loss on leptin signaling in hypothalamic cells. Leptin plays a crucial role in regulating appetite and energy expenditure by modulating the activity of POMC and AgRP neurons in the hypothalamus (Baldini & Phelan, 2019; Toda et al., 2017). Binding of leptin to its receptor, LepR-B, inhibits AgRP neurons and activates POMC neurons to regulate appetite and energy balance (Baldini & Phelan, 2019; Toda et al., 2017). Alterations in the primary cilium disrupt the availability of LepR-B at the cell surface resulting in dysregulation of appetite and energy balance (Berbari et al., 2013; Oh et al., 2015).

To assess the impact of Gal-8 on leptin signaling, we examined STAT3 phosphorylation at the Y705 residue, a downstream event of leptin binding to its receptor. Treatment with leptin (100 nM) for 1 hour resulted in 73% increase in pSTAT3 levels compared to untreated control cells (Figure 16). However, cells pretreated with Gal-8 exhibited only a modest 17% increase in pSTAT3 levels (Figure 16). Therefore, Gal-8-induced loss of the primary cilium negatively affects leptin/LepR-B signaling.

These findings suggest that Gal-8-mediated disruption of the primary cilium interferes with leptin signaling pathways in hypothalamic cells. This effect can potentially contribute to dysregulation of appetite and energy balance.

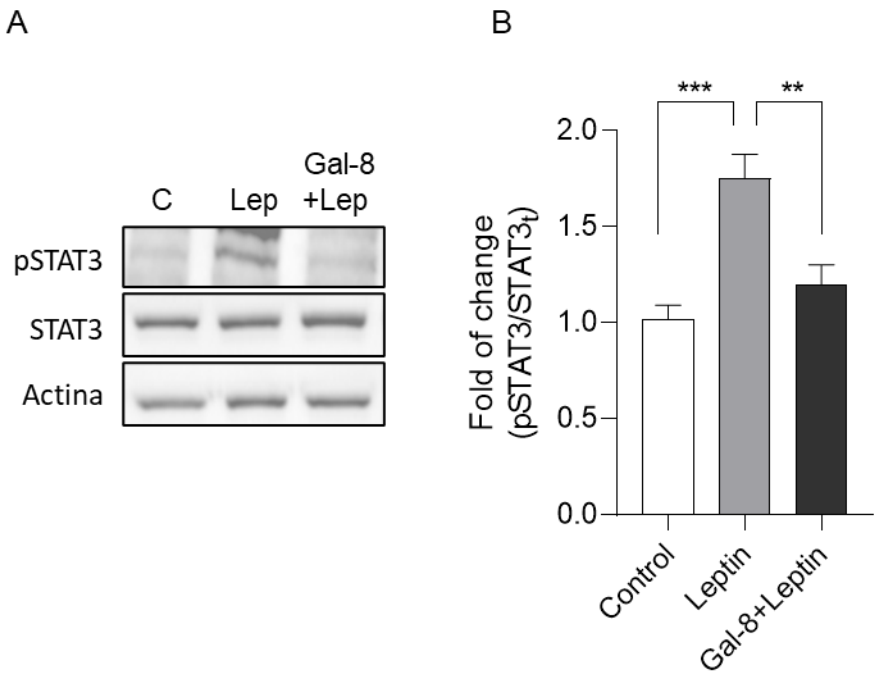


**Figure 16.- Gal-8 pre-treatment avoids leptin signaling in Clu-177 cells.**

Clu-177 cells were pre-treated with Gal-8 or vehicle for 2 h, washed and then incubated with 100 nM leptin for 1 hr. Western blot showing that Gal-8 pretreatment avoided STAT3 phosphorylation induced by leptin treatment, as quantified in the Graph. (One-way Anova, **P<.01, ***P<.001).

### 4.3.2.- Metabolic effects of Gal-8 in mice.

The leptin-melanocortin circuit in the hypothalamus plays a crucial role in regulating appetite and energy expenditure (Baldini & Phelan, 2019; Toda et al., 2017). As Gal-8-induced reduction in leptin signaling in Clu-177 cells, we investigated the metabolic and activity parameters in our previously characterized Gal-8 knockout (KO) mice (Figure 17 and 18) (Pardo et al., 2017).

Our results revealed that Gal-8 KO mice exhibited lower body weight and food intake compared to wild-type (WT) mice (Figure 17A and B). Interestingly, Gal-8 KO mice also displayed a significant increase of approximately 30% in locomotor activity compared to WT mice (Figure 17C). Previous studies had shown that POMC and AgRP arcuate neurons can regulate peripheral glucose homeostasis (Alsina et al., 2018; Burke et al., 2017; Steculorum et al., 2016; Zhou et al., 2007). To assess glucose metabolism, we performed a glucose tolerance test (GTT). Gal-8 KO mice demonstrated a 33% decrease in glycemia 30 minutes after intraperitoneal glucose injection compared to WT mice (Figure 17D), while no significant differences were observed at basal levels or at 60-, 90-, and 120-minutes post-glucose administration.

We investigated the respiratory exchange ratio (RER), which reflects the type of energy metabolism occurring in the body (Marvyn et al., 2016). An RER close to 0.7 is associated with fat oxidative metabolism, whereas RER close to 1 is indicative of glycolytic metabolism (Marvyn et al., 2016; Ramos-Jiménez et al., 2008). Leptin-treated and trained animals under a standard diet also exhibit an RER near 1 (Choi et al., 2015; DiSilvestro et al., 2016; Marvyn et al., 2016; Ramos-Jiménez et al., 2008). We found a mean RER for WT mice of 0.87, while Gal-8 KO mice had a higher RER of 0.95 (Figure 17E). This suggests a more glycolytic metabolism in Gal-8 KO mice, consistent with the GTT results.

Strikingly, intranasal administration of Gal-8 resulted in a reduction of RER to 0.88 in Gal-8 KO mice, resembling the WT phenotype. This recovery of the WT RER phenotype in Gal-8 KO mice provides additional evidence supporting a role of Gal-8 in the brain regulation of metabolism and energy expenditure control.

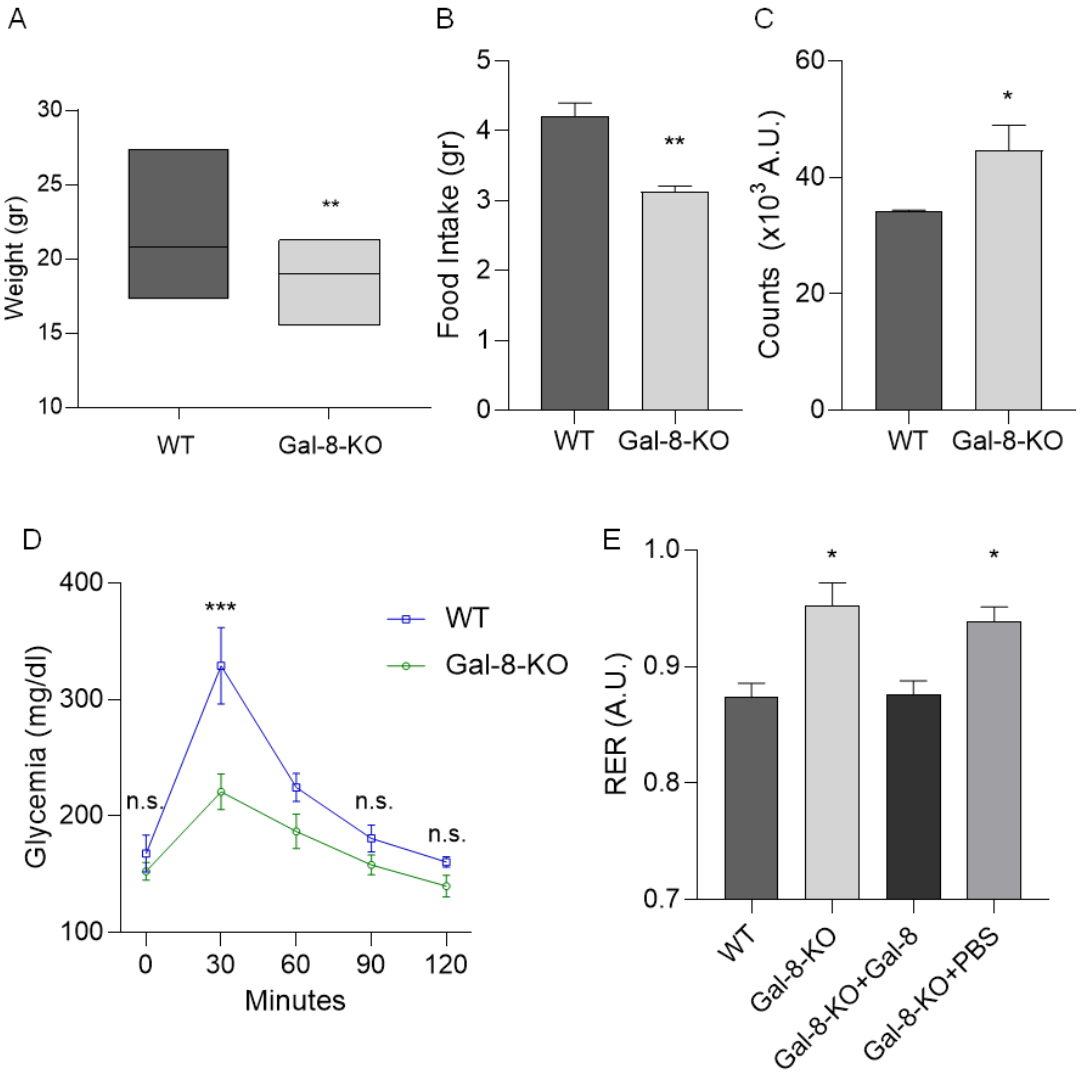


**Figure 17.- Metabolic differences between WT and Gal-8-KO mice**

Gal-8 KO mice have lower weight (A) and food intake (B) than WT mice, but increased locomotor activity (C), (t-test, P<.01**, P<.05*). D) Glucose tolerance test shows Gal-8 KO mice decreases more rapidly the glucose levels than WT mice, displaying clear difference 30 min after the glucose intraperitoneal injection (Two-way Anova, ***P<.001; n=4); E) Gal-8-KO mice showed an increased Respiratory Exchange Ratio compared with WT mice, and this difference disappeared upon intranasal Gal-8 treatment (One way Anova: * P<.05; n=4)

### 4.3.3. Conclusions

In summary, our findings in Gal-8 KO mice indicate a requirement of Gal-8 in the maintenance of body weight and food intake, locomotor activity, glucose metabolism, and RER profile. The restoration of the WT phenotype in Gal-8 KO mice after intranasal Gal-8 administration is in concordance with the involvement of Gal-8 in the brain regulation of metabolism and energy expenditure. Together with the effects of Gal-8 in ciliogenesis and leptin-mediated signaling found in Clu-177 cells, all these results support a role of Gal-8 in the leptin-melanocortin circuit that regulates energy homeostasis.

## 5.0- Discussion.

In this study, we aimed to investigate the role of Gal-8 in PC structure and function in hypothalamic neurons involved in energy balance (Berbari et al., 2013; Guo et al., 2016; Jacobs et al., 2016; C. H. Lee et al., 2020; D. J. Yang et al., 2022). Previous studies have highlighted the significance of the PC in regulating leptin receptor functions and energy balance (Guo et al., 2016, 2019; Seo et al., 2009). Our findings in CLU177 cells, serving as a model system, demonstrate that Gal-8 negatively regulates PC function by inducing structural resorption. This mechanism involves downstream activation of Src and FAK, leading to calcium influx and subsequent activation of the AurkA/HDAC6 axis. Based on the previous data on Gal-8 and potential downstream elements of src and FAK, we identified the L-type calcium channel as a crucial regulator of PC sensitivity to Gal-8. As anticipated, Gal-8-induced PC resorption was associated with reduced responsiveness to leptin.

### 5.1.- Galectin-8 causes primary cilium loss.

We first determined that 30 nM of Gal-8 treatment is the minimum concentration that elicits noticeable PC loss in CLU177 cells. Approximately 20% of the cells loss cilium after 4h of Gal-8 treatment. The remaining cilium displays ~22.5% length reduction. While Gal-8 has been identified in human cerebrospinal fluid with variations among individuals, its concentration in this compartment remains unknown (Barake et al., 2020; John & Mishra, 2016; Pardo et al., 2017, 2019). However, measurements in the plasma of healthy individuals have shown Gal-8 concentrations to reach approximately 5 nM, while elevated levels of 15 nM have been detected in proinflammatory conditions such as Crohn's disease or colorectal cancer (Barrow et al., 2011; Kessel et al., 2021). Considering the high expression of Gal-8 in the choroid plexus (Barake et al., 2020; John & Mishra, 2016; Pardo et al., 2017, 2019), where cerebrospinal fluid is produced, and the thalamus, which is near the hypothalamus, it is likely that Gal-8 concentrations in these compartments surpass those detected in the serum. Hence, the concentration of 30 nM that induces PC changes in cultured cells very likely falls within physiological or physiopathological ranges.

As we used recombinant Gal-8 produced in bacteria, it was necessary to test whether the observed effects were due to potential contamination with LPS in Gal-8 preparations. Studies in mice and HT22 cells have shown that LPS reduces cilium length via activation of Toll-Like receptor (TLR) 4 (Baek et al., 2017). Also, bacterial cell wall can activate TLR 2/6 and cause cilia loss by suppressing ciliogenesis, (Mann et al., 2023). Here we exploited the thermostability of *E. coli* LPS, which resists above 200°C (B. Gao et al., 2006; Rodriguez-Torres et al., 1993), to denature recombinant Gal-8 while leaving operative a potential LPS contaminant. Conditions that denature Gal-8 but not LPS completely abolished the effects on PC, thus discarding potential LPS effects.

PC loss has been associated with cell proliferation (Izawa et al., 2015; Mirvis et al., 2019; Senatore et al., 2022). Previous studies, including our own, have shown that Gal-8 induces proliferation in various cell types, though at higher concentrations (Jiménez, 2022; Metz et al., 2016; Oyanadel et al., 2018; Tribulatti et al., 2009). However, under the experimental conditions leading to PC loss we did not detect an increase in DNA synthesis or in cell number. Therefore, we can confidently exclude the possibility that the effects of Gal-8 on PC are mediated by an induction of cell proliferation pathways.

Entrance of cells into the cell cycle triggers PC resorption through the activation of AurkA (Ford et al., 2018; Izawa et al., 2015; Mirvis et al., 2019; Senatore et al., 2022). In response to mitotic stimuli, AurkA is activated by Ca2+/Calmodulin (CaM) at the basal body of the cilium and subsequently activates HDAC6 (Plotnikova et al., 2012; Pugacheva et al., 2007). This activation leads to the deacetylation of axoneme microtubules causing disassembly of the primary cilium (Hubbert et al., 2002; Izawa et al., 2015; Pugacheva et al., 2007). Our results show that Gal-8 treatment induces HDAC6 activation and that the effects of Gal-8 on the cilium can be prevented by inhibiting AurkA. Collectively, these findings indicate that Gal-8-mediated cilia loss occurs through the AurkA/HDAC6 pathway without inducing cell proliferation.

Galectins are carbohydrate-binding proteins that interact with N-acetylactosamines present in glycoproteins and glycolipids (Nabi et al., 2015). The N-terminal

carbohydrate recognition domain (CRD) of Gal-8 exhibits a unique preference for terminal α-2,3-sialyllactose, whereas its C-terminal CRD shares with other galectins the general preference for N-acetylactosamines (Ideo et al., 2003, 2011). Galectins are also engaged in protein-protein interactions, as demonstrated by Gal-8 interaction with the autophagy adaptor NDP52 (Kim et al., 2013; S. Li et al., 2013). To assess the role of carbohydrate interactions in Gal-8-mediated PC effects, we utilized β-lactose to block both carbohydrate-binding domains and α-2,3-sialyllactose to interfere with the N-terminal domain (Ideo et al., 2003, 2011; Oyanadel et al., 2018). Both β-lactose and α-2,3-sialyllactose effectively prevented Gal-8-induced PC loss, indicating dependency of Gal-8 effects on carbohydrate interactions.

These results reveal the potential of Gal-8 to exert a negative regulatory role on PC ciliogenesis in hypothalamic cells, through interactions with cell surface glycans.

### 5.2.- Gal-8-induced PC loss involves a b1-integrin-Src-FAK pathway leading to calcium influx

Integrins have been identified as major counterreceptors of Gal-8 (Barake et al., 2020; Nabi et al., 2015). Previous studies conducted by our laboratory and others have demonstrated that Gal-8 activates the Src/FAK pathways downstream of integrins (Barake, 2021; Cárcamo et al., 2005; Diskin et al., 2012; Levy et al., 2001; Norambuena et al., 2009; Oyanadel et al., 2018; Pardo et al., 2019). Investigations in various cell types have shown that Src activation inhibits ciliogenesis (Bershteyn et al., 2010; Drummond et al., 2018) and activates Aurora kinase A (AurkA) (Barretta et al., 2016), which plays a role in primary cilium (PC) resorption during the cell cycle (Barretta et al., 2016; Izawa et al., 2015; Plotnikova et al., 2010, 2012). We conducted pulldown assays and show that Gal-8 binds to α3β1 and α5β1 integrins in Clu-177 hipothalamic cells, consistent with previous findings in other cell types (Cárcamo et al., 2005; Oyanadel et al., 2018; Pardo et al., 2019). Furthermore, we also show that Gal-8 treatment induces Src kinase activation and inhibition of this kinase prevents Gal-8-induced loss of cilia.

Previous studies have also shown that stimulation of α4β1 and α5β1 integrins with RGD peptides leads to calcium influx through L-type calcium channels following Src and FAK activation (Gui et al., 2006, 2010; Waitkus-Edwards et al., 2002; X. Wu et al., 2001; Yan Yang et al., 2010). In platelet cells, Gal-8 has been shown to induce calcium influx through an unknown mechanism (Romaniuk et al., 2010). Considering that AurkA is activated by Ca2+/CaM (Plotnikova et al., 2010, 2012), we investigated these possibilities in relation to the effect of Gal-8 on PC. We demonstrated that Gal-8 induces calcium influx in CLU177 cells, which together the PC loss can be prevented by EGTA. Calcium influx is then required in the pathway leading to PC loss induced by Gal-8 treatment.

Other galectins have also been reported to induce intracellular calcium changes (Fan et al., 2019; X. Gao et al., 2014; Hu et al., 2018; Kashio et al., 2003; J. Wang et al., 2011) For instance, Galectin-1 interacts intracellularly with L-type calcium channels and promotes their degradation via the proteasome in arterial tissue and

cardiomyocytes (Fan et al., 2019; Hu et al., 2018; J. Wang et al., 2011). Gal-3 and Gal-9 induce intracellular calcium mobilization but not calcium influx (X. Gao et al., 2014; Kashio et al., 2003). Among various calcium channels, we focused specifically on the voltage-dependent L-type calcium channels, including Cav1.1, Cav1.2, Cav1.3, and Cav1.4 (Feng et al., 2018; Striessnig et al., 2015). Src and FAK activity have been demonstrated to open Cav1.2 and Cav1.3 L-type calcium channels (Chao et al., 2008; Waitkus-Edwards et al., 2002; X. Wu et al., 2001). We found that inhibition of Src and FAK prevented calcium influx and the loss of primary cilia induced by Gal-8. Additionally, the inhibition of L-type calcium channels using Nifedipine also prevented Gal-8-induced calcium influx. The mechanism involves HDAC6 activation downstream of calcium influx. AurkA is a known activator of HDAC6, and we previously demonstrated that HDAC6 becomes activated upon Gal-8 treatment in an AurkA-dependent manner, as it is prevented by the AurkA inhibitor VX-680.

Collectively, these findings suggest a mechanism by which Gal-8 induces changes in primary cilia. This mechanism initiates with carbohydrate-mediated interaction of Gal-8 with β1-integrin leading to Src/FAK activation and subsequent calcium influx through CAV1.3 calcium channels. Calcium then activates AurkA that in turn activates HDAC6, which is known to deacetylate α-tubulin resulting in depolymerization and PC shortening (Figure 18) (Hubbert et al., 2002; Izawa et al., 2015; Mirvis et al., 2019).

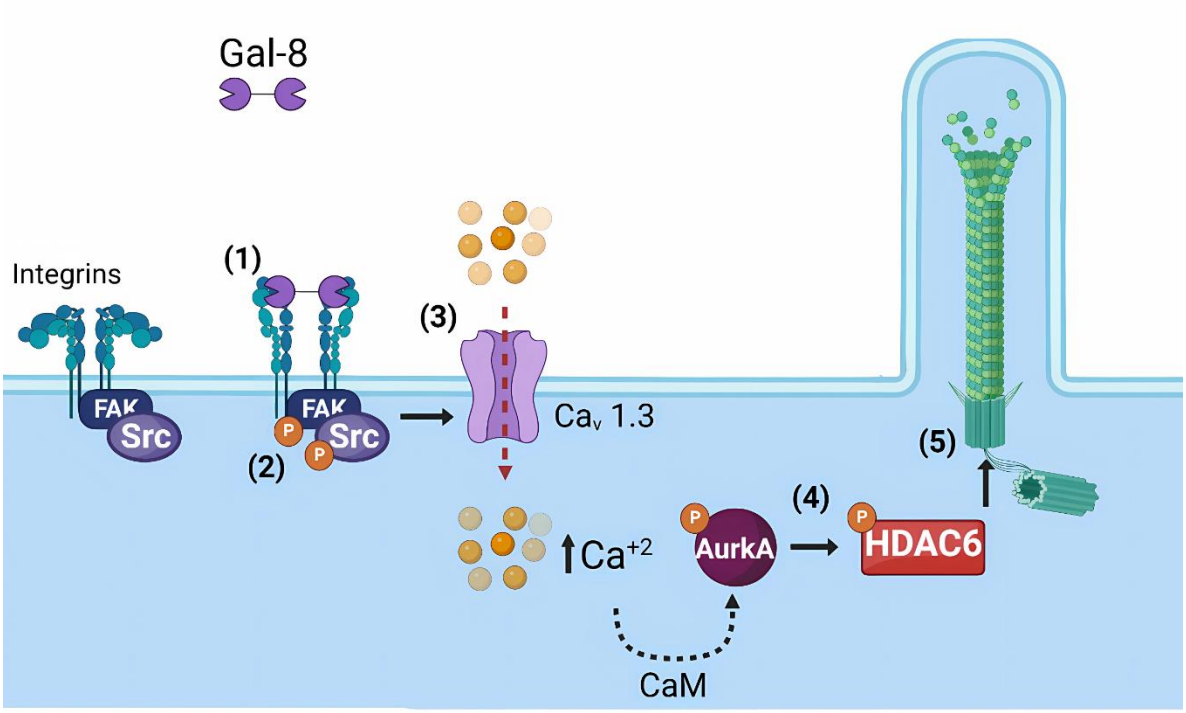


**Figure 18: Model of the mechanism of Gal-8-induced effects.** Gal-8 binds and activates $\alpha 3\beta 1$ and $\alpha 5\beta 1$ integrins (1) leading to the activation of FAK and *Src* (2), which then induce the opening of L-Type calcium channel Cav1.3 and calcium influx (3). The increased intracellular level of calcium activates the AurKA/HDAC6 axis (4) that deacetylate the a-tubulin resulting in its depolymerization and PC reabsorption (5).

### 5.3.- Gal-8 effects in metabolism.

PC plays a crucial role in LepR-B function (Berbari et al., 2013; Guo et al., 2016, 2019; Jacobs et al., 2016; C. H. Lee et al., 2020; Seo et al., 2009). While the presence of LepR-B in the cilium remains controversial (Oh et al., 2015), the evidence strongly suggests that PC is necessary for LepR-B trafficking to the plasma membrane (Guo et al., 2016; Jacobs et al., 2016; Seo et al., 2009). Loss or morphological alterations of PC associates with decreased localization of the LepR-B at the plasma membrane and a diminished signaling of this receptor (Guo et al., 2016; Jacobs et al., 2016; Seo et al., 2009).

Our results indicate that PC decrease induced by Gal-8 in Clu-177 cells results in less leptin signaling, as reflected in lower levels of phosphorylated STAT3 (pSTAT3). This *in vitro* effect is consistent with phenotype of the Gal-8-KO mice. We found that these mice have lower weight and diminished food intake, increased locomotor activity and higher respiratory exchange ratio (RER) compared with WT mice. RER of 0.7 U.A. is associated with lipid oxidation metabolism while a value near 1 U.A. indicates glycolytic metabolism (Marvyn et al., 2016). High-fat diets tend to reduce RER in mice (Marvyn et al., 2016), whereas food intake or leptin treatment increases this ratio in animals under standard diet (Choi et al., 2015; DiSilvestro et al., 2016; Marvyn et al., 2016; Ramos-Jiménez et al., 2008). We found an increased RER of 0.95 U.A in Gal-8 mice indicating a predominant glycolytic metabolism. This is presumably due to an elevated sensitivity to leptin. Further experiments would be necessary to substantiate this point.

Intranasal administration is an interesting alternative to introduce proteins into the brain (Ducharme et al., 2010; Falcone et al., 2014). Radioactive albumin and small peptides results have been detected in the hypothalamus within 5 minutes after their intranasal administration, reaching peak concentrations after 1 hour (Ducharme et al., 2010; Falcone et al., 2014). We showed that intranasal administration of Gal-8 in KO mice restores the RER phenotype of WT mice. This result provides further evidence supporting a role of Gal-8 in the brain-dependent regulation of metabolism, very likely involving the leptin-mediated function.

The regulation of energy balance in the hypothalamus is controlled by POMC, AgRP and MC4R neurons (Ahmed et al., 2021; Vercruysse et al., 2016; Yongjie Yang & Xu, 2020). LepR-B signaling inhibits the orexigenic activity of AgRP neurons, while POMC activity stimulates MC4R anorexigenic neurons (Sweeney et al., 2023). Dysfunctional cilia of hypothalamic neurons or LepR-B neurons of the hypothalamus and other brain regions result in hyperphagia and resistance to leptin and insulin stimuli, leading to obesity (Berbari et al., 2013; Guo et al., 2016; Jacobs et al., 2016; D. J. Yang et al., 2022). In contrast, ciliary dysgenesis in adult mice POMC neurons do not affect feeding behavior, food intake and weight (C. H. Lee et al., 2020). In this condition, the preservation of cilia and LepR-B activity in AgRP neurons is sufficient to maintain metabolic homeostasis above the cilia alteration in POMC neurons. Our findings in Gal-8 KO mice models and intranasal Gal-8 administration on RER, food intake, locomotor activity and weight may compromise the ciliary function and leptin signaling in all these neurons.

Additionally, we observe that Gal-8-KO mice have an improved response to GTT, reflected in a faster recovery of basal glycemia after intraperitoneal injection of glucose than WT mice. GTT response depends on both central and peripheral regulation systems. In the brain, the melanocortin system contributes to regulate the peripheral glucose homeostasis through the activity of 5-HT2CR (Burke et al., 2017; Zhou et al., 2007) and POMC gene expression (Alsina et al., 2018), both improving the insulin response and glucose uptake by peripheral tissue. In contrast, an increased activity of AgRP neurons induces insulin tolerance and decreased glucose uptake in brown adiposite tissue (Steculorum et al., 2016). On the other hand, the response of pancreatic beta cells to glucose levels secreting insulin would also contribute to the GTT response (Concepción et al., 2023)**.** Because the Gal-8-KO mice lack Gal-8 expression in all tissues it possible that our glycemia tests may reflect functional changes of the hypothalamic neurons and the activity of pancreatic beta cells.

### 5.4.- Possible role of Gal-8 in metabolic syndrome and obesity.

Our findings, demonstrating that Gal-8 induces PC resorption and reduces leptin signaling in Clu-177 cells, coupled with the observation that Gal-8 knockout animals exhibit lower weight and increased RER compared to WT mice, suggest a potential role of Gal-8 in energy balance. Dysfunction of Gal-8 in the brain could potentially contribute to the progression of metabolic syndrome and obesity, although no direct data on the involvement of Gal-8 in these pathologies exist. Nevertheless, several studies report alterations in α-2,3-sialic acid glycosylation or in proteins mediating this glycosylation process (Fougerat et al., 2018; Fradin et al., 2017; Ollikainen et al., 2015). For example, epigenetic studies in obese individuals reveal lower methylation levels in the ST3Gal4 gene that encodes a sialyltransferase responsible for α-2,3-sialylation of membrane proteins (Ollikainen et al., 2015). Children with obesity exhibit higher methylation levels in Neu1, a sialidase responsible for cleaving α-2,3-sialic acid residues from membrane proteins (Barake et al., 2020; Fradin et al., 2017). Mice receiving high-fat diet show reduced Neu1 expression, insulin resistance and hyperglycemia, effects that become reversed by pharmacological treatment with Ambroxol which increases Neu1 expression (Fougerat et al., 2018). It is possible that pathogenic conditions affecting the glycosylation patterns and α-2,3-sialic acid content of cell surface glycoproteins may alter Gal-8 binding and function (Barake et al., 2020; Nabi et al., 2015).

Our laboratory reported function-blocking autoantibodies against Gal-8 in patients with systemic lupus erythematosus or with multiple sclerosis (Cárcamo et al., 2005; Norambuena et al., 2009; Pardo et al., 2017). These autoantibodies block Gal-8 binding to cell surface glycans (Cárcamo et al., 2005; Norambuena et al., 2009; Pardo et al., 2017) and might eventually exert pathogenic effects in the brain under conditions that compromise the blood-brain barrier. This is suggested by the worse prognostic showed by multiple sclerosis patients bearing these antibodies at the moment of diagnosis (Pardo et al., 2017). It would be interesting to assess whether function-blocking anti-Gal-8 autoantibodies exert pathogenic effects at the level of the hypothalamus.

## 6.0.- Conclusions.

1. A Gal-8-sensitive pathway involving integrin/FAK/Src activation, calcium influx through L-type calcium channel $Ca_v1.3$ and downstream AurKA/HDAC6 axis is operative in ciliogenesis/PC disassembling in hypothalamic Clu-177 cells.
2. The metabolic phenotype of Gal-8 KO mice and the effects of Gal-8 nasal administration suggest a similar pathway may exist in the hypothalamus.
3. Gal-8 has the potential to exert modulating roles upon ciliogenesis and metabolic function of PC in the hypothalamus.
4. A Gal-8-dependent signaling pathway that controls PC ciliogenesis and function in hypothalamic cells may be involved in metabolic alterations offering new therapeutic opportunities.

## 7.0.- References.


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