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EL NÚCLEO ARQUEADO DEL HIPOTÁLAMO: UN SITIO DE RETROALIMENTACIÓN NEGATIVA PARA LA SECRECIÓN DE GLUCOSA Y CORTICOSTERONA

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All things appear and disappear because of the concurrence of causes and conditions. Nothing ever exists entirely alone; everything is in relation to everything else.

Gautama Buddha

Believe nothing just because a so-called wise person said it

Believe nothing just because a belief is generally held

Believe nothing just because it is said in ancient books

Believe nothing just because it is said to be of divine origin

Believe nothing just because someone else believes it

Believe only what you yourself test and judge to be true

Gautama Buddha

Contents

Resumen

El núcleo supraquiasmático (SCN) organiza las variaciones diarias de glucosa y corticosterona (Cort) en la circulación. Para regular con precisión la producción de glucosa y Cort, el SCN requiere información proveniente de otras regiones cerebrales para modular la respuesta a los cambios en estas dos variables. El SCN posee conexiones recíprocas con uno de los principales sensores metabólicos del hipotálamo, el Núcleo Arqueado (ARC). La información metabólica catabólica activa el ARC e inhibe la actividad neuronal del SCN. Sin embargo, se sabe poco acerca de la influencia de la hora del día sobre los procesos fisiológicos regulados por el ARC.

En este trabajo, se investigó si el ARC puede detectar cambios en los niveles de Cort y la disponibilidad de glucosa. Para probar si el SCN es capaz de modular la sensibilidad del ARC a las condiciones metabólicas catabólicas, se administró 2DG y se evaluó la actividad neuronal en el ARC, determinada por la inmunoreactividad (IR) a c-Fos. La inmunoreactividad a c-Fos se incrementó después de la administración de 2-desoxiglucosa (2DG), un estímulo que simula un estado hipoglucémico. Después de la administración de 2DG, se encontró una mayor actividad neuronal del ARC al final del período de luz (zeitgeber 11, ZT11) comparada con la activación al inicio del período de luz (zeitgeber 2, ZT2), lo que sugiere la participación del SCN. Después de la administración de 2DG, la mayor activación de las neuronas del ARC en ZT11 estuvo asociada con mayores niveles de glucosa en sangre en comparación con ZT2. Se observó que en animales con lesión unilateral del SCN, la activación de las neuronas del ARC fue mayor en el lado ipsilateral a la lesión, lo que sugiere un papel inhibitorio del SCN sobre las neuronas del ARC. La respuesta contraregulatoria sobre los niveles de glucosa inducida por 2DG correlacionó con una mayor actividad neuronal del ARC y fue significativamente mayor en animales con lesión unilateral del SCN.

El papel del ARC como sitio donde la 2DG puede inducir una respuesta contrarregulatoria fue confirmado por microdiálisis local de 2DG. La administración de 2DG en el ARC produjo un mayor incremento en la glucosa circulante en comparación con la administración de 2DG en áreas circundantes tales como el núcleo ventromedial del hipotálamo (VMH). A partir de estos resultados, llegamos a la conclusión de que el SCN utiliza una vía neuronal hacia el ARC para modificar el paso de información metabólica sensorial al cerebro, regulando la sensibilidad del ARC a la glucosa y las respuestas contrarregulatorias a las condiciones hipoglucemiantes.

En una etapa siguiente, se investigó si el ARC también puede detectar y producir ajustes rápidos de Cort en la circulación. Desde el punto de vista clásico, las variaciones de Cort son determinadas por el núcleo paraventricular del hipotálamo (PVN), a través de la CRH liberada a la adenohipófisis para secretar ACTH, y a través del sistema nervioso autónomo simpático (ANS), estimulando directamente la liberación de Cort desde la glándula suprarrenal. La Cort da retroalimentación a través de receptores de glucocorticoides (GR) expresados en el cerebro. En este trabajo se mostró que las neuronas del PVN que proyectan a la glándula suprarrenal no expresan GR, generando la pregunta de cómo es que las neuronas del ANS en el PVN obtienen información sobre la Cort presente en la circulación para controlar la suprarrenal. Dado que el ARC posee una barrera hematoencefálica menos restrictiva, expresa GR y proyecta al PVN, se planteó la hipótesis de que el ARC puede dar retroalimentación negativa a la liberación de Cort. En primer lugar, se demostró que el ARC tiene la capacidad de detectar los cambios de Cort más rápido que otras estructuras. En animales suprarrenalectomizados, la IR para GR se pierde. Cuando se administró Cort vía intravenosa

(IV), la IR para GR se recuperó rápidamente en el ARC, pero no en otras regiones del cerebro como la corteza o el hipocampo. Dentro del ARC, las células positivas para GR se ubican principalmente en la región cercana al tercer ventrículo, que es la localización clásica de las neuronas AgRP /NPY. Posteriormente, se identificó las neuronas sensibles a la Cort circulante por medio de hibridación *in situ* para AgRP o POMC combinada con inmunohistoquímica para detectar GR. Se observó que las neuronas positivas a GR también fueron positivas para AgRP, pero no para POMC. Además, en condiciones bajas de Cort (mañana), la microdiálisis local en el ARC con un antagonista de GR tipo I produjo un aumento rápido y sostenido de Cort. Esto no se observó con el antagonista de GR tipo II. En el pico circadiano de Cort (ZT 11), la administración del antagonista de GR tipo II, pero no del antagonista de GR tipo I, aumentó los niveles de Cort sin incrementar los niveles de ACTH. Las infusiones del antagonista a GR tipo II en el PVN no modificó los niveles circulantes de Cort, demostrando la capacidad específica del ARC para dar retroalimentación negativa a la Cort. Asimismo, la administración de los agonistas de GR tipo I y II en el ARC impidieron el aumento de Cort después de un estímulo estresante, demostrando el papel del ARC como sensor para modular la liberación de Cort.

Nuestros hallazgos demuestran que el ARC detecta los niveles sanguíneos de Cort y glucosa. Adicionalmente, es capaz de producir respuestas contrarregulatorias rápidas y ajustarlas en función del momento del día.

Abstract

The suprachiasmatic nucleus (SCN) organizes daily variations of circulating glucose and corticosterone (Cort). To accurately control the production of glucose and Cort, the SCN requires input coming from other brain regions to modulate the response to changes in those two variables. Hereto the SCN possesses reciprocal connections with one of the major metabolic sensors in the hypothalamus, the Arcuate Nucleus (ARC). Catabolic metabolic information activates the ARC and inhibits SCN neuronal activity. However, little is known about the influence of the time of the day on physiological processes regulated by the ARC.

Here, we investigated whether the ARC can sense changes in glucose availability and variations of Cort. First, to test if the SCN can modulate the sensitivity of the ARC to catabolic metabolic conditions, we administered 2DG and evaluated ARC neuronal activity, as determined by c-Fos immunoreactivity (IR). C-Fos IR was increased after a hypoglycemic stimulus by 2-deoxyglucose (2DG). The highest ARC neuronal activity after 2DG was found at the end of the light period (zeitgeber 11, ZT11) with a lower activity in the beginning of the light period (zeitgeber 2, ZT2), suggesting the involvement of the SCN. The higher activation of ARC neurons after 2DG at ZT11 was associated with higher 2DG induced blood glucose levels as compared with ZT2. Unilateral SCN-lesioned animals, gave a mainly ipsilateral activation of ARC neurons at the lesioned side, suggesting an inhibitory role of the SCN on ARC neurons. The 2DG-induced counterregulatory glucose response correlated with increased ARC neuronal activity and was significantly higher in unilateral SCN lesioned animals. The role of the ARC as the site where 2DG induce a counterregulatory response was confirmed by local microdialysis of 2DG. 2DG administration in the ARC produced a higher increase in circulating glucose compared with 2DG administration in surrounding areas such as the ventromedial nucleus of the hypothalamus (VMH). From this part, we concluded that the SCN uses neuronal pathways to the ARC to gate sensory metabolic information to the brain, regulating ARC glucose sensitivity and counterregulatory responses to hypoglycemic conditions.

In a next step, we investigated whether the ARC also can detect and produce fast adjustments of circulating Cort. From a classical point of view, Cort variations are driven by the Paraventricular nucleus of the hypothalamus (PVN), via CRH targeting the adenohypophysis to release ACTH, and via the sympathetic autonomic nervous system (ANS), directly stimulating Cort release from the adrenal gland. Cort feeds back through glucocorticoid receptors (GR) expressed in the brain. Here we show that PVN neurons projecting to the adrenal gland do not express GR, leaving the question how the ANS in the PVN gets information about circulating Cort to control the adrenal. Since the ARC shows less restrictive blood brain barrier, expresses GR and projects to the PVN, we hypothesized the ARC can give negative feedback to Cort release from the adrenal. First, we demonstrated that the ARC has the capacity to sense changes of Cort faster than other structures. In adrenalectomized animals, GR staining is lost in the absence of Cort. When we administered Cort IV, GR was rapidly recovered in the ARC but not in other brain regions as the cortex or the hippocampus. Within the ARC, GR-positive cells were distributed mainly in the region close to the third ventricle, the classical AgRP/NPY neuronal location. Subsequently, we characterized the neurons responsive to circulating Cort by means of in situ hybridization for AgRP or POMC combined with GR immunohistochemistry.

We observed that GR-positive neurons were also positive for AgRP but not POMC. Additionally, in low Cort conditions (morning), local microdialysis in the ARC with type I GR antagonist produced a fast and sustained increase of Cort. This was not observed with type II antagonist. At the circadian peak of Cort (afternoon), type II GR antagonist but not type I antagonist increased Cort levels but not ACTH levels. Antagonist infusions in the PVN did not modify circulating Cort levels, demonstrating the specificity of the ARC to give Cort negative feedback. Furthermore, type I and II GR agonists in the ARC prevented the increase of Cort after stress, demonstrating the role of the ARC as a sensor to modulate Cort release.

Our findings show that the ARC may be essential to sense blood levels of Cort and glucose, to adapt counterregulatory responses depending on the time of the day.

Foreword

The hypothalamus is involved in most processes related to secure the homeostasis and survival of organisms and species, which requires precise integration of stimuli originated inside and outside the body to accurately regulate behavioral, metabolic, autonomic and endocrine outputs. There is a wide range of hormones controlled by the hypothalamus, including vasopressin and oxytocin synthesized in the hypothalamus and released into the general circulation, and releasing hormones secreted into the portal circulation, directed to the pituitary gland to control release of prolactin and growth hormone and stimulating hormones which in turn control the release of corticosterone, testosterone, estrogen, progesterone and thyroid hormones from peripheral glands. This endocrine control is coupled to metabolites such as glucose, triglycerides, proteins and electrolytes as sodium and calcium. Given the ample range of hormones and metabolites present in the blood, the present work focused on the capacity of the hypothalamus to sense and give counter-regulatory responses to two substances related to metabolic and endocrine function, i.e. glucose and corticosterone. The hypothalamus regulates the day-night variation of their level in the circulation and once it detects - via the Arcuate nucleus (ARC)- levels that deviate from these set points, it adjusts endocrine and autonomic output to preserve homeostasis

The present work is divided into three general sections, the first is the introduction, that gives an overview of the circadian system, the structures involved in glucose homeostasis and the regulation of corticosterone release. It will focus on the ARC as a metabolic and endocrine sensor in the hypothalamus, and possible ways of interaction with the autonomic nervous system to control corticosterone and glucose production.

The second part consists of the results of the experimental approaches to investigate if the arcuate nucleus can sense glucose deficient states and is based on the article entitled: “The Suprachiasmatic Nucleus Modulates the Sensitivity of Arcuate Nucleus to Hypoglycemia in the Male Rat” (Endocrinology, September 2016, 157(9):3439–3451) and the evaluation of the capacity of the ARC to sense Cort and produce a fast feedback to maintain the Cort production based on the article entitled: “ The Arcuate Nucleus: A Site of Fast Negative Feedback for Corticosterone Secretion in Male Rats” (eNeuro 2017; 10.1523/ENEURO.0350-16.2017)

The final part of the manuscript consists of the discussion of the different results obtained and provides a tentative model to integrate the changes observed after the different treatments.

Chapter I: General Introduction and aims

The Suprachiasmatic nucleus and endocrine rhythms

To maintain the organization of daily physiology, the variation of diverse functions as behavior, food intake, metabolism and hormone release is highly regulated in a 24-hour rhythm. To control the oscillation of these variables, a series of feedback systems are distributed over different organs, neural networks, and molecular mechanisms known together as the circadian system. Herein the SCN takes central place generating a day-night rhythm in neuronal activity that is transmitted to hypothalamic target structures. This allows the brain to generate autonomous oscillations that are later transmitted to independent peripheral oscillators (e.g. the liver). At a molecular level, the circadian clock consists of a network of transcriptional–translational feedback loops that drive rhythmic, ~24-hrs expression patterns of core clock components. In the primary feedback loop, CLOCK and BMAL1 heterodimerize and initiate transcription of target genes containing E-box cis-regulatory enhancer sequences, including Period (in mice, Per1, Per2, and Per3) and Cryptochrome (Cry1 and Cry2). Negative feedback is achieved by PER: CRY heterodimers that translocate back to the nucleus to repress their own transcription by acting on the CLOCK: BMAL1 complex[1]. At the level of neuronal networks, the suprachiasmatic nucleus (SCN) receives photic information from the retinohypothalamic tract, non-photic stimuli from brainstem and thalamus to entrain its endogenous rhythm and then distributes the signal to further hypothalamic nuclei (**Fig. 1**) (e.g. the paraventricular nucleus (PVN) and dorsomedial hypothalamus (DMH)).

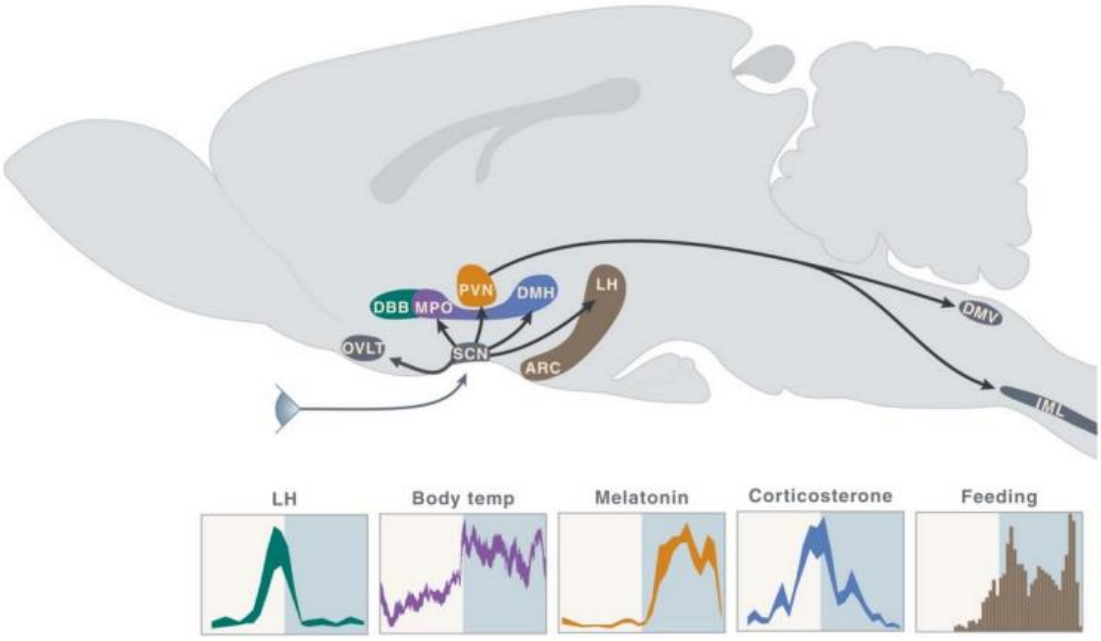

Figure 1. The SCN projects to other hypothalamic nuclei to modulate daily hormonal and other physiological set points[2].

Photic stimuli produce glutamate release from the retinohypothalamic tract to the ventral part of the SCN, glutamate, in turn, promotes the increase of nitric oxide (NO) to reset the autonomous rhythms of the neurons in the SCN [3]. Glutamate is also released in this nucleus from nerve terminals originated in the NTS in response to changes in blood pressure [4],

granting and additional feedback of the internal state of the body. In contrast to glutamatergic action the geniculohypothalamic tract release NPY in the SCN, and can prevent phase shifts after glutamate release[5] in response to metabolic[6] and photic stimulation. Similarly, serotoninergic terminals in the SCN coming from the raphe nucleus counteract the excitatory effects of light[7]. All the above-mentioned afferents mainly converge in the ventral portion of the SCN, where gastrin-releasing peptide (GRP), substance P (SP), calbindin (CalB), calretinin, neurotensin (NT), vasoactive intestinal peptide (VIP) and GABA (among others) are abundantly expressed. Clock genes expressed in these neurons are not intrinsically rhythmic in the absence of light, however, alteration of VIP[8], GRP or CalB release from the ventrolateral to the dorsomedial portion of the SCN, generates a disruption of many biological rhythms[9]. Interestingly, exposure to long day photoperiods changes GABAergic activity from inhibitory to excitatory, without changing the GABAergic synaptic tone. This process is caused by seasonal changes and it destabilizes the SCN rhythmicity [10].

Neurons expressing GABA, Angiotensin II, met-enkephalin and Vasopressin (VP) (among others) are present in the dorsomedial portion of the SCN and form a network with the ventrolateral portion. This network is responsible for robust self-sustained rhythms in clock gene expression that is necessary for the neuronal activity of SCN neurons [11]. Accordingly, neuromedin S (NMS) has been shown to be important for keeping the SCN pacemaker-neurons synchronized[12]

The rhythmic signal from the SCN is transmitted to hypothalamic target structures results in daily rhythms in behavior associated with the rhythmic release of many different hormones: leptin, ghrelin, melatonin, thyroid hormones, gonadal hormones, and corticosteroid hormones; in addition to other metabolic processes as the hepatic glucose production and insulin sensitivity.

It is important to note that the inappropriate timing of these signals is associated with a deficient metabolic feedback, and has a deleterious effect on the circadian system and on health[13,14]. For example, high fat diet or food intake during the rest phase has been shown to desynchronize and dampen clock gene rhythmicity[13], leading to obesity, insulin resistance[15], and cardiovascular disease[16], thus providing a link as to why these diseases, including cancer, have a high incidence in shift workers[17,18]. Even though the SCN gives rhythmicity to the endocrine and metabolic output, the neurons in the SCN depend on other structures to sense and adjust the levels of most hormones and metabolites in the circulation. Although the ARC is known to sense metabolic information, it has not yet been studied as a component of the circadian system in control of daily hormone secretion. Since the ARC has receptors to most hormones and metabolites[19], we hypothesize that the ARC can play a role as the sensor on the circadian control of glucose and glucocorticoid production. A more detailed explanation of the control of glucose and corticosterone (Cort) is given in the following sections.

The control of glucose production

From a classical point of view, circulating blood glucose results from intestinal absorption from food, glucose production (gluconeogenesis), glycogen breakdown (glycogenolysis) and glucose uptake by the different tissues. In general, pancreatic β-cells release insulin to inhibit gluconeogenesis and glycogenolysis in the liver after feeding, thus decreasing glucose output to the circulation. Insulin also acts at peripheral tissues to increase glucose uptake, resulting in decreased blood glucose levels. During fasting, pancreatic α-cells release glucagon to increase gluconeogenesis and glycogenolysis in the liver; at the same time, epinephrine is released from the adrenal gland, both increasing circulating blood glucose levels[20] (**Fig. 2**). Glucose must be transported from the circulation to target cells by two different type of transporters: the sodium-dependent glucose cotransporters (SGLTs) and the family of facilitative sodium-independent glucose transporters (GLUTs)[21] expressed differentially across tissues and possessing different substrate specificities[22] (**Supplementary table 1**)**,** from the 12 family members, only GLUT 4 and 12 are insulin sensitive. At the level of the liver itself, glucose production can be regulated directly by the local concentration of glucose[23].

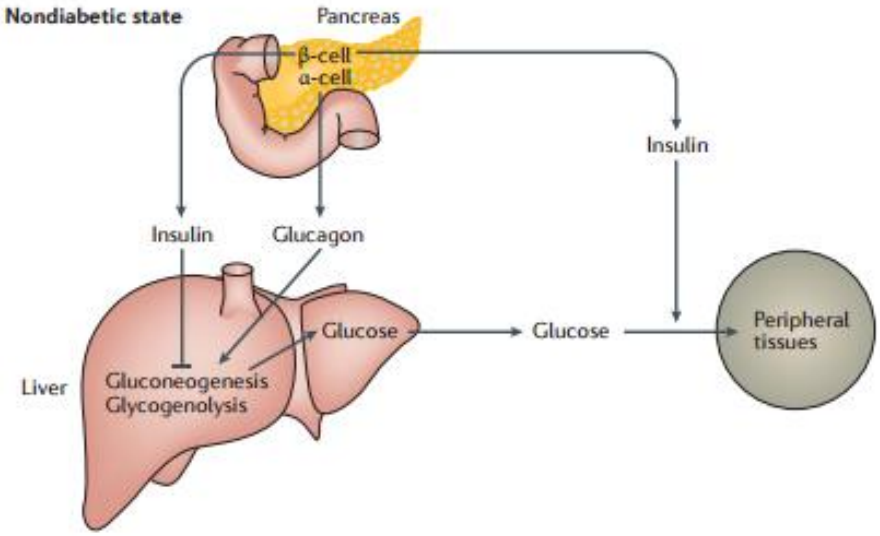

Figure 2. Schematic of the classic model of glucose homeostasis and its main components [20].

In addition to the endocrine inhibition of glucose production via insulin, the nervous system plays a crucial role in controlling glucose homeostasis. To maintain adequate glucose levels, the CNS detects variations via different sensory systems: 1) the hepatic portal-mesenteric veins (PMV)[24]signalling to the vagal nerve[25], and 2) the spinal nerve pathway[26] activating the brainstem[27] which can convey metabolic information to the hypothalamus[28] and the 3) glucose sensitive neurons in the hypothalamus.

The hypothalamus exerts an important control on glucose homeostasis in response to diverse signals as the time of the day[29], in addition to metabolic and hormonal clues, e.g. hypothalamic glucagon signaling inhibits hepatic glucose production (HGP)[30] and GCs induce severe hepatic insulin resistance[31]. This is because the liver receives sympathetic and parasympathetic innervation to adjust glucose metabolism in response to different stimuli detected in the hypothalamus. Specifically, it has been shown that the VMH can stimulate the sympathetic nerves towards the liver[32–34], the LH the parasympathetic pathway[35,36] and the PVN can

stimulate both sympathetic and parasympathetic outputs[37,38]. Sympathetic output nerves increase glucose production[39,40] in response to local signals as well as inputs from the ARC[41]. These processes vary along the day to allow a circadian rhythm of glucose driven by the master clock located in the hypothalamus, the SCN (see below).

Glucose feedback to the hypothalamus

Plasma glucose concentrations are narrowly maintained between 3.0 and 5.6 mM, however, since it is considered the main fuel for neurons[42], glucose levels must be maintained within narrow values inside the CNS to secure the sufficient supply of fuel to avoid permanent damage and compromise vital functions. Within the cerebrospinal fluid, the concentration is much lower and the range of variation is narrower, between 0.5 and 2.5 mM[43]. This difference in the levels of glucose between the general circulation and the CNS is maintained by the Blood Brain Barrier (BBB), preventing the free access of glucose into the brain. Alterations of the glucose transport mechanisms to the brain are associated with obesity[44], diabetes[45] and metabolic deseases[46].

As the presence of the BBB limits the access of cells, hormones and metabolites from the circulation into the central nervous system[47], it creates a compartmentalization that serves three basic functions: 1) maintain the composition of the extracellular environment within the central nervous system, 2) limit/prevent the entrance of cells, metabolites and hormones to the brain and 3) respond with local inflammation after changes in the microenvironment[48]. These functions are maintained by the endothelial cells lining cerebral microvessels and the presence of tight junctions (TJs)[49] and adherent junctions (AJs) [50]. These unions limit the access paracellularly as it occurs in most endothelia and force molecules to take alternative routes as the transcellular lipophilic pathway, transport via specialized proteins, receptor-mediated transcytosis and adsorptive transcytosis. TJ are a complex of proteins spanning the intercellular cleft (occludin and claudins) and junctional adhesion molecules (JAMs). Claudins and occludin in turn bind to cytoplasmic scaffolding/regulatory proteins Zonula Occludens 1-3 (ZO1-3) -also known as Tight junction proteins 1-3-. TJs restrict the paracellular diffusion between endothelial cells to polar molecules, ions and macromolecules (**Fig. 3**), generating an *in vivo* electrical resistance of 1800 Ω cm2, demonstrating the effectiveness of the BBB to block the dissemination of small molecules and ion flux.

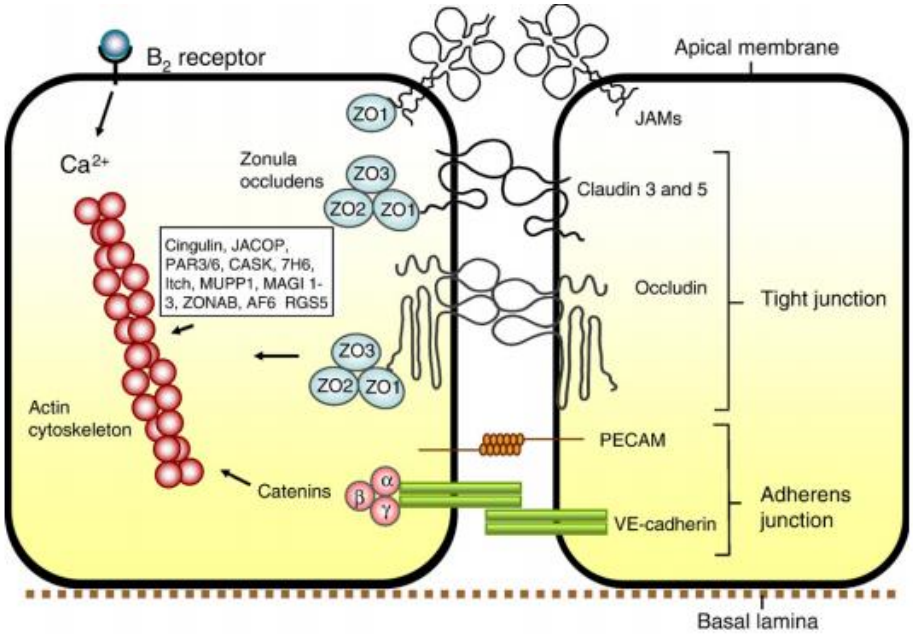

Figure 3. The structure of BBB tight junctions. The tight junctional complex comprises occludin, claudins 3 and 5, and possibly other claudins. Cadherins of the adherens junctions provide structural integrity and attachment between the cells and are necessary for the formation of tight junctions. The barrier to diffusion and the high electrical resistance of the BBB appear to be largely due to the properties of claudins 3 and 5. The claudins and occludin are linked to the scaffolding proteins ZO-1, ZO-2, and ZO-3, linked in turn via cingulin dimers to the actin/myosin cytoskeletal system within the cell.

In addition, other cellular types are associated with the endothelial cells to form the neurovascular unit. These cells are the pericytes, that contact the capillary wall and communicate with endothelial cells through synapse-like contacts, the astrocytes, that encase the large majority of the capillary wall and communicate with neurons at the tripartite synapse and microglia[51]. This configuration of cells, the presence of TJs and AJs and the very little pinocytosis observed in the epithelial cells, restricts enormously the pass of hydrophilic molecules to the CNS. Therefore, the epithelial cells need special mechanisms to translocate hydrophilic compounds as glucose, amino acids and other compounds across the BBB (**Supplementary table 2**).

Glucose transport into the brain relays principally on sodium-independent bidirectional facilitative transporters from the solute carrier family known as glucose transporters (GLUT) 1 to 8 expressed differentially at the many cellular types present in the neurovascular unit (**Supplementary table 3**)[21,52]. It has been proposed, therefore, that part of the role of GLUTs expressed there may not only serve as a mechanism to transport glucose into the brain but also as sensors to monitor the availability of glucose in the brain and the periphery[53]. The limited access due to the presence of the BBB and the saturation of GLUTs suggests the necessity of additional mechanisms to sense glucose from the periphery.

A possible way to blood born molecules to rapidly signal into the brain is to access via small regions demarcated in the periphery of the third and fourth ventricles, called Circumventricular Organs (CVOs)[54]. CVOs are essential to the adequate communication of peripheral information to the hypothalamus, since they possess a more permissive structure in their BBB[55], demonstrated by the presence fenestrations in the capillaries[54] and the transference of barrier properties as AJs and TJs to ependymal cells[56] as observed in the area postrema (AP), the subfornical organ (SFO), the vascular organ of lamina terminalis (OVLT) and the ARC-median eminence complex (ARC-ME)[55,57].

In early studies, IV administration of gold thioglucose -a toxic glucose analog- destroyed the ARC, but surprisingly, left most of the brain structures unaffected. These results are evidence of the privileged access to the blood components, glucose in this case, into the ARC, and the limited access of glucose directly into other brain regions. After ablation of the ARC with gold thioglucose, animals increased food intake and body weight[58], linking the glucose action in the ARC to the regulation of metabolism. Posterior research associated the control of metabolism and glucose sensing to two neuronal populations in the ARC, one with orexigenic effects expressing NPY[59] that highly colocalizes with AgRP[60] and GABA[61], and other with anorexigenic effects expressing POMC[60] gene and CART[62] (**Fig. 4**). Since glucose and feeding follow a circadian rhythm, we could hypothesize the ARC is an ideal structure to sense and control circulating Cort and glucose variations along the day.

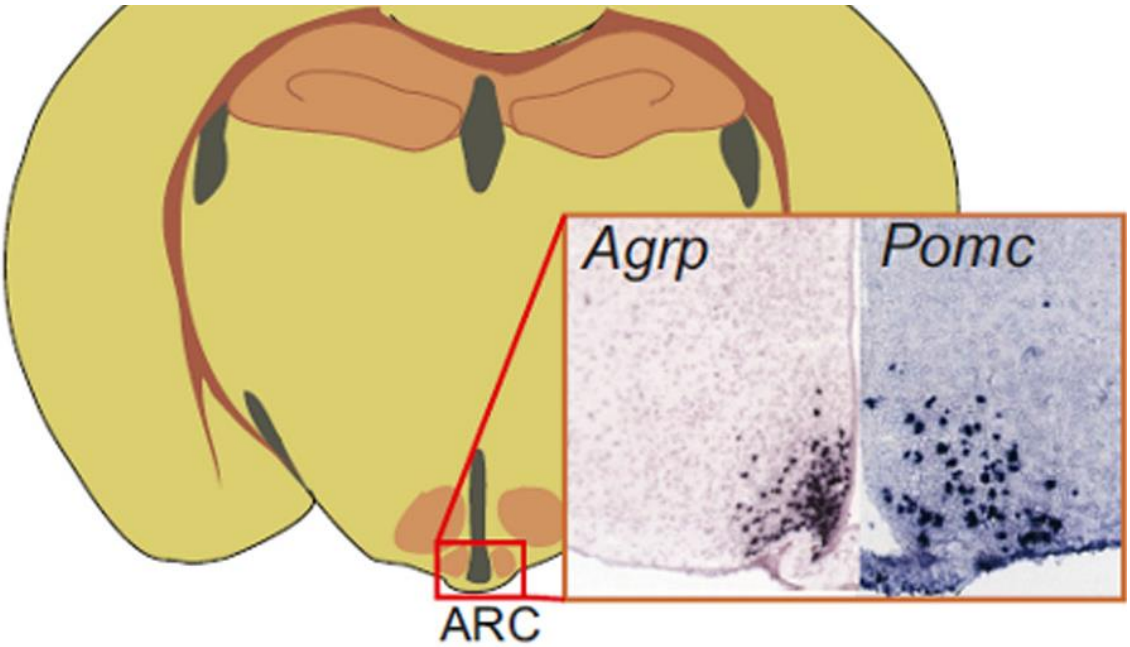

Figure 4: Localization of the ARC in the hypothalamus, and identification of the two main neuronal populations linked to food intake and energy metabolism. *In situ* Hybridization shows AgRP neurons concentrated in the ventromedial ARC, while POMC neurons are scattered mostly in the lateral part if the ARC.

Of interest, the BBB properties at the level of the ARC can change according to the availability of glucose in the periphery as result of fasting and refeeding[63]. To mimic the effects of fasting, researchers have drawn upon the use of glucose analogs for many years to induce a fake state of cellular hypoglycemia[64–68]. The most extensively used is 2-deoxy-D-glucose (2DG), a structural analog of glucose differing at the second carbon atom by the substitution of hydrogen for a hydroxyl group. 2DG enters the cell through the glucose transporter and is phosphorylated by hexokinase. Due to low levels of intracellular phosphatase, 2-DG-PO4 is trapped in the cell. 2-DG-PO4 is unable to undergo further metabolism. High intracellular levels of 2-DG-6-PO4 cause allosteric and competitive inhibition of hexokinase. This results in inhibition of glucose metabolism (**Fig. 5**).

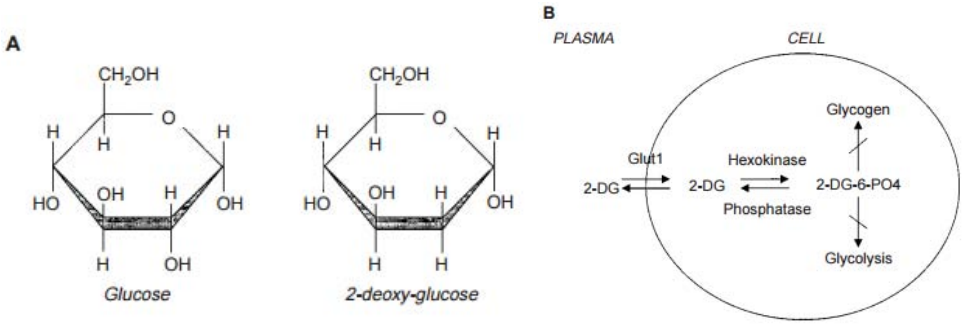

Figure 5. (A) Structural comparison of glucose and 2-deoxy-D-glucose. 2DG and glucose differ at the second carbon. (B) Simplified diagram of 2- DG action in the cell[69].

2DG administration causes BBB changes resembling those observed due to fasting at the level of the BBB, suggesting there is a glucose sensing mechanism able to modify permeability to the hypothalamus in response to metabolic changes. One possible mechanism is the glucose detection at the level of ependymal-glial cells called tanycytes, since they express GLUT2[70,71]

and exert other physiological changes after hypoglycemia. It has been observed that destruction of tanycytes impairs the hyperglycemic response and the feeding behavior induced by administration of 2-DG[72]. These phenomena, in addition to the possible BBB changes, can also be due to chemical transmitters released by the tanycytes in response to glucose levels, e.g. the case of endozepines, peptides known to bind to benzodiazepine receptors, that are stimulated by glucose, and antagonists to this peptides produce increased blood levels of glucose, suggesting and endozepinergic tone to reduce glycaemia via the melanocortinergic system residing in the POMC and AgRP neurons in the ARC[73].

Together with the opening of the BBB due to fasting, there is also an increase in glucose concentration in the ARC in comparison with other hypothalamic nuclei as the VMH[63] (**Fig. 6**). This increase may be a fist mechanism to allow a correct signaling of hypoglycemia. On low glucose availability and fasting, NPY/AgRP neurons display higher firing rate[74], which apparently is supported by an increase in their number of mitochondria, and the glucose elevation may serve to sustain their increased metabolic demand[75,76]. A possible consequence of this glucose increases is the high abundance of an unusual subtype of astrocyte known as Gomori-positive astrocytes in the ARC, characterized by a large number of mitochondria engulfed by lysosomes[77] and the expression of GLUT2[71]. The phenotype of Gomori-positive astrocytes could be possibly due to oxidative stress[78] related to increased rate of glucose uptake and oxidation[71] induced by the higher glucose in the ARC.

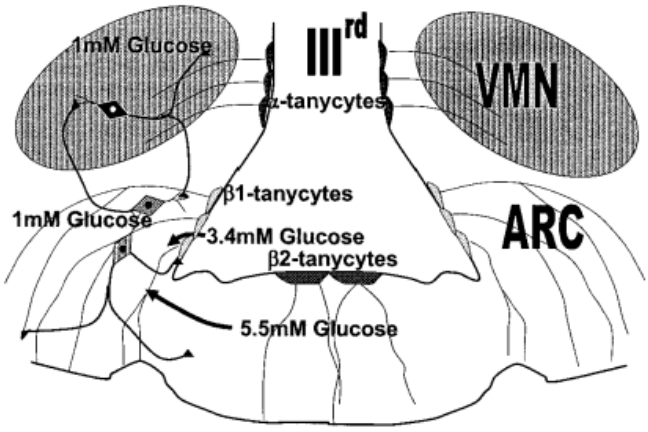

Figure 6. ARC neurons are exposed to different levels of glucose. From the CSF, glucose diffuses across the tanycytes lining the third ventricle; from the blood, glucose diffuses across the fenestrated capillaries in the median eminence. Additionally, glucose is transported across the blood-brain barrier via GLUTs. Some of these neurons synapse with VMN neurons, which are exposed primarily to glucose transported from blood and diffusing from CSF[79].

Astrocytes also seem to participate in glucose regulation, since inactivation of glut2 in these cells causes fed hyperglucagonemia and suppresses the glucagon secretory response to hypoglycemia induced by 2-DG injections. When glut2 in is restored, glucagon secretion also normalizes[80]. Furthermore, sustained hyperglycemia reduces glut1 in hypothalamic astrocytes, associated with increased hypothalamic glucose concentrations and lower glucose production, while overexpression of glut1 normalized plasma glucose levels and restored hypothalamic glucose signaling[81].

Once glucose has entered the hypothalamus, it can signal directly to glucose sensitive neurons identified within several nuclei as the ARC[44], VMH[82], LH[83], PVN, and DMH[84]; however, it has

been demonstrated that GLUT2 dependent glucose sensitive neurons in most hypothalamic nuclei send nerve terminals to contact NPY and POMC neurons[85], suggesting that although other hypothalamic nuclei may sense glucose changes, the ARC has to integrate the sensing of other areas, and then produce a counterregulatory response.

After glucose changes are detected by the ARC, it can initiate a counter-regulatory response and integrate other physiological relevant conditions (e.g. the time of the day or the inflammatory state), because it has connections with hypothalamic nuclei related to autonomic and endocrine control[86] (**Fig.7**). The projections towards the LH, DMH, and PVN may form part of a circuit necessary to mount an efficient response to changes on glycemia. For example, AgRP neurons contribute to increased HGP via GABAergic input to pre-autonomic neurons in the PVN[40], from the PVN, there is evidence that catecholaminergic neurons modulate glucose metabolism and energy expenditure[87]. Furthermore, AgRP neurons can control parasympathetic input to the pancreas, simultaneously modify insulin release and as consequence glucose metabolism[41].

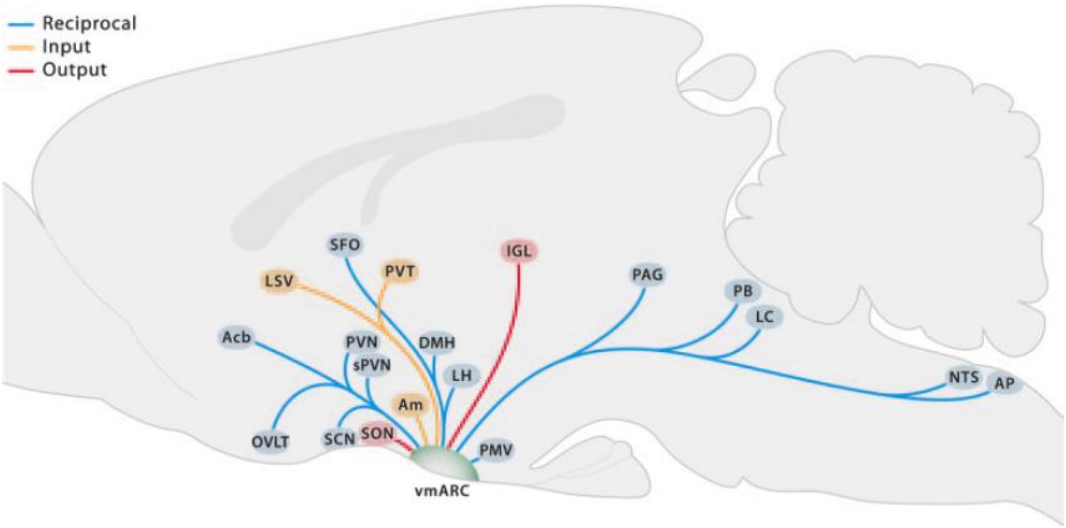

Figure 7: Sagittal scheme illustrates the main connections of ARC within the CNS. Reciprocal connections are shown in blue; yellow areas represent input to ARC; red areas represent output from ARC [86].

Glucose levels vary in a circadian manner

Glucose utilization and production vary along the circadian cycle, increasing glucose values before the beginning of the activity phase[37,88,89] (**Fig. 8**). This daily pattern may originate by the feeding schedule together with the influence of the SCN. It is remarkable that fasting or a six-meals-a-day feeding schedule is not able to prevent the circadian variation of glucose, however, bilateral lesion of the SCN prevents completely circadian oscillations[37]. This evidence demonstrates that the functionality of the SCN is necessary to maintain the rhythm. In these conditions, hepatic insulin sensitivity is greatly impaired and may be one factor to account for the loss of rhythmicity[90]. In the same sense, exposition to permanent light, which causes a disruption of the function of the SCN and as consequence arrhythmic physiology, leads to dysbalanced metabolism characterized by impaired glucose tolerance[91]

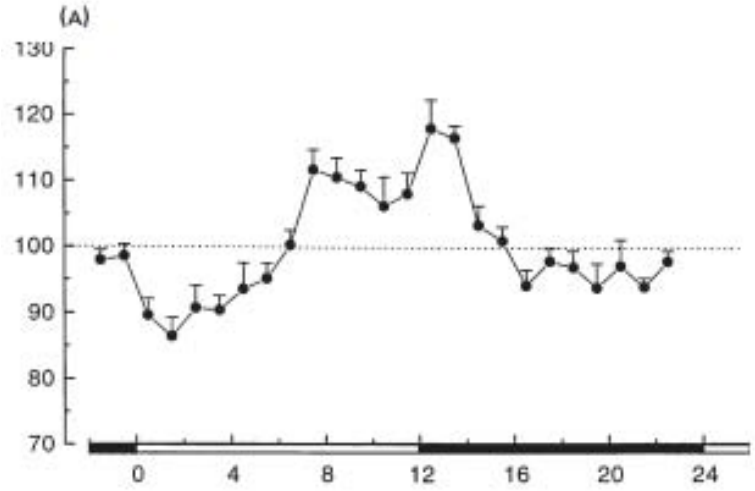

Figure 8. Circadian pattern of circulating glucose expressed as percentage of the basal level observed ad ZT 0 [37]

Later experiments demonstrated that the SCN controls glucose production via polysynaptic connection to the sympathetic output to the liver[92] since ablation of sympathetic input to the liver obliterates the circadian rhythm of glucose[93]. The suggested mechanism controlling daily variations of glucose consist of glutamatergic connections to the pre-autonomic portion of the PVN projecting to the sympathetic branch of the autonomic nervous system towards the liver, maintaining a sustained glutamatergic tone, and GABAergic inputs varying activity differentially along the day (**Fig. 9**) [94].

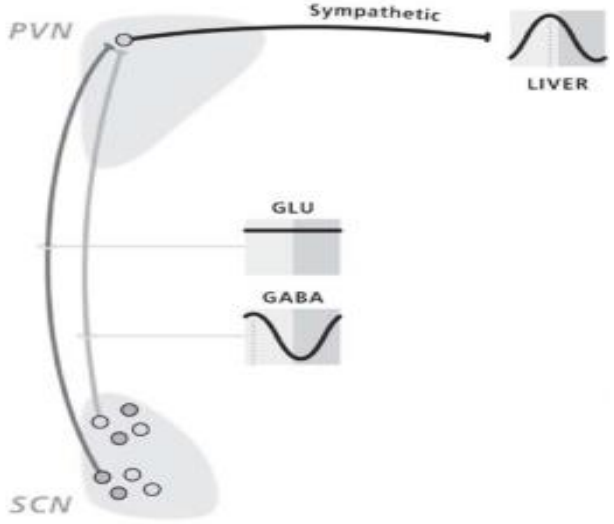

Figure 9. Schematic representation of the daily activity pattern of hypothalamic populations of GABAergic and glutamatergic neurons implicated in the autonomic control of the daily rhythms in hepatic glucose production.

Additionally, it has been demonstrated that the SCN projects to other hypothalamic nuclei containing glucose sensitive neurons as the VMH, DMH, and ARC[86]. Specifically, it has been demonstrated that activity of the ARC neurons follows a circadian rhythm and this activation can be modified by light exposure[95], demonstrating a functional connection between the SCN and the ARC. In the same sense, the response of ARC neurons to metabolic stimuli varies

depending on the time of the day[86]. This evidence suggests that the communication between the SCN and the ARC may be crucial to establishing a differential hepatic glucose production and a counterregulatory response to hypoglycemia depending on the time of the day. (**See hypothesis 1**)

The HPA axis and the control of glucocorticoid production

Glucocorticoids (GCs) -cortisol and Cort- are steroid hormones synthesized from cholesterol, and have profound effects on metabolism[96], including feeding behavior[97,98], glucose production[31,99,100], fatty acid storage[101–103], mitochondrial activity [104], insulin sensitivity[31,105,106] and the transcription of several genes after translocation to the nucleus [107]. Early studies demonstrated the relationship between glucocorticoids and hepatic glucose after treatment to patients with Addison disease[108], and more recent studies have demonstrated the decrease of glucose production after antagonizing these hormones[109].

GCs release is classically considered to be controlled by the hypothalamus-pituitary-adrenal (HPA) axis. This axis was first described to be composed of a population of parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) that send axons to the portal vein, the anterior pituitary gland and the cortex of the adrenal gland, where Cort is synthesized and then released into the general bloodstream (**Fig. 10**). The activity of the HPA axis is one of the clearest examples of circadian rhythmicity in the body since the release of Cort takes place daily at the end of the resting phase to prepare the organisms to their active phase[110].

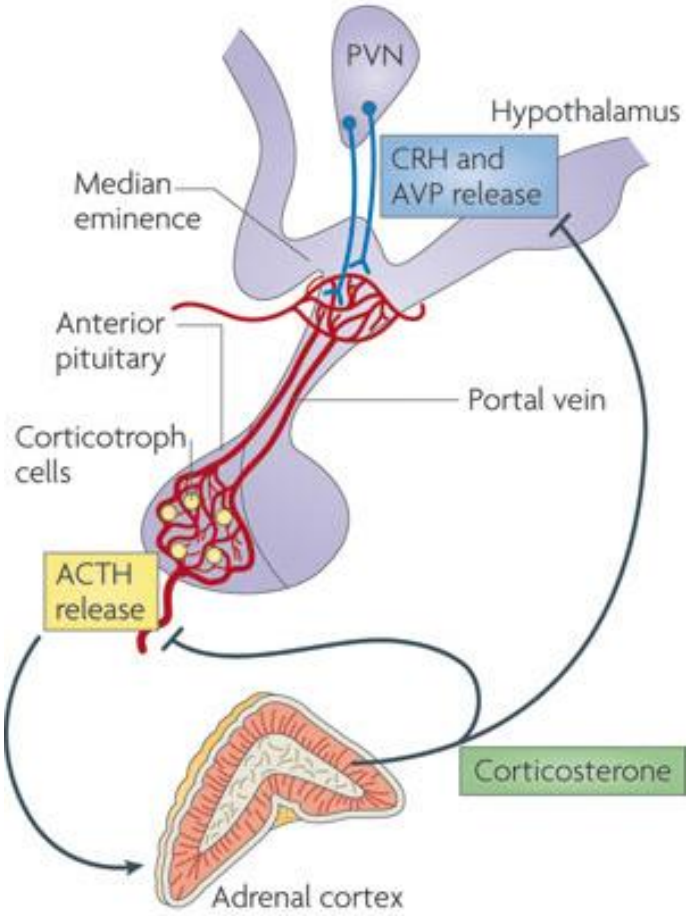

Figure 10: scheme depicting the main components of the HPA axis.[111]

The release of corticosteroids can also be induced by stimuli interpreted as potentially noxious to the organism[112]. From a general point of view, activation of the HPA axis is mediated by the

input that the PVN receives from many components of the limbic system as the hippocampus, medial prefrontal cortex, and amygdala as well as the brainstem[113].

Although these structures are clearly involved in the modulation of the activity of the HPA axis, it is in the hypothalamus, specifically in the PVN, where most of the inputs are integrated and the first output of the axis resides. The PVN is divided into three magnocellular and five parvocellular subdivisions. Neurons from the magnocellular portion project to the neurohypophysis to release oxytocin and vasopressin, while the parvocellular subdivision project to the autonomic nuclei in the brainstem and spinal cord and are responsible for the activation of the autonomic nervous system[114]. In addition, there is a set of parvocellular neurons projecting to the anterior hypophysis through the median eminence that expresses peptides known to influence the anterior pituitary, the corticotrophin releasing hormone (CRF) (**Fig. 11**) and the thyroid releasing hormone (TRH), influencing the release of Cort ant thyroid hormone respectively.

CRF is a member of the same family of the urocortins and is recognized by G protein-coupled receptors (CRFR1) -alpha and beta- and CRFR2 -alpha, beta, and gamma-. Once released into the portal circulation to the pituitary, CRF binds to CRFR1 expressed in the corticotropes and elicits the release of adrenocorticotrophic hormone (ACTH) to the bloodstream. In addition to the stimulatory action of CRF, vasopressin (AVP) also has a stimulatory effect on the release of ACTH; in this case, it is via the activation of the V 1b receptors in the corticotropes.

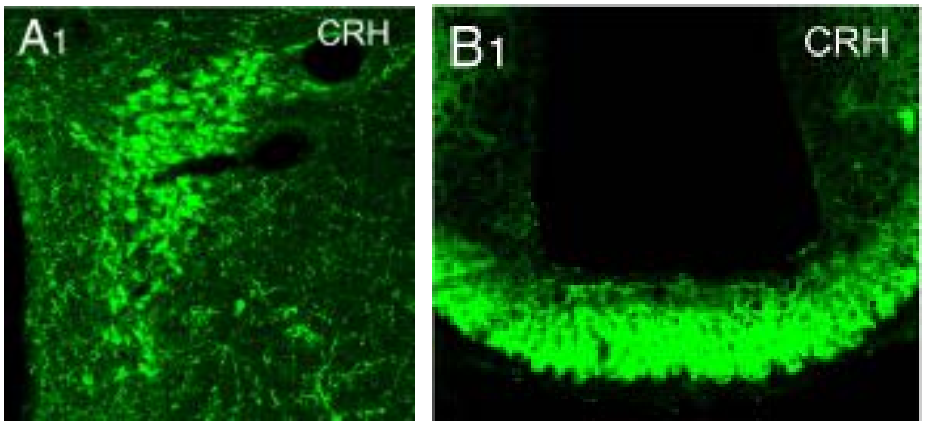

Fig 11. Parvocellular neurons in the PVN contain CRH (A). Secretory axons travel to the hypophysis via the median eminence(B) [115]

ACTH is derived from a larger precursor known as Pro-opiomelanocortin (POMC) by the action of specific prohormone convertase enzymes PC1 also known as PC 3 and gives origin to other peptides when processed by PC2 in other tissues different from the pituitary (**Supplementary table 4**). Once ACTH enters the systemic circulation, it exerts different effects depending on the tissue and receptor it is bound to (**Supplementary table 5**). The correct signaling of ACTH in the adrenal gland requires the presence of the melanocortin receptor 2 (MC2R) in conjunction with the melanocortin receptor 2 accessory protein (MRAP), that is necessary to the traffic of MC2R from the endoplasmic reticulum to the cell surface. At physiological levels, ACTH binds only the MC2R, expressed uniquely in the cortex of the adrenal gland to promote its growth. In addition, ACTH stimulates the cells located in the zona fasciculata of the adrenal gland to release glucocorticoids, and to some very limited extent the zona glomerulosa and reticularis to release mineralocorticoids or adrenal androgens respectively.

Binding of ACTH to MC2R stimulates adenylate cyclase, elevating cyclic adenosine monophosphate (cAMP) and leading to activation of protein kinase A (PKA), causing, in turn, the increased expression of steroidogenic enzymes. Biosynthesis of steroid hormones in the adrenal gland begins with the transport of cholesterol to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR). Afterward, cytochrome P450 family 11 subfamily A member 1 (CYP11A1) cleaves cholesterol into pregnenolone; this is the rate-limiting step in steroidogenesis.

The main difference on glucocorticoid synthesis pathway in rodents and non-rodent mammals: Cort is the dominant GC in rodents and cortisol in non-rodent mammals. This is due to the absence of the enzyme steroid 17 alpha-hydroxylase/17,20 lyase (P450c17) in the rodent adrenal, leaving progesterone as a precursor to being converted to Cort by 21-hydroxylase and 11 beta-hydroxylase enzymes (**Fig. 12**).

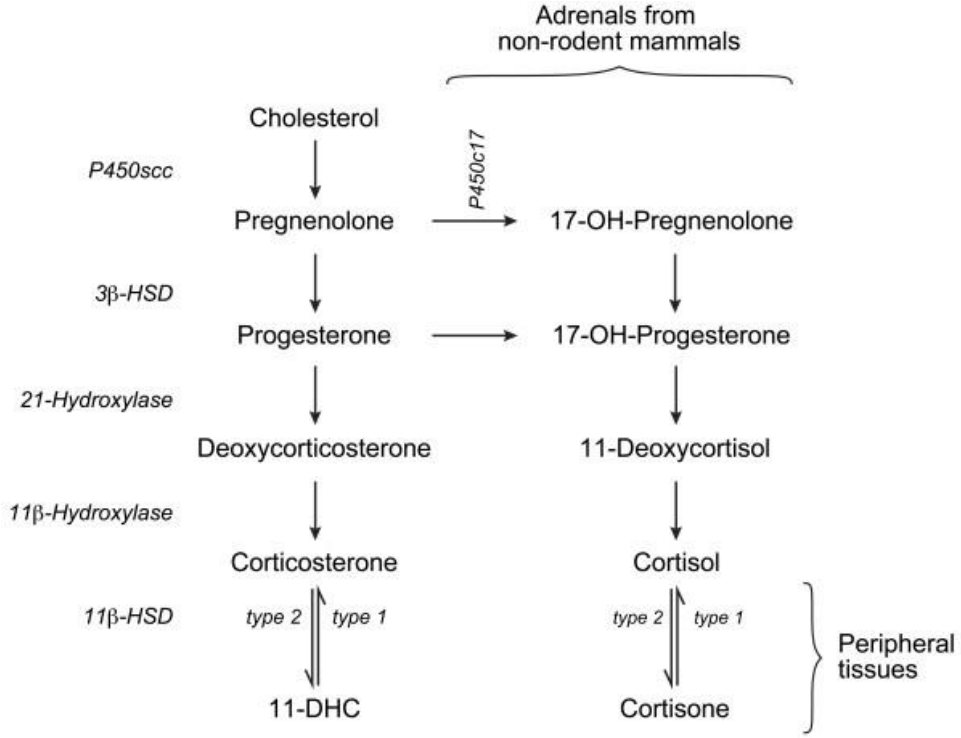

Figure 12: Biosynthetic pathway of glucocorticoids in human and rodents.

Since free steroid hormones can pass through most biological membranes, Cort is synthesized and released on demand directly into the bloodstream. Once in the circulation, more than 75% of Cort is bound to transcortin, also known as corticosteroid-binding globulin (CBG), a protein with high affinity and low capacity for Cort and to some extent to albumin, which has low affinity and high capacity and binds to approximately 15% of Cort. Since CBG forms an inactive complex with GCs in the plasma, CBG has an important role in GCs bioavailability and access to target tissues. It has been proposed a double role of CBG acting as a buffer and reservoir and delivery molecule in the blood[116].

GCs exert their effects via two sets of receptors: the glucocorticoid receptor and the mineralocorticoid receptor. In the hypothalamus, Cort produces its effects mainly via two receptors: the type I or mineralocorticoid receptor (MR) and type II or glucocorticoid receptor (GR)[117]. When Cort binds to these receptors, the complex is quickly translocated from the cytoplasm to the nucleus in a dose and time-dependent manner [107]. Type I receptor has ten

times more affinity for Cort and is rapidly saturated, while type II has lower affinity to Cort and is a high capacity receptor. Whereas type I has been described as an important component of basal Cort homeostasis due to the high occupation under low Cort situations, type II receptor is thought to respond mainly to high levels of Cort[117–120].

Finally, cell-specific 11β-HSD-catalyzed reactions occur in a tissue-specific manner. Active glucocorticoid -Cort- is metabolized by 11b-hydroxysteroid dehydrogenase 2 (11b-HSD2) to its inactive form 11-dehydrocorticosterone while regeneration can occur via 11b-hydroxysteroid dehydrogenase 1 (11b-HSD1) (**Fig. 13**). However, the physiological effects of the action of both enzymes remain elusive due to inconsistencies found between different genetic backgrounds[121,122].

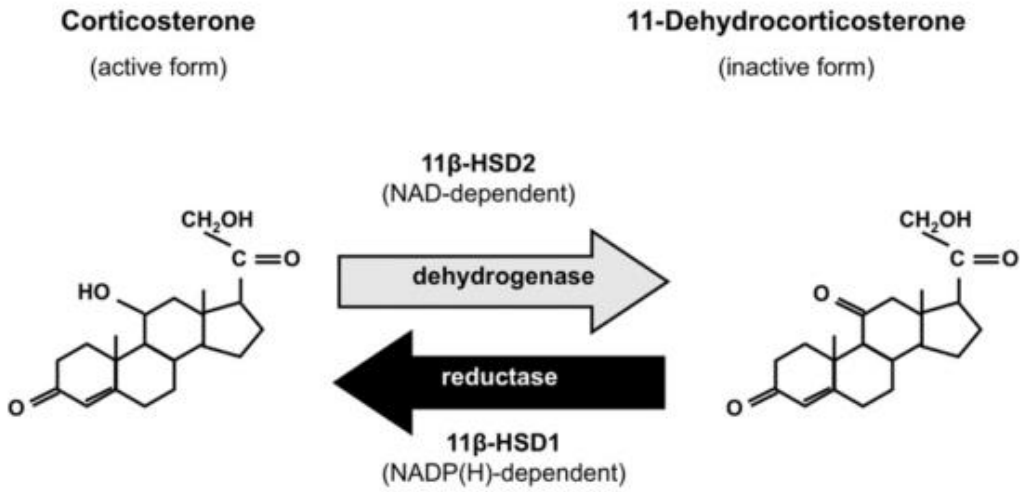

Figure 13. The enzymatic actions of 11b-HSD in inter-conversion of active and inactive glucocorticoids in rodents.

Circadian control of glucocorticoids

GC have a marked rhythm in plasma of about 24 hours; this pattern of production is organized by the influence of the SCN with a maximum just at the beginning of the activity phase even in the absence of photic cues, while ACTH variation is minimal[123] (**Fig. 14**).

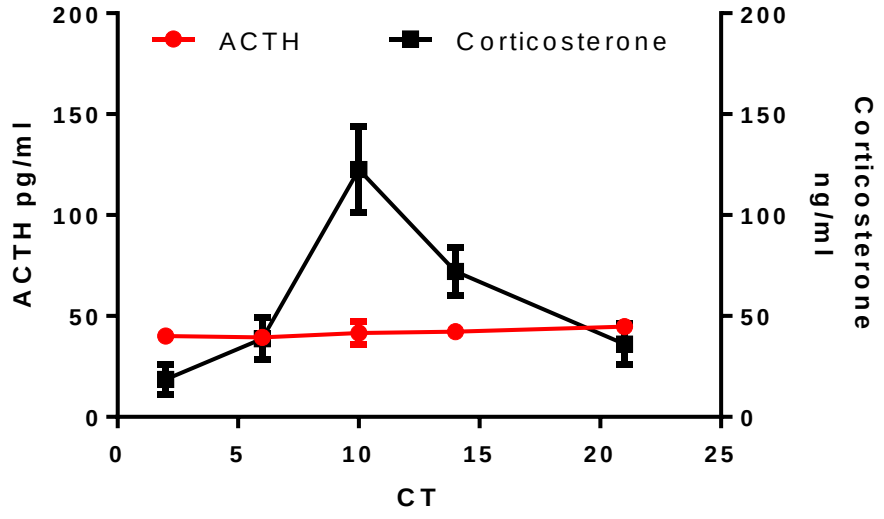

Figure 14. Cort presents a daily peak just before the activity period; this release is maintained even under conditions of constant darkness, in absence of light -the classical Zeitgeber (ZT)-, the time is indicated as Circadian Time (CT)[124].

GC levels have been reported to increase after ablation of the SCN in rats, indicating that at least part of the circadian control might operate in an inhibitory fashion[124]. In addition, infusions of AVP -one of the main neurotransmitters synthesized in the SCN- into the paraventricular/ dorsomedial hypothalamus area inhibits Cort release in rats with or without double SCN lesion[125]. Likewise, Cort release is stimulated when AVP is blocked by antagonist infusion into these brain regions during the circadian peak times of AVP release [125,126], suggesting that projections from SCN towards the PVN/DMH containing AVP[123,127], modulate the release of Cort in a circadian way[128].

In addition to the inhibitory effect, the SCN can also stimulate Cort release when appropriate. The increases in ACTH and Cort secretion upon AVP antagonist infusion are most pronounced during the second half of the light period, consistent with the stimulatory activity appearing during this period (**Fig. 15**) [129].

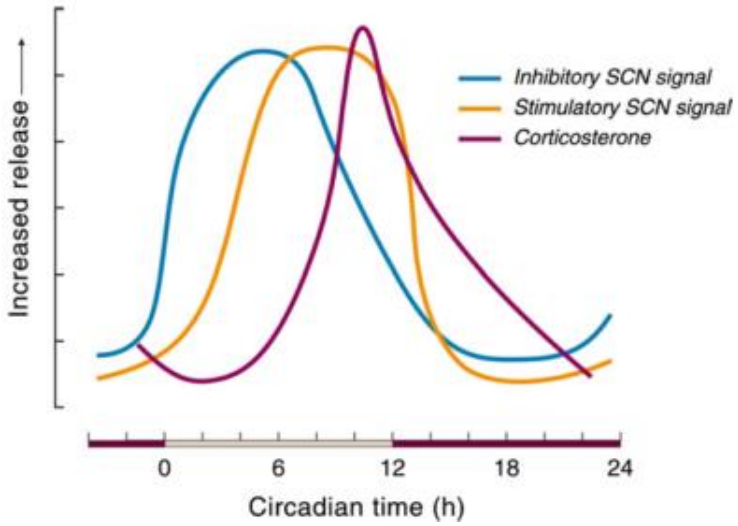

Figure 15. Schematic representation of the diurnal release pattern of SCN transmitters involved in the circadian control of Cort release.[130]

At the level of the adrenal gland, responsiveness to ACTH is greater in the evening than in the morning, despite similar levels of circulating ACTH[131,132]. However, *in vitro* experiments to test the endogenous rhythm of sensitivity to ACHT fail to show circadian rhythm, indicating that ACTH levels or the cellular machinery in the adrenal alone are not sufficient to drive the rhythm of Cort [133]. A plausible mechanism to control circadian Cort release is the autonomic control of the adrenal gland since the circadian rise depends on the integrity of the sympathetic neural input to the gland (**Fig. 16**) [134]. Simultaneously, this autonomic control strongly influences the circadian rhythm that exists in the stress response. Early in the rat sleep period, stress results in high ACTH and GC levels while early in the active period the same stress hardly results in a rise of ACTH while plasma GC still show pronounced increases though significantly less than in the beginning of the sleep phase. These observations indicate that the autonomic control of the adrenal not only serves to control the circadian rise of GC but also to regulate stress-induced GC levels depending on the time of the day.

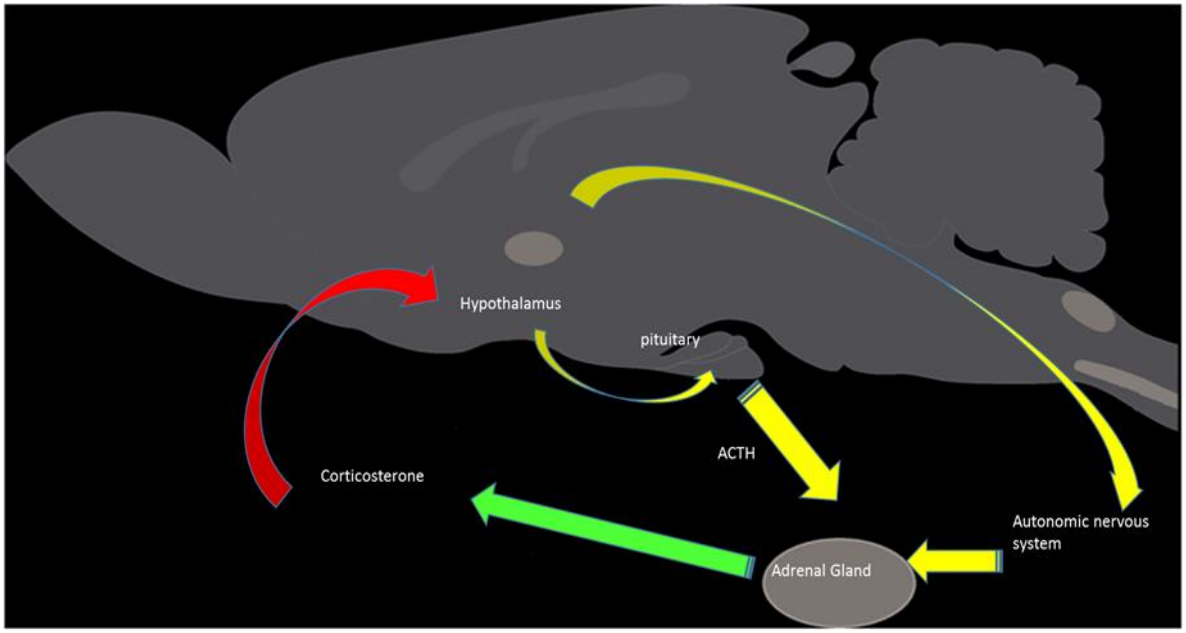

Figure 16. Two important pathways exist to control Cort secretion: via CRH and ACTH and polysynaptic neural connections from the hypothalamus to the adrenal gland.

Glucocorticoids in the ARC: signaling and metabolism

The ARC has two neuronal populations involved in energy expenditure and food intake, one expresses neuropeptide Y (NPY), Agouti Related Peptide (AgRP) and Gamma Aminobutyric Acid (GABA), and exerts orexigenic effects when stimulated. The other population contains neurons expressing cocaine and amphetamine regulated transcript (CART) and POMC, which translates into alpha-melanocyte stimulating hormone (Alpha-MSH) to inhibit food intake and energy expenditure. These neuronal populations can be accessed by components of the circulation due to the presence of fenestrations in the ARC-ME complex[135]. In addition, glial cells in this nucleus have been reported to facilitate fenestration in the ARC and participate in the transport of many molecules as thyroid hormone[75], ghrelin[136], leptin[46], and insulin[137].

Glucocorticoids have profound effects on metabolism, including feeding behavior, glucose production, fatty acid storage, mitochondrial activity [104], insulin sensitivity and the transcription of several genes after translocation to the nucleus [107]. In view of the important role of glucocorticoids on metabolism, and the high expression of MR and GR in the ARC, a site of metabolic integration, we hypothesized the ARC requires the action of GR and MR to correctly integrate food intake and energy expenditure[138,139]. An example of this is a chronic stress condition, typically accompanied by high GCs levels, which is linked to increased body weight gain and finally obesity [97,140,141]. In addition, the induction of high GCs via exogenous administration, increases caloric intake and voluntary intake of palatable food [142–145] in a dose-related way [146], indicating that the development of obesity could be a consequence of the change in the diet and the amount of food ingested in addition to other metabolic changes in the periphery caused by the increase of GCs.

In the ARC, about 50% of the GR-IR neurons also expressed MR-IR [147]. After adrenalectomy (ADX), GR- IR disappears in most of the forebrain, including the PVN and ARC, in which GR-IR is dramatically reduced without affecting GR mRNA. The disappearance of GR-IR neurons after ADX is due to a lack of activating effects of endogenous GCs on GRs rather than to decreased levels or loss of GR protein since the removal of GCs elevates the intracellular levels of GRs[148].

One hour after treatment with Dex, GR-IR returned to normal levels in the ARC but not in other brain structures [148], demonstrating the fast effects of GC on this nucleus.

The effects of GCs on feeding must be mediated at least in part by their interaction with neurons co-expressing glucocorticoid receptors and NPY/AgRP or POMC/CART in the ARC [19,62,149,150]. Early studies demonstrated the presence of GR in half the neurons positive for NPY in the ARC and indicated the presence of synaptic contacts of NPY axons upon PVN neurons containing CRF[151]. This evidence provides an anatomical pathway by which NPY may integrate metabolic information sending mainly inhibitory signals to the PVN[152 153], linking CRH, metabolism, and stress[154]. Hereby, metabolic changes can be sensed by the ARC and then conveyed to the PVN to modify the synthesis of CRF, and consequently the activity of the HPA axis.

At the molecular level, Cort impacts on the transcription of peptides in the ARC. For example, the daily increase of Cort coincides with the circadian increase of AgRP mRNA expression. This effect is also observed after administration of high doses of Cort, which significantly increase AgRP mRNA[155]. In ADX animals, the diurnal rhythm is abolished by suppressing nighttime expression, show reduced AgRP and NPY in fasting conditions and decreased POMC after feeding[98]. More recent studies have shown that the gene encoding AgRP is a direct target for Cort, and GR together with the brain-specific homeobox factor synergize to directly activate AgRP transcription[156]. Using the same experimental approach, CART mRNA levels in the Arcuate nucleus were diminished and partially restored after Dex administration [157].

At a cellular level, Cort absence changes the resting membrane potentials and synaptic arrangement in the ARC, reducing the number of inhibitory synapses onto POMC neurons and the number of excitatory synapses onto NPY AgRP neurons, causing hyperpolarization of NPY and a depolarization of POMC neurons and is reversed by Cort treatment[158]. The effects of GC can be mediated also via intracellular metabolic signals, as the AMP-activated protein kinase (AMPK) or the mammalian Target of Rapamycin (mTOR). AMPK activity is increased in negative metabolic conditions functions as a consequence of ATP diminished availability within the cells[159] and inhibits mTOR activity, another nutrient sensing enzyme implicated in leptin signaling in the hypothalamus[160]. In organotypic cultures, it has been demonstrated that GCs stimulate AMPK signaling in the ARC, increasing NPY and AgRP mRNA and inhibition of AMPK signaling attenuates the effects of GCs on NPY/AgRP expression. AMPK signaling is decreased in ADX animals and is stimulated by peripheral administration of GC[161]. Administration of Dex decreased mTOR signaling, and it was accompanied by an increase in the amount of NPY mRNA[162]. This demonstrates that GCs can function as an inductor of a negative metabolic state in the neurons by two pathways, increasing intracellular signals of fasting and diminishing the efficiency of leptin in the ARC to signal satiety, leading to a combined effect to increase the consumption of calories and increased hepatic glucose production.

As mentioned above, Cort is necessary for correct signaling of other hormones to ARC neurons. Although ghrelin induces orexigenic effects, the increased synthesis of NPY and AgRP [163] by this hormone also requires the presence of Cort. In addition, Cort can counteract leptin effects in a dose-dependent manner [164]. Importantly, there is evidence that the levels of GR in the ARC can be modified by insulin, resulting in a stimulation of GR transcript after an acute dose,[106] and repeated insulin-induced hypoglycemia [165]. Consequently, GCs may increase food intake and or lower metabolic efficiency via such mechanisms, suggesting combined effects between the signaling of GC and other metabolic related hormones, amplifying the effects of ghrelin to signal a negative metabolic state and drive for search and food consumption, aside from

diminishing the effect of the signals of energetic availability related to leptin (**Fig. 17**). These effects also may explain why the changes in metabolic signaling are emphasized by augmenting the amount of GR after hypoglycemia.

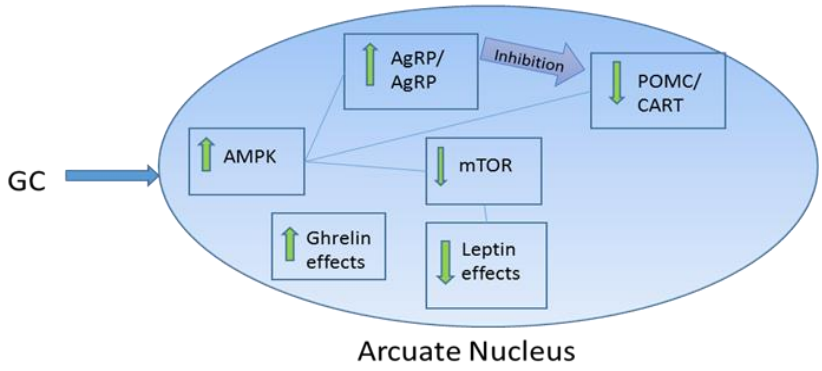

Figure 17. Intracellular changes in ARC neurons produced by GCs, enhancing the signals of negative metabolic state to increase the transcription of orexigenic peptides as AgRP and NPY

As discussed previously, the feeding conditions can also modify glucocorticoid production [166,167]; however, the type of nutrients consumed seems to be also important. High-fat diet interferes with the ARC[168] and HPA axis function under basal and stress conditions [169,170], although the effect may depend also on other factors [171] Palatable food consumed chronically induces an increase in sympathetic activity, demonstrated by changes in heart rate, blood pressure [172], temperature [173], and accumulation of adipose tissue[174]. These changes are paralleled by changes in the circulating Cort[175]. High glucose levels can promote a state similar to glucocorticoid resistance [176] probably due to the increased autonomic tone.

Activation of the GR in the Arcuate by administration of Dex via retrodialysis may stimulate NPY release, which in turn stimulates sympathetic pre-autonomic output from hypothalamus[31] This clearly points to the need for an accurate GC input to the ARC, to maintain the set point of peptide production and release, as well as the responsiveness to incoming stimuli and the autonomic tone to the liver, fat storage and other tissues involved in energetic homeostasis.

Glucocorticoids feedback to the hypothalamus

Once GC are released due to circadian or other stimuli, there must be a mechanism to control the magnitude of the response to maintain the homeostasis of the body. This is especially true for conditions such as the chronic stress or metabolic and endocrine disorders. In fact, an inadequate feedback of GC is observed in a series of chronic illnesses as depression [177], chronic fatigue syndrome [178], obesity and resistance to satiety signals such as insulin and leptin [101,164], which in turn can lead to diabetes[179].

Recent studies have shown the importance of regulatory mechanisms independent of the HPA axis for steroidogenesis, as has been demonstrated in the control of glucocorticoid variations by gut microbiota [180] and the coupling of CYP11B1 activity -the enzyme responsible for GC synthesis in the adrenal gland- to peroxiredoxin III (PrxIII) inactivation[181]; however, for the largest part of known physiology, the central nervous system has been proposed as the main site of glucocorticoid feedback.

At the level of the pituitary gland, POMC has a known negative glucocorticoid response element that promotes direct transcriptional repression by glucocorticoids[182], which may account for delayed feedback effects.

In the brain, the hippocampus, PVN, and Arcuate nucleus are the areas with most prominent expression of GR, [183,184] and therefore, these brain areas have been studied as possible regulators of GC feedback; however, the evidence is not conclusive and it is suggested that feedback is distributed into a network, and therefore integrative neuronal circuits mediating glucocorticoid negative feedback are yet to be clearly defined [185,186]

At the level of the hypothalamus, there is evidence of the PVN mediating the negative feedback to some degree, since implantation of GC agonist in this region induced changes in AVP and CRF expression, while this was ineffective in other areas such as the amygdala, hippocampus, subiculum, cerebral cortex and lateral septum[187,188]. In addition, GCs rapidly inhibit PVN activity and presynaptic glutamate release[189,190]. The inhibition may relay in various mechanism, as retrograde opioid signaling [191], endocannabinoids [190,192] or nitric oxide [193]. Importantly, this negative feedback may also be mediated by a mechanism independent of GR occupation and mRNA synthesis[194,195], suggesting the necessity of an additional mechanism to directly and accurately sense Cort levels.

It is important to note that GR and MR may play different roles in relationship with the circadian maintenance of GC circulating levels. It has been demonstrated that MR but not GR receptor can mediate fast feedback mechanism for glucocorticoids in the morning[119,196] and systemic pharmacological blockade of the MR produced an increase in circulating Cort in the morning but not differences in the response to stress or evening production[197]. In contrast, direct retrodialysis in the ARC or the PVN with a GC agonist is able to prevent the daily surge of Cort[139]. Consequently, is seems that occupation of MR is important to maintain low GC levels in the resting phase, and the occupation of GR in PVN or ARC may be important to modulate the magnitude of the surge. (**See hypothesis 2**).

Aim of the studies

In this work, we aimed to investigate the participation of the ARC in the sensing of different signals related to energy homeostasis, i.e. glucose and Cort. In addition, we focused on the role of the SCN to modulate this sensing processes and produce their respective counterregulatory response in a time-of-the-day dependent way.

We hypothesized that the SCN provides rhythmicity to the circadian responses of the ARC via a monosynaptic pathway, this connection is essential to sense and produce changes in glucose production and that produce a counterregulatory response.

For Cort, we hypothesized that the ARC transmits a negative feedback to GCs depending on the time of the day, via an autonomic polysynaptic pathway to the adrenal gland

Objectives:

- Characterize the sensitivity of the ARC to hypoglycemia along the day.
- Evaluate the possible inhibitory role of the SCN in the counterregulatory response to hypoglycemia or fasting initiated by the ARC.
- Characterize the response of the ARC to Cort.
- Investigate the contribution of type I and type II GR to the negative feedback at different time points of the day
- Explore a possible autonomic, ACTH-independent pathway to control daily changes on Cort secretion.

Hypotheses:

Hypothesis 1: The communication between SCN and ARC may be crucial to establishing a differential hepatic glucose production and a counterregulatory response to hypoglycemia depending on the time of the day.

Hypothesis 2: The ARC participates in the negative feedback to glucocorticoids; this participation is differential depending on the time of the day

Chapter II. Based on:

The Suprachiasmatic nucleus modulates the sensitivity of Arcuate nucleus to hypoglycemia

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Abstract

The suprachiasmatic nucleus (SCN) and arcuate nucleus (ARC) have reciprocal connections; catabolic metabolic information activates the ARC and inhibits SCN neuronal activity. Little is known about the influence of the SCN on the ARC. Here, we investigated whether the SCN modulated the sensitivity of the ARC to catabolic metabolic conditions. ARC neuronal activity, as determined by c-Fos immunoreactivity, was increased after a hypoglycemic stimulus by 2-deoxyglucose (2DG). The highest ARC neuronal activity after 2DG was found at the end of the light period (zeitgeber 11, ZT11) with a lower activity in the beginning of the light period (zeitgeber 2, ZT2), suggesting the involvement of the SCN. The higher activation of ARC neurons after 2DG at ZT11 was associated with higher 2DG induced blood glucose levels as compared with ZT2. Unilateral SCN-lesioned animals, gave a mainly ipsilateral activation of ARC neurons at the lesioned side, suggesting an inhibitory role of the SCN on ARC neurons. The 2DG-induced counterregulatory glucose response correlated with increased ARC neuronal activity and was significantly higher in unilateral SCN lesioned animals. Finally, the ARC as the site where 2DG may, at least partly, induce a counterregulatory response was confirmed by local microdialysis of 2DG. 2DG administration in the ARC produced a higher increase in circulating glucose compared with 2DG administration in surrounding areas such as the ventromedial nucleus of the hypothalamus (VMH). We conclude that the SCN uses neuronal pathways to the ARC to gate sensory metabolic information to the brain, regulating ARC glucose sensitivity and counterregulatory responses to hypoglycemic conditions.

Introduction

The SCN, located in the basal hypothalamus, organizes the daily physiology allowing the organism to anticipate regular changes in the environment. The SCN is directly involved in glucose metabolism[29,198], providing a daily rhythm of hepatic glucose production (HGP)[89,199] and of glucose uptake in the peripheral tissues [200,201]. These events are principally mediated by SCN projections to the autonomic nervous system (ANS) [29,89,201] and show their nadir and peak in the beginning (ZT2) and the end (ZT11) of the light period respectively[199,200]. This results in high glucose tolerance and high plasma glucose concentrations at ZT11, and low tolerance and low plasma glucose concentrations at ZT2. This circadian regulation shows that the SCN organizes glucose metabolism to prepare the organism for its rest and activity period [202].

On the other hand, the ARC, also situated in the basal hypothalamus, is involved in sensing circulating metabolites and hormones and regulating energy homeostasis [57,203]. Two neuronal populations have been implicated in this role; NPY/AGRP/GABA and alpha-MSH/CART

neurons[203]. Both populations are proposed to exert antagonistic roles; orexigenic NPY neurons are activated during low energy states such as fasting[204,205] and promote energy conservation, and anorexigenic alpha-MSH neurons are activated by high energy states such as refeeding and satiety[206] and promote energy expenditure. These neurons also were identified as sensitive to different levels of extracellular glucose concentrations and have been classified as glucose-excited neurons (GE) and glucose-inhibited neurons (GI) [207]. The GI neurons, identified in their majority as NPY neurons, increase their firing rate to a decrease in extracellular glucose concentrations[59], which agrees with their role in counterregulatory responses to compensate reduced energy supplies[208,209].

Hypoglycemia can be induced experimentally by the peripheral or central administration of 2DG, a glucose analog that cannot be metabolized. Induced hypoglycemia is accompanied by a series of counterregulatory responses characterized by an increase in hepatic glucose production, Cort, epinephrine, and glucagon secretion; supported by an increase in food intake [210–212]. Hereby the ventromedial hypothalamus was proposed as one of the glucose sensitive nuclei responsible for sensing a fast decrease in glucose and inducing the counterregulatory responses to hypoglycemia[66,68,213,214].

These observations combined with the SCN influence on glucose uptake and release, together with the observed reciprocal connections between ARC and SCN[86,215] suggests a functional relationship between both nuclei. For example, it was shown that intravenous ghrelin injection activates ARC NPY neurons and at the same time inhibits SCN neuronal activity[86,216]. To investigate the possible influence of the SCN on the ARC we hypothesized that the SCN may influence the ARC sensitivity to low levels of glucose in a circadian manner. Hereto we explored two different time points; ZT2 and ZT11 when the SCN has a minimal and maximal influence on glucose production of the liver[200]. We hypothesized that the SCN influences the neuronal response of the ARC to low energy states through neural projections. We observed a time-dependent inhibitory influence of the SCN on the neuronal activation in the ARC to hypoglycemia indicating a gating role of the SCN for metabolic information reaching the brain via the ARC. This conclusion was corroborated by the observation in unilateral SCN lesioned animals that a 2DG challenge results in a higher neuronal activation in the ARC at the lesioned site followed by a higher glucose counterregulatory response. The ARC as site sensitive for hypoglycemic conditions was demonstrated by local microdialysis of 2DG in the ARC; 2DG microdialysis in the ARC resulted in a higher increase in circulating glucose levels compared to animals administrated with 2DG in the VMH. Thus, an intriguing interaction is revealed between SCN and ARC whereby the SCN influences the sensitivity of the ARC such, that its response to the same hypoglycemic challenge is dependent on the time of the day.

Materials and Methods

Animals

Male Wistar rats (250-300g) were housed in individual cages under constant temperature (23°C ± 2 °C) and 12/12h light/dark conditions; lights on 7:00h (ZT0), off 19:00h (ZT12). Food and water were provided ad libitum to all the animals before and after surgery. When fasted, the food was removed for 2 days prior the experiment. All animals were handled every day to

avoid stress effects during the experiment. The Committee for Ethical Evaluation at Institute for Biomedical Research, UNAM, approved experiments in accordance with the Mexican norms for animal handling (Norma Oficial Mexicana NOM-062-ZOO-1999).

Experimental design

ARC activation under hypoglycemic conditions at ZT2 and ZT11.

To explore the ARC activation during hypoglycemia, expression of c-Fos in the ARC after induction of cerebral or systemic hypoglycemia due to 2DG (2 Deoxy-D-Glucose Grade III, Sigma) injection was assessed. To induce cerebral hypoglycemia, rats received an intracerebroventricular cannula (ICV) and a catheter in the jugular vein. After 7 days of recovery, the food was removed one hour before the infusions were performed. The animals were ICV infused with either saline (n=6) or 2DG 2.5 mg/kg (n=6) or 2DG 5 mg/kg (n=6). The infusions were performed at ZT2 or ZT11, after 120 minutes the animals were sacrificed. For systemic hypoglycemia rats received a catheter in the jugular vein, intravenous (IV) infusions of saline (n= 5) or 2DG (n=6) (250 mg/kg) were performed at ZT2 or ZT11. After 120 minutes the animals were sacrificed. For the cerebral and systemic hypoglycemic groups blood samples were obtained at 0, 15, 30, 60 and 120 minutes after the ICV or IV injection.

After 2DG induced hypoglycemia, animals were sacrificed by deep pentobarbital anesthesia (100 mg/Kg, Pisabental, Pisa Agropecuaria) followed by perfusion fixation with 4% phosphate-buffered paraformaldehyde pH 7.2. Afterward, the brains were processed for immunohistochemistry for c-Fos and NPY or alpha-MSH.

SCN unilateral lesions

To assess the influence of the SCN on ARC activation to catabolic metabolic conditions, unilateral lesions of the SCN were performed to conserve the activity and hormonal rhythms and to eliminate unilateral projections from the SCN to the ARC. After a 3-week interval for activity recording, sham (n=10) and unilateral SCN lesioned rats (n=11) were infused with saline (sham group n=5, unilateral SCN group n= 6) or 2DG IV (250 mg/kg) (sham group n=5, unilateral SCN group n= 6) at ZT2. Both groups of rats received a jugular catheter. Blood samples were taken 0, 15, 30, 60 and 120 minutes after infusion, animals were then sacrificed by deep pentobarbital anesthesia followed by perfusion fixation. Food intake was measured during 120 minutes in all saline and 2DG induced hypoglycemia groups. Neuronal activation of the ARC ipsilateral and contralateral to the SCN lesioned side was evaluated with immunohistochemistry for c-Fos and NPY and alpha-MSH. The completeness of the unilateral lesion was verified histologically, and all animals without a complete lesion discarded, as well as animals whereby the other side of the SCN was damaged (**Fig. 22**).

To test if the SCN was also regulating the sensitivity of the ARC to fasting, sham-operated rats (n=10) and unilateral SCN lesioned rats (n=12) were exposed to 48h fasting, afterwards animals were sacrificed by deep pentobarbital anesthesia followed by perfusion fixation at ZT 2 (Sham group n=5, unilateral SCN group n= 6) or ZT11 (Sham group n=5, unilateral SCN group n= 6). Neuronal activation of the ARC ipsilateral and contralateral to the SCN lesioned side was evaluated with immunohistochemistry for c-Fos and NPY and alpha-MSH.

2DG Retrodialysis in the ARC

To explore if the ARC could initiate 2DG hypoglycemia counter-regulatory responses, we performed 2DG retrodialysis in the ARC at ZT2. Homemade microdialysis probes were placed unilaterally using a stereotactic apparatus into the ARC (n=9), animals with misplaced cannulas (e.g. in the Ventromedial nucleus of the hypothalamus= VMH) were analyzed in a separate group (n=5). Coordinates were adapted from the atlas of Paxinos and Watson[217] (coordinates for ARC implantation: -2.4mm anterior from bregma, +1.7mm lateral from midline and V -9.5 mm below brain surface, tooth bar -3.4°). Next in the same operation procedure silicon catheters (Silastic) were inserted into the right jugular vein for blood sampling. The catheter and microdialysis probes were fixed on top of the skull with stainless steel screws and secured with dental acrylic. The jugular catheter was maintained permeable with a solution of 45% glycerol, 40% isotonic saline, 10% heparin (5000 IU/ml, Inhepar, Pisa Agropecuaria) and 5% Antibiotic Mixture 100X, (Gibco) replaced every two days. The position of cannulas was verified at the end of the protocol by histological examination with Nissl staining.

After one week of recovery, animals were connected to a multichannel fluid infusion swivel (Instech Laboratories), one day before the experiment, to prevent any effect of stress. The day of the experiment, retrodialysis initiated with ringer solution (3 µL/min) from ZT0 to ZT2. At ZT2, ringer solution retrodialysis continued in the vehicle group (n=5) or was replaced by 2 DG (n=4). Blood samples were taken at 0, 10, 20, 40 and 80 min after the starting of drug retrodialysis. At the end of the experiments, all rats were deeply anesthetized with a lethal dose of sodium pentobarbital and the brains were obtained for histological analysis.

SCN projections to ARC NPY and alpha-MSH neurons.

To explore the SCN projections to the ARC NPY and alpha-MSH neurons, Cholera Toxin b (CTb) tracing experiments were performed. After successful (n=3) CTb injections in the SCN, animals were allowed to recover for 10 days. Afterward, they were fasted for 48 hours and sacrificed by deep pentobarbital anesthesia followed by perfusion fixation at ZT12. Immunofluorescence was then performed for c-Fos, NPY, alpha-MSH, GAD67, and vGLUT1.

Surgical procedures

Ketamine 100mg/ml (0.18 ml/kg im; Anesket, Pisa Agropecuaria SA de CV, Mexico) and Xylazine 20 mg/ml (0.1ml/kg ip; Procin, Pisa Agropecuaria SA de CV, Mexico) were injected i.m. to anesthetize the animals. A stainless-steel guide cannula was stereotactically implanted in the 3rd ventricle (coordinates: -5mm anterior from bregma, 0mm lateral from midline and V -6.6mm below brain surface). The tooth bar was set at -3.5mm. The sinus vein was laterally pulled to introduce the guide cannula. Additionally, a silicon catheter (0.025 in. i.d. and 0.047 in. o.d, Silastic, Dow Corning, USA) was inserted into the left jugular vein to obtain blood samples. The cannula was filled with 0.3 ml PVP solution (Polyvinylpyrrolidone K25 Polyvinylpyrrolidone, Aldrich, .7mg; Heparin 5000 UI/ml, Inhepar, PiSA) and 0.7 ml antibiotic (Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture 100X, Gibco) and the outer end of the cannula was fixed on the back of the animals and blocked with a small needle.

Unilateral electrolytic SCN lesions were performed using the following stereotactic coordinates: -4mm anterior from bregma, 0.9mm lateral from midline and V -8.2mm below brain surface. A current of 0.35 mA was applied for 45 seconds. In the sham groups, the electrode was introduced without electric current. After a 3-week period in which the activity

was recorded, animals were inserted with a silicon catheter in the left jugular vein to facilitate blood sampling.

0.1 µl of Cholera Toxin subunit b (conjugated with Alexa Fluor 555, Molecular Probes, Eugene, OR) was unilaterally injected in the SCN using the following coordinates: -3mm anterior from bregma, 10mm lateral from midline and V -8.4mm below brain surface with an angle of 4°.

Tissue processing

After an overdose of sodium pentobarbital (Sedalphorte, 63mg/ml) the animals were transcardially perfused with 250 ml of 0.9% saline solution and 250 ml of 4 % paraformaldehyde in phosphate buffer (0.1 M, pH 7.2). Brains were removed afterward and post-fixed for 24 hours in 4% paraformaldehyde at 4°C. After this period, the brains were cryoprotected in 30% sucrose for 2 days. 40 µm sections were cut and immunohistochemistry was performed. C-Fos primary antibody made in rabbit was incubated in free floating sections (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight. After the primary incubation, the sections were rinsed with PBS 0.1M and incubated with donkey anti-rabbit biotin-SP-conjugated secondary antibody (1:200, Jackson laboratories, Maine, USA) for 1 hour and again rinsed for 10 min. Next avidin-biotin complex incubation was performed (1:500, Vector Laboratories, CA, USA) for an hour and then rinsed 3 times with PBS for 10 min. Finally, tissues were reacted with diaminobenzidine (10mg/100 ml TBS), hydrogen peroxide (10µl, 30% H_2O_2) and a 250 µL of nickel 10% TBS solution (Sigma, 98% ammonium nickel sulfate hexahydrate) for 6 min and rinsed again with PBS for 10 min. Tissues were mounted, dehydrated and coverslipped with microscopy Entellan (Merck).

Immunofluorescence was also performed for C-Fos, NPY, alpha-MSH (Millipore, MA, USA), GAD67 (Millipore, MA, USA) and vGLUT1 (Millipore, MA, USA). Free-floating sections were incubated overnight with primary antibodies for rabbit c-Fos (1:1000), sheep NPY (1:1000), sheep alpha-MSH (1:1000), mouse GAD67 (1:200) and guinea pig vGLUT1 (1:2000). The next day secondary antibodies (1:400) conjugated with fluorescent dyes: donkey anti-rabbit Cy3, donkey anti-sheep Cy2/Cy5, donkey anti-mouse Cy2 and donkey anti-guinea pig Cy5 were incubated for 2 hours. After the rinsing, the sections were mounted and covered with glycerol and examined with an LSM 5 Pascal (Zeiss, Jena, Germany) confocal laser scanning microscope.

General activity monitoring

Rats were housed in individual cages (45 X 30 X 35cm) placed on plates with movement sensors in soundproof lockers with controlled lighting conditions. The movement was registered in a digital format and automatically stored at 1 min intervals for further analysis with SPAD9 based on MATLAB. Due to the different sensitivity of individual sensors, movement counts were normalized to the proportional percentage whereby the total daily activity is 100%. The mean of 10-minute normalized data intervals was obtained per group and plotted in daily activity profiles where the 2-week period of baseline and the 3-week period after the unilateral SCN lesion are indicated.

Cell counts

For the quantification of the C-Fos-IR positive cells, 2 anterior, 2 medial and 2 posterior sections of the ARC were selected based on a stereotactic atlas (28). Images of selected

sections were digitalized at 20X magnification using a computerized images system (DP2-BSW, Olympus Corporation) and the number of c-Fos-positive profiles was obtained using Image J (NIH) bilaterally for the 2DG ICV and sham 2 DG IV. The number of c-Fos-positive profiles was selected using Image J unilaterally in the case of the lesioned and non-lesioned side of the ARC of the 2 DG IV sham and unilaterally SCN lesioned animals, as well for the unilateral SCN lesioned and sham fasted animals. For the unilateral SCN lesioned and sham 2DG IV infusion animals, the percentage of C-Fos IR nuclei per side was also calculated.

Confocal images analysis

After obtaining the images for the confocal analysis, colocalizations were analyzed with the software LSM 5 Pascal software (version 2.8). The overlap between C-Fos and NPY or alpha-MSH was examined. Images including overlapping on fibers marked with CTb and GAD67 or GLUT1 were also analyzed.

Glucose measurements

Blood samples were centrifuged (4000 rpm/8 minutes) and the serum was collected and stored at -20°C for glucose measurements. A commercial colorimetric kit (N 70478; Hycel de México) which is based on the reaction between glucose and fenol-4-aminofenazona as chromogen, was used. Serums were analyzed at 500 nm in a spectrophotometer.

Statistical analysis

Results are expressed as the mean ± the standard error of the mean (SEM). The cell counts, percentages, and food intake were analyzed with a 2-way analysis of variance (ANOVA). ZT, substance, and lesions were used as factors. Pairwise comparisons were evaluated with a Tukey posthoc test. Significant values were set at $p<0.05$. Glucose concentrations were analyzed by an ANOVA of repeated measures with 2 between subject factors: lesions and substance. An adjustment of Bonferroni was performed with significant values set as $p<0.05$. Statistical analyses were performed with Systat 12 (SYSTAT, version 12.00.08).

Results

The sensitivity of the ARC to hypoglycemia varies along the day.

To determine if there is a time dependent regulation of the activation of the ARC to hypoglycemia, we examined whether animals showed a difference in ARC neuronal activity as measured by c-Fos expression at two different time points after 2DG induced hypoglycemia, 2DG induced hypoglycemia was induced at ZT2 (the time point with lowest diurnal glucose levels) and compared with ZT11 (the time point with highest diurnal glucose levels). No differences were found in saline administered animals, yet a temporal regulation was evident in the animals that received 2DG ICV ($p<0.001$) with a higher activation at ZT11 than ZT2 after 2DG induced hypoglycemia (**Fig. 18a, 18b**). The number of c-Fos-positive cells after ICV 2DG infusions showed a significant effect of dose ($F(1,35) = 19.37$; $p<0.001$) and time ($F(2,34) = 119.168$; $p<.001$). In these conditions, c-Fos nuclei in the ARC seem to be present in NPY positive neurons (**Fig. 19**). Based on these findings, we hypothesized that the SCN inhibits the

sensitivity of the ARC to low plasma glucose levels at ZT2 as compared to ZT11, thus exerting a possible impact on the counterregulatory response to hypoglycemia. Indeed, the plasma glucose levels before and after the 2DG and saline ICV infusions also showed a significant effect of time after the infusion ($F(4,10) = 4.851$; $p<0.003$) and for substance administered ($F(1,13) = 679.063$; $p<0.001$). Before the ICV infusions in both saline and 2DG groups, higher basal glucose levels were measured at ZT11 as compared to ZT2, indicating an intact circadian plasma glucose rhythm. 15 minutes after 2DG injections, a significant increase in plasma glucose was evident in both ZT11 and ZT2 groups ($p<0.001$) (**Fig. 18c**). The increase in plasma glucose suggests that glucose-homeostatic mechanisms were activated in concert with ARC neuronal activation after ICV 2DG infusions. However, no differences were observed between plasma glucose levels at ZT2 and ZT11 after central induced hypoglycemia.

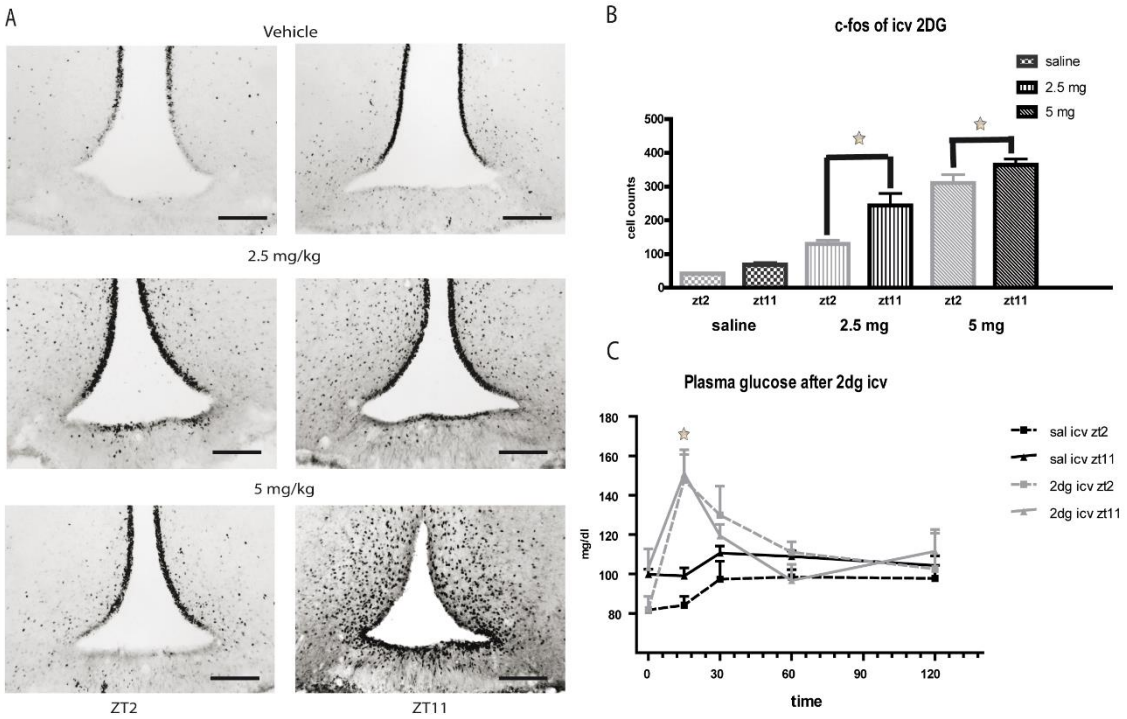

Figure 18. The neuronal response of the ARC to brain hypoglycemia is time modulated. a. c-Fos immunoreactivity in the ARC at ZT2 and ZT11 after saline or 5 mg/kg 2-deoxyglucose (2DG) or 2.5 mg/kg 2DG ICV infusions. There is a clear increase of activation at ZT11 as compared to ZT2. b. Cell counts of c-Fos protein immunoreactivity after ICV infusions of saline or 5 mg/kg or 2.5mg/kg 2DG at ZT2 and ZT11. There was a significant difference between the 2 time-points, with a higher c-Fos expression at ZT11 than ZT2 with both concentrations of 2DG. c. Plasma glucose levels after 2.5 mg ICV infusion of 2DG at ZT2 and ZT11. The increase in plasma glucose after 2DG ICV infusions is not different between time points. The black lines indicate 2DG infusions and the gray lines indicate saline infusions. In all ARC pictures, the scale bar represents 0.5mm.

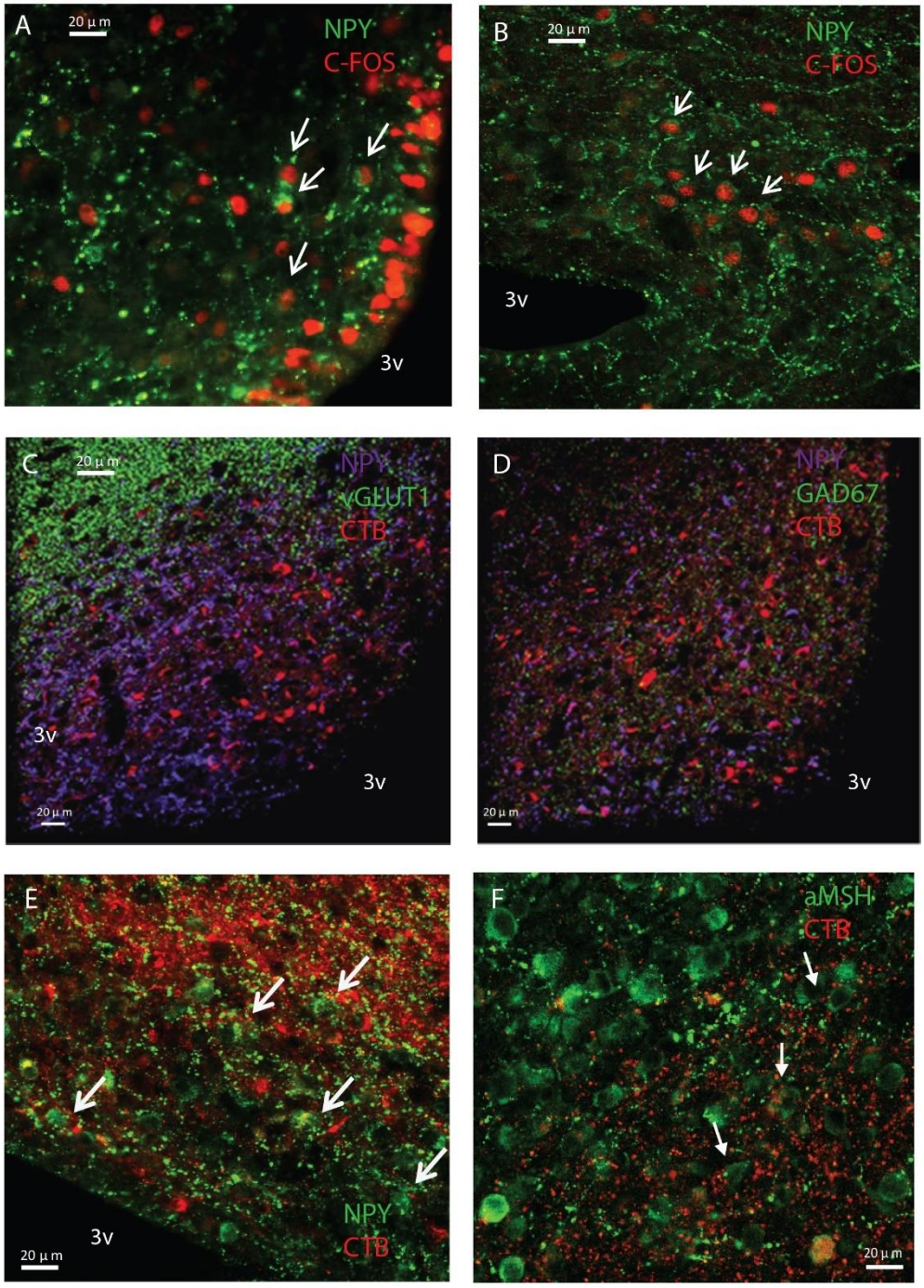

Figure 19. NPY neurons are activated at SCN lesioned site during negative metabolic conditions. a. Double immunofluorescence of c-Fos (red) and NPY (green) immunoreactivity in the ARC. Some NPY neurons after 2DG ICV show c-Fos. b. Double immunofluorescence of c-Fos (red) and NPY (green) expression in the ARC of fasted SCN unilateral lesioned animals. Activated neurons in the ARC are NPY positive at the site corresponding to the lesion of the SCN. c. Triple immunofluorescence of NPY (blue), vGLUT1 (green) and CTb (red). vGLUT1 immunoreactivity is more distributed in the lateral part of the ARC. d. Triple immunofluorescence of NPY (blue), GAD67 (green) and CTb (red). GAD67 is more distributed in the vm part of the ARC. e. Double immunofluorescence for NPY (green) and CTb (red). CTb fibers have contact with NPY neurons (arrows). f. Double immunofluorescence for alpha-MSH (green) and CTb (red). CTb fibers contact alpha-MSH neurons**.**

To compare the activation of the ARC to central and systemic hypoglycemia, we then applied a systemic administration of 2DG. Reflecting the hypoglycemia sensed by the ARC, 2DG IV administration caused an increase in c-Fos expression in the ARC compared to saline infused animals ($F(1,10) = 136.015$; $p<0.001$), showing also a clear time of infusion difference ($F(1,10) = 136.015$; $p<0.001$) with higher c-Fos-IR at ZT11 compared to ZT2 (**Fig 20a, 20b**). Also, here we observed co-localization of c-Fos with NPY in the vm ARC, while we could not find co-localization with alpha-MSH neurons (**Fig. 21**). This indicates that NPY neurons are activated after both cerebral and systemic hypoglycemia and that another, yet unidentified, the population of neurons in the ARC is also glucosensitive and may facilitate the response to systemic hypoglycemia.

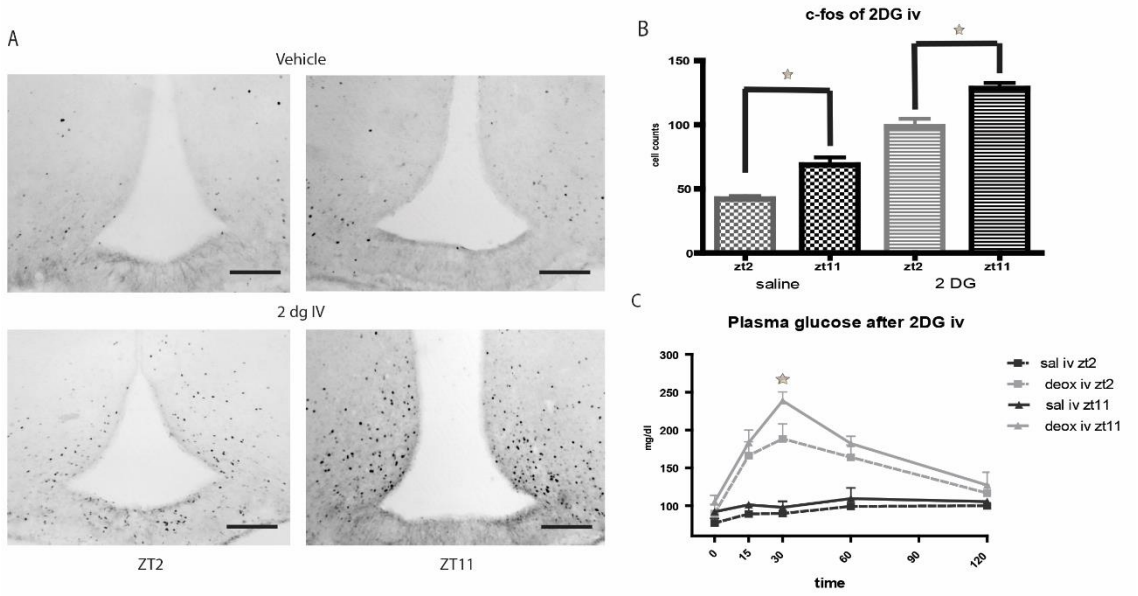

Figure 20. The response of the ARC to systemic hypoglycemia is time modulated. a. c-Fos immunoreactivity in the ARC at ZT2 and ZT11 after saline or 250 mg/kg 2-deoxyglucose (2DG) IV infusion. There is a clear increase of activation at ZT11 compared to ZT2. b. Cell counts of c-Fos protein immunoreactivity after 250 mg/kg 2DG IV infusions at ZT2 and ZT11. There was a significant difference between the two time-points, with a higher expression at ZT11 than ZT2 in saline and 2DG infused animals. Black color indicates ZT11 and gray color indicates ZT2. c. Plasma glucose levels after 250 mg/kg IV infusion of 2DG in ZT2 and ZT11. There is an increase in plasma glucose after 2DG IV infusions at both time points, with a higher increase at ZT11 infusion. The black lines indicate saline infusions and the gray lines indicate 2DG infusions. In all ARC pictures, the scale bar represents 0.5mm.

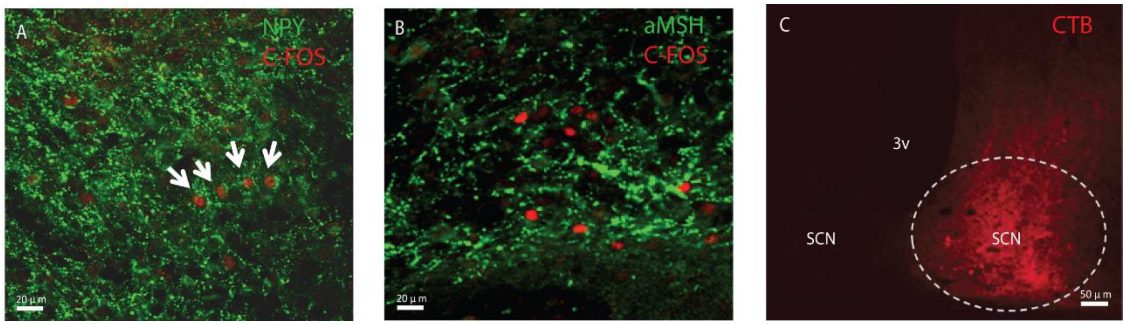

Figure 21. NPY neurons are activated by systemic hypoglycemia. a. Double immunofluorescence of c-Fos (red) and NPY (green) demonstrating c-Fos in NPY neurons after 2 DG IV infusion and b. c-Fos (red) and M S H α (green) immunoreactivity in the ARC after 2 DG IV infusion no presence of c-Fos in M S H α cells could be observed. c. An example of CTb injection into the SCN. The dotted line indicates the area of the SCN.

Similar as with the ICV injections the basal plasma glucose levels after the IV infusions showed time of the day differences ($F(1,15) = 5.82$; $p<0.033$) illustrating the intact daily glucose plasma rhythm. The 2DG infusions induced an increase in plasma glucose levels that was absent in the saline infusion groups ($F(1,15) = 26.218$; $p<0.001$), indicating counterregulatory responses to systemic hypoglycemia. Post hoc test indicated differences in time, in plasma glucose levels at 15 ($p<0.002$), 30 ($p<0.001$) and 60 ($p<0.003$) minutes after the injections, as well as differences between groups infused at ZT2 and ZT11 ($p<0.006$), with higher levels of plasma glucose levels at ZT11 than ZT2 (**Fig 20c**). The higher increase in plasma glucose levels after systemic hypoglycemia at ZT11 was in concert with a higher activation of the ARC, suggesting that the animals were more sensitive to hypoglycemia at the end of the light period than at the beginning of the light period. 2 Hour food intake after the 2DG IV infusions at both time points was increased ($p<0.001$) (ZT2: 4.62 g SEM ± 0.29; ZT11: 5.83 grams SEM ± 0.19) compared to saline IV infused animals (ZT2: 1.05 g SEM ± 0.05; ZT11: 1.68 g SEM ± 0.39); again, indicating a counter-regulatory response to hypoglycemia. Food intake between the 2 time points did not differ significantly (**Fig 22c**).

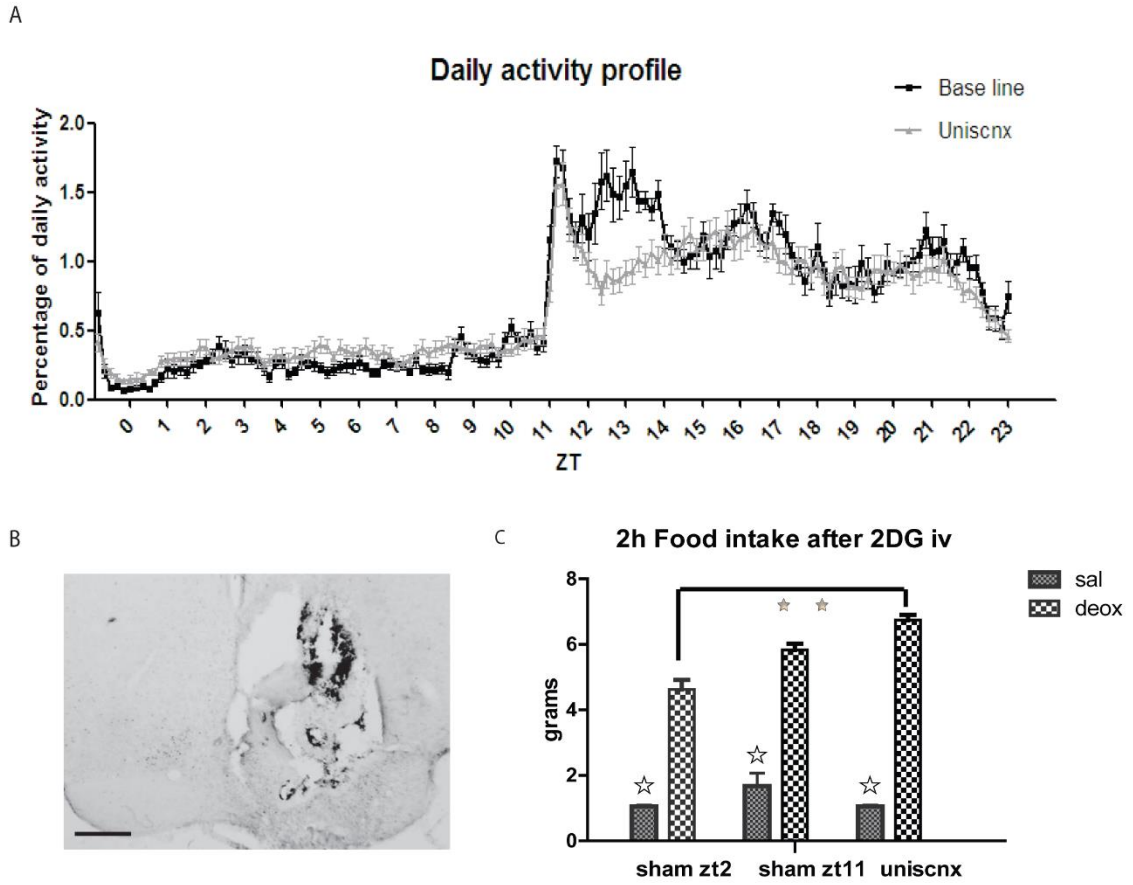

Figure 22. a. Activity profile of sham and unilateral SCN lesioned animals showing the nearly identical activity pattern of these animals. b. An example of a unilateral SCN lesion. The scale

bar represents 0.3mm. c. 2h food intake after 2DG IV in sham animals sacrificed at ZT2 and ZT11 and unilateral SCN lesioned animals sacrificed at ZT2. The unilateral SCN lesioned group (uniSCNX) shows a higher increase in food intake after 2DG IV infusions. The star indicates differences between 2DG IV and saline IV infusions. The double star indicates differences between unilateral SCN lesioned and sham 2DG IV infused animals.

The SCN inhibits the sensitivity of the ARC to hypoglycemia.

Time differences found in the activation of the ARC and counterregulatory responses to hypoglycemia may depend on the SCN directly or may be associated with hormonal or behavioral rhythms driven by the SCN. Therefore, we performed unilateral lesions of the SCN, to conserve the locomotor activity rhythms and hormonal rhythms but to eliminate unilateral projections from the SCN to the ARC. It is already reported that the SCN maintains mainly ipsilateral projections to the ARC (29), so we hypothesized that after a unilateral lesion, the neuronal response to hypoglycemia in the ipsilateral ARC will be largely eliminated.

To test if the SCN was regulating the sensitivity of the ARC to 2DG induced hypoglycemia, we analyzed the activation between the ipsilateral and contralateral sides of the ARC after a unilateral SCN lesion and saline or 2DG IV infusions. Only animals with a complete unilateral lesion of the SCN and an intact SCN at the contralateral side were considered correctly lesioned. To confirm that the unilateral SCN lesioned animals were rhythmic, daily locomotor activity patterns were monitored before and after the lesion. The pattern of activity in sham and unilaterally lesioned animals showed a clear daily rhythm with low activity counts during the day and high activity counts during the night. No difference was observed between groups (**Fig. 22**).

Both sides of the ARC increased their neuronal activity to systemic hypoglycemia as compared to saline infused animals ($F(1, 21) = 76.957$; $p<0.001$) (**Fig 23a, 23b**). A comparison between the percentage of activation of the lesioned and non-lesioned side of the ARC both with infusion of 2DG and saline, showed significant differences ($F(3, 29) = 41.488$; $p<0.001$), suggesting a higher activation of the lesioned side versus the non-lesioned side in both cases ($p<0.001$) (**Fig 23c**). The double immunofluorescence staining for c-Fos and NPY also showed co-localization of the neurons activated and NPY (**Fig 19b**).

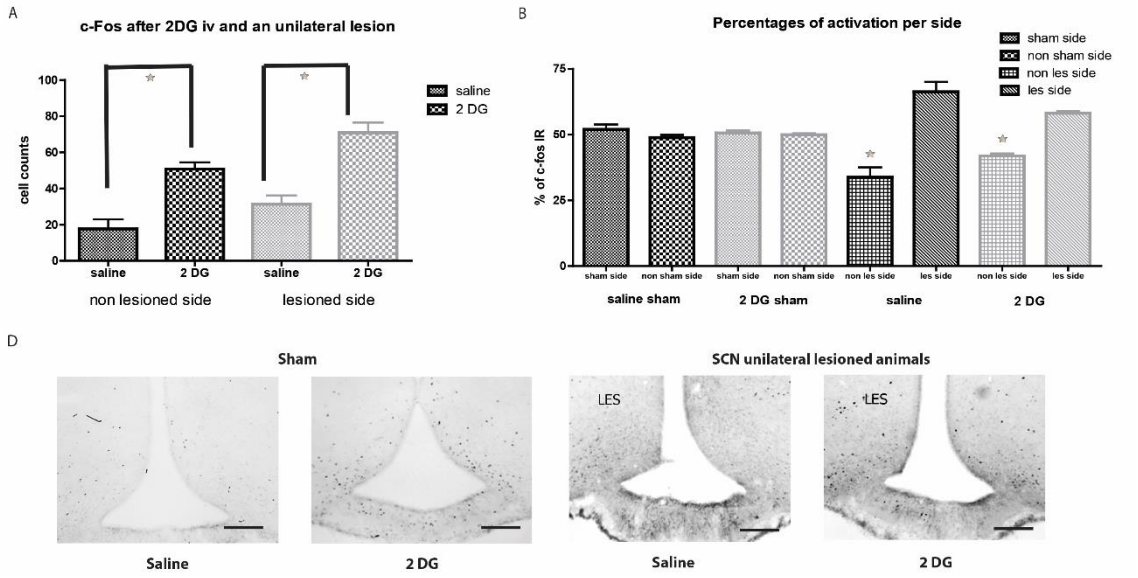

Figure 23. Unilateral lesion of the SCN increases c-Fos unilateral in the ARC after 2DG.

a. Number of c-Fos-IR neurons per side of the ARC after 2DG or saline IV injections in SCN of unilaterally lesioned animals. We found a higher activation with 2DG as compared to the saline infused animals. The black bars indicate the ARC side corresponding to the non-lesioned side of the SCN, while the gray bars indicate the ARC side corresponding to the lesioned side of the SCN. b. Percentages of activation of the ARC side corresponding to the lesioned and non-lesioned side of the SCN of SCN unilateral lesioned animals. We found a difference between sides in saline and 2DG injected animals. The black bars indicate saline IV infusions while the gray color bars indicate 2DG IV infusions. c. c-Fos expression in the ARC of sham and unilaterally lesioned animals sacrificed at ZT2 after an infusion of 250 mg/kg of 2DG or saline IV. An increase in activation in the ARC side corresponding to the lesioned side of the SCN compared to the ARC side corresponding to the non-lesioned side of the SCN is evident with both infusions of 2DG and saline. In all ARC pictures, the scale bar represents 0.5mm.

To assess whether the changes in activation of the ARC after unilateral lesion of the SCN affect counterregulatory responses to hypoglycemia, plasma glucose levels, and food intake were evaluated. The basal plasma glucose levels showed no difference between the unilaterally lesioned groups and the non-lesioned animals, with the normal circadian variation in blood glucose levels. After the 2DG IV infusion, the unilaterally lesioned animals developed hyperglycemia, with a notorious increase at 15, 30 and 60 minutes after the infusion (**Fig. 24a**). The ANOVA indicated differences in time ($F(4,12) = 26.417$; $p<0.001$), substance administered ($F(1,15) = 27.957$; $p<0.001$) and lesion ($F(1,15) = .299$; $p<0.049$). The food intake after the 2DG IV infusion also showed significant differences ($F(3, 29) = 102.080$; $p<0.001$) in the unilaterally lesioned animals compared to sham-lesioned. In the unilaterally lesioned animals, 2DG IV induced more food intake than in the sham group at ZT2 ($p<0.001$), no differences in food intake were found between unilateral SCN lesioned animals infused at ZT2 and sham animals after 2DG IV at ZT11 ($p<0.563$) (**Fig. 22c**). These results indicate that the SCN regulates the sensitivity of the animals to hypoglycemia, probably inhibiting the neural response of NPY neurons in the ARC under hypoglycemic and euglycemic conditions.

To assess if indeed, as the c-Fos data suggested, the ARC was responsible for the 2DG induced hyperglycemia observed after a unilateral disinhibition of the ARC due to the unilateral removal of the inhibitory influence of the SCN, we performed unilateral ARC 2DG retrodialysis at ZT2. After assessment of correct ARC probe placement by Nissl histology (**Fig. 24c**), we investigated the effects of 2DG or ringer solution retrodialysis in the ARC on plasma glucose. An additional misplaced probe group (**Fig. 24d**), mainly in the VMH, was also considered. ANOVA indicated differences in time ($F(4, 60) = 11.75$; $p<0.0001$) and treatment ($F(3, 15) = 49.25$; $p<0.0001$) between the ringer and 2DG infused groups. An increase of plasma glucose was only observed in the 2DG infused groups at 10 minutes after the starting of infusion (**Fig. 24b**). We observed a steady plasma glucose increase until the end of the infusion after 80 minutes; indicating that 2DG direct infusions in the ARC and VMH result in stimulation of hypoglycemia counterregulatory responses. When comparing the increase of plasma glucose between the ARC probe placed and the VMH probe placed groups, a higher plasma glucose increase was observed in the ARC probe placed group after 20 minutes of starting the 2DG infusion ($p<0.001$), the ARC plasma glucose stimulation persisted until 80 minutes ($p<0.001$), the end of the infusion (**Fig. 24b**). These results demonstrate that the ARC can provide hypoglycemia counterregulatory responses and may mediate the 2DG induced plasma glucose increase observed after SCN unilateral lesions.

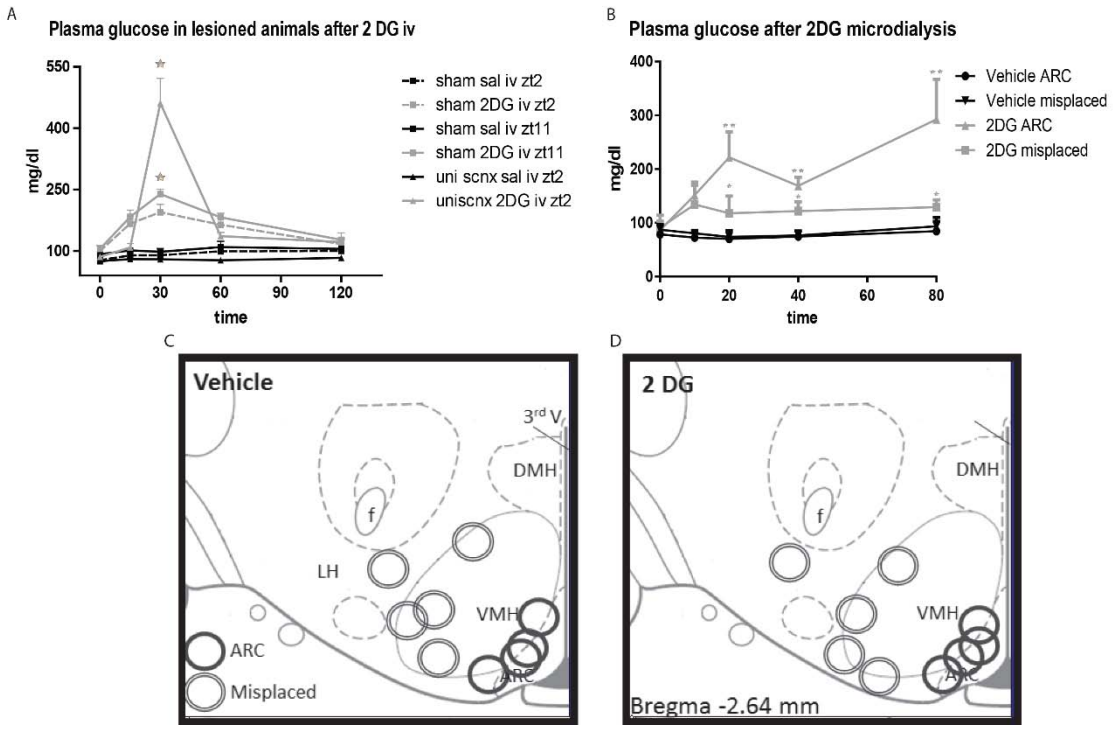

Figure 24. Unilateral lesion of the SCN enhances plasma glucose counterregulatory response to 2DG. a. Plasma glucose of sham and unilateral SCN lesioned 2DG IV infused animals. The increase in glucose after 2 DG is higher at ZT11 in sham animals. The major increase observed is present in the unilateral SCN lesioned animals. Unilateral SCN lesioned animals (uniSCNX). b. Plasma glucose during unilateral 2DG microdialysis in the ARC. 2DG increases plasma glucose after 2DG infusions in the ARC and the VMH (misplaced). ARC 2DG administration enhances the rise in plasma glucose. One star symbolizes differences with the vehicle group, two stars symbolize differences between ARC and VMH 2DG administered groups. c. Representative image of the

ARC microdialysis probe placements. d. Representative image of the VMH microdialysis probe placements.

The SCN inhibits the sensitivity of the ARC to fasting.

To assess if the SCN inhibitory influence observed during hypoglycemia, was also present in a normal catabolic physiological condition, the neuronal activation of the ARC ipsilateral and contralateral to the SCN lesioned side after 48 hours fasting, was evaluated in sham and unilateral SCN lesioned animals. The ANOVA indicated differences between the unilateral lesioned and the sham group ($F(2, 20) = 31.857$; $p<0.001$) in the number of cells activated in the ARC after fasting at zt2 (**Fig. 25a, 24c**) and zt11 (**Fig. 25b, 25d**). Differences between the lesioned and the non-lesioned side were observed in the animals sacrificed at both time points ($p<0.01$), whereby the ARC ipsilateral to the SCN-lesioned side (LS) showed the highest c-Fos as compared to the ARC contralateral to the lesioned site (non-LS). Moreover, the SCN-intact site of the ARC at ZT2 showed a higher number of c-Fos-IR than the ARC of the sham fasted group ($p<0.03$). The higher c-Fos staining in the ARC at the SCN lesioned site indicates that the SCN inhibits via neuronal pathways the response of the ipsilateral ARC to fasting. The difference observed between the intact side of the lesioned and the sham animals at ZT2 indicates that the SCN also inhibits the contralateral side but to a lesser extent.

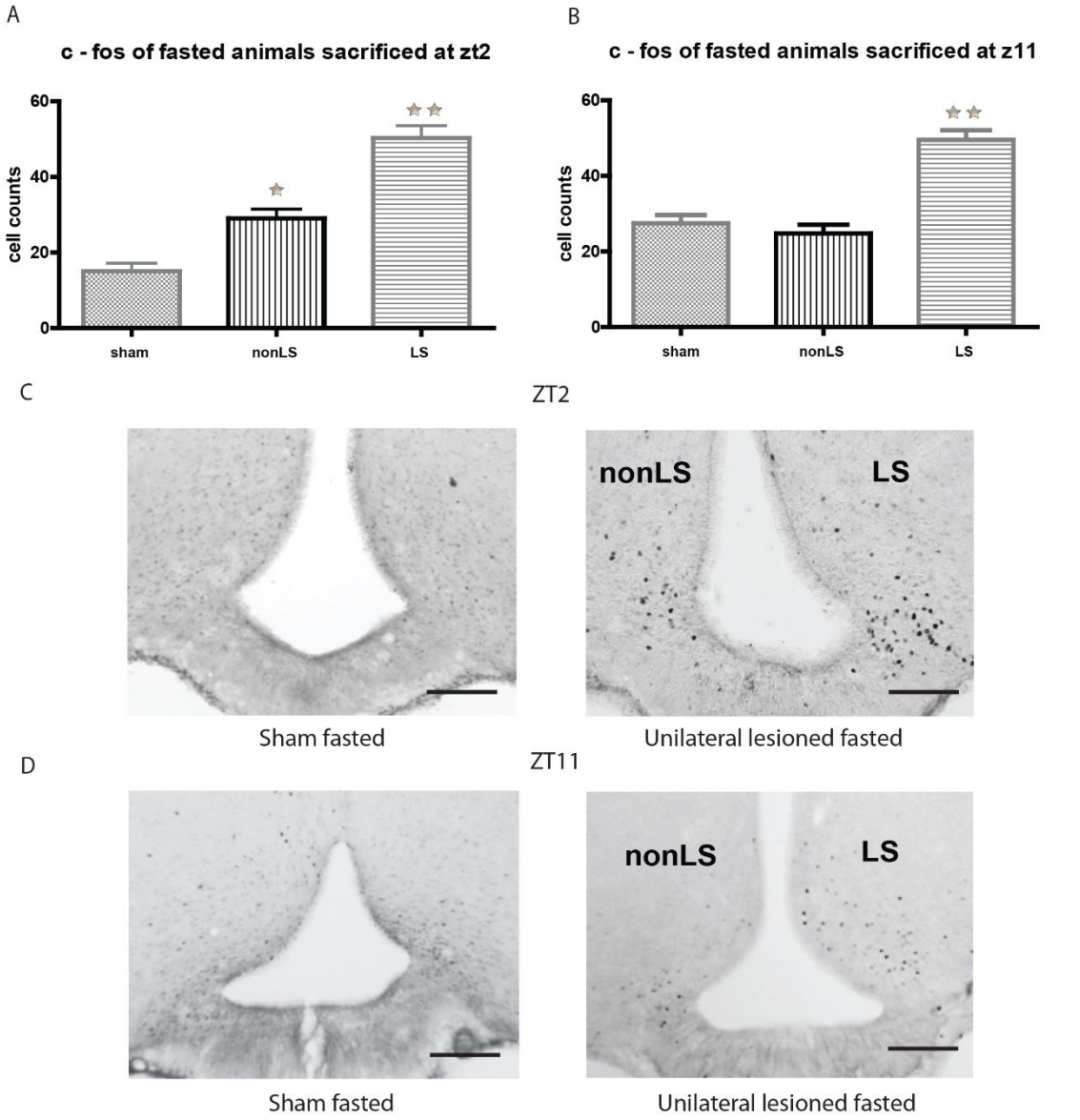

Figure 25. Unilateral lesion of the SCN increases c-Fos in the ARC unilaterally after fasting. a. Cell counts of c-Fos-immunoreactive neurons in the ARC of SCN unilateral lesioned animals fasted for 48h and sacrificed at ZT2. We found an increase of c-Fos immunoreactivity at the lesioned side and non- lesioned side. The star indicates differences between sham and non-lesioned site. The double star indicates differences between lesioned and non-lesioned site. b. Cell counts of c-Fos-immunoreactive neurons in the ARC of SCN unilateral lesioned animals fasted for 48h and sacrificed at ZT11. We found an increase of c-Fos immunoreactivity at the lesioned side and non-lesioned side. The double star indicates differences between lesioned and non-lesioned site. c. c-Fos immunoreactivity in the ARC of sham and unilateral lesioned fasted animals sacrificed at ZT2. An increase of c-Fos in the lesioned side compared to the non-lesioned side was evident in the lesioned animals. Non-LS stands for non-lesion side and LS stands for lesion side. d. c-Fos immunoreactivity in the ARC of sham and unilateral lesioned fasted animals sacrificed at ZT11. An increase of c-Fos in the lesioned side compared to the non-lesioned side was evident in the lesioned animals. Non-LS stands for non-lesion side and LS stands for lesion side. In all ARC pictures, the scale bar represents 0.5mm.

The SCN projects to ARC NPY and alpha-MSH neurons.

To explore a possible mechanism mediating the modulation of the SCN over the ARC glucose sensing, we performed CTb tracing experiments, with the hypothesis that the SCN will project to the neuronal populations NPY and alpha-MSH in the ARC. The immunofluorescence staining for v-GLUT1 and GAD-67 showed a predominance of GABA processes in the VM ARC, while glutamatergic processes were observed predominantly in the lateral part of the ARC (**Fig. 19 c, 19d**), indicating that the main inhibitory input to the ARC is in the VM part, where mainly the NPY/AgRP neurons are located. The pattern of innervation in the ARC coming from the SCN, illustrated by the presence of CTb fibers, was mainly ipsilateral and directed to the lateral part of the ARC, with some lesser axons directed to the VM part of the ARC. This suggests that the main input from the SCN to the ARC is in the lateral part where mainly the alpha-MSH neurons are located. Combining immunofluorescence for alpha-MSH and NPY with CTb staining we observed that both neuronal populations were in contact with fibers coming from the SCN (**Fig. 19e, 19f**), confirming the interaction between the SCN and ARC NPY and alpha-MSH neurons.

Discussion

Here we demonstrated a time-dependent hypoglycemic induced activation of the ARC as result of a direct neuronal interaction between the SCN and the ARC, suggesting that the SCN may gate sensory information at the level of the ARC. Evidence for the gating of this information is that after unilateral lesioning of the SCN not only the ARC shows a higher neuronal activity but that also the glucose counterregulatory response to the hypoglycemic stimulus is enhanced.

The time-dependent neuronal activation in the ARC after systemic hypoglycemia, correlated with the counter-regulatory response as observed by the increase of food intake and plasma glucose levels. The time of the day difference in activation after the same hypoglycemic stimulus indicates that there is a rhythm in the central capacity to respond to low plasma glucose levels. This rhythm, characterized by a higher sensitivity of the ARC to hypoglycemia at the end of the resting period, correlates with the already reported higher tolerance to glucose and higher plasma glucose levels at this time[198–200]. Strikingly, the temporal modulation of the ARC neural activity after systemic hypoglycemia was accompanied by a clear time of the day related increase in plasma glucose levels, which was not observed when central hypoglycemia was induced. We hypothesize that when peripheral glucose levels fall, the decrease in the plasma glucose is first detected by glucose sensitive neurons located in or close to the circumventricular organs to regulate the counterregulatory responses to the hypoglycemic event. This hypothesis was supported by retrodialysis administration of 2DG directly in the ARC, which resulted in a more pronounced increase of plasma glucose levels than when 2DG was administered in the VMH.

Areas like the VMH, the Paraventricular nucleus of the hypothalamus (PVN), the Dorsomedial nucleus of the hypothalamus (DMH) and the Lateral hypothalamus (LH) have been described as glucose sensitive areas, part of circuits that contribute to the triggering of counterregulatory responses to low glucose levels, but that only later may contribute later to the response[207,218–221]. This agrees with the observation of an absence of a time of the day difference in glucose response after intracerebral induced hypoglycemia and the absence of a clear time of the day

difference in the activation of other hypothalamic nuclei, except the ARC, after central 2DG induced hypoglycemia (data not shown). Therefore, we propose that specifically, the SCN targets the ARC to change its sensitivity for a time of the day dependent hypoglycemic event, allowing the ARC to counteract hypoglycemia more aggressively when glucose levels are expected to be high. When 2DG is administered ICV, 2DG can access all hypothalamic areas like the LH, PVN, and VMH simultaneously triggering counterregulatory responses to hypoglycemia breaking the normal sequence of events as occurs after systemic hypoglycemia.

In addition, the present results indicate that time-dependent modulation of sensing hypoglycemia is directed by the SCN in an inhibitory manner. This was illustrated after fasting and hypoglycemia in unilateral SCN lesioned animals by the increase of NPY activation on the lesioned side of the ARC nucleus. This increase in ARC activation was accompanied by higher plasma glucose levels and more food intake after the administration of 2DG, indicating that the animals were more sensitive to hypoglycemia. This agrees with observations that HGP, plasma glucose levels, and glucose tolerance have a circadian rhythm driven by the SCN, and that the SCN influences the glucose homeostasis in an inhibitory manner[198–200]. Furthermore, it was shown that the SCN and the ventromedial ARC have reciprocal projections[216]. The reciprocal interaction between these two nuclei suggests that the SCN can also modulate the functionality of the ARC. Our data showed that the SCN can inhibit the response of the ARC to low energy states and modulate the counterregulatory responses to hypoglycemia.

The sensing of hypoglycemia and fasting might be mediated by different mechanisms. Besides the decrease of glucose levels during fasting conditions, there is a clear increase in circulating ghrelin which can activate NPY neurons in the ARC to motivate food intake [65,222]. Satoh[223] showed that the administration of ghrelin at the beginning of the light period generates less c-Fos-IR in the ARC than at other time points, which not only supports our data but also suggests that apart from the low glucose levels during fasting, the SCN may also influence the sensitivity of the ARC to plasma ghrelin levels. Still, during the 2DG induced hypoglycemia, the ARC may sense primarily the decrease in glucose levels, which is not accompanied by an increase in plasma ghrelin levels [222]. Interestingly the ARC also shows a rhythm of c-Fos-IR during ad libitum conditions which shows a clear peak in the dark period (ZT22), mainly in the alpha-MSH area of the ARC. During light exposure at night and in the light phase of the animals the ARC c-Fos was significantly lower[95]. Our data confirmed an inhibitory influence of the SCN over the ARC response to negative metabolic conditions during the light phase. At the other hand in the study of Guzman et al[224], not only an inhibitory influence of the SCN on the ARC was illustrated but also a stimulatory input at night, this stimulatory input was shown especially relevant for an adequate diurnal temperature control. Consequently, these and our studies illustrate the presence of both excitatory and inhibitory projections of the SCN to the ARC, that vary over the day-night cycle, and the importance of SCN-ARC interaction for the control of different physiological functions.

In addition to the ARC, also the liver[68] and brain areas like the area postrema and NTS in the brainstem might be involved indirectly sensing decreased circulating glucose levels. It is known that the NTS has GI neurons that respond to hypoglycemia and that the hypothalamus and NTS maintain a functional interaction that can modify the primary activation of the ARC to hypoglycaemia[225,226]. Our results indicate that under 2DG induced hypoglycemic conditions,

the inhibitory influence of the SCN over the activation of ARC neurons to hypoglycemia during the resting period is an important contributor to the counterregulatory response.

In conclusion, our results showed that next to the rhythm in plasma glucose, the SCN also controls a time modulated glucose sensing in the ARC which can influence the responses to low energy like hypoglycemia and fasting. Therefore, we propose that the SCN-ARC interaction serves to gate information of the metabolic state of the body to the brain, to adjust the physiological response in a circadian manner.

Chapter III. Based On:

The arcuate nucleus: a site of fast negative feedback for corticosterone secretion

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Abstract

Variations in circulating Cort are driven by the Paraventricular nucleus of the hypothalamus (PVN), mainly via the sympathetic autonomic nervous system (ANS) directly stimulating Cort release from the adrenal gland and via CRH targeting the adenohypophysis to release ACTH. Cort feeds back through glucocorticoid receptors (GR). Here we show in male Wistar rats that PVN neurons projecting to the adrenal gland do not express GR, leaving the question how the ANS in the PVN gets information about circulating Cort to control the adrenal. Since the arcuate nucleus (ARC) shows less restrictive blood brain barrier, expresses GR and projects to the PVN, we investigated whether the ARC can detect and produce fast adjustments of circulating Cort. In low Cort conditions (morning), local microdialysis in the ARC with type I GR antagonist produced a fast and sustained increase of Cort. This was not observed with type II antagonist. At the circadian peak of Cort (afternoon), type II GR antagonist but not type I antagonist increased Cort levels but not ACTH levels. Antagonist infusions in the PVN did not modify circulating Cort levels, demonstrating the specificity of the ARC to give Cort negative feedback. Furthermore, type I and II GR agonists in the ARC prevented the increase of Cort after stress, demonstrating the role of the ARC as a sensor to modulate Cort release. Our findings show that the ARC may be essential to sense blood levels of Cort and adapt Cort secretion depending on conditions as stress or time of the day.

Significance Statement: Cort secretion is importantly driven by pre-autonomic sympathetic neurons in the PVN, while negative feedback of glucocorticoids is generally expected to be mediated via the interaction of CRH neurons in the PVN and corticotrophs in the hypophysis. Here, we demonstrate that the arcuate nucleus rapidly senses circulating Cort and thus adjusts the endocrine adrenal output via the pre-autonomic neurons in the PVN. This fast control may be an alternative mechanism used by circulating hormones and metabolites to communicate with control centers in the brain via the arcuate nucleus for fast feedback.

Keywords: Glucocorticoids, arcuate, paraventricular nucleus, glucocorticoid receptor, negative feedback, ACTH

Introduction

The control of glucocorticoids (GCs) -cortisol and Cort- is known to be mediated by the endocrine portion of the PVN modulating the secretion of ACTH from the pituitary via the release of corticotrophin releasing hormone (CRH) and vasopressin in the median eminence to the portal circulation [125,227]. However, rats maintained with constant circulating ACTH after hypophysectomy still display a clear circadian peak of Cort before the activity period [228]. In contrast, this peak is lost after denervation of the adrenal gland [229,230], demonstrating the

necessity of the autonomic input to the adrenal gland to release Cort in a circadian manner, probably due to modifications of sensitivity to ACTH in the adrenal gland [230–232].

In the hypothalamus, Cort binds to two receptors: Type I or mineralocorticoid receptor (MR) and Type II or glucocorticoid receptor (GR) [117]. When Cort binds to these receptors, the complex is quickly translocated from the cytoplasm to the nucleus in a dose and time-dependent manner [107]. Type I receptor has ten times more affinity for Cort and is rapidly saturated, while type II has lower affinity to Cort and is a high capacity receptor. Type I has been described as an important component of basal Cort homeostasis due to the high occupation under low Cort situations, type II receptor is thought to respond mainly to high levels of Cort [117–120].

Cort regulation also requires a system of negative feedback to the central nervous system, which has been predominantly attributed to the cortex, amygdala, hippocampus, PVN or the pituitary gland[233] where GR is highly expressed[184,234]. Although the pituitary gland expresses receptors to GCs, the fact that Cort can increase independently of appreciable modification of ACTH levels indicates the presence of additional regulatory mechanisms[127,235]. Moreover, when GR is knocked down selectively in the PVN, the circadian release of Cort remains unaffected and the stress response is only slightly modified [236]. These results challenge the view of the PVN sensing GCs to give an effective negative feedback.

An indication for alternative control mechanisms for Cort secretion came after polysynaptic retrograde neuronal tracing from the adrenal cortex, identifying first order neurons in the intermediolateral column (IML), followed by neurons in the PVN and third order neurons in the Arcuate Nucleus (ARC). Consequently, we hypothesized that the identified projections from the ARC to PVN neurons projecting to the adrenal may serve to integrate information about circulating Cort to fine-tune endocrine function[237].

In the ARC, Type I and Type II glucocorticoid receptors are highly expressed [147,183,234]. ARC Type II receptor concentration is one of the highest within the brain together with the hippocampus and PVN[183]. In the ARC, two major subsets of neurons have been reported to be responsive to Cort with electrophysiological and transcriptional changes [158,161,162]: one co-expresses the orexigenic Agouti Related peptide (AgRP) and neuropeptide Y (NPY), while alpha-melanocyte stimulating hormone (alpha-MSH), an anorexigenic peptide, is present in the other population together with the cocaine and amphetamine-related transcript (CART)[238].

Here, we provide evidence that GR dispersion of GR after adrenalectomy is rapidly reversed in the ARC after IV Cort administration, whereas in other brain regions including the PVN it is reversed later. In addition, we illustrate the absence of GR in pre-autonomic neurons of the PVN projecting to the adrenal gland. This result, together with a specific response to GR antagonist in the ARC but not in the PVN, points the ARC as the site to control Cort release via the ANS. Retrodialysis in the ARC with GR agonists and antagonists at different time points or after stress indeed established the role of the ARC as a sensor of hormonal fluctuations in the circulation to fine tune Cort release under different physiological conditions. This circuit may comprise projections from AgRP neurons to the pre-autonomic part of the PVN, which is connected by a polysynaptic pathway to the adrenal gland.

Materials and methods

Animal Handling

All animal experiments were performed in accordance with the authors' university animal care committee's regulations. Male Wistar rats weighing 250–350 g (Animal facility of the authors' university) were housed in individual cages, with a 12/12-h light-dark schedule (lights on at 0700 h, as Zeitgeber time [ZT] 0). The number of animals used was reduced according to ethical dispositions. Standard laboratory rodent diet (Lab diet, 5013) and tap water were provided ad libitum. At the end of the protocols, all rats were deeply anesthetized with pentobarbital (100 mg/Kg, Pisabental, Pisa Agropecuaria SA de CV) and perfused transcardially with 0.9% saline followed by a solution of 4% paraformaldehyde (PFA) in 0.1M PB (pH 7.4). Brains were removed and kept in PFA 4% at 4°C for 24 hrs. and subsequently changed to 30% sucrose with 0.02% sodium azide in 0.1M PBS for cryoprotection until cut.

Experimental design

To determine the GR's rapid response to circulating CORT rats were randomly assigned to 1 of 4 groups. Intact (n=6), adrenalectomy + vehicle (8), adrenalectomy + Cort (n=6), adrenalectomy + Dex (n=10). After one week of recovery, the animals were given intravenous (IV) sterile saline (100 µL), dexamethasone (100 µL, 0.05 mg/kg, Sigma-Aldrich) or Cort: HBC complex (100 µL, 0.5 mg /kg of Cort, Sigma-Aldrich).

To determine the role of the ARC or PVN for GCs negative feedback rats were randomly assigned to one of the following groups: rats bearing bilateral cannulas in the PVN retrodialyzed with vehicle (n=4) or Type I GR antagonist (n=4) (eplerenone 160 µmol/L, Sigma-Aldrich), rats bearing bilateral cannulas in the ARC retrodialyzed with vehicle (n=4) or Type I GR antagonist (n=4) (eplerenone 160 µmol/L, Sigma-Aldrich).

In order to determine the participation of type I and II GR in the ARC for circadian regulation of Cort, rats bearing bilateral cannulas in the ARC were assigned to one of the following groups: Vehicle (n=4), Type I GR agonist (n=4) (fludrocortisone 180 µmol/L, Sigma-Aldrich) or Type I GR antagonist (n=4) (eplerenone 160 µmol/L, Sigma-Aldrich); Type II GR agonist (n=5) (dexamethasone, 200 µmol/L, Sigma-Aldrich), Type II GR antagonist (n=4) (mifepristone, 180 µmol/L, Sigma-Aldrich) infusion at ZT2. At ZT 10, rats bearing bilateral cannulas in the ARC were assigned to treatment with vehicle (n=4), type I GR agonist (n=4), Type I GR antagonist (n=4), Type II GR agonist (n=5) Type II GR antagonist (n=4).

To further explore the capacity of GC's to give negative feedback in the ARC animals were exposed to mild stress. Animals bearing bilateral cannulas in the ARC were assigned randomly to the following groups: No stress + vehicle (n=4), no stress + Dex (n=5), no stress + Fludrocortisone (n=4), Stress + Vehicle (n=4), stress + Dex (n=4), Stress + Fludrocortisone (n=4).

Transneuronal tracing.

Animals were anesthetized with sodium pentobarbital (50 mg/kg). 3ul of the viral suspension of pseudorabies virus (PRV), Bartha strain (containing 1 x 10^6 plaque-forming units, was pressure injected into the left adrenal with a 30-gauge needle attached to a Hamilton syringe. Evans blue was added to the viral suspension to allow visual inspection of the injection site. Animals survived for 3-4 days.

Adrenalectomy.

Animals underwent surgeries according to the different experimental designs under anesthesia with intramuscular Ketamine (40-80 mg/kg, Anesket, Pisa Agropecuaria SA de CV) and intraperitoneal Xylazine (5-8 mg/kg, Procin, Pisa Agropecuaria SA de CV). The adrenal glands were removed bilaterally by a cut on the back of the animal and silicon catheters (Silastic) were inserted into the right jugular vein. After one week of recovery, the animals were given IV sterile saline (100 µL), dexamethasone (0.05 mg/kg, Sigma-Aldrich) or Cort: HBC complex (0.5 mg /kg of Cort, Sigma-Aldrich). The animals were sacrificed 7 min after the injection as described before.

Cannulation and stereotactic surgery for retrodialysis.

Animals were anesthetized with Ketamine and Xylazine as described before. Silicon catheters (Silastic) were inserted into the right jugular vein for blood sampling. Home-made microdialysis probes were placed bilaterally into the ARC or the PVN with a standard Kopf stereotaxic apparatus. Coordinates were adapted from the atlas of Paxinos and Watson 2007[217] (PVN: Anteroposterior -1.1 mm, lateral + 0.9 mm, ventral -6.9 mm, tooth bar -2.5°, lateral angle 4°. ARC: Anteroposterior -2.4 mm, lateral +1.7 mm, ventral -9.5 mm, tooth bar -3.4°). The catheter and microdialysis probes were fixed on top of the skull with stainless steel screws and secured with dental acrylic. The jugular catheter was maintained permeable with a solution of 45% glycerol, 40% isotonic saline, 10% heparin (5000 IU/ml, Inhepar, Pisa Agropecuaria SA de CV, México) and 5% Antibiotic Mixture 100X, (Gibco) replaced each two days.

Retrodialysis

For bilateral retrodialysis, the probes were placed into the PVN or ARC. The position of the cannulas was verified at the end of the protocol by histological examination with Nissl staining. Animals with misplaced probes were excluded from analysis. After one week of recovery, animals were connected to a multichannel fluid infusion swivel (Instech Laboratories) 1 day before the experiment for adaptation to prevent any effect of stress on the day of the experiment.

Morning experiments: The day of the experiment, the animals with the bilateral probe in the ARC received retrodialysis of ringer solution (3 µL/min) from ZT0 to ZT2. Next, the ringer solution of retrodialysis was replaced by the corresponding drugs at the following concentrations at the same infusion rate: Vehicle, Type II GR agonist (dexamethasone, 200 µmol/L, Sigma-Aldrich), Type II GR antagonist (mifepristone, 180 µmol/L, Sigma-Aldrich), Type I GR agonist (fludrocortisone 180 µmol/L, Sigma-Aldrich) or Type I GR antagonist (eplerenone 160 µmol/L, Sigma-Aldrich). 200 µliters of blood were taken 5 minutes before and 5, 10, 20, 40 and 80 min after starting the drug retrodialysis. Drugs were dissolved in EtOH 0.5% in Ringer used as vehicle solution.

Animals with PVN bilateral probe received retrodialysis of ringer followed by Vehicle or eplerenone as described before.

Afternoon experiments: The day of the experiment, the animals received retrodialysis of ringer solution from ZT 8 to ZT 10. After, the ringer solution was replaced by the corresponding drugs and blood samples were taken as described before.

Stress experiments: Animals received retrodialysis of ringer solution (3 µL/min) from ZT0 to ZT2. Subsequently, stress was induced by transferring the animal to a new clean home cage, just before the change of drug infusion by retrodialysis. Animals were assigned randomly to the following groups: No stress + vehicle, no stress + Dex, no stress + Fludrocortisone, Stress + Vehicle, stress + Dex, Stress + Fludrocortisone.

Immunohistochemistry:

Brains were cut in 40 µm coronal sections with a cryostat (Microm HM550) and stored in 30% sucrose with 0.02% sodium azide in 0.1 M PBS until staining. Sections for immunolabeling (Six per region per animal) were collected and rinsed in 0.1M PBS and then incubated 10 min with hydrogen peroxide 3% in 0.1 PBS, followed by overnight incubation with blocking solution (0.1% fraction V bovine albumin, 0.2% triton X-100 in 0.1M PBS). All the antibodies were diluted in blocking solution. The primary antibodies were used as follows: rabbit anti-PRV (1:150 000, anti-alpha Aujerszky, a generous donation of Dr. H. Pol, ID.DLO, Lelystad, the Netherlands), rabbit anti-GR (1: 16000; homemade[183]). The primary antibody was incubated 1hr at room temperature and then 24-48 hrs. under constant shaking at 4°C; afterward, sections were rinsed with wash buffer and incubated 1 hr. at room temperature with the corresponding biotinylated secondary antibody (donkey anti-rabbit,1:1000; Jackson, donkey anti-goat,1:1000; Jackson), rinsed, and incubated in avidin- biotin complex (1:500 Vector Laboratories) for 1 hour. Product visualization was obtained with 0.01% diaminobenzidine (DAB), 0.05% nickel ammonium sulfate, and 0.01% hydrogen peroxide in TBS 0.1M for 8 to 10 minutes. When a second staining was performed, the same sections were incubated as described before with primary antibody followed by incubation with biotinylated secondary antibody and after incubated in the avidin-biotin complex. Product visualization was obtained with 0.01% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in TBS 0.1 M for 5 to 8 minutes. The sections were rinsed and changed to a solution of gelatin 0.25% and 0.04% sodium azide in PBS 0.1 M and placed on gelatinized glass slides. After drying, the sections were dehydrated with alcohol and xylene and coverslipped with Entellan (Merk).

For fluorescent immunohistochemistry, after incubation with the primary antibody, sections were incubated 1hr with donkey anti-rabbit Cy2 (1:500 Jackson), donkey anti-goat (1:500 Jackson) or donkey anti-sheep (1:500 Jackson) as convenient, rinsed with washing buffer and mounted on gelatin-coated slides. Finally, slides were covered slipped with Mowiol mounting medium. Bright-field photomicrographs were taken with a digital camera (Lumenera, Infinity 2-5 URFC color) and the software Infinity Capture and Analyze, fluorescent photomicrographs were acquired with confocal microscope (Nikon, A1R+STORM)

In situ hybridization

Probes: The sequences used to AgRP were: Forward primer TCCCAGAGTTCTCAGGTCTAAGTC (Sigma-Aldrich), Reverse primer ACAGCGACGCGGAGAACGAGA (Sigma-Aldrich), the product length was 134 bp. To POMC were: Forward primer AGGTGTACCCCAATGTTCGC (Sigma-Aldrich), Reverse primer ACCCTCACTGGCCCTTCTTG (Sigma-Aldrich), the final product length was 476 bp. The reverse primers were labeled in 5' with T7 polymerase promoter.

DIG-RNA labeled was obtained using 200 ng purified primer, 2 µL DIG labeling Mix (Roche), 2 µL T7 RNA and polymerase (Roche) 2 µL 10x concentrated transcription buffer (Roche), 40U

RNase out (Invitrogen) and MiliQ DEPC water to reach 20µ of total reaction volume. The mix was incubated for 2 hr. at 37°C in water bath. To improve labeling reaction, 1µ of RNA polymerase was added to the reaction mixture and was incubated 1 hr. at 37°C. Afterwards, 2µL of 0.2 M EDTA pH 8.0, 2.5 µL 4 M LiCl and 75 µL pre-chilled (in -20°C freezer) 100% ethanol were added to the reaction mix and incubated overnight at -20°C. 50 µL of pre-chilled ethanol were added, the mix was centrifuged 10 min at 14000 rpm at 4 °C, the supernatant was removed and the tube was left open in ice to dry for 10 min. The samples were resuspended in 100 µL of sterile RNase-free water.

The DIG-labeling reaction efficiency was determined by spot assay. The RNA dilutions were spotted on Nylon Membrane (Zeta Probe Bio-Rad) incubated with anti-DIG-POD (Roche) and revealed with DAB. The color intensity was compared between labeled control RNA (Roche) and problem samples. Optimal probe concentration was confirmed in brain slices.

Hybridization: Brains were removed and kept in PFA 4% at 4°C for 24 hrs., and subsequently changed to a solution of 30% sucrose RNase-free with 0.02% sodium azide in 0.1M PBS for cryoprotection until cut. Brains were cut in 40 µm coronal sections with a cryostat (Microm HM550) and stored in 0.1 M PBS until staining.

Sections (Six per region per animal) were collected and rinsed 10 min in 0.1M PBS, then fixed in 4% PFA in PBS for 15 min and rinsed with triton x100 0.03% in PBS RNase-free (washing buffer). Afterwards, the sections were treated 30 min with active DEPC 0.1% in 0.1 M PBS, rinsed with washing buffer and incubated 30 min with hydrogen peroxide 3% in 0.1 PBS, rinsed with washing buffer and incubated 10 min at RT with 0.25% acetic anhydride in 0.1M 0.1 M triethanolamine/MiliQ DEPC water, rinsed and incubate 1 hr. at 63°C with hybridization solution without probe (4x SSC, 50% deionized formamide, 1x Denhardt's solution, 0.02% ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin) in a hybridization oven (Boekel scientific, model 230402). The sections were then incubated overnight at 63°c with RNA probes previously denatured in hybridization solution for 5 minutes at 85°C in a water bath followed by ice immersion for 5 min. After incubation, the sections were washed with 2x SSC without formamide at RT, and successive washings with 5x SSC with 50% formamide, 2x SSC with 50% formamide and 0.2x SSC 50% formamide for 15 minutes at 63°C and finally 2x SSC for 5 min at RT. Subsequently, the tissue was incubated in buffer 1 (100 mL Tris-HCl 1M pH 7.4, 30 mL NaCl 5M and 870 mL miliQ water treated with DEPC) for 5 minutes and incubated with blocking solution 1% (Roche) in buffer 1 for 30 min. Next, the sections were incubated with sheep anti-DIG-POD antibody (1:1000, Roche) in buffer 1 with blocking solution 2hr at RT. Sections were rinsed with washing buffer and the signal was amplified with TSA Plus Biotin Kit (Perkin Elmer) as indicated by the provider. Finally, the sections were incubated with streptavidin-AP 1 hr. at RT (1:000, Jackson). After, the sections were rinsed with washing buffer and the color was revealed with liquid Fast-Red substrate kit (Abcam). The incubation time was approximately 20 min. Immunohistochemistry was performed after hybridization as described before.

Cell counts

For the quantification of the GR IR positive cells, 2 anterior, 2 medial and 2 posterior sections of the cingulate cortex, central and medial amygdala, dentate gyrus of the hippocampus, PVN

and ARC were selected based on a stereotactic atlas. Images of selected sections were digitalized at 20X magnification using a computer. Images were taken with a digital camera (Lumenera, Infinity 2-5 URFC color) and the software Infinity Capture and Analyze and the number of GR-positive profiles was selected using Image J, (NIH) bilaterally for the Intact, Adrenalectomy +vehicle, adrenalectomy + Dex and Adrenalectomy + Cort.

Analytical methods:

Blood samples were kept on ice and centrifuged at 4000 rpm/8 minutes. The serum was collected and stored at -20°C. Serum Cort concentrations were measured using RIA kits (MP Biomedical, USA), serum ACTH levels were measured with EIA kit (Phoenix Pharmaceuticals, USA) and aldosterone with ELISA (Alpco, USA) as indicated by the providers.

Statistical analysis

Values are shown as means ±SEM for each group. Comparisons were performed using two-way RM ANOVA, followed by Tuckey multiple comparison test (INStat; Graph Pad Prism 6.01 Software, Inc., San Diego, CA). Association between variables was explored by simple linear regression. P values <0.05 indicate a statistically significant difference. Table 1 contains more information regarding the statistical results presented.

Table 1: Statistical data

Figure	Panel	Data structure	Test type	P value
26	P	Normally distributed	one-way ANOVA	< 0,0001
26	Q	Normally distributed	one-way ANOVA	< 0,0001
26	R	Normally distributed	one-way ANOVA	< 0,0001
26	S	Normally distributed	one-way ANOVA	< 0,0001
26	T	Normally distributed	one-way ANOVA	< 0,0001
28	A	Normally distributed	two-way RM ANOVA	0,0002
29	A	Normally distributed	two-way RM ANOVA	0.0012
29	B	Normally distributed	two-way RM ANOVA	0.3361
29	C	Normally distributed	two-way RM ANOVA	0.0297
29	D	Normally distributed	two-way RM ANOVA	0.7184
30	A	Normally distributed	two-way RM ANOVA	0.2709
30	B	Normally distributed	two-way RM ANOVA	0.4094
30	C	Normally distributed	two-way RM ANOVA	0.0012
30	D	Normally distributed	two-way RM ANOVA	0.0106
31	A	Normally distributed	two-way RM ANOVA	0,4819
31	B	Normally distributed	two-way RM ANOVA	0,8865
32	A Vehicle ZT2	Normally distributed	Linear regression	0,0741
32	B Dexamethasone ZT2	Normally distributed	Linear regression	0,6525

32	A Fludrocortisone ZT2	Normally distributed	Linear regression	0,4217
32	B Mifepristone ZT2	Normally distributed	Linear regression	0,6743
32	A Eplerenone ZT2	Normally distributed	Linear regression	0,0318
32	C Vehicle ZT 10	Normally distributed	Linear regression	0,1518
32	D Dexamethasone ZT10	Normally distributed	Linear regression	0,242
32	C Fludrocortisone ZT 10	Normally distributed	Linear regression	0,504
32	D Mifepristone ZT 10	Normally distributed	Linear regression	0,4581
32	C Eplerenone ZT10	Normally distributed	Linear regression	0,9576
33	A	Normally distributed	two-way RM ANOVA	0,0005
33	B	Normally distributed	two-way RM ANOVA	0,003

Table 1. Data distribution and statistical results.Normality tested using Kolmogorov-Smirnov test with Dallal-Wilkinson-Lille for P value

Results

The GRs in the ARC rapidly respond to circulating Cort

In intact animals treated with IV vehicle, GR IR is ubiquitously present in the brain, e.g. cortex, hippocampus, amygdala, PVN and ARC (**Fig. 26 A-E**). As GR nuclear immunoreactivity (IR) is lost in absence of Cort and rapidly recovered within minutes after agonists binding to GR [107], we decided to use the reappearance of GR IR 7 minutes after IV administration of Cort or Dex to investigate the speed of access of circulating Cort. Adrenalectomy caused complete loss of GR IR in the brain as reported previously [148], and IV administration of saline, Cort or Dex did not produce GR IR in the cortex ($F=116.1$, $P<0.0001$), amygdala ($F=120.9$, $P<0.0001$) and hippocampus ($F=599.6$, $P=0.0001$) (**Fig. 26 F-H, K-M**). GR IR positive cells decreased after adrenalectomy and GR IR was recovered especially in the ventromedial ARC ($F=49.76$, $P<0.0001$) and to some extent in the PVN ($F= 40.03$, $P<0.0001$) with no differences between Dex or Cort (**Fig. 26 I, J, N-T**) seven minutes but not earlier after IV Cort or Dex administration. GR-IR was recovered ubiquitously in the brain 50 minutes after administration of Dex (data not shown).

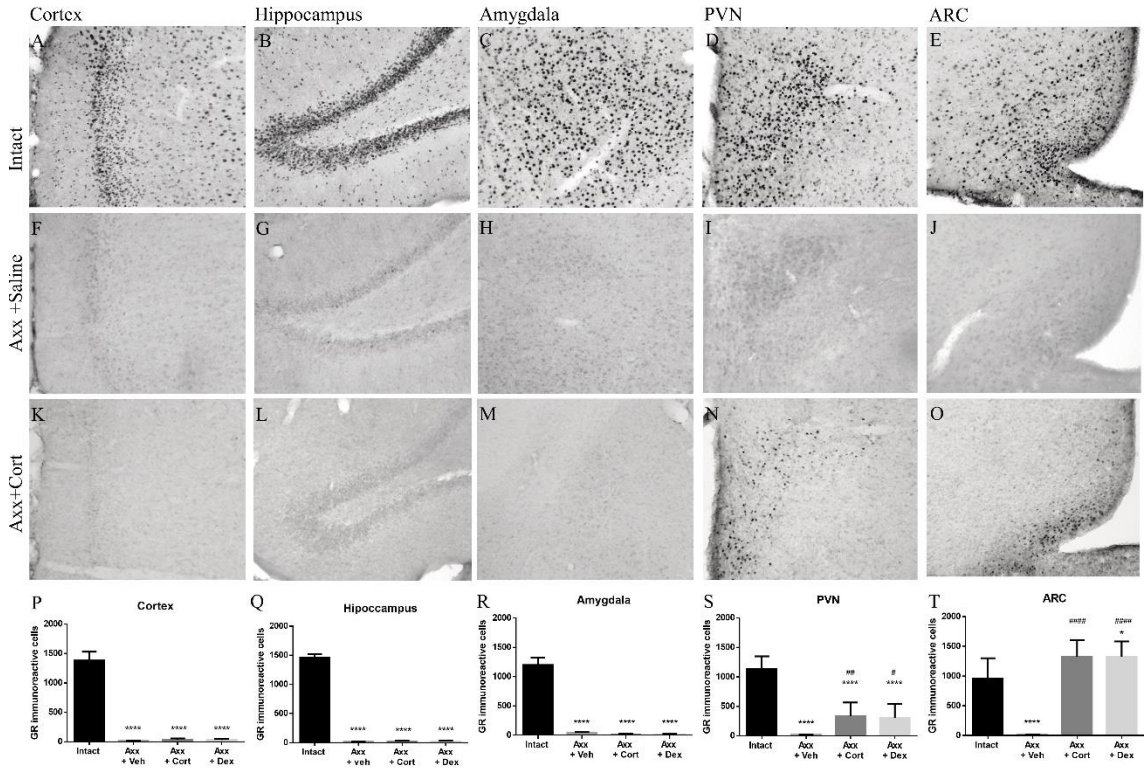

Figure 26. GR IR is abundant in intact animals (A-E) (n=6), blunted after adrenalectomy (F-J) and is selectively recovered after Dex or Cort administration. 7 minutes after injection into the circulation of Vehicle (n=6), Cort (n=6) or Dex (n=10) into adrenalectomized animals, GR is not recovered in the Cortex (K, P), hippocampus (L, Q) or amygdala (M, R). In contrast, IV injection of Cort or Dex partly recovers GR IR in the PVN (N, S) and the ARC GR IR is fully recovered shortly after administration of Cort and Dex (O, T). Asterisks indicate statistically significant differences from the intact group (*P < 0.05; **P < 0.01; *** P < 0.001 ****P<0.0001). Hashes indicate significant differences from the adrenalectomized + vehicle group ((#P < 0.05; ##P < 0.01; ###P < 0.001 ####P<0.0001) All data are presented as the mean ± SEM. (Scale bar = 100µm).

Within the ARC, GR-positive cells were distributed mainly in the region close to the third ventricle, the classical AgRP/NPY neuronal location. Subsequently, since GR was not recovered homogenously in the ARC, we characterized the neurons responsive to circulating Cort by means of in situ hybridization for AgRP or POMC combined with GR immunohistochemistry. We observed that GR-positive neurons were also positive for AgRP (**Fig. 27 A-D**) but not POMC (**Fig. 27 F-I**).

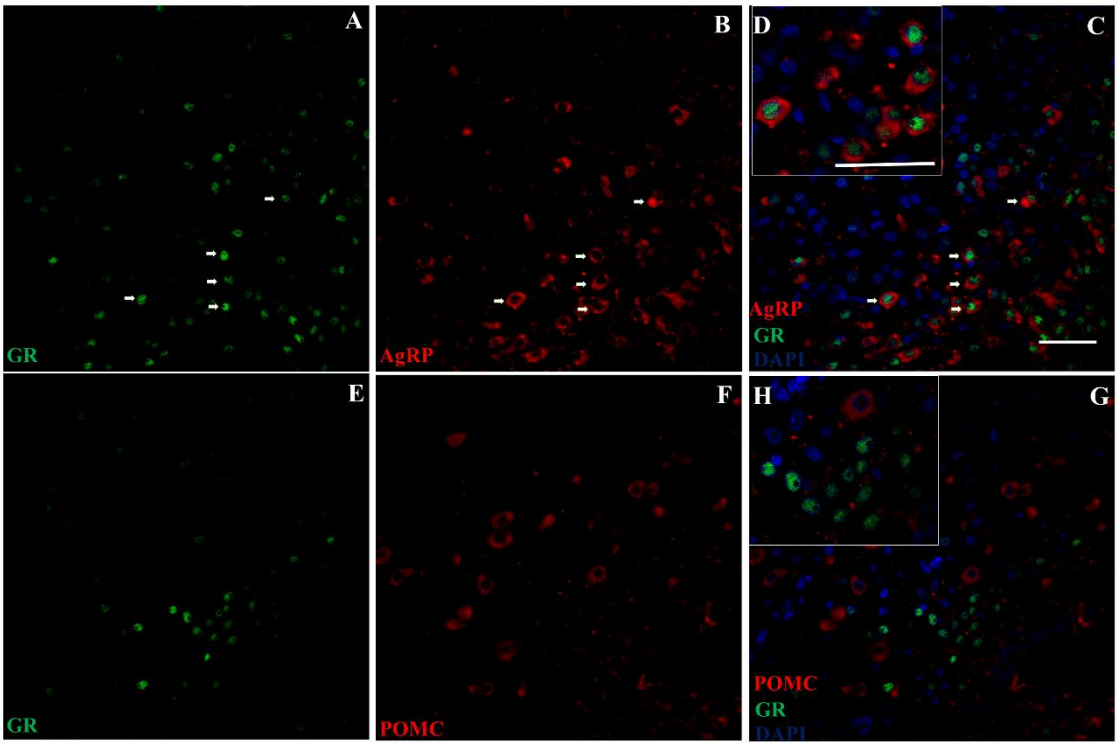

Figure 27. Photomicrographs showing recovery of GR in AgRP (A-C) but not in POMC neurons 7 minutes after Cort injection into the circulation (E-G) (Scale Bar = 50 µm). Higher magnification of AgRP (D) and POMC (H) sections (scale bar = 50µm). White arrows indicate neurons with GR and AgRP or POMC. DAPI (blue), GR (green), POMC or AgRP (red).

These results illustrate the privileged capacity of the ARC to rapidly sense circulating molecules, either via the fenestrations of the blood brain barrier in the case of ARC or later for the PVN via the cerebrospinal fluid, once these molecules have entered the third ventricle via the ARC-Median Eminence complex. This phenomenon has been previously reported for no steroid hormones, such as ghrelin and leptin [46,136,239].

ARC, but not PVN, gives negative feedback to GCs

Based on the recovery of GR IR in both the ARC and PVN shortly after Cort IV administration, we investigated their capacity to produce a negative feedback on Cort. We compared the effects of reverse microdialysis with type I GR antagonist (eplerenone) either in the ARC or PVN and obtained blood samples at 5 min before and 10, 20, 40 and 80 min during the infusion. The repeated measures ANOVA pointed to differences in time ($F(5,60) = 20.17$, $P<0.0001$) and place of drug infusion ($F(3,12) = 16.43$; $P=0.0002$). Infusion in the ARC caused an increase in Cort at 10 min and continued to the end of the infusion. In contrast, retrodialysis in the PVN caused an increase of Cort only after 80 min (**Fig. 28 A**).

Since type I antagonist infusion in the PVN produced a late change of Cort production, we investigated whether both pre-autonomic and endocrine portions in the PVN express GR, given that both neuronal populations are described to contribute to GC production. Following inoculation of pseudorabies virus (PRV) in the cortex of the adrenal gland, we identified neurons positive to GR and those positive to PRV by double immunohistochemistry. We observed two separate populations, with the highest amount of GR present in the medial

parvocellular part of the PVN and absent in the pre-autonomic PVN neurons polysynaptically connected to the adrenal gland (**Fig. 27 B, C**).

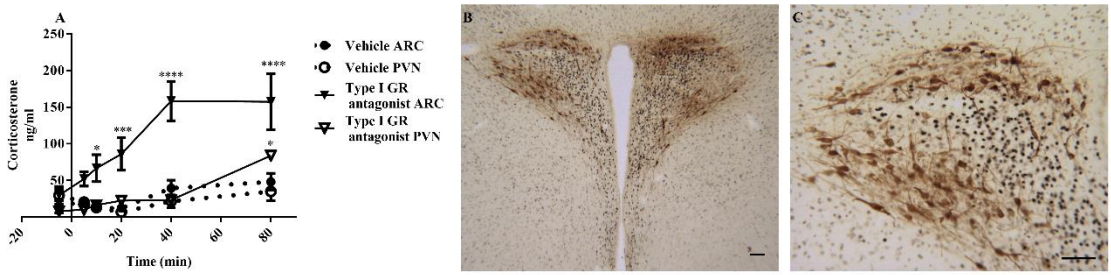

Figure 28. ARC, but not PVN, provides negative feedback to GC release from adrenals. (A) Plasma concentration of Cort during retrodialysis in the ARC (closed figures) or PVN (open figures) with vehicle (dotted lines ARC n=4, PVN n=4) or eplerenone (continuous lines ARC n=4, PVN n=4). (B) photomicrographs of the PVN for identification of GR-positive cells (black nuclear staining) and PRV-positive cells (brown, cytosolic staining) demonstrating the absence of GR in PVN neurons projecting polysynaptically to the adrenal(n=6) (Scale bar = 100 µm). (C) Higher magnification of PVN (scale bar = 100 µm). Asterisks indicate statistically significant differences (*P < 0.05; **P < 0.01; *** P < 0.001 ****P<0.0001). All data are presented as the mean ± SEM.

Taken together, these results suggest that circulating Cort reaches rapidly GR-positive neurons in the ARC to quickly increase circulating Cort. This fast negative feedback seems to involve ARC-AgRP projections to pre-autonomic neurons in the PVN. The delayed response of the PVN to the GR antagonist seems to be due to the absence of GR in the pre-autonomic neurons projecting to the adrenal gland.

Occupancy of type I and Type II GR in the ARC gives differential negative feedback during circadian trough and peak of Cort

Since the sympathetic input to the adrenal gland is necessary to induce adequate circadian rhythm of Cort, we hypothesized that the ARC could participate in the circadian regulation of Cort release transmitting the signal about circulating Cort to the autonomic portion of the PVN.

GC act via two different receptors along the circadian cycle. Previous studies have demonstrated that type I and not type II is occupied with low concentrations of Cort while type II receptor is occupied when Cort is higher[118]. We, therefore, tested the pharmacological agonist for type I or type II GR (fludrocortisone and dexamethasone respectively) or their antagonists (mifepristone and eplerenone) under unstressed conditions in the morning (ZT 2) or afternoon (ZT 10) with retrodialysis in the ARC.

In the morning, at ZT2, retrodialysis with type I GR agonist did not change Cort, while type I GR antagonist produced a fast increase of Cort compared to the vehicle ($F(2,9) = 15.67$ $P = 0.0012$) (**Fig. 29 A**). In contrast, neither type II GR antagonist or agonist produced any effect on Cort when compared to vehicle infusion ($F(2,10) = 1.493$ $P = 0.279$) (**Fig. 30A**). Retrodialysis with type I receptor agonist or antagonist did not cause changes in ACTH ($F(2,9) = 1.234$ $P =$

0.3361) (**Fig. 29B**). Retrodialysis with type II receptor agonist or antagonist did not cause changes in ACTH (F (2,9) = 0.9880 P = 0.4094) (**Fig. 30B**).

Taking into account previous reports suggesting the increase of other corticosteroids after sympathetic stimulation or increased ACTH[240–242], we measured aldosterone during the retrodialysis. We did not find any significant variation in aldosterone production after stimulation or inhibition of type I (F (2,6) = 0.8265 P= 0.4819) or type II (F (2,6) = 0.1229 P = 0.8865) GR in the ARC, indicating that the induced adrenal stimulation is specific for Cort (**Fig. 31 A, B**) despite the use of Type I receptor agonist and antagonist.

At ZT 10 (late afternoon), when Cort nearly reaches its daily peak[129], the change of Cort in the group treated with the Type I receptor antagonist differed from the vehicle group at 20 and 40 minutes after the beginning of the treatment and was similar to the Cort levels of the vehicle treated group at 80 min (F (2,11) = 4.925 P = 0.0297) (**Fig. 29C**). In contrast to ZT 2, the group infused with type II GR antagonist showed a steady increase of Cort immediately after the beginning of the treatment (F (2,10) = 14.11 P = 0.0012) (**Fig. 30C**). Type I GR agonist or antagonist in the ARC did not change blood concentration of ACTH (F (2,8) = 0.3448 P = 0.7184) (**Fig. 29D**), while type II agonist and antagonist-induced significant changes in circulating ACTH (F (2,7) = 9.343 P = 0.0106) (**Fig. 30D**), interestingly, these changes were not paralleled by Cort (Fig. 5C).

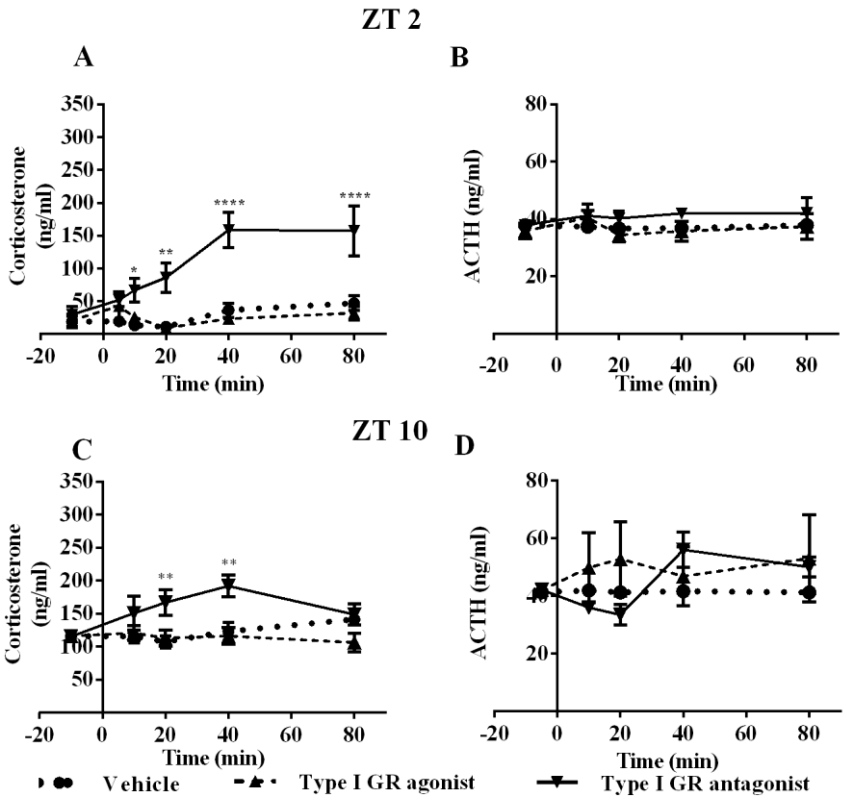

Figure 29. Differential involvement of type I receptor during the circadian trough and peak of Cort. Plasma concentration of Cort (A) and ACTH (B) during retrodialysis with vehicle (dotted line, n=4) agonist (dashed line, n=4) or antagonist (continuous line n=4) to type I receptor At ZT 2. Plasma concentration of Cort (C) and ACTH (D) during retrodialysis with vehicle (dotted line,

n=4), agonist (dashed line, n=4) or antagonist (dotted line n=4) to type I receptor At ZT 10 (agonist n= 5, antagonist n=4). Asterisks indicate significant differences (*P < 0.05; **P < 0.01; *** P < 0.001 ****P<0.0001). All data are presented as the mean ± SEM.

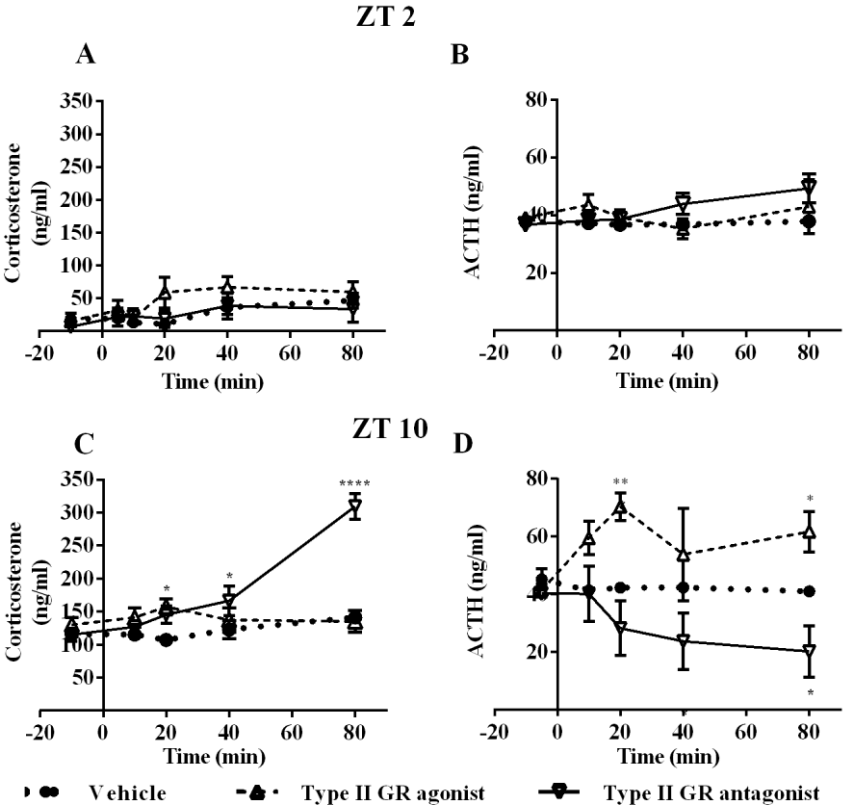

Figure 30. Differential involvement of type II receptor during the circadian trough and peak of Cort. Plasma concentration of Cort (A) and ACTH (B) during retrodialysis with vehicle (dotted line, n=4) type II receptor agonist (dashed line, n= 5) or antagonist (continuous line n=4) At ZT 2. Plasma concentration of Cort (C) and ACTH (D) during retrodialysis with vehicle (dotted line, n=4), agonist (dashed line, n=4) or antagonist (continuous line n=4) At ZT 10. Asterisks indicate significant differences (*P < 0.05; **P < 0.01; *** P < 0.001 ****P<0.0001). All data are presented as the mean ± SEM.

Simple linear regression shows there is no relationship between ACTH and Cort at ZT 2 for vehicle (P = 0.0742, R square = 0.1665), Type I GR agonist (P = 0.4217, R square = 0.05443), Type I GR antagonist (P = 0.0318, R square = 0.2312), Type II GR agonist (P = 0.06525, R square = 0.0089) or type II GR antagonist (P = 0.6743, R square = 0.01004) (**Fig. 32 A, B**). In the afternoon, dialysis with vehicle (P = 0.1518, R square = 0.1513), Type I GR agonist (P = 0.5040, R square = 0.0350), Type I GR antagonist, Type II GR agonist, and Type II GR antagonist did not show any correlation between ACTH and Cort (**Fig. 32 C, D**).

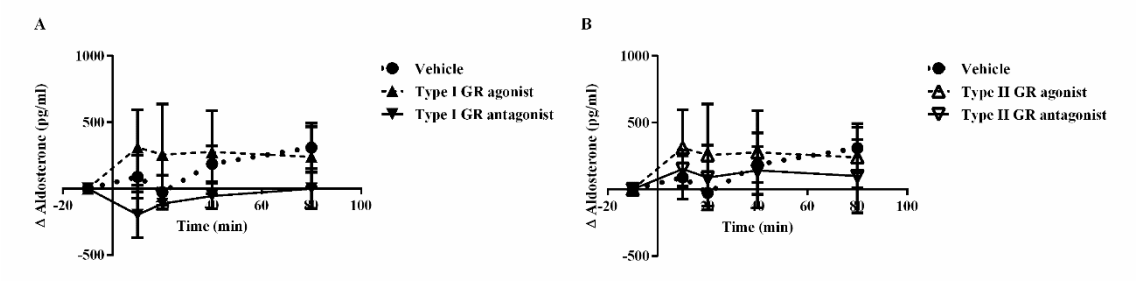

Figure 31. Aldosterone levels do not change by GCs in the ARC. Plasma concentration of aldosterone during retrodialysis with agonist (dashed line) or antagonist (dotted line) to type I receptor (A) and type II receptor (B) At ZT 2). All data are presented as the mean ± SEM.

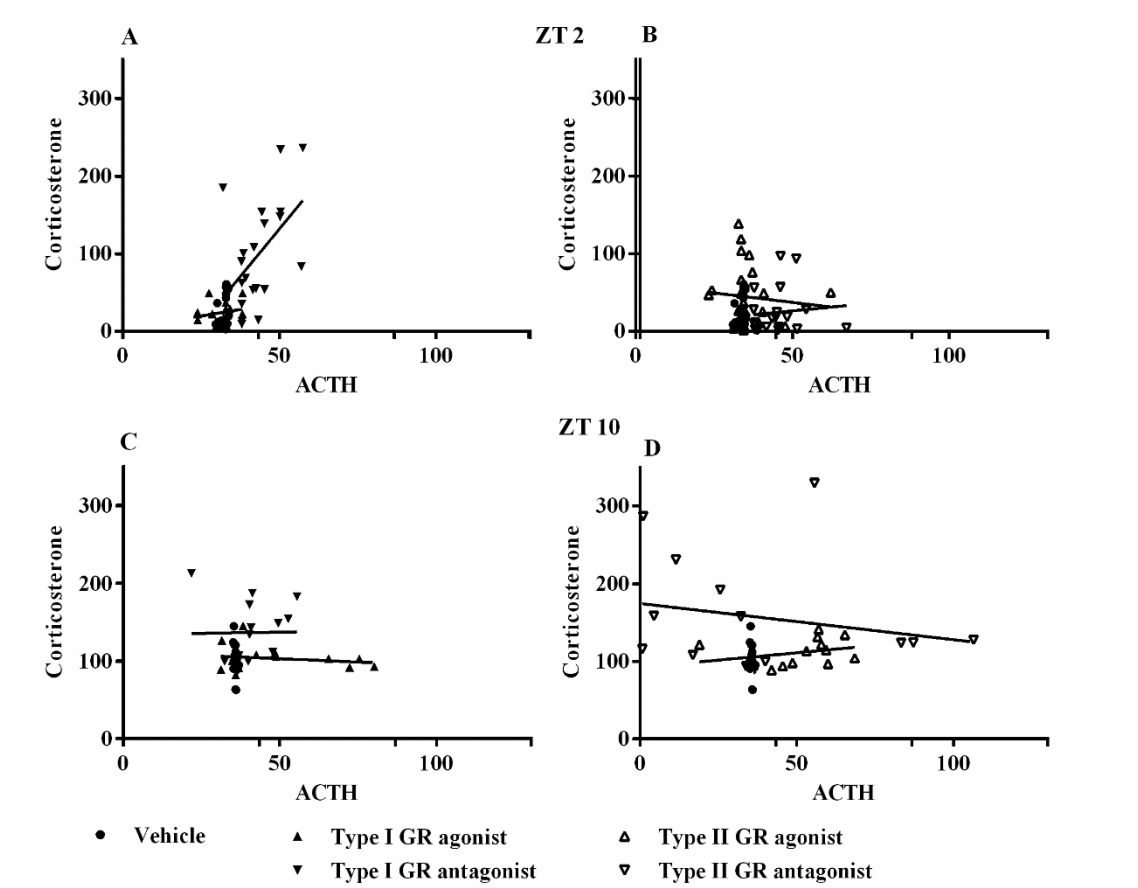

Figure 32. Linear regression of ACTH and Cort in plasma during dialysis with Type I GR and type II GR agonist or antagonists at ZT 2 (A, B) and ZT 10 (C, D) illustrating the absence of correlation between ATCH and Cort levels.

These results suggest that Cort release is mediated by the autonomic nervous system rather than via ACTH. Moreover, the present results provide physiological evidence of the preferential occupation of type I receptor in the morning and the occupation of type II in the afternoon and demonstrate for the first time the importance of GR occupation in the ARC to regulate Cort. In addition, the contrast between ACTH and Cort circulating levels, as observed with type II GR antagonist at ZT 10, supports the autonomic control of Cort release.

GR agonist in the ARC blunts the stress response.

To further explore the capacity of GCs to give negative feedback in the ARC, we compared Cort release of unstressed animals with the release after exposing the animals to a novel environment as stressor at ZT2, when Cort levels are normally low[123]. Simultaneously with the stress, we administered either vehicle, type I or type II receptor agonist in the ARC by means of retrodialysis. The treatment with vehicle or agonists alone did not increase circulating levels of Cort. As expected, the animals exposed to stress and treated with vehicle displayed a rapid increase in circulating Cort, while the group treated with Type I GR agonist after stress displayed only a peak at 20 min and returned to values comparable to vehicle treated animals ($F(3,12) = 12.59$ $P = 0.0005$) (**Fig. 33A**). Similarly, the group treated with Type II GR agonist after stress showed only a small increase of Cort at 10 min and reached vehicle values in later samples ($F(3,13) = 9.461$ $P = 0.0014$) (**Fig. 33B**). These results further demonstrate the roles of type I and type II GRs in the ARC to monitor circulating Cort levels under diverse conditions providing fast negative feedback, as demonstrated in this experiment, preventing the expected elevation of Cort.

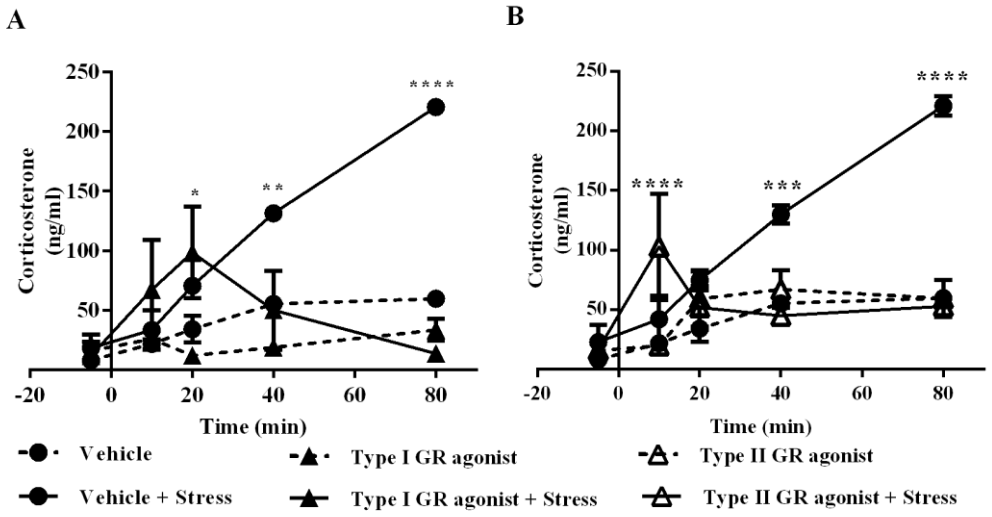

Figure 33. GR agonists in the ARC blunt the stress response. Plasma concentration of Cort during retrodialysis with agonist to type I receptor (closed triangles; A, n=4), type II receptor (open triangles; B, n=5) or vehicle (closed circles n = 4) At ZT 2 to control (dotted line) or stressed animals (vehicle, n=4, type I agonist n=4, type II agonist n=5) (continuous line). Asterisks indicate significant differences (*P < 0.05; **P < 0.01; *** P < 0.001 ****P<0.0001). All data are presented as the mean ± SEM.

Discussion

Since the discovery that Cort secretion is for an important part regulated by the autonomic output of the PVN, studies were missing answering how the negative feedback for this arm of the PVN could take place. The present study adds to the negative feedback of Cort evidence of a fast feedback via neurons in the ARC. Here, low and high levels of Cort occupy respectively Type I and Type II GR and do not change Cort via ACTH. Consequently, this information is transmitted polysynaptically to the adrenals. Present results confirm for the first time the early hypothesis that in the beginning of the rest period (ZT 2) when circulating levels of Cort are low, only the Type I GRs are occupied and contribute to the negative feedback of blood Cort

levels. At the end of the rest period (ZT 10), when Cort levels rise and the Type II GRs are occupied, the Type I GR do not play a role anymore in the feedback which is then taken over by the Type II GR[117,118,192].

A few minutes after IV administration of Cort in adrenalectomized rats, the staining for GR is recovered mainly in ARC AgRP neurons, indicating that circulating Cort penetrates most probably immediately via fenestrated capillaries in the ARC - median eminence complex (**Fig. 26**). The delay in GR staining to 7 minutes is most probably due to the delay in translocation of GR from the cytoplasm to the nucleus[107]. The delay in the appearance of GR staining in the cortex and hippocampus agrees with previous studies showing a delay of approximately 20 min between the increase of Cort in the circulation and inside the hippocampus after stress [243]. This observation demonstrates that brain structures other than the ARC are affected later by the changes of Cort. The immediate appearance of GR in AgRP neurons but not POMC neurons (**Fig. 27**) agrees with previous observations of changing levels of AgRP mRNA in the ARC along the day mediated by Cort [155], by the action of type II GR[244] and AMPK[161], and the identification of a brain-specific GR homeobox factor to regulate AgRP[156]. Considering this and the projections of AgRP going from ARC to PVN[245], suggest that AgRP cells may transmit information related to GC status from the circulation to other hypothalamic structures such as the PVN.

The absence of a response after GR antagonist infusion in the PVN, together with the lack of GR in pre-autonomic neurons (**Fig. 28**) indicates that Cort in the PVN is not enough to give a fast negative feedback to GCs. This is supported by observations that animals with specific KO of GR in the PVN still present similar levels of Cort before the activity period and under stress [236], confirming that PVN neurons mediating GC release in a circadian manner do not express GR. The pre-autonomic portion of the PVN, although devoid of GR, can stimulate glucocorticoid release independently of the amount of ACTH via the activity of the splanchnic nerve [124,127,231]. Thus, as we showed here, AgRP input from the ARC to the pre-autonomic portion of the PVN may transmit GC state to control splanchnic nerve tone and ACTH sensitivity in the adrenal gland.

Evidence for specific effects of the microdialysis probes placement comes from the experiments comparing the effect on the PVN or on the ARC since both hypothalamic nuclei are close enough to allow some effect of diffusion. However, our data clearly shows that only cannulas placed correctly in the arcuate nucleus provide a negative feedback when the drug is applied (**Fig. 28**). In addition, we have observed that misplaced cannulas outside the ARC (mostly in the VMH) did not have any effect on the release of Cort (Data not shown).

The absence of a relationship between ACTH and Cort levels as presently observed has also been reported previously in several conditions as restricted feeding schedules or disruption of the resting/active phases via light exposure [235,246]. In the evening, the adrenal gland is more responsive to ACTH than in the morning, despite similar levels of circulating ACTH [131,132]. In the same sense, experiments conducted in SCN lesioned animals or *in vitro* did not show this rhythm [124,133]. Taken together these observations can be interpreted as differences in adrenal responsiveness to ACTH-mediated by the autonomic output to the adrenal cortex. This could be confirmed in further experiments manipulating the GR in the ARC of hypophysectomized animals maintained with constant levels of ACTH.

We show that the use of agonists does not change significantly the amount of ACTH released during the dialysis at any condition at ZT 2. Interestingly, the use of type II GR agonist clearly produces an increase of ACTH at ZT 10. Here the question arises whether the effects of the agonists and antagonists can be due to diffusion of the microdialysed drugs into the portal system and then to the pituitary to reach the corticotrophs. However, the increase of ACTH at ZT 10 cannot be due to the action of GR agonist on the corticotrophs in the pituitary, because the agonist would inhibit ACTH release there [247]. In the same sense, the action of antagonists on corticotrophs should increase the release of ACTH [248]. This effect is not observed after type I GR antagonist administration at ZT 2 or 10. Moreover, at ZT 10, when the highest Cort values are achieved after antagonist is applied, we observed a drop of ACTH instead of the expected increase if the drug went to the portal system, which suggests that corticotrophs are not sensing the drug applied to the ARC, but rather respond to the actual increased Cort values. The changes of ACTH at ZT 10 during dialysis with type II GR agonist and antagonist may be explained in part by the effects of glucocorticoid non-genomic action on the pituitary gland [247,249].

Previous experiments have demonstrated the action of both type I and II GR to regulate Cort production [118,119,197], however, the site of action remained elusive. Here, we show that the ARC is importantly involved in the Cort feedback by a differential action of type I and Type II GR, dependent on the time and basal levels of Cort (**Fig. 29**). At ZT2, when mostly type I GRs are occupied, an antagonist for this receptor and not for Type II GR in the ARC produced a fast and sustained increase of Cort as previously reported for IV injections [119,250]. On the other hand, At ZT 10, when Cort is already high, the occupation of type II GR is more important to control GCs, as demonstrated by the increase of Cort after infusion of Type II GR antagonist. At this time, in contrast to ZT 2, type I receptor antagonist is not enough to increase Cort production.

We observed a transient increase of Cort after antagonizing Type I GR at ZT 10, during the circadian peak. This transient increase would indicate the response of low-affinity Type I GR to high concentrations of Cort [196] to monitor the beginning of the surge of Cort at an early stage. Deferred effects may be mediated by Type II GR as suggested by the later effects observed with Type II antagonist at the same time point. In the same sense, our experiment with stress conditions shows that Type I GR agonist does not produce an immediate negative feedback at low Cort, suggesting that occupation of low-affinity Type I GR may be necessary to initiate a counterregulatory effect.

In conclusion, the fast feedback mechanism demonstrated in the present experiments suggests the existence of at least two mechanisms to control circulating Cort levels. First, a fast sensing of circulating Cort by the ARC gives immediately a negative feedback on the secretion of Cort from the adrenal gland. Second, a slow feedback mediated by hampered penetration of Cort into other brain areas due to the presence of MDR1-type P-glycoprotein in the vasculature that slows down the transport of Cort into the brain[251,252]. Consequently, areas such as the hippocampus and the bed nucleus of the stria terminalis are only reached by Cort many minutes after the ARC. This mechanism may influence both the direct control of the adrenals well as the control of ACTH secretion via their projections to the PVN[253]. Such a two phases control system may initially prevent too high increases of Cort and next allow longer

term modifications and setting of balance by other brain regions [86,254]. It is attractive to propose similar mechanisms to control the circulating levels of these molecules.

Chapter IV: General Discussion

Neuronal Pathways for glucose and Cort regulation

In the present work, we evaluated the importance of the different circadian response to changes in glucocorticoid microdialysis in the ARC and to hypoglycemia induced by 2DG. We demonstrated that the ARC can sense both glucose availability and Cort levels, and rapidly produce a differential response to the occupation of GRs or to cellular induced hypoglycemia in a time-of-the-day dependent manner. This is important because previous studies have focused on the influence of the ARC on the SCN [4,6,216] to adjust the circadian rhythm within the SCN; however, little was known about how the SCN controls the response to the variation of metabolites sensed by the ARC.

Different responses between ZT 2 and ZT 10

In our experiments, we observed different responses between ZT 2 and ZT 10 to 2DG and GR agonists and antagonist. One of the mechanisms behind these different responses may be the monosynaptic input from the SCN to the ARC that we have described here. As reported previously, this connection between the central clock and the ARC may serve to keep systemic energy metabolism in agreement with the time of the day and orchestrate physiological responses to the metabolic stimuli [86]. This is particularly evident in the rhythms of glucose and Cort since both are synchronized to peak values before the beginning of the activity period. Furthermore, after the SCN is destroyed, the circadian rhythm is lost and the response to stress and hypoglycemia is impaired.

One possible explanation of the observed different responses between ZT 2 and ZT 11 may be a variation between inhibitory and excitatory signals coming from the SCN towards neurons in the ARC. It has been previously reported that neurons in the SCN are activated by light[255], this activation of the SCN is proposed to inhibit the neurons in the ARC, since the higher expression of c-Fos in the ARC is observed at night, when activity in the SCN is minimal, and light exposure at night diminishes the ARC activity[95]. Accordingly, blood glucose and Cort levels are low at the beginning of the light period, while they start to increase when SCN activity diminishes. Additional suggestions for the neuronal control mechanism are provided by SCN lesions. After complete ablation of the SCN, the activation in the ARC at the end of the dark period is lost[95], possibly because of the lack of glutamatergic input. Furthermore, in our experiments, unilateral ablation of the SCN produces a marked increase of activation in the ipsilateral ARC after 2DG administration (Chapter II) [254], probably due to the lack of GABAergic inputs. These two apparently contradictory results can be conciliated if we propose different activity patterns of these two populations in the SCN. As described previously for the control of the PVN, the SCN maintains a tonic glutamatergic output to the PVN, while the GABAergic output fluctuates along the day [94]. Therefore, it is possible that the ARC also receives from the SCN a tonic glutamatergic input and increased GABAergic tone at the beginning of the light phase, that is diminished at ZT11 or lost after SCN lesions, causing as consequence an increased activation in the ARC in response to hypoglycemia due to the lack of the oscillating inhibitory input.

AgRP/NPY/GABA neurons of the ARC are targeted by Corticosterone and glucose

In our experiments, we found colocalization of c-Fos and NPY in the ARC after the administration of 2DG. This coincides with previous descriptions of NPY neurons responding to changes in extracellular glucose. Since glucose sensing mechanism include effects on the uncoupling protein 2 (UCP2)[44], the AMP-activated protein kinase (AMPK)[256], ATP-sensitive potassium channels[209], the activity of the glucokinase[257], the Na/K-ATPase[218] and mTORC1[258], the exact mechanism to detect glucose availability in these neurons is yet to be elucidated.

This activation of NPY neurons may induce the increase of glucose release as a counterregulatory response since previous studies have demonstrated the increase of glucose production from the liver after central administration of NPY, assessed both by circulating glucose and different rate-limiting enzymes involved in hepatic glucose production[259].Also supporting the role in increasing glucose levels in the circulation is the observation that NPY neurons can diminish the hepatic insulin sensitivity via the ANS [31]. This diminishment of insulin sensitivity may, in turn, increase the gluconeogenic activity of the liver.

After administration of Cort or Dex in ADX animals, we found GR-positive signal only in AgRP neurons. As described before, NPY/AgRP neurons are important for glucose production in negative metabolic conditions. Glucocorticoid action on NPY/AgRP neurons is important for energy homeostasis, and an increase of GCs leads to obesity, liver steatosis, and insulin resistance[31,260,261], while the absence of endogenous Cort causes anorexia and body weight loss [143,262]. Consequently, these metabolic conditions may be related to the effects of Cort on the synthesis and release of NPY/AgRP from ARC neurons, suggesting that this neuronal population is particularly sensitive to the changes in Cort. Their activations by Cort and hypoglycemia, together with their anatomical position in the ARC indicates that AgRP neurons are the first order neurons to detect changes of GCs and glucose in the circulation, resulting in endocrine and metabolic adjustments. The complete circuit by which the SCN-ARC may control the counterregulatory glucose response has yet to be investigated in future experiments.

Now, we propose that the SCN controls both POMC and AgRP neurons to regulate the ratio of energy intake and expenditure. While POMC neurons have a prevalent role in the control of energy expenditure and the thermogenic response via projections to the median preoptic nucleus [263], AgRP/NPY neurons may control the autonomic sympathetic output from the PVN towards the liver to control gluconeogenesis and towards the adrenal gland to control Cort release in response to negative metabolic states. This bimodal output from the ARC may control the circadian variations of basal metabolism, preparing the animal for the necessities of the active or resting phase.

The PVN and its control on Corticosterone and ACTH secretion

Despite some fast recovery of GR in the PVN after Cort or Dex administration, retrodialysis of Cort antagonist in the PVN did not cause considerable changes of circulating Cort during the treatment, indicating that the sensing of Cort in the PVN does not participate in the rapid control of Cort secretion. Although we did not measure CRF or ACTH in this condition, it is likely that the infusion of the antagonist in the PVN changed the activity of CRH neurons, and consequently the release of ACTH from the pituitary gland. However, these possible changes

were not reflected in changes in circulating Cort, suggesting that the GRs in the PVN possibly function in a later stage of the feedback or after a stressing event, when ACTH levels are often increased dramatically[123]. This is supported by the recent finding that lack of GR in PVN neurons does not change the circadian peak of Cort or the response to chronic stress in male rats [236]. This, together with our observations that pre-autonomic neurons of the PVN projecting to the adrenal gland do not contain GRs, and NPY activates sympathetic output to the adrenal and the liver, indicates that the induced fast changes of Cort by the retrodialysis in the ARC are induced via these AgRP neurons in the ARC projecting to the PVN. This is supported by observations indicating that animals maintained with chronic release of ACTH after hypophysectomy from an implant still continued to produce the circadian peak of Cort[228]. Consequently, we propose a change of adrenal sensitivity to ACTH-driven by the autonomic nervous system in response to Cort changes detected in the ARC. This indicates a dual control of Cort secretion, one based on CRH neurons in the PVN to modulate ACTH release from the pituitary, and a parallel mechanism via the ARC projections controlling the pre-autonomic neurons in the PVN projecting to the sympathetic ANS inducing Cort release from the adrenal gland.

The control of hormone release via the ARC-PVN-ANS may not be restricted to Cort and could indicate a general mechanism to timely control hormone release from other glands. In the ARC, besides the GRs, there is also expression of receptors to most hormones controlled by the hypothalamic-hypophysis-glands axes (e.g. estrogen receptors[106,264], androgen receptors[265], growth hormone receptors[266] and thyroid hormone receptors[267]) as well as hormones secreted by the stomach, pancreas[106], liver[268] and adipose tissue[269] (e.g. ghrelin, insulin, somatomedins and leptin). The ARC may serve as a general endocrine sensor to monitor multiple hormonal signals, and transmit this information to the pre-autonomic neurons in the PVN, providing additional input to glands throughout the body for adjusting hormone release. Tracing studies have demonstrated that the pre-autonomic portion of the PVN projects to the thyroid gland and gonads, demonstrating the presence of the autonomic input to several glands. It is reported that denervation of a gland leads to atrophy and dysfunction. Denervation of the thyroid gland affects the magnitude and duration of thyroid hormone release, in the same way, the gonads are severely affected by the lack of neuronal input: when ovaries are denervated, the sexual cycle is impaired and the offspring reduced, while in the testes, lack of innervation results in impairment of steroidogenesis. In addition, this innervation may serve as a mechanism to stimulate or inhibit hormonal release in the absence of changes of hormones released from the hypophysis. Here, we propose that adequate sensing at the ARC and the transmission of the signal via the autonomic nervous system may be a mechanism to maintain the hormonal production and give negative feedback.

Is there a role for the BBB on the sensing of glucose and Cort in the ARC?

Corticosterone and the BBB

Our experiments show clear differences in the penetration of GCs into the CNS after administration of GCs (i.e. Cort and Dex). The lack of GR immunoreactivity in most brain regions analyzed, as the cerebral cortex, amygdala, and hippocampus may be due to the

presence of the BBB, which is one of the most important factors to restrict access of circulating factors to the brain. On the contrary, since BBB in the ARC is less restrictive, glucose and Cort from the circulation can pass more freely to this nucleus in the hypothalamus, allowing a faster sensing and, in the case of Cort, the recovery of GR IR in the ARC and to some extent in the PVN, probably after diffusion of Cort into the cerebrospinal fluid. As mentioned in the introduction, the access of different molecules to the brain depends on many mechanisms. There is evidence that not all steroid hormones have the same access to the brain [270]. Cort is one of the steroid hormones with lower penetration to the brain, together with aldosterone and cortisol. These three hormones are natural ligands for GRs, and therefore, GR occupation is highly restricted compared to other steroid receptors. In addition, when penetration of Cort is assessed in the hippocampus, there is a delay of 20 minutes between the increase of Cort in the circulation and the increase in the hippocampus measured by microdialysis [243]. Furthermore, when total penetration of Cort is compared between diverse regions of the CNS, it has been found that the access of Cort to the complete hypothalamus is more restricted than for other regions of the central nervous system[270]. These differences may depend on the BBB, that in addition to the presence of TJs and AJs to prevent the pass of molecules as small as ions, also has efflux membrane transporters, that can intercept some of the passively penetrating substances and pump them out of the endothelial cell[271]. The ABCB1-type multidrug resistance efflux transporter P-glycoprotein (P-gp) transports Cort against diffusion gradient, from the cytoplasm to the blood, putatively to prevent the cytotoxic effect of Cort and several solutes present in the circulation[272273]. Therefore, because of the expression of ABCB1, a large part of the brain detects changes of Cort later than the CVOs. These factors support the idea that the CVOs have a prominent role in the detection and fast response to deviating levels of hormones and metabolites in the circulation.

Glucose and the BBB

The access of glucose into the brain is also restricted by the BBB. Previous reports indicate that the values of glucose levels are substantially lower in the brain compared to the circulation. Although not measured systematically, IV administration of 2DG produced an increase of c-Fos-IR mainly in the ARC, while other neighboring nuclei as the VMH or LH remained largely unaffected. This fact suggests that the effects of 2DG were mainly in the ARC, indicating that the ARC has faster access to circulating 2DG probably due to the lack of a complete BBB. In the same sense, when 2DG is administered ICV, one of the most surprising results is the lack of differences in glucose levels between ZT2 and ZT11. One possibility to explain this outcome is that when 2DG is administered directly into the cerebrospinal fluid, it bypasses the physiological restrictions imposed by the BBB, and acts at the same time on most neurons and glial cells, overriding the circadian control of glucose production to cope with a general state of cellular hypoglycemia. In contrast, when administered intravenously, the hypoglycemia may affect in different ways regions where the BBB is complete and the CVOs. Therefore, 2DG effects may be observed first in the nuclei most exposed to the general circulation, while other nuclei still depend on transport across the BBB. Therefore, the counterregulatory processes are initiated after 2DG is sensed in the ARC, while other hypothalamic glucose sensitive structures may detect later the hypoglycemic stimulus, allowing another level of integration to organize changes in metabolism and energy expenditure.

Another element to consider is the possible restructuration of the main components of the BBB, as fenestrated capillaries, TJs and AJs. It has been described that ZO1 and fenestrations in the capillaries can undergo rearrangements depending on the metabolic status of the individuals[63]. These effects appear to be mediated by vascular endothelial growth factor A

(VEGF-A) released by tanycytes in the ARC-ME. VEGF-A expression is highly regulated by glucose levels and is impaired in patients with diabetes[274]; so it is likely that circadian variations in glucose and hormones drive changes in the BBB mediated by tanycytic VEGF-A. This restructuration of the properties of the BBB at the ARC takes place when an animal is fasted and circulating glucose levels are low. As consequence, there is an opening of the BBB at the level of the ARC. The result is a counterintuitive increase of glucose concentration in the ARC in comparison with other hypothalamic nuclei such as the VMH [63]. This increase can be explained by the fact that glucose concentrations in the brain are much lower than in the circulation, and the restructuration of the BBB allows glucose to diffuse from the circulation. The increase of glucose may be a first mechanism to allow a correct signaling of neurons after hypoglycemia. On low glucose availability and fasting, NPY/AgRP neurons display higher firing rate[74], which apparently is supported by an increase in their number of mitochondria, and the glucose elevation may serve to sustain their increased metabolic demand[75,76]. The results presented in this thesis suggest that similar reorganization occurs along the day, granting differential access to all kind of blood-borne molecules depending on the status of the BBB at a certain time point. Further studies will need to be done to establish this phenomenon and examine the underlying mechanism.

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List of acronyms and abbreviations

11b-HSD	11b-hydroxysteroid dehydrogenase
2 DG	2 deoxy-D-glucose
ABCB1	ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 1
ACTH	Adrenocorticotropic hormone
ADX	Adrenalectomy
AgRP	Agouti-related peptide
AJ	Adherent junction
Alpha-MSH	Alpha-melanocyte stimulating hormone
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AP	Area postrema
ARC	Arcuate nucleus
AVP	Arginine vasopressin
BBB	Blood-brain barrier
CalB	Calbindin
cAMP	Cyclic adenosine monophosphate
CART	Cocaine and amphetamine regulated peptide
CBG	Corticosteroid binding globulin
Cort	Corticosterone
CRFR	Corticotrophin-releasing-factor receptor
CRH	Corticotrophin-releasing factor
CRH	Corticotrophin releasing hormone
Cry	Cryptochrome
CTb	Cholera toxin subunit b
CVO	Circumventricular organ
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
DAB	Diaminobenzidine

Dex	Dexamethasone
DMH	Dorsomedial hypothalamic nucleus
GABA	Gamma amino butyric acid
GAD67	Glutamate decarboxylase/glutamic acid decarboxylase 67
GC	Glucocorticoid
GE	Glucose excited neurons
GI	Glucose-inhibited neurons
GLUT	Sodium-independent glucose transporter
GR	Glucocorticoid receptor
GRP	Gastric Releasing Peptide
HGP	Hepatic glucose production
HPA	Hypothalamus-Pituitary-Adrenal
HPG	Hypothalamus-pituitary-Gonads
HPT	Hypothalamus-pituitary-Thyroid
ICV	Intracerebroventricular
IML	Intermediolateral column
IR	Immunoreactivity
IV	Intravenous
JAM	Junctional Adhesion Molecule
LH	lateral hypothalamus
MC2R	Melanocortin receptor 2
ME	Median eminence
MR	Mineralocorticoid receptor
MRAP	Melanocortin receptor 2 accessory protein
NMS	Neuromedin S
NO	Nitric Oxide
NPY	neuropeptide Y
NT	Neurotensin
NTS	Nucleus of the Solitary Tract
OVLT	Vascular organ of lamina terminalis

Per	Period
PFA	paraformaldehyde
P-gP	P-glycoprotein
PKA	Protein kinase A
PMV	Hepatic Portal Mesenteric Vein
PRV	pseudorabies virus
PSN	penicillin-streptomycin-neomycin
PVN	paraventricular nucleus
PVP	polyvinylpyrrolidone
SCN	suprachiasmatic nucleus
SEM	the standard error of the mean
SFO	Subfornical Organ
SGLT	Sodium-dependent glucose cotransporter
SP	Substance P
StAR	Steroidogenic acute regulatory protein
TJ	Tight junction
TRH	Thyrotropin Releasing Hormone
TSH	Thyroid Stimulating Hormone
UCP2	Uncoupling protein 2
uniSCNX	unilateral lesion of the suprachiasmatic nucleus
VEGF-A	vascular endothelial growth factor A
vGLUT1	vesicular glutamate transporter 1
VIP	Vasoactive Intestinal Peptide
VMH	ventromedial nucleus of the hypothalamus
VP	Vasopressin
Zonula Occludens	ZO
ZT	Zeitgeber

Supplementary Data

Isoform	Previous name	Class	Main tissue localization	Insulin sensitive	Functional characteristics
GLUT 1	-	I	Erythrocytes, brain, ubiquitous	No	Glucose
GLUT 2	-	I	Liver, pancreas, intestine, kidney	No	Glucose (low affinity), fructose
GLUT 3	-	I	Brain	No	Glucose (high affinity)
GLUT 4	-	I	Heart, muscle, WAT, BAT, brain	Yes	Glucose (high affinity)
GLUT 5	-	II	Intestine, testes, kidney	No	Fructose, glucose (very low affinity)
GLUT 6	GLUT 9	III	Brain, spleen, leukocytes	No	Glucose
GLUT 7		II	n.d.	n.d.	n.d.
GLUT 8	GLUT X1	III	Testes, brain, other tissues	No (yes in blastocysts)	Glucose
GLUT 9	GLUT X	II	Liver, kidney	n.d.	n.d.
GLUT 10	-	III	Liver, pancreas	No	Glucose
GLUT 11	GLUT 10	II	Heart, muscle	No	Glucose (low affinity), fructose (long form)
GLUT 12	GLUT 8	III	Heart, prostate, muscle, small intestine, WAT, brain	Yes	n.d.
HMIT		III	Brain	n.d.	H+-myo-inositol

Supplementary table 1: The glucose transporter (GLUT) family of facilitative sugar transporter. not described (n.d.) (modified from Wood 2003).

Route of transport across the BBB	Main component transported	Description
Passive diffusion	Lipid soluble, non-polar molecules	Higher lipid solubility and several other physicochemical factors favor this process

ABC transporter efflux	Lipid soluble, non-polar molecules and conjugates	Intercept some of the passively penetrating solutes and pump them out of the endothelial cell
Solute carriers SLC	Glucose, amino acids, glycosides, monocarboxylates, small peptides, free fatty acids, organic anions, organic cations	Passive, primarily or secondarily active, and can transport many essential polar molecules
Transcytosis: **Receptor-mediated** **Adsorptive-mediated**	Transferrin, melanotransferrin, lipoproteins, Amyloid beta, glycosylated proteins, IgG, insulin, leptin, tumor necrosis factor alpha, epidermal growth factor	RMT requires receptor binding of ligand and can transport a variety of macromolecules such as peptides and proteins across the cerebral endothelium (transcytosis). Appears to be a vesicular-based system which carries their macromolecule content across the endothelial cells
Mononuclear cell migration	Leukocytes	Leukocytes cross the BBB either by a process of diapedesis through the endothelial cells (penetrating close to the tight junctional regions) or via modified tight junctions

Supplementary table 2: Main routes of transport across the BBB[51]

Transporter	**Location**	**Expression/conditions**
GLUT 1	Endothelial cells	Major
GLUT 1	Astrocytes	Major
	Microglia	Low levels
	Neurons	Fetal brain
GLUT 2	Astrocytes	Low levels
	Neurons	Low levels
GLUT 3	Neurons (high affinity, high capacity)	Major
	Endothelial cells	Low levels
GLUT 4	Astrocytes	Low levels
	Endothelial cells	Low levels
	Neurons	Low levels

GLUT 5	Microglia (Fructose, low affinity for glucose) Endothelial cells	Low levels Low levels
GLUT 6	Neurons	Low levels
GLUT 7	Astrocytes	Low levels
GLUT 8	Endothelial cells Neurons	Low levels Low levels

Supplementary table 3. Location and expression of glucose transporters at the blood-brain barrier[52]

Peptide	**Convertase**	**Described functions**	**Year of discovery**
ACTH	PC 1/PC2	Stimulation of the adrenal gland	1955
Beta-MSH	PC2	Food intake regulation	1956
Alpha-MSH	PC2	Food intake regulation	1957
Beta-LPH	PC1	Melanin production/ lipolysis/ steroidogenesis	1964
Gamma-LPH	PC2	Melanin production/ lipolysis	1967
Corticotrophin like intermediate peptide (CLIP)	PC2	Insulin secretagogue/ paradoxical sleep	1972
Beta-endorphin	PC2	Pain management and reward	1975
N POMC fragment	PC1		1979

Supplementary table 4. POMC products, prohormone convertase enzyme, and main effects reported for each product.

Receptor	Major site of expression	Ligand preference	Function	Effect of deletion	Comments
MC1R	Melanocytes	Alpha MSH> ACTH> gamma MSH	Pigmentation of hair and skin	Red hair, pale skin	Agouti antagonizes
MC2R	Adrenal cortex	ACTH	Steroidogenesis and adrenal growth	Adrenal failure	Absolute dependency on MRAP
MC3R	Brain, spinal cord	Gamma MSH> alpha MSH = ACTH	Complex, inhibits POMC neurons	Obesity	Enhanced action with MRAP2
MC4R	Brain, spinal cord	Alpha MSH> ACTH> gamma MSH	Appetite regulation	Obesity	AgRP is an inverse agonist
MC5R	Multiple tissues	Alpha MSH> ACTH> gamma MSH	Exocrine gland function	Defective sebum production and thermoregulation	Also expressed in immune cells

Supplementary table 5. Main features of the melanocortin receptors

Publications

Scientific articles:

Luis Leon-Mercado, Daniela Herrera Moro Chao, María del Carmen Basualdo, Carolina Escobar, Mitsuhiro Kawata, Ruud M Buijs. **The arcuate nucleus: a site of fast negative feedback for corticosterone secretion. eNeuro,** 13 February 2017, 4 (1) ENEURO.0350-16.2017

Daniela Herrera-Moro Chao*, **Luis Leon Mercado***, Ewout Foppen, Mara Guzmán Ruiz, María del Carmen Basualdo, Carolina Escobar, Ruud M Buijs. **The Suprachiasmatic nucleus modulates the sensitivity of Arcuate nucleus to hypoglycemia in the male rat. Endocrinology**, 2016. 157 (9), 3439-3451. 10.1210/en.2015-1751

Frederik Buijs, Mara Guzmán Ruiz, **Luis Leon Mercado**, María del Carmen Basualdo, Carolina Escobar, Andries Kalsbeek, Dick Swaab, Buijs. **Interaction between the Arcuate and Suprachiasmatic nucleus is essential for rhythmicity in physiology**. **eNeuro**. 17 March 2017, 4 (2) ENEURO.0028-17.201

Mara Guzman Ruiz, Arlen Ramirez Corona, Natali Guerrero-Vargas, Elizabeth Sabath, Oscar Ramirez Plascencia, Rebeca Fuentes Romero, **Luis Leon Mercado**, María del Carmen Basualdo Sigales, Carolina Escobar, Ruud M Buijs. **Role of the Suprachiasmatic and Arcuate Nuclei in Diurnal Temperature Regulation in the Rat**. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 2015. *35*(46), 15419–15429. doi:10.1523/JNEUROSCI.1449-15.2015

Scientific Review:

Frederik Buijs, **Luis Leon Mercado**, Mara Guzmán Ruiz, Natali Guerrero-Vargas, Francisco Romo Nava, Ruud M. Buijs. **The circadian system: A regulatory feedback network of periphery and brain**. Physiology, 2016. 31 (3), 170-181. 10.1152/physiol.00037.2015

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Presentations in congresses:

May 21-25, 2016. 15th Biennial Meeting of the Society for Research on Biological Rhythms, Palm Harbor, Florida, USA. Poster. The Arcuate nucleus: Site for Time-Of-Day-Dependent Negative Feedback on Corticosterone Secretion. **Luis Leon-Mercado**, Daniela Herrera Moro Chao, María del Carmen Basualdo, Ruud M Buijs

October 17-21, 2015. Society for Neuroscience 45th Annual Meeting. Chicago, Illinois, USA. Poster. Glucocorticoid receptors in the arcuate nucleus mediate a fast feedback of corticosterone secretion. **Luis Leon-Mercado**, Daniela Herrera Moro Chao, María del Carmen Basualdo, Ruud M Buijs

November 9-13, 2013. Society for Neuroscience 43td Annual Meeting. San Diego, California, USA. Poster. The Arcuate Nucleus participates in glucocorticoid negative feedback. **Luis Leon-Mercado**, Daniela Herrera Moro Chao, María del Carmen Basualdo, Ruud M Buijs

November 4-9, 2012. 1st FALAN- IBRO congress- 55th Congreso Nacional de Ciencias Fisiológicas, Neurociencias y Neurobiología de México. Cancún, Quintana Roo, México. Poster. The Arcuate Nucleus Mediates Glucocorticoid Fast Feedback through a neural Pathway. **Luis Leon-Mercado**, Daniela Herrera Moro Chao, María del Carmen Basualdo, Ruud M Buijs

May 19-23, 2012. 13th Biennial Meeting of the Society for Research on Biological Rhythms, Destin, Florida, USA. Poster. The paraventricular nucleus of the hypothalamus, a target for SCN mediated food intake? **Luis Leon-Mercado**, María del Carmen Basualdo, Ruud M Buijs

April 25-27 2012. Second congress of graduate students, UNAM, DF, México. Oral Presentation. El núcleo Paraventricular del hipotálamo, un sitio de blanco para la ingesta de alimento.