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Action of ciliary neurotrophic factor on mouse brain feeding centers

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1. RIASSUNTO

Il fattore neurotrofico ciliare (CNTF) è un fattore neurotrofico in grado di indurre sazietà e aumentare la spesa energetica sia nell'uomo che in roditori. Esso agisce a livello cellulare con un meccanismo simile a quello della più nota leptina, attraverso la via di trasduzione Jak-STAT3. Studi recenti hanno evidenziato che nell'ipotalamo tuberale di topo il CNTF è normalmente espresso da cellule gliali e il suo meccanismo d'azione è potenziato in modelli sperimentali di obesità. Mediante studi di immunoistochimica, abbiamo dimostrato per la prima volta che la somministrazione di CNTF induce l'attivazione non solo di STAT3, ma anche di STAT1 e STAT5 nell'ipotalamo tuberale, in particolare nelle cellule ependimali del terzo ventricolo, nei beta-taniciti e in cellule gliali dell'eminenza mediana. In questa sede, inoltre, il CNTF attiva nei beta-taniciti e nelle cellule dell'eminenza mediana il c-Fos, marker di attivazione cellulare. Abbiamo quindi verificato l'interessante ipotesi che il CNTF, oltre ad un'azione a livello ipotalamico, potesse avere un'azione anche a livello dei centri truncali, i quali costituiscono un'altra importante sede per il controllo del comportamento alimentare. Abbiamo dimostrato in questo successivo studio, che il CNTF nell'area postrema, così come nell'eminenza mediana, attiva le diverse isoforme STAT3, STAT1 e STAT5. Studi di co-localizzazione hanno evidenziato che gran parte delle cellule gliali responsive al CNTF nell'area postrema esprimono i tipici marker di immaturità, tra i quali nestina e vimentina. Dopo 120 minuti dal trattamento con CNTF abbiamo osservato una forte attivazione di c-Fos nei neuroni del nucleo del tratto solitario e una debole attivazione dello stesso marker in alcuni neuroni colinergici del nucleo motore dorsale del vago. Il doppio trattamento con CNTF (120 minuti, per indurre espressione di c-Fos) e con leptina (25 minuti, per indurre fosforilazione di STAT3) ha permesso di dimostrare la co-localizzazione dei due marker in una piccola popolazione di neuroni nella porzione caudale del nucleo del tratto solitario. Inoltre, nell'area postrema, si evidenzia una notevole discrepanza tra le numerose cellule che rispondono al ligando e quelle che lo producono, le quali sono poche cellule gliali situate nel funiculus separans e nelle meningi. Questo disaccoppiamento tra espressione

del CNTF e del suo recettore è stato confermato anche da studi di RT-qPCR, sia a livello dell'area postrema che dell'ipotalamo. In conclusione, il CNTF rappresenta un nuovo fattore di sazietà coinvolto nella regolazione del bilancio energetico che, oltre ad una principale azione a livello dell'ipotalamo, esercita un'azione parallela e ridondante anche a livello del tronco encefalico. Esso, inoltre, non agisce solamente su neuroni ma anche su cellule gliali, dove modula non solo il sistema di trasduzione Jak-STAT3, ma anche Jak-STAT1 e Jak-STAT5.

1. SUMMARY

The ciliary neurotrophic factor (CNTF) induces satiety and increase of energy expenditure in rodents and humans through a leptin-like activation of the Jak-STAT3 signaling pathway. Recent studies demonstrated that CNTF is constitutively produced by hypothalamic glial cells and that its expression is up-regulated in obese mice. By immunohistochemistry studies, here we show for the first time that after systemic treatment, rat recombinant CNTF induced activation of STAT1 and STAT5 in the tuberal hypothalamus in mice, in particular in ependymal cells bordering the third ventricle floor and lateral recesses, and in median eminence β-tanycytes and glial cells. Moreover, STAT activation was accompanied by c-Fos expression in β-tanycytes and median eminence cells of CNTF- treated mice. We also tested the hypothesis that CNTF affects the brainstem centers involved in feeding and energy homeostasis. In the area postrema of mice, as well as in the median eminence, CNTF activates STAT3, STAT1 and STAT5. Co-localization studies showed that a significant proportion of CNTF-responsive glial cells were also positive for immaturity and plasticity markers, such as nestin and vimentin. After 120 min from the treatment, we observed a strong c-Fos expression in several neurons of the rostral and caudal solitary tract nucleus (NTS), and a weak c-Fos immunostaining in some cholinergic neurons of the dorsal motor nucleus of the vagus. Treatment with CNTF (120 min, to induce c-Fos expression) and leptin (25 min, to induce STAT3 phosphorylation) demonstrated the co-localization of the two markers in a small percentage of neurons in the caudal NTS portion. In contrast to the high responsiveness to CNTF, in the area postrema CNTF immunoreactivity is weak and sparse, and mainly detected in glial cells of the funiculus separans and the meninges. RT-qPCR in micropunched area postrema and hypothalamus mouse tissues confirms the high discrepancy between the CNTF and CNTF receptor expressions in both the two brain regions examined.

In conclusion, CNTF represents a novel satiety factor involved in the pathophysiological regulation of the energy balance that exerts a parallel and redundant action in hypothalamic and brainstem feeding centers. In addition, it

acts not only on neuronal cells but also on glial cells, where it modulates not only the Jak-STAT3 transduction system, but also Jak-STAT1 and Jak-STAT5 activities.

2. INTRODUCTION

2.1. Central control of food intake

Obesity has reached epidemic levels worldwide, accompanied by the increased prevalence of multiple obesity-associated clinical conditions such as insulin resistance and diabetes, cardiovascular diseases, some cancers, mood related disorders, and numerous other health problems (Kenny, 2011). The brain plays a central role in energy homeostasis by integrating multiple peripheral metabolic inputs, such as nutrients, gut-derived hormones, and adiposity-related signals. Central nervous system regulates diverse aspects of body metabolism and feeding behaviour, including hunger and satiety, food-seeking behaviour, gastric emptying, nutrient uptake in the gut, thermogenesis, pancreatic insulin secretion, as well as the effects of insulin in the liver, adipose tissue, and skeletal muscle (Morton *et al*., 2006; Broberger, 2005).

Lesioning studies performed in several species in the late 1930's through the 1950's highlighted the importance of hypothalamus in the regulation of body weight and demonstrated the presence in the hypothalamus of two opposite neuronal centers: the lateral (lateral hypothalamic area, LHA) and dorsal (paraventricular nucleus of hypothalamus, PVN) areas of the hypothalamus were described as "feeding centers", whereas more ventral structures (arcuate nucleus, ARC and ventromedial nucleus, VMN) were described as "satiety centers" (Hetherington and Ranson, 1939, 1940, 1942a,b). The validity of this "dual center" model has been discussed and questioned for twenty years. More recently, the notion of specific centers of the brain that control food intake has been replaced by that of discrete and complex neuronal pathways that generate integrated responses to various central and peripheral afferent inputs (Schwartz *et al*., 2000).

After the discovery of leptin (Zhang *et al*., 1994), an anorexigenic adipose tissue-derived hormone that circulates in proportion to fat mass, numerous studies focused on the neuronal hypothalamic structures mediating the leptin dependent energy homeostasis regulation. The identification of leptin receptors in hypothalamus (Tartaglia *et al*., 1995) confirmed the role of the

hypothalamus as a crucial site of central energy sensing, with central leptin-insensivity being a hallmark of obesity (Frederich *et al*., 1995; Seeley *et al*., 1996).

Recently, also brainstem neurons have been described as involved in central control of energy balance, by processing energy status information and having an important role in ingestive control (Schwartz, 2006; Schwartz, 2009).

Collectively, circulating signaling peptides that modulate food intake and energy expenditure provide metabolic and nutritional information to the CNS by acting on hypothalamic as well as brainstem neuronal circuits (Young, 2012; Morton *et al*., 2014; Schneeberger *et al*., 2014).

2.1.1. Hypothalamic circuit involved in energy homeostasis

The hypothalamus, a small structure representing less of 1% of the total volume of the brain, is an important center that controls many physiological aspects critical for survival including temperature, reproduction, biological cycles, hormonal balance and energy regulation.

It is constituted by distinct hypothalamic nuclei involved in energy balance, including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), the dorsolmedial nucleus (DMN) and the ventromedial nucleus (VMN) (Schwartz *et al*., 2000).

ARC is situated in the mediobasal hypothalamus, adjacent to the floor of the third ventricle and it's the best-studied region for the neuronal control of energy balance. ARC is particularly sensitive to hormonal and nutrient fluctuations in the bloodstream thanks to its position, immediately adjacent to the median eminence (ME), a circumventricular organ that has a fenestrated blood-brain barrier (Johnson and Gross, 1993). Moreover, ARC like many of the key nuclei controlling energy balance, is in close proximity to the third ventricle. The ventricle wall contains layers of ciliated ependymal cells and tanycytes (Rodríguez *et al*., 2005). Tanycytes are unique glial cell type of the mature brain with a distinct morphology: they are directly in contact with the cerebrospinal fluid at their apical surface, and send a single and long basal process into the brain parenchyma. They form a blood-cerebrospinal fluid

barrier at the level of circumventricular organs (Langlet *et al*., 2013a) and they play a crucial role in brain homeostasis, regulating the access of peripheral metabolic signals to the ARC (Langlet, 2014).

Additionally tanycytes, similarly to various types of glial cells, may interact with other neural cells, communicating *via* changes in intracellular Ca^{2+}, responding to several transmitters and releasing ATP (Dale, 2011).

In the ARC, distinct and antagonistic neuronal populations coordinate various peripheral and central signals as hormones, neuropeptides and neurotransmitters to control the hunger/satiety status (Schwartz *et al*., 2000). There are two major populations of neurons controlling appetite and energy expenditure: a subset of neurons that co-express orexigenic neuropeptide Y (NPY) and agouti-related peptide (AGRP) and a population of neurons that co-express the anorexigenic neuropeptides cocaine-and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC) (Hahn *et al*., 1998). Both types of neurons (NPY/AGRP and POMC/CART) are regulated by leptin and insulin but in an opposing manner.

Thus, NPY/AGRP neurons are inhibited by leptin and insulin, and consequently are activated in conditions where leptin or insulin levels are low (Schwartz *et al*., 1996, Hahn *et al*., 1998, Sipols *et al*., 1995). Conversely, conditions characterized by reduced insulin or leptin inhibit POMC and CART expression in the ARC, and administration of these hormones can prevent or attenuate these neuropeptide response (Schwartz *et al*., 1997, Kristensen *et al*., 1998). NPY/AGRP and POMC/CART neurons of the ARC are defined as "first-order neurons"and project to "second-order neurons" of the PVN, the perifornical area (PFA) adjacent to the fornix and the LHA, and to brainstem neurons of nucleus of solitary tract (NTS) (Schwartz *et al*., 2000) (**Fig. 1**).

POMC neurons produce the anorectic peptide α-melanocyte stimulating hormone (α-MSH) by post-transcriptional processing of POMC. α-MSH binds to the melanocortin receptors 3 and 4 (MC3R and MC4R) on second-order neurons and activates catabolic pathways, leading to reduced food intake and increased energy expenditure (Cowley *et al*., 1999). Different modifications in the POMC or melanocortin receptor genes have been studied for several years in mice and many different variants have been linked to obesity and metabolic

syndrome in both rodents and humans (Huszar *et al*., 1997; Butler *et al*., 2000; Gropp *et al*., 2005).

On the other hand, central administration of NPY increases food intake via Y1 or Y5 receptors, which are highly expressed in the ARC, PVN, and VMH (Raposinho *et al*., 2001). Likewise, central administration of AgRP induces hyperphagia and weight gain by inhibiting the binding of α-MSH to MC3R/MC4R (Schwartz *et al*., 2000). Selective ablation of NPY/AgRP neurons in adult mice results in anorexia and weight loss (Luquet *et al*., 2005), confirming a critical role of these neurons in the regulation of energy homeostasis.

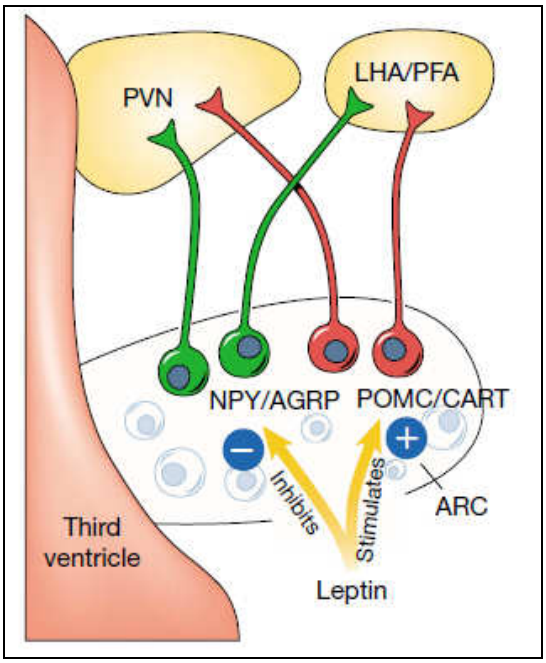

Fig. 1 - First order NPY/AgRP and POMC/CART neurons regulated by leptin and projections to the PVN, LHA and PFA (Schwartz *et al*., 2000).

2.1.2. Brainstem circuits involved in energy homeostasis

Much of the credit for recognition of the contribution of brainstem structures in control of food intake goes to Grill and colleagues. They showed that sapid stimuli administered by mouth elicited the same mechanical ingestive responses in de-cerebrated rats and in control ones (Grill and Norgren, 1978a, b). They also demonstrated that the glucoprivic feeding resulting from insulin-induced hypoglycemia was evident even in de-cerebrated rats (Flynn and Grill, 1983), suggesting that glucose related ingestive behaviors could also be generated in caudal brain structures. Moreover, the same group showed that the satiety hormone cholecystokinin reduced food intake in de-cerebrated rats, compelling further evidence that caudal brainstem can regulate food intake in the absence of hypothalamic influences (Grill and Smith, 1988).

The brainstem contains heterogeneous populations of neurons with distinct biophysical and neurochemical properties that express appetite-modulatory neuropeptides such as NPY and POMC; these neurons also express receptors mediating the effects of the same neuropeptides, indicating the existence of local circuits that contribute to the regulation of ingestive behaviors (Schneeberger *et al*., 2014). Moreover, in the brainstem receptors for a variety of circulating hormones such as leptin, ghrelin, glucagone-like peptide 1 (GLP1), amylin, and cholecystokinin (CCK) have been described (Schwartz *et al*., 2006).

Vagal signaling from the gastrointestinal tract is an important afferent to the brainstem, conveying information about luminal distension and nutritional content (Travagli *et al*., 2006). The importance of vagus nerve transmission has been demonstrated with several studies by eliminating or stimulating its activity. For example, chronic or acute vagal stimulation leads to a reduction in body weight and food intake (Gil *et al*., 2011). Vagal afferents also play important roles in the regulation of meal size and duration (Schwartz *et al*., 1999).

In the brainstem, three main structures are involved in regulating energy homeostasis: the area postrema (AP), the nucleus of solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMX). These structures are strongly

anatomically and functionally integrated to provide autonomic, behavioral and endocrine responses to energy-related peripheral cues and are collectively referred to as the dorsal vagal complex (DVC) (Young, 2012).

NTS is a structure bordering the posterior part of the 4th ventricle and it is the main sensory relay for the viscera, that integrates sensory information from gastrointestinal tract and abdominal organs, as well as taste information from the oral cavity (Travers *et al*., 1987). Afferent inputs related to satiety from the liver, the gastrointestinal tract during food ingestion and from peptides that are released upon nutrient stimulation, such as CCK, are transmitted through the vagus nerve and sympathetic fibres to the NTS, where they are integrated with descending hypothalamic inputs (Schwartz *et al.,* 2000) (**Fig. 2**). In particular, ARC POMC neurons project into NTS, where high expression of MC4R has been reported (Kishi *et al*., 2003).

The NTS also receives projections from orexin neurons located in the LHA (Ciriello *et al*., 2003), and the delivery of orexin A into the brainstem increases food intake (Parise *et al*., 2011). The orexigenic nature of the LHA and the anatomical connection with the NTS suggest that this system may serve as a mechanism to limit satiety signals from gastrointestinal tract.

Another hypothalamic nucleus sending projections into the NTS is the PVN: contralateral disruption of PVN outputs and related NTS inputs causes hyperphagia and obesity (Kirchgessner and Sclafani, 1988), indicating that PVN-brainstem pathways potentiate satiety signals at the level of NTS and play a significant role in the regulation of energy balance.

Adiposity signals such as leptin and insulin potentiate the satiating effect of CCK to activate NTS neurons (Emond *et al*., 1999).

Administration of MC4 receptor agonists or antagonists in the 4th ventricle elicits the same feeding response of those induced by injecting these compounds into the third ventricle (Grill *et al*., 1998).

These findings, combined with the evidence that leptin receptors and POMC neurons are both present in NTS, demonstrate definitively the involvement of NTS in energy homeostasis. Thus, the NTS like the ARC may contain neurons that respond to leptin and, through ascending projections to key forebrain

areas, contribute to adaptive feeding responses to changes in body fat content (Schwartz *et al*., 2000).

DMX is directly ventral to the NTS, adjacent to the central canal and is the main source of vagal innervation of various organs within the gastrointestinal tract, including the stomach, gut and pancreas (Kalia, 1981). It has an important role in the regulation of gut motility and secretion (Young, 2012), but it is also a major brain region involved in mediating pancreatic secretion, as revealed by more recent studies (Mussa and Verberne, 2013).

AP is a circumventricular organ adjacent to the NTS at the posterior extreme of the 4th ventricle. Circumventricular organs (CVOs) have been described as the "windows of the brain" because they have a fenestrated vasculature, so they lack a complete blood-brain barrier (BBB) (Johnson and Gross, 1993). For this reason, CVOs allow chemical messages to leave the brain and permit substances that do not normally cross the BBB elsewhere to alter brain functions (Horsburgh and Massoud, 2013). The vasculature of the CVOs differs from general brain vasculature. Firstly CVOs lack the expression of the tight junction proteins such as claudin-5, occludin and zonula occludens-1 (ZO-1); secondly the expression of the transport proteins such as glucose transporter 1 and transferrin receptor is not detectable (Morita and Miyata, 2012).

In addition to AP and ME mentioned above, other CVOs are the organ vasculosum of the lamina terminalis (OVLT), the subfornical organ (SFO), the neurohypophysis (NH), the pineal gland (PG) and the subcommissural organ (SCO).

CVOs are generally connected with adjacent neuronal nuclei: for example, the ME is in contact with the ARC through numerous reciprocal innervations and the AP is strictly interconnected with the NTS.

The border zone between the AP and the NTS is made of a continuous monolayer of columnar cells immunoreactive for both the tight junction protein ZO-1 and the astrocyte marker glial fibrillary acidic protein (GFAP) (Wang *et al*., 2008). The function of this diffusion barrier between AP and NTS is to reinforce the endothelial barrier of the BBB and to mediate the interactions between circulating feeding-related peptides and the region

adjacent to the CVO (Wang *et al*., 2008). In this border zone, called "funiculus separans" also extracellular matrix markers are highly expressed: extracellular matrix is indeed important for CVOs function, for diffusion of molecules between AP and NTS and for the maintenance of extracellular matrix hydration (Pócsai and Kálmán, 2014).

In the early 1950's Borison and colleagues identified the AP as the chemoreceptor trigger zone involved in controlling the emetic reflex (Borison and Wang, 1953). Chemosensitive receptors in the AP detect emetic agents in the blood and relay their information to neurons of adjacent brain regions to cause vomiting and nausea (Hornby, 2001). Other functions related to cardiorespiratory and fluid homeostasis were identified over the ensuing 40 years (Young, 2012). Moreover, the position of AP within the brain, the lack of BBB and other ultrastructural peculiarities suggested that this CVO was highly specialized to sense blood- and CSF-borne chemical information. In addition, AP receives neuronal information directly from various thoracic and abdominal viscera via several cranial nerves, and projections from PVN (Riediger, 2012). Another property of AP is the presence of a large variety of receptors for a number of neuroactive compounds and chemical substances such as neurotransmitters, hormones and toxins (Young, 2012). All these features underscore the importance of AP in feeding behaviour and energy homeostasis. This role in ingestion was proposed for the first time in 1981 (Edwards and Ritter 1981); studies on AP ablation, demonstrated that AP-lesioned (APX) rats were hypophagic and showed lower body weight compared to sham-lesions animals, although they over-consume highly palatable food. Furthermore, APX leads to loss of hypoglycaemic or glucoprivic feeding responses induced by insulin, suggesting a role of AP in central nervous glucose sensing (Hyde and Miselis, 1983). Therefore, AP is not simply a conduit through which signals flow into the brain, but it is now being recognized as the initial site of integration for these signals as they enter the circuitry of the central nervous system (Price *et al*., 2008).

In conclusion, brainstem neurons make key contributions to the control of energy balance by processing energy status informations at different levels; by sensing circulating metabolites and hormones released in the bloodstream by

peripheral organs; by receiving vagal inputs from gastrointestinal tract; by receiving neuronal inputs from midbrain and forebrain nuclei and by projecting into local brainstem circuits and other brain regions to provide information that will be integrated to control energy balance (Schneeberger *et al*., 2014).

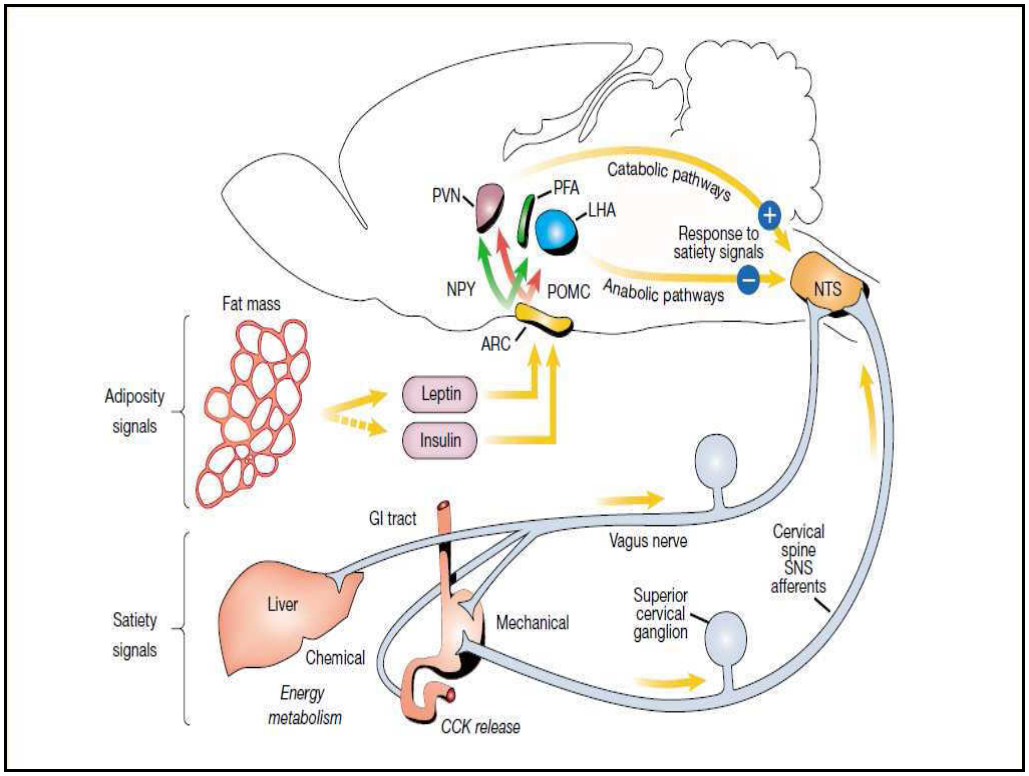

Fig. 2 - Neuroanatomical model of pathways by which adiposity signals, leptin and insulin, interact with central autonomic circuits regulating meal size. Leptin and insulin in the ARC stimulate POMC/CART neurons and inhibit NPY/AGRP neurons. These pathways projects to PVN and LHA/PFA, that are connected with hindbrain autonomic centres involved in energy balance. NTS integrates these hypothalamic inputs with vagal afferent signals from the liver, the gastrointestinal tract and from peptides such as CCK. Net neuronal output from the NTS leads to the termination of meals and is potentiated by catabolic projections from the PVN and inhibited by input from the LHA/PFA (Schwartz *et al*., 2000).

2.1.3. Appetite and satiety peripheral signals

Peripheral signals play important role in the regulation of food intake, as well as glucose homeostasis and energy expenditure. To be an 'adiposity' signal, a circulating compound must meet several criteria: 1) it must circulate in proportion to the total amount of stored fat, 2) it should interact with the brain directly, presumably by crossing the blood-brain barrier, to act on receptors in the CNS that are involved in the regulation of food intake and energy expenditure, and 3) changes in its level or activity should produce predictable changes in energy balance, by altering food intake and energy expenditure (Seeley and Woods, 2003). All these criteria are suited exactly by leptin, the hormone discovered in 1994 (Zhang *et al*., 1994) that changed the landscape in the understanding of the central regulation of energy balance.

Leptin, the product of the *ob* gene, is the anorexigenic adipose tissue-derived hormone that circulates in proportion to fat mass (Considine *et al*., 1996). This adipokine can interact with the CNS crossing the BBB through a saturable transport system (Schwartz *et al*., 1996).

Tartaglia and co-workers in 1995 identified and cloned the rodent and human leptin receptor, a molecule belonging to the gp130 family of cytokine receptors. After leptin binding, the homodimerization of the receptor activates preferentially the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, a mechanism of action in common with other cytokines (Cattaneo *et al*., 1999). Leptin also acts through phosphatidylinositol-3-OH kinase (PI3K), a pathway important for both food intake and glucose homeostasis (Niswender *et al*., 2001). There are multiple leptin receptor isoforms, with the long form (LEPRb) being essential for the effect of leptin. The lack of leptin or LEPRb in both rodents and human causes a phenotype characterized by hyperphagia, reduced energy expenditure and severe obesity (Tartaglia *et al*., 1995; Lee *et al*., 1996; Montague *et al*., 1997). Obese patients generally exhibit leptin resistance, which is the inability of high circulating leptin levels to exert central anorexigenic actions, which precludes the use of leptin as a therapeutical anti-obesity agent.

The leptin receptor is highly expressed in hypothalamic nuclei; in the ARC it's expressed in both AgRP and POMC neurons, where leptin produces opposite effects, stimulating POMC neurons and inhibiting AgRP neurons (Cowley *et al*., 2001). More recent studies demonstrate the presence of leptin receptor also in the DVC, in particular in a subpopulation of glial cells located in the AP/NTS border, suggesting the action on leptin also at the level of brainstem (Dallaporta *et al*., 2009).

Insulin, a hormone produced by β-cells, has traditionally been associated with glucose metabolism, but compelling evidence indicates that it also acts as an anorectic signal within the CNS. Its involvement in the control of food intake was suggested by Woods *et al*., in 1979. Insulin, similarly to leptin, is secreted in the bloodstream in proportion to fat stores and its receptor is expressed in hypothalamic areas involved in the control of appetite; insulin up-regulates arcuate POMC and decreases NPY and AgRP expression (Marks *et al*., 1990; Schwartz *et al*., 1996). Deletion of leptin receptors in POMC neurons results in moderate obesity without any effect on glucose homeostasis (Balthasar *et al*., 2004), while deletion of insulin receptors alone from POMC neurons fails to influence energy or glucose homeostasis (Konner *et al*., 2007); these studies highlight different functional role of both signals on melanocortin neurons. In rat brain, insulin-receptors were also detected in AP, NTS and DMX (Pardini *et al*., 2006).

Ghrelin is a hormone mainly produced by the stomach and regulated by the ingestion of nutrients (Ariyasu *et al*., 2001). Circulating ghrelin levels are increased under fasting conditions and reduced after refeeding. It has been described as a "hunger signal" because its secretion stimulate appetite (Cone, 2005). Ghrelin activates arcuate NPY/AgRP neurons, while inhibits POMC neurons by increasing GABA release onto them (Cowley *et al*., 2003; Zigman and Elmquist, 2003). Ghrelin exerts its biological actions on energy balance through the growth hormone segretagogue receptor (GHSR) that is present in hypothalamic nuclei, but also in the AP, where two different subpopulation of neurons coexist, one of which is depolarized by ghrelin, whereas the second one is hyperpolarized by the hormone (Hoyda *et al*., 2009).

Amylin is an anorexigenic hormone co-secreted with insulin by pancreatic β-cells in response to meal-related stimuli (Young, 2005). Amylin reduces food intake at near physiological doses via a reduction of meal size (Lutz *et al*., 1994). Numerous studies demonstrated that AP mediates amylin's suppressive effect on food intake; in fact, while vagal afferents are not required for amylin's anorexigenic actions, APX blocks feeding inhibitory action of amylin (Lutz *et al*., 2001). Amylin receptors are highly expressed in the AP and electrophysiological studies demonstrate that amylin exert strong excitatory effects in 44% of the recorded spontaneously active AP neurons; these effects were blocked by the amylin receptor antagonist AC187 and appear to be mediated by the intracellular second messenger cyclic GMP (Riediger *et al*., 2001). Moreover, amylin induces the expression of immediate early gene c-Fos, a marker of neuronal activation, in the AP neurons and about 50% of these neurons are noradrenergic (Potes *et al*., 2010). Interestingly, in the AP at least a proportion of the neurons responsive to amylin are also glucose responsive (Riediger *et al*., 2002). Recent studies indicated that amylin reduced eating in rats under euglycemic and hyperglycemic, but not hypoglycemic, conditions (Lutz, 2012).

CCK is postprandially secreted from the mucosa of the small intestine and its systemic delivery suppresses food intake in both animals models and humans (Gibbs and Smith, 1977; Kissileff *et al*., 1981). CCK receptors are expressed in the brainstem and hypothalamus, but the anorectic effects of CCK are critically mediated by vagal sensory neurons that project into the NTS/AP (Moran *et al*., 1997). NTS POMC neurons are activated by CCK and brainstem MC4R signalling is required for CCK-induced suppression of appetite (Fan *et al*., 2004). Moreover, other studies demonstrate that ghrelin attenuates, and leptin synergistically potentiates, the effect of CCK on appetite (Barrachina *et al*., 1997; Lee *et al*., 2011).

GLP-1 is another peripheral anorectic signal manly secreted from intestinal mucosa that acts directly in the CNS to control food intake; GLP-1 levels are high following a meal and are low under fasting conditions. GLP-1 receptor is expressed in key CNS areas involved in the control of energy balance such as the hypothalamus and brainstem (Merchenthaler *et al*., 1999).

Peptide YY similarly to GLP-1, is manly released from intestinal mucosa in response to nutrient ingestion (Adrian *et al*., 1985). It exerts anorexigenic effects in the ARC (Challis *et al*., 2003), but also in the brainstem and vagal-brainstem circuits (Koda *et al*., 2005). The peripheral delivery of this peptide has been shown to increase neuronal activity in NTS and AP neurons (Blevins *et al*., 2008).

2.2 The Ciliary Neurotrophic Factor (CNTF)

The ciliary neurotrophic factor (CNTF) is a peptide originally described by Adler *et al*., in 1979 for its ability to support survival of parasympathetic neurons from chick ciliary ganglion. It exerts important effects on neuronal and glial precursors during the development of the central and peripheral nervous system, and on the postnatal maintenance of sensory, sympathetic and motor neurons (Sendtner *et al*., 1994; Sleeman *et al*., 2000). Its expression is particularly high in peripheral nerve Schwann cells and in cerebral white matter astocytes (Stöckli *et al*., 1991; Guthrie *et al*., 1997).

CNTF is a 23 kDa molecule of 200 amino acid belonging to the interleukin-6 (IL-6)-cytokine family. This family includes IL-6, interleukin 11 (IL-11), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), leptin, cardiotrophin-like-cytokine (CLC) and neuropoietin (Bauer *et al*., 2007). These multifunctional cytokines exhibit a similar helical structure characterized by four antiparallel α-helices (Bazan, 1990). Members of the IL-6 family activate the signal transducing receptor protein, glycoprotein 130 (gp130) and for this reason these molecules can display overlapping biological activities (Heinrich *et al*., 2003). Indeed, cytokines act on many different target cells (pleiotropism) and frequently affect the action of other cytokines in an additive, synergistic or antagonistic manner; their actions are often redundant, i.e. similar biological responses can be achieved by several different cytokines (Heinrich *et al*., 1998).

Cytokines belonging to the IL-6 family usually signal through tyrosine kinases of the JAK family and transcription factors of the STAT family, but they can also activate the mitogen-activated protein kinase (MAPK) signal or a cascade involving PI3K (Heinrich *et al*., 2003). CNTF binds to a three-part receptor complex (CNTFR) consisting of the ligand-specific binding subunit receptor α (CNTFRα), which is attached to the cell membrane by a glycosylphosphatidylinositol linkage, and the signal-transducing subunits gp130 and LIF receptor b (LIFRb) (Ip *et al*., 1993).

CNTF binding to CNTFRα triggers gp130 and LIFRb heterodimerization, giving rise to the active receptor complex (Simi and Ibanez, 2010); this

receptor complex activates the Janus family of tyrosine kinases (Jak1/Jak2), thereby leading to tyrosine phosphorylation, dimerization and nuclear translocation of signal transducers and activators of transcription (STATs), mainly STAT3 (Bonni *et al*., 1997). Phosphrylated STAT3 (PSTAT3) dimers bind to specific response elements in DNA promoter regions to activate the transcription of target genes (**Fig. 3**). Besides STAT3, six other members of STAT family have been described: STAT1, STAT2, STAT4, STAT5a and STAT5b, and STAT6 (Cattaneo *et al*., 1999; Kisseleva *et al*., 2002). Activation of JAK/STATs stimulates cell proliferation, differentiation, migration and inhibits apoptosis (Kisseleva *et al*., 2002). The signal transducers gp130 and LIFR share sequence similarity and signalling capabilities with the leptin receptor and leptin acts through the same signalling pathway (Gloaguen *et al*., 1997).

CNTF shows distinctive features compared to other growth factors acting on the nervous system. First, its amino acid sequence lacks a signal peptide, suggesting a citosolic rather than a vesicular-mediated secretory process (Lin *et al*., 1989); therefore, its release from cells requires a non-conventional and as yet unidentified mechanism. In addition, CNTF is hardly detectable during embryonic development and its synthesis begins, or increase greatly, in the nervous system after birth, reaching adult levels during the postnatal period (Stöckli *et al*., 1991). Masu and colleagues in 1993 firstly demonstrated that the ablation of the CNTF gene by homologous recombination resulted in a progressive atrophy and loss of motor neurons in adult mice, but this modification did not affect embryonic development and first postnatal weeks of life (Masu *et al*., 1993). On the contrary, CNTFRα null mice died within 24h postnatally because of a suckling defect caused by severe motor neuron deficits in the brainstem and spinal cord motor nuclei (DeChiara *et al*., 1995). This evidence suggests the existence of a second putative ligand for the CNTF receptor whose role is critical at least in embryonic and neonatal periods (Derouet *et al*., 2004).

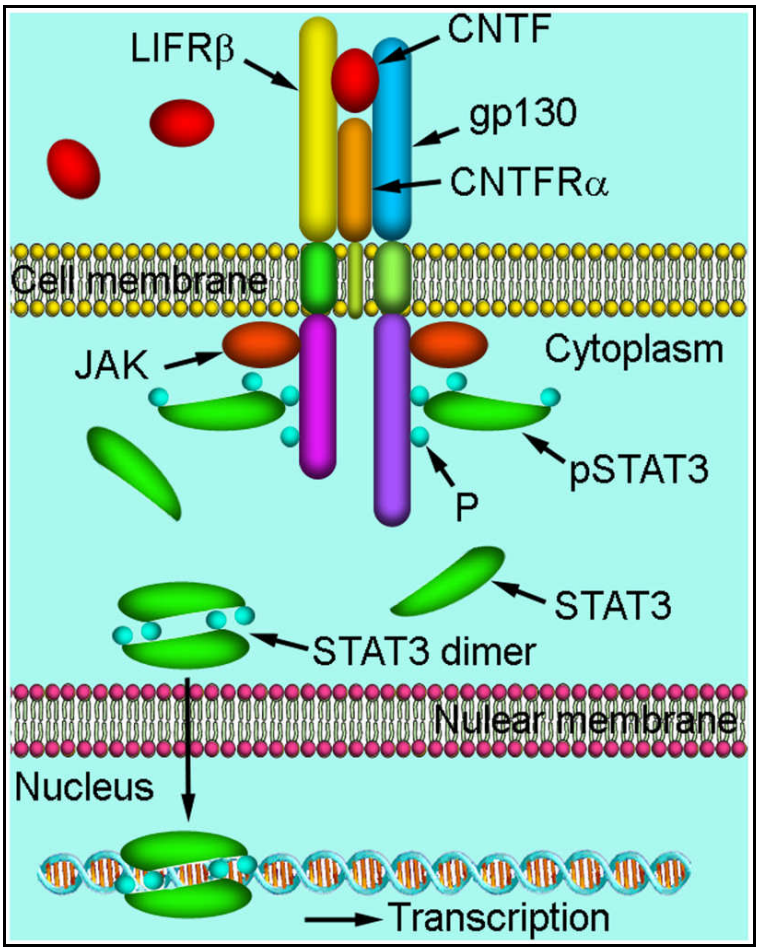

Fig. 3 - Schematic illustration of CNTF signaling through STAT3. CNTF binds to the receptor complex of CNTFRα, gp130, and LIFRβ and activates JAK kinase. Activated JAK kinase phosphorylates tyrosine residues (P) of the intracellular domain of gp130 and LIF, which provide docking sites for STAT3. After STAT3 is phosphorylated at the docking sites by JAK kinase, phospho-STAT3 (pSTAT3) forms dimers and translocates to the nucleus to induce gene transcription (Wen *et al*., 2012).

2.2.1. CNTF and CNTFRα distribution

CNTF was described for the first time as present in high amount in chick ciliary neurons (Adler *et al*., 1979); subsequent studies demonstrated CNTF presence and bioactivity in the adult rat sciatic nerve and in peripheral nerves of rabbit and rat (Manthorpe *et al*., 1986).

Immunohystochemical studies on rats and mice showed a high expression of CNTF in glial cells of central and peripheral nervous system, in particular in peripheral Schwann cells and in white matter astrocytes (Stöckli *et al*., 1991;

Sendtner *et al*., 1992; Henderson *et al*., 1994). CNTF was also detected in the olfactory bulb and optic nerve (Stöckli *et al*., 1991).

Whereas CNTF expression is low in normal CNS grey areas, it is up-regulated after mechanical or ischemic lesions (Guthrie *et al*., 1997; Lee *et al*., 1997b), suggesting a role of CNTF in the protection of injured neurons and of axonal projections (Stöckli *et al*., 1991; Dallner *et al*., 2002).

CNTFRα shows a more widespread expression respect to its ligand and is widely present in the hypothalamus, thalamus, brainstem, cerebral cortex, olfactory bulb and other areas (Lee *et al*., 1997a). CNTFRα is expressed mainly from neurons, in both the developing and adult nervous system (Ip *et al*., 1993; Lee *et al*., 1996; MacLennan *et al*., 2000); the expression of this receptor by cultured astrocytes suggested that glial cells might also represent CNTF targets (Rudge *et al*., 1994).

CNTFRα was also found in peripheral organs, such as heart, lungs, skeletal muscle, adrenal gland, skin, kidney, liver and testis (MacLennan *et al*., 2000).

CNTF injected intraperitoneally or intravenously may reach distinctive areas of the brain parenchyma by diffusing through the circumventricular organs which lacks the BBB; as a consequence, detection of nuclear P-STAT3 immunoreactivity after CNTF treatment has been proposed as a reliable tool for the characterization of CNTF responsive cells, bearing the CNTFRα (MacLennan *et al*., 2000). In the hypothalamus of mice and rats systemic injection of recombinant CNFT induced P-STAT3 expression in the brain meninges and in some perivascular cells, in ARC neurons, in the ependymal wall of the ventricular system and in the circumventricular organs, such as the vascular organ of the lamina terminalis (VOLT) and the ME (Lambert *et al*., 2001; Anderson *et al*., 2003).

Recent immunohistochemistry studies provide evidence that tanycytes and ependimal cells of the third ventricle produce CNTF and contain its functional receptor. The close spatial relationship of CNTF-producing and CNTF-responsive cells in the ependymal layer is consistent with the possibility that the mouse ependyma is provided with CNTF-dependent paracrine and/or autocrine loops involved in the CNTF-mediated responses to physiologic or pathological stimuli (Severi *et al*., 2012).

2.2.2. CNTF and energy balance

Because CNTF can promote the survival of motor neurons (Sendtner *et al*., 1990), it has been tested as a therapeutic agent for amyotrophic lateral sclerosis (ALS). Unfortunately, CNTF did not alter disease progression, leading instead to unexpected side effects like weight loss and anorexia (Miller *et al*., 1996). However, most of the patients developed anti-CNTF antibodies (ALS CNTF Treatment Study Group, 1996). Some years later, a variant of CNTF with improved potency and stability, Axokine ($CNTF_{Ax15}$), was tested in a randomized dose-ranging trial on obese patients, leading to a significant weight loss after treatment. Unfortunately, nearly 70% of the treated subjects again developed antibodies against Axokine after approximately three months of treatment (Ettinger *et al*., 2003) and the trials were stopped.

From these observations, many research groups started to investigate the role of CNTF in energy balance and they described the ability of this cytokine to mimic leptin action, reducing appetite and activating similar molecular pathways in the CNS (Anderson *et al*., 2003; Kelly *et al*., 2004). In addition to weight loss, $CNTF_{Ax15}$ administration lowered hyperinsulinemia, hyperglycemia, and hyperlipidemia associated with obesity (Gloaguen *et al*., 1997; Lambert *et al*., 2001; Watt *et al*., 2006).

Studies aimed at elucidating the mechanism of action of exogenously administered CNTF, or Axokine, stressed the role of this molecule in the hypothalamus and it was suggested that the anorectic response to CNTF could be due to a leptin-like action in the hypothalamus via activation of Jak1/Jak2-STAT3 signaling in the ARC (Lambert *et al*., 2001).

Interestingly, CNTF can also be effective in suppressing food intake in conditions in which leptin is ineffective, like in diet-induced obese mice, in *ob/ob* and *db/db* mice and in MC4R- deficient mice (Gloaguen *et al*., 1997; Lambert *et al*., 2001; Xu *et al*., 1998). Moreover, CNTFRα deletion in hypothalamic leptin receptor-expressing neurons failed to impair the anorectic effect of $CNTF_{AX15}$ (Stefater *et al*., 2012); thus CNTF may also act on hypothalamic targets that are partially different from leptin's ones.

Another important benefit of central administration of CNTF, or CNTF analogs, is the ability to remain effective after therapy has terminated: in a study by Lambert and co-workers (2001), mice maintained a decreased body weight after the CNTF treatment was finished. One possible explanation is that the delay in weight gain could be linked to neurogenesis in the hypothalamus. In fact, central administration of CNTF was found to promote cell proliferation in the adult mouse hypothalamus and many of these newborn cells expressed neuronal markers and can respond to leptin. In addition, killing the newborn cells blocks the long-term, but not the short term, effects of CNTF on body weight (Kokoeva *et al*., 2005).

In the normal mouse hypothalamus, CNTF is expressed in the ependyma of the third ventricle and in astrocytes (Severi *et al*., 2012). Subsequent studies showed that CNTF expression significantly increases in the ependymal layer and tanycytes of the tuberal and mammillary regions of mice rendered obese by an high fat diet (HFD) and that it decreases in mice kept in calorie restriction (CR) conditions. Interestingly, changes in CNTF expression were paralleled by changes in its receptor, CNTFRα. Collectively, these data suggest that in mice an HFD is associated with increased CNTF signalling in the hypothalamus, whereas CR is associated with reduced hypothalamic CNTF signalling. These findings support the notion that CNTF is a novel, glial-derived modulator of the energy balance and upregulation of CNTF signalling in the hypothalamus of HFD mice can be a compensatory mechanism counteracting the positive energy balance (Severi *et al*., 2013). In contrast, *ob*/*ob* mice showed no evidence of increased hypothalamic CNTF signalling; this suggests that leptin does not affect hypothalamic CNTF signalling and that diet composition may affect CNTF expression and signalling more than food amount in the mouse hypothalamus. (Severi *et al*., 2013).

CNTF has metabolic effects not only in the brain, but also in peripheral tissues, such as muscle, liver and adipose tissue. In the skeletal muscle, it improves insulin sensitivity enhancing oxidation of fatty acids and decreasing the synthesis and deposition of lipids and metabolites (Watt *et al*., 2006). CNTF also acts in the liver by reducing hepatic steatosis (Sleeman *et al*., 2003). Moreover it has been described that CNTFRα is expressed in adipose

tissue (Zvonic *et al*., 2003) and studies with *db/db* mice treated with recombinant CNTF, showed an up-regulation of UCP1 expression in brown adipose tissue (Liu *et al*., 2007). Ott and colleagues (2002) demonstrated also a direct role for CNTF signalling in brown adipocytes *in vitro*, with treatment with CNTF leading to the phosphorylation of STAT3 and p42/44 MAP kinase.

In conclusion, CNTF has a dual action: it reduces appetite at the level of hypothalamus and has metabolic effects on peripheral tissues, increasing insulin sensitivity on muscle and liver and stimulating thermogenesis in the adipose tissue (Matthews and Febbraio 2008).

Collectively these data suggest that endogenous CNTF has an important role in regulating energy homeostasis, and that unravelling its mechanism of action may reveal novel targets for the pharmacological treatment of obesity.

3. AIMS OF THE PROJECT

The experimental evidences detailed above suggest that CNTF, expressed by hypothalamic glial cells and tanycytes, could be a novel, endogenous, glial-derived peptide involved in the control of energy balance. Moreover, the strong mismatch between CNTF-producing and CNTF-responsive cells in coronal sections of the mouse tuberal hypothalamus suggests that CNTF could be a circulating satiety factor. In order to better elucidate the action of CNTF on mouse hypothalamus, this study aimed to assess whether circulating CNTF, besides activating STAT3, also activates other cellular pathways.

Furthermore, we tested the hypothesis that, similar to other better-characterized satiety factors, circulating CNTF may act not only at hypothalamic level, but also on brainstem centers, that are further important brain regions involved in the regulation of energy homeostasis.

4. MATERIALS AND METHODS

4.1. Animals and experimental conditions

Adult Swiss CD-1 mice and adult *ob/ob*, *db/db*, and wild type C57BL/6 mice were purchased from Charles River Laboratories (Calco, Italy). CNTF-deficient mice, where the CNTF gene has been eliminated by homologous recombination (Masu *et al*., 1993), were provided by Dr. M. Sendtner (Wuerzburg, Germany). All animals were housed in plastic cages under constant environmental conditions in a 12 h light/dark cycle at 22 °C. They had *ad libitum* access to food and water and handling was limited to cage cleaning. They were killed for experimental procedures at 12-14 weeks of age. All efforts were made to minimize animal suffering and to reduce the number of animals used. Experiments were carried out in accordance with Italian Istitutional Guidelines (EC Council Directive 86/609/EEC of 24 November 1986).

4.2. Tissue processing

For morphological analyses, mice were anesthetized with 2,2,2-tribromoethanol (Avertin) (Sigma-Aldrich, Saint Louis, MO, USA) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were carefully removed from the skull, postfixed with the same fixative solution for 24 h at 4°C and washed in PB. Free-floating coronal sections (40-µm-thick) of brainstem and hypothalamus were cut with a Leica VT1200S vibratome (Leica Microsystems, Vienna, Austria) and kept in phosphate buffered saline (PBS), pH 7.4, at 4°C until use. Adjacent brain sections were used to identify the exact location of individual hypothalamic nuclei and areas by Nissl staining (Paxinos and Franklin, 2001).

Before sacrifice, some mice received a single intraperitoneal injection (0.3 mg/kg of body weight) of recombinant rat CNTF (R&D Systems, Minneapolis, MN, USA) and/or mouse recombinant leptin (Sigma-Aldrich, Saint Louis, MO, USA; 3 mg/kg of body weight) for different periods of time

(see Results). Control mice were injected with pyrogen-free saline. The volumes of CNTF, leptin and vehicle ranged from 180 to 220 µl according to body weight; injections were performed using a Hamilton syringe.

For RT-qPCR assays animals were anesthetized and decapitated, the brain was rapidly removed from the skull and placed with its ventral side up in a pre-cooled adult mouse coronal brain matrix (ASI Instruments, Warren, MI, USA). A 2 mm-thick midsagittal slice was cut from each brain; the AP and the bottom portion of the tuberal hypothalamus, containing the ME and the ARC, were micropunched with a size 1.0 mm Harris Uni-Core device (Electron Microscopy Sciences, Hatfield, PA, USA). Samples were snap-frozen in liquid nitrogen and stored at -80°C. The remaining part of the slice was fixed, cut and stained according to standard procedures to assess whether micropunching was successful. Samples from sections where the AP or the mediobasal hypothalamus were not precisely dissected out were discarded.

4.3. Primary antibodies

The primary antibodies used in the study are shown in Tab.1:

Description	Marker	Host/isotype	IHC	IF	Manufacturer
Signaling	anti-phospho-specific-(Tyr701)-STAT3	Rabbit/IgG	1:1000	1:700	9131, Cell Signaling Technology Inc. (Beverly, MA, USA)
		Goat/IgG	1:1000	1:700	Sc-7993, Santa Cruz Biotech. (Santa Cruz, CA, USA)
	anti-phospho-specific-(Tyr694)-STAT1	Rabbit/IgG	1:1000	1:700	9167, Cell Signaling TechnologyInc.

	anti-phospho-specific-(Tyr705)-STAT5	Rabbit/IgG	1:1000	1:700	9314, Cell Signaling Technology Inc
Cell Marker	Choline Acetyltransferase (ChAT)	Rabbit/IgG		1:1000	AB143, Merk Millipore (Darmstadt, Germania)
	Dopamine β Hydroxylase (DBH)	Rabbit/IgG		1:500	PA5-34664, Thermo Fisher Scientific (Waltham, Massachusetts, USA)
	Glial Fibrillary Acidic Protein (GFAP)	Mouse/IgG		1:1000	G3893, Sigma-Aldrich (St Louis, MO, USA)
	Glutamic acid decarboxylase 67 (GAD67)	Mouse/IgG		1:800	MAB 5406, Merk Millipore
	Human Neuronal Protein (HuC/D)	Mouse/IgG		1:50	A21271, Life technologies (Carlsbad, CA, USA)
	Nestin	Mouse/IgG		1:300	MAB353, Merk Millipore
	Tryptophan hydroxylase 2 (TPH2)	Rabbit/IgG		1:700	51124, Cell Signaling Technology Inc.
	Vimentin	Goat/IgG		1:300	sc-7557, Santa Cruz Biotech.
Activity marker	c-Fos	Goat/IgG	1:5000	1:4000	sc-52-G, Santa Cruz Biotech.

anti-CNTF	CNTF	Goat/IgG	1:100	1:50	AF-557-NA, R&D Systems (Minneapolis, MN, USA)

Tab.1 - Primary antibodies used for immunohistochemistry (IHC) and immunofluorescence (IF).

4.4 Peroxidase immunohistochemistry

Immunohistochemical detection of CNTF was performed according to standard procedures. In brief, free-floating sections were reacted with 0.3% H_2O_2 (in dH_2O; 30 min) to block endogenous peroxidase, rinsed with PBS and incubated with 3% normal serum blocking solution (in PBS; 60 min). Then they were incubated with the primary antibody in PBS, overnight at 4°C. After a thorough rinse in PBS, sections were incubated in 1:200 v/v biotinylated secondary antibody solution (in PBS; 30 min), rinsed in PBS and incubated in avidin-biotin peroxidase complex (ABC Elite PK6100, Vector), washed several times in PBS and finally incubated in 3,3' diaminobenzidine tetrahydrochloride (0.05% in 0.05 M Tris with 0.03% H_2O_2; 5 min). After immunohistochemical staining, sections were mounted on slides, air-dried, dehydrated in ethanol, cleared with xylene and covered with Eukitt. Staining was not detected when the primary antibody was omitted.

Unmasking procedures were used for P-STAT3, P-STAT1 and P-STAT5 immunohistochemical detection (Frontini *et al*., 2008). Free-floating sections were reacted with 1% NaOH and 1% H_2O_2 (20 min), 0.3% glycine (10 min) and 0.03% sodium dodecyl sulphate (10 min). After rinsing in PBS, they were blocked with 3% normal goat serum (in 0.2% Triton X-100; 60 min) and incubated with the primary antibodies at appropriate dilutions (Tab. 1) in PBS, overnight at 4°C. The next day, the procedure was performed as described above.

4.5. Immunofluorescence and confocal microscopy

For double-labelling experiments, free-floating sections were processed according to the P-STAT3 protocol until the incubation with the primary antibody, when P-STAT3 was evaluated. In all other double labelling experiments standard immunoistochemical protocol was used. Sections were incubated overnight in a mixture of two primary antibodies raise in different species and diluted as shown in Tab.1.

The next day sections were washed twice with PBS and incubated in a cocktail of fluorophore-linked secondary antibodies at a dilution of 1:100 v/v in PBS for 1 h at room temperature. The secondary antibodies were Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 555 donkey anti-mouse IgG, Alexa Fluor 647 donkey anti-mouse IgG and Alexa Fluor 555 donkey anti-rabbit IgG (all from Invitrogen, Carlsbad, CA, USA). All blocking solutions were done with normal donkey sera.

Sections were subsequently washed twice with PBS, mounted on standard glass slides, air-dried and coverslipped using Vectashield mounting medium (Vector). Sections were viewed under a motorized Leica DM6000 microscope at different magnifications. Fluorescence was detected with a Leica TCS-SL spectral confocal microscope (Leica Microsystems) equipped with an Argon and He/Ne mixed gas laser. Fluorophores were excited with the 488 nm, 543 nm and 649 nm lines and imaged separately. Images (1024 x 1024 pixels) were obtained sequentially from two channels using a confocal pinhole of 1.1200 and stored as TIFF files. Brightness and contrast of the final images were adjusted using Photoshop 6 (Adobe Systems, Mountain View, CA, USA).

4.6. Morphometric Analysis

The percentage of P-STAT3-positive cells also expressing P-STAT1, P-STAT5 or cell markers (listed in Tab. 1), was calculated in 5 alternate double-stained coronal sections of the brainstem and of the tuberal portion of the hypothalamus from 3 mice per experimental group. For all measurements, the results were expressed as the mean ± standard error of the mean (SEM).

4.7. RNA isolation, cDNA Synthesis, and RT-qPCR

Total RNA was extracted from micropunched tissue after homogenization using RNeasy Micro kit (Qiagen, Milano, Italy) according to the manufacturer's instructions. Three separate micropunches of the AP and mediobasal hypothalamus were pooled by pipetting samples onto the same purification column to increase RNA yield. The quality and quantity of isolated total RNA was evaluated using the 2100 BioAnalyzer (Agilent Technologies, Milano, Italy). One microliter from each isolated RNA sample was analyzed with RNA 6000 Pico LabChips (Agilent Technologies). To determine mRNA levels, 500 ng of RNA was reverse-transcribed with a High-Capacity cDNA RT Kit with RNase Inhibitor (Applied BioSystems, Foster City, CA, USA) in a total volume of 20 µl. Real time gene expression was analyzed in triplicate by using TaqMan Gene Expression Assays (Applied BioSystems) as listed: TATA box binding protein (TBP): Mm00446973_m1; CNTF: Mm00446373_m1; CNTFRα: Mm00516693_m1; POMC: Mm00435874_ml; Vimentin: Mm01333430_ml, and Master Mix TaqMan (all from Thermo Fisher Scientific). The efficiency of each assay was evaluated using a standard curve with serial dilutions of a known template, and the equation of the linear regression line, along with the coefficient of determination (R^2), were calculated. The reaction efficiency was 96.84% for CNTF, 92.56% for CNTFR, 93.71% for POMC, and 95.4% for vimentin.
Reactions were carried out in a Step One Plus instrument (Thermo Fisher Scientific) using 25 ng of cDNA in a final reaction volume of 20 µl and the following thermal cycle protocol: initial incubation at 95 °C for 10 min,

followed by 40 cycles of 95 °C for 15 sec and 60 °C for 20 sec. In order to rule out genomic contamination, a control reaction where reverse transcriptase was omitted in the amplification mixture was included for each sample. Relative mRNA expression was determined by the Δ-Ct method ($2^{-\Delta Ct}$) using TBP levels as an endogenous control. Differences in starting total RNA and in cDNA synthesis efficiency among samples were normalized using TBP expression. Data are presented as histograms $\pm$ SEM.

5. RESULTS

5.1. CNTF-responsive cells in the hypothalamus

In the mouse hypothalamus, CNTF administration activates STAT3 in arcuate nucleus neurons, in ME cells and diffusely in the third ventricle ependyma (Severi *et al*., 2012). We demonstrated that besides STAT3, other STAT isoforms are activated by CNTF. In particular, immunohistochemical analyses of coronal brain sections from treated and control mice were performed using polyclonal anti-P-STAT1 antibody. P-STAT1 immunoreactive cells were absent in control mouse, suggesting that STAT1 signaling is not activated in the hypothalamus in basal conditions. By contrast, systemic injection of recombinant CNTF induced STAT1 phosphorylation in cells of the ME (**Fig. 4A**) and other brain circumventricular organs, including the vascular organ of the lamina terminalis (VOLT, **Fig. 4B**) and the subfornical organ (**Fig. 4C**). In particular, in the ME of treated mice P-STAT1 staining was found in the ependymal cells making up the floor of the third ventricle (roof of the ME) and in those forming the ventrolateral recesses of the third ventricle and facing the medial portion of the ARC (**Fig. 4A, D** and **E**); several cells scattered throughout the ME layers were also positive (**Fig. 4D**). P-STAT1 staining was mainly detected in cell nuclei, as also confirmed by double-staining and confocal microscopy analysis with the TO-PRO3 nuclear stain (**Fig. 4F**). However, in some cells, the cytoplasm was also variably stained; this was especially evident in the ependymal cells of the ventrolateral recesses of the third ventricle, where P-STAT1-positive tanycyte-like cells exhibited long, faintly positive processes extending to the adjacent nervous tissue (**Fig. 4E**). Positive cells in the ME usually displayed an elongated nucleus without evident nucleoli (**Fig. 4D**).

Double-staining experiments and confocal microscopy demonstrated that P-STAT1-positive ependymal cells on the floor of the third ventricle were nestin-positive β2-tanycytes (**Fig. 4G-I**), whereas those forming the ventrolateral recesses of the third ventricle were vimentin-positive β1-tanycytes (**Fig**. **4J-L**). Also a proportion of CNTF-responsive ME cells were

positive for nestin (25.20% ± 2.77, n=5; **Fig. 4G-I**, insets) or vimentin (13.20% ± 0.84, n=5; **Fig. 4J-L**) and only occasionally expressed GFAP, the marker of mature astrocytes (**Fig. 5A-C**); their morphological features were similar to radial glial cells, with long projections spanning the ME, from the ependyma to the pial layer. None of these cells co-localized with the neuronal markers NeuN or HuC/D (**Fig. 5D-F**).

We demonstrated that CNTF treatment activated also P-STAT5 in mouse hypothalamus: as previously reported in rats (Lerant *et al*., 2001; Mutze *et al*., 2007) and mice (Ladyman *et al*., 2012), the tuberal hypothalamus of control mice contained a variable number of faintly P-STAT5-positive neurons, mainly located in the ARC but also scattered in the ventromedial and dorsomedial nuclei and lateral hypothalamus. However, no P-STAT5 staining was detected in the ME, whereas punctate immunolabeling was frequently found in its internal portion, suggesting the presence of P-STAT5 immunoreactive axons (**Fig. 6A**). As previously observed with P-STAT1, CNTF injection induced P-STAT5 nuclear expression in the VOLT, the subfornical organ and the ME, where ependymal and glial cells exhibited a variable degree of nuclear staining (**Fig. 6B**). However, unlike P-STAT1 staining, some astrocytes-like cells in the medial portion of the ARC also appeared to be strongly responsive to CNTF (**Fig. 6B**). Co-localization studies confirmed that ependymal CNTF-responsive cells were nestin-positive β2-tanycytes (**Fig. 6C-E**) and vimentin-positive β1-tanycytes (**Fig. 6F-H**), whereas some of those detected in the ME were nestin-positive (32.80 ± 2.28, n=5; **Fig. 6C-E**) or vimentin-positive (16.00 ± 1.58, n=5; **Fig. 6F-H**) immature glial cells. In the ARC of treated mice, the P-STAT5-positive cells were GFAP-positive astrocytes (**Fig. 6I-K**).

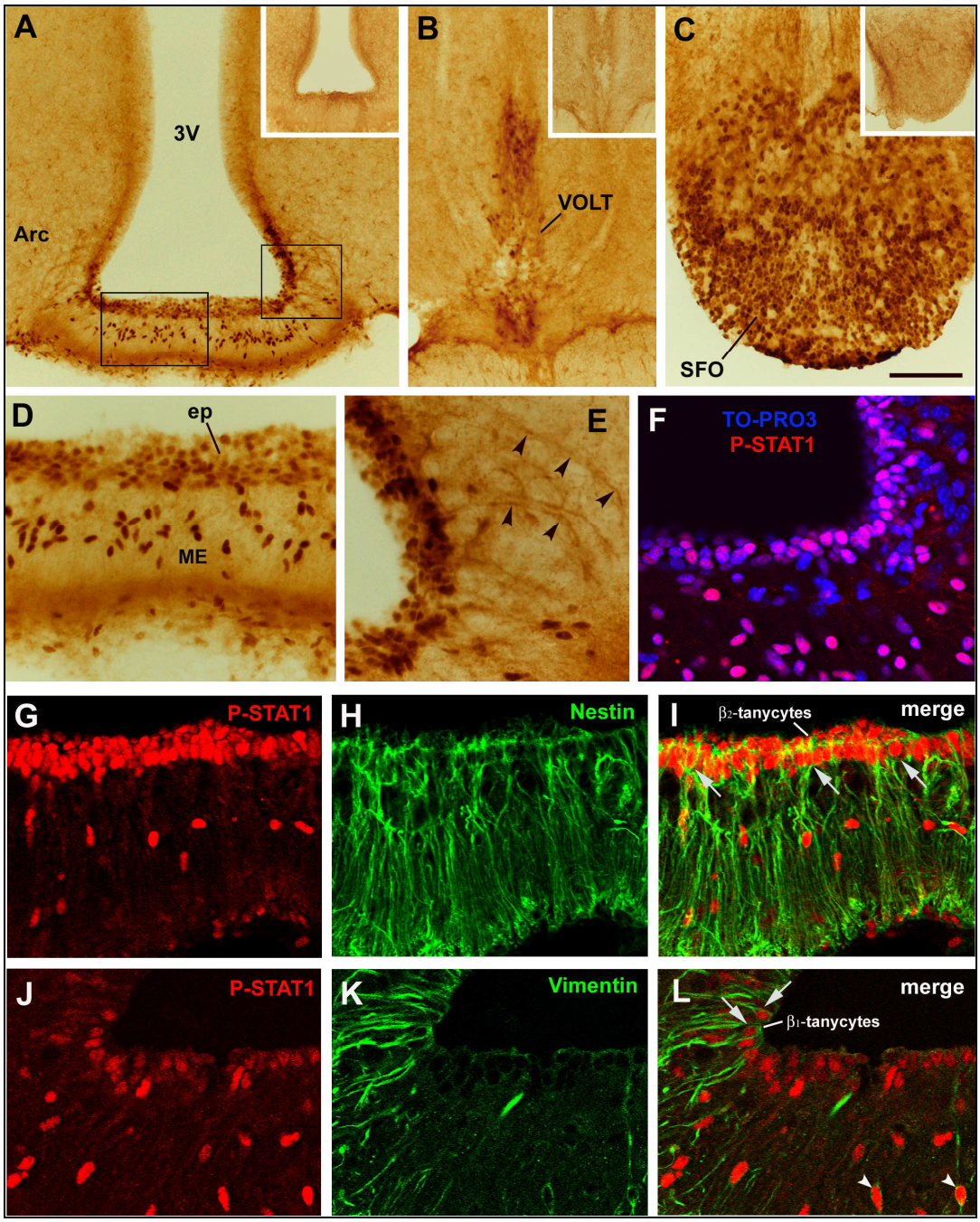

Fig.4 – P-STAT1 immunohistochemistry in coronal sections of mouse brain. In CNTF treated mice, P-STAT1 staining was detected in ME (A), VOLT (B) and subfornical organ (SFO; C); insets show P-STAT1 immunohistochemistry in the corresponding structures of control mice. In the ME, P-STAT1 staining was detected in cell nuclei of ependymal (ep) and underlying cells (D and E); ependymal cells of the ventrolateral wall of the third ventricle (3V) exhibited long P-STAT1-positive processes (arrowheads). D and E are enlargements of the areas framed in A. In F is represented the co-localization of P-STAT1 with the nuclear stain TO-PRO3. By double-staining experiments and confocal microscopy, P-STAT1 positive ME cells showed clear nestin (G-I) and vimentin (J-L) positivity. Bar: A=150 µm; B and C=100 µm; inset of A-C=300 µm; D=60 µm; E=40 µm; F-L=30 µm; insets of G-I= 5 µm.

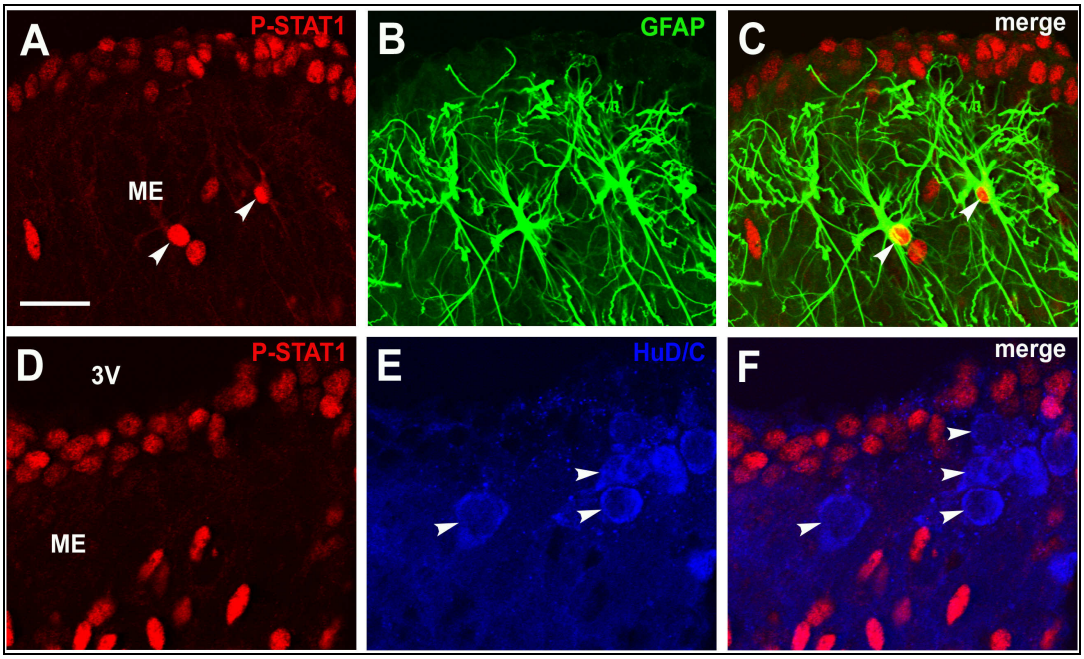

Fig.5 – Double label confocal microscopy showing some P-STAT1-positive ME cells also expressing GFAP (A-C, arrowheads). None of these cells co-localized with the neuronal marker HuC/D (D-F, arrowheads).
Bar: A-F= 30 µm

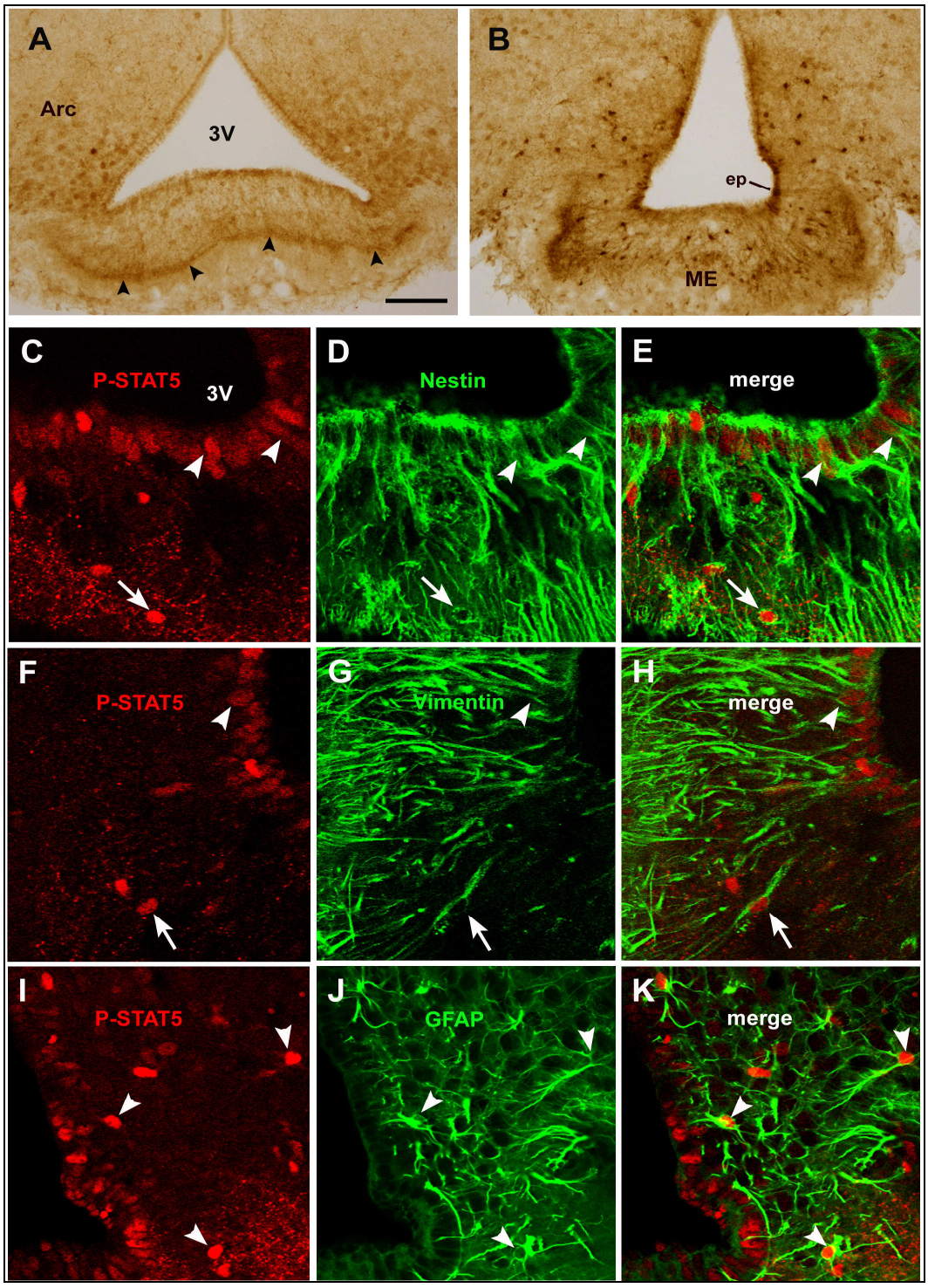

Fig.6 - P-STAT5 immunohistochemistry in coronal sections of mouse brain. In control mice (A) weak P-STAT5 nuclear staining was detected in neurons of the ARC and in fibers of ME (arrowheads). In CNTF-treated mice (B), P-STAT5 positive cells appeared along the ependyma (ep), in the ME and in the ARC. By double-staining experiments and confocal microscopy, P-STAT5-positive ependymal (arrowheads) and underlying (arrows) cells were also positive for nestin (C-E) or vimentin (F-H). In the Arc P-STAT5-positive cells co-localized with GFAP (I-K, arrowheads). Bar: A and B=150 µm; C-K=25 µm.

The percentage of P-STAT1- and P-STAT5-positive cells also exhibiting P-STAT3 immunoreactivity in the tuberal hypothalamus of treated mice was evaluated by double staining experiments and confocal microscopy. Results showed that 92.62% $\pm$ 4.75 (n=5) of P-STAT1-positive β-tanycytes also exhibited P-STAT3 immunoreactivity, and 72.08% $\pm$ 4.82 (n=5) of P-STAT1-immunoreactive ME cells were also positive for P-STAT3 (**Fig. 7A-D**). Concerning P-STAT5, virtually all ependymal and ME cells expressing it in treated mice were also positive for P-STAT3 (**Fig. 7E-G**).

Thus, systemic CNTF administration leads to activation of multiple STAT isoform in β-tanycytes and ME glial cells, which both exhibit markers of immaturity and plasticity; on the contrary, in the ARC neurons and in the few ME neurons found just below the ependyma only STAT3 is activated (**Fig. 7A-D).**

To obtain further confirmation of the CNTF action in β-tanycytes and ME glial cells, the hypothalamic expression of P-STAT1, P-STAT5 and c-Fos, a marker of neuronal activation, was evaluated in control and treated mice 20, 40, 80 and 120 min after the injection. Results showed that as early as 20 min from treatment, ME glial cells already displayed P-STAT1 and P-STAT5 immunoreactivity, whereas the ependymal cells stained faintly or patchily (**Fig. 8A** and **E**). Full activation of the Jak-STAT1 and -5 pathways was found at 40 min (**Fig. 8B** and **F**) and 80 min (**Fig. 8C** and **G**). Interestingly, at 80 min c-Fos staining was also detected in β-tanycytes and ME cells (**Fig. 9B**) of treated but not of control mice (**Fig. 9A**). Double-labeling experiments demonstrated that in treated mice numerous P-STAT1 (**Fig. 9C-E**) and P-STAT5-positive (**Fig. 9F-H**) cells also expressed c-Fos, suggesting that CNTF determines immediate early gene activation. At 120 min P-STAT immunoreactivity declined in tanycytes and ME cells (**Fig. 8D** and **H**) whereas some GFAP-positive astrocytes mainly located on the lateral border of the ME and in the inferomedial portion of the ARC exhibited P-STAT5 and P-STAT1 immunoreactivity (**Fig. 8D,H** and **I-K**). At 120 min, c-Fos continued to be detected not only in tanycytes and ME cells, but also in the ARC. In conclusion, the action of CNTF in β-tanycytes and ME glial cells is time-dependent and induces c-Fos expression.

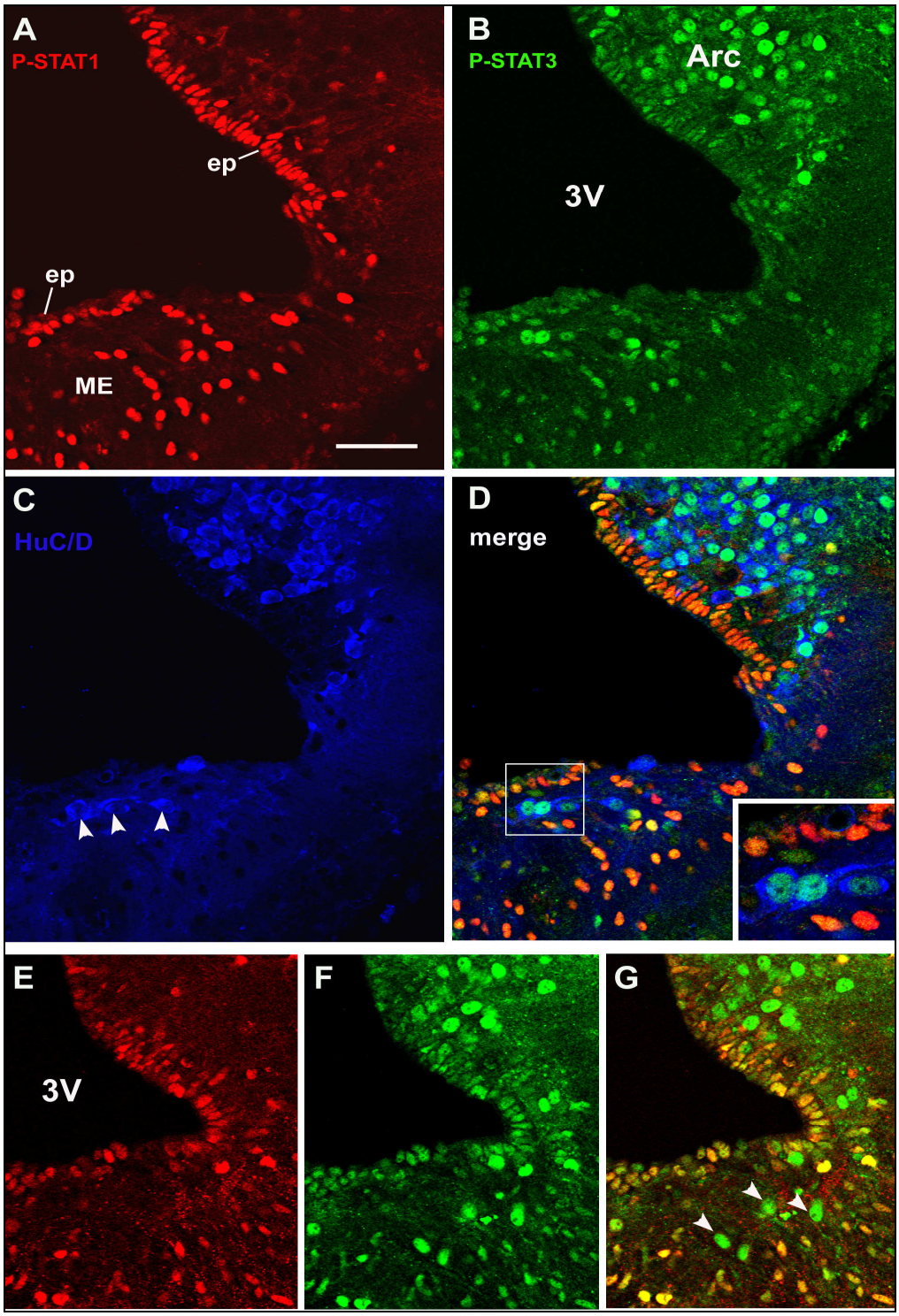

Fig.7 – Multiple labelling and confocal microscopy analysis of the tuberal hypothalamus from a CNTF-treated mouse. CNTF activated STAT1 (A) and STAT3 (B) in ependymal (ep) and ME cells. Some HuC/D-positive neurons (B-C, some indicated by arrowheads) in the ME and ARC were also positive for P-STAT3 but not for P-STAT1 (D). In the botton panels, all ependymal and ME cells co-expressed P-STAT5 and P-STAT3 (E-G). Some cells (arrowheads), likely neurons, in ME and ARC are positive only for P-STAT3. Bar: A-D=30 µm; inset of D=15 µm; E-G=35 µm.

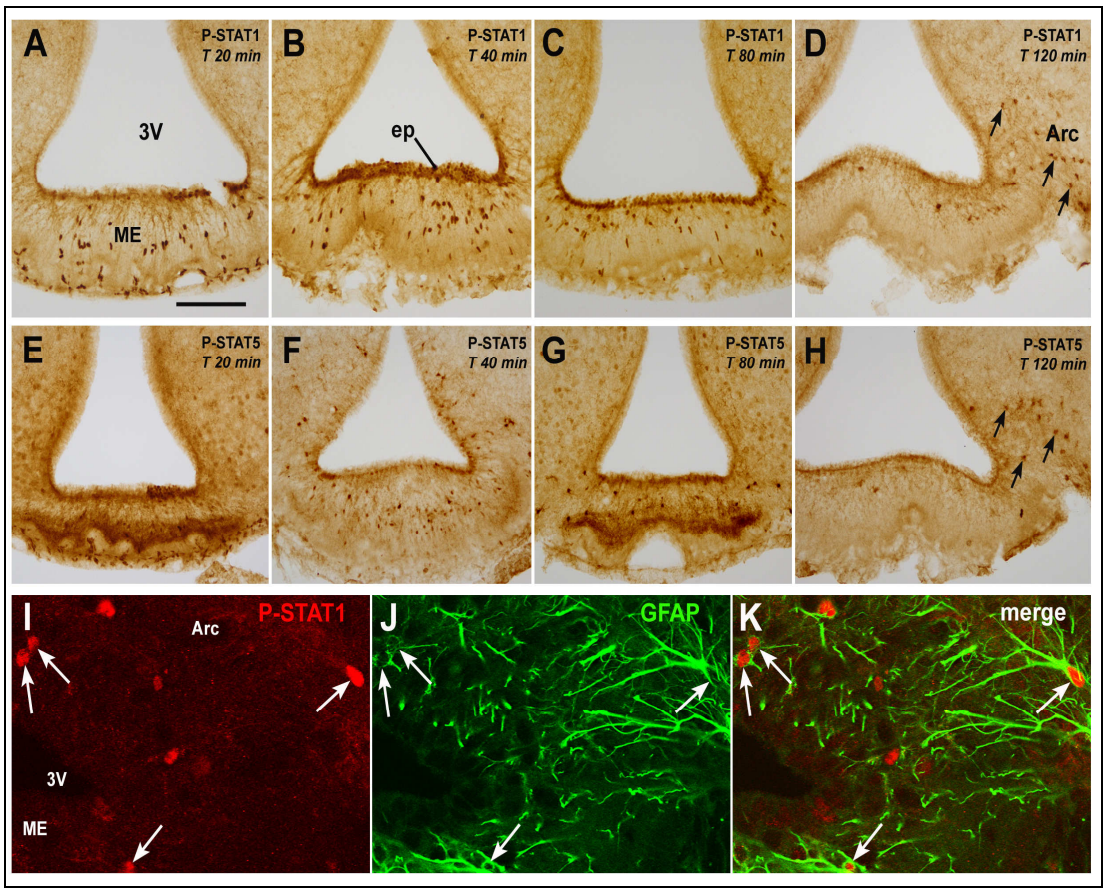

Fig.8 – P-STAT1 (A-D) and P-STAT5 (E-H) immunohistochemistry at different timepoints after CNTF-administration. At 120 min from CNTF administration, some cells in the ARC exhibited P-STAT1 and P-STAT5 positivity (D and H, arrows); these cells co-localized with GFAP (I-K, arrows). Bar: A-H= 180 µm; I-K=30 µm.

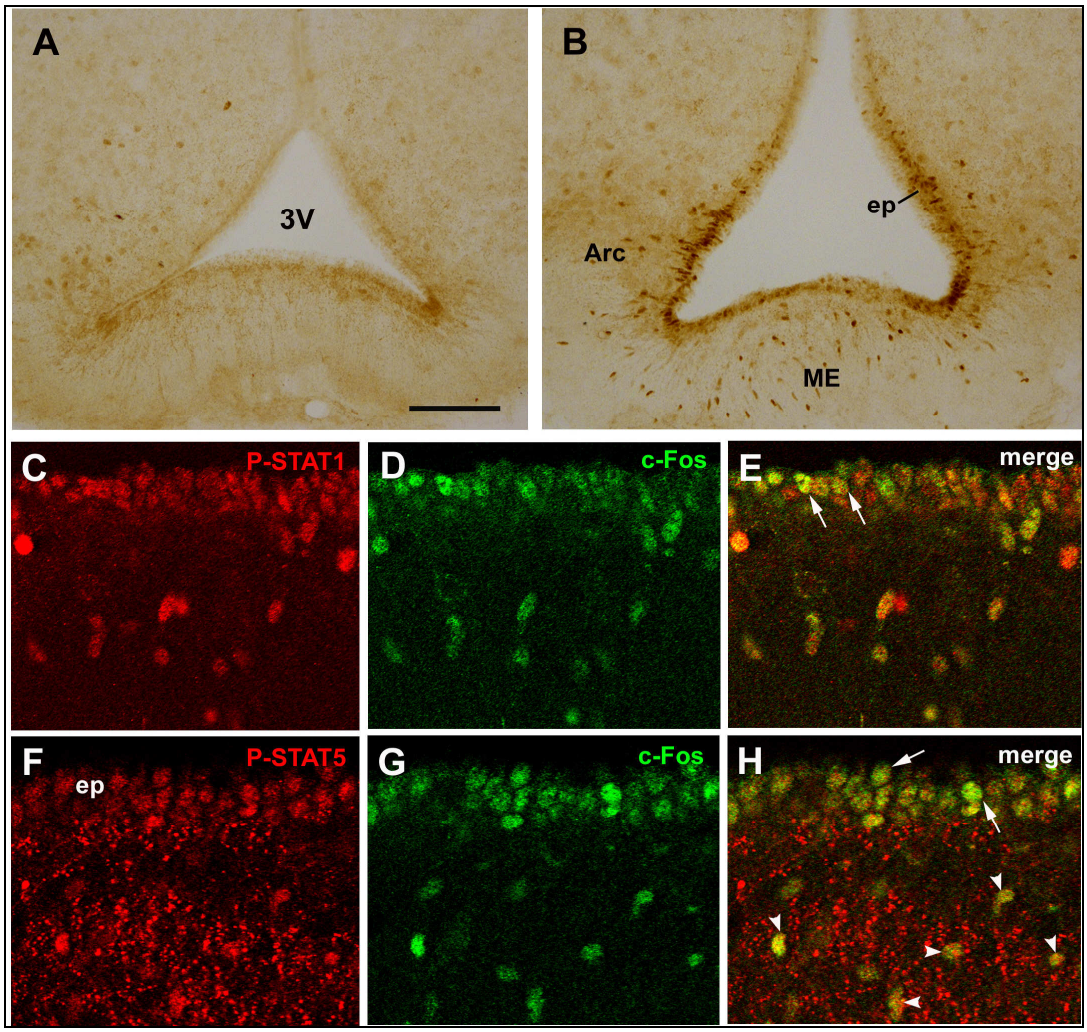

Fig.9 – c-Fos immunohistochemistry in the mouse ME. In control animal (A), faint c-Fos staining was detected in some scattered cell nuclei, whereas at 80 min from CNTF administration (B) ependymal (ep) and subependymal ME cells and few cells in the Arc showed c-Fos expression. By double-staining experiments, P-STAT1-positive (C-E) and P-STAT5-positive (F-H) ependymal (arrows) and ME (arrowheads) cells also expressed c-Fos. Bar: A and B=150 µm; C-H=25 µm.

5.2. Mismatch of CNTF-responsive and CNTF-producing cells in the tuberal hypothalamus

As reported previously (Severi *et al.*, 2012, 2013), CNTF was mainly expressed in the ependyma bordering the upper portion of the lateral wall of the third ventricle, where numerous α-tanycytes were also stained. CNTF was never expressed in underlying β-tanycytes or ME cells. As a result, there was always a considerable mismatch between overlying CNTF-producing cells and underlying CNTF-responsive cells (**Fig. 10A-C**). This suggests that in physiological conditions ME β-tanycytes and glial cells may be stimulated by α-tanycyte-derived CNTF secreted into the cerebrospinal fluid and/or that they may be selectively targeted by circulating CNTF.

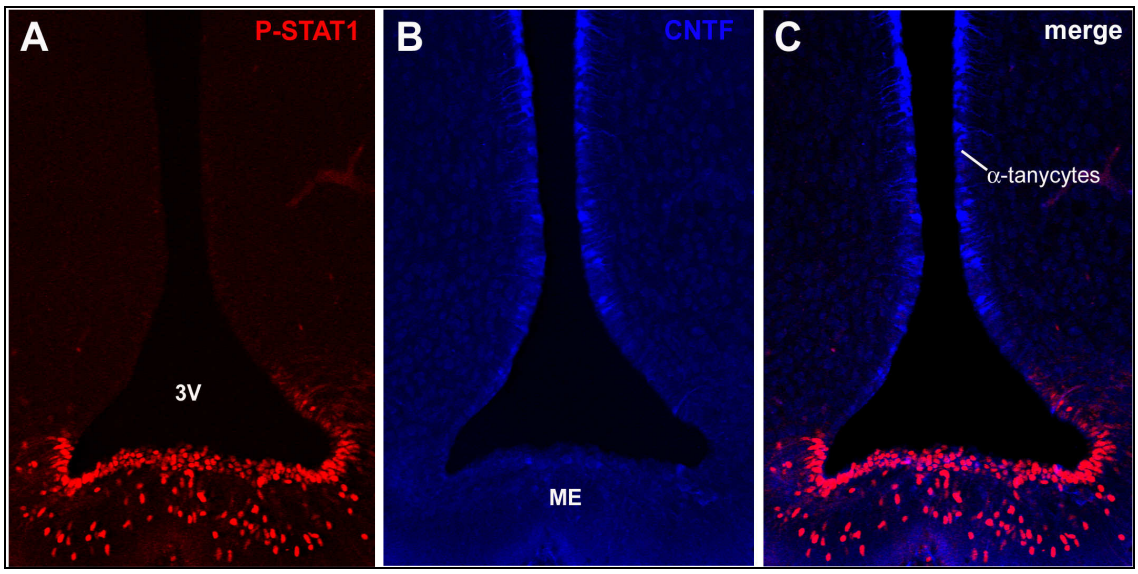

Fig.10 – P-STAT1/CNTF double labelling of mouse tuberal hypothalamus showing the mismatch between CNTF-responding (A) and CNTF-producing (B) cells 40 min after CNTF injection.

5.3. CNTF-responsive cells in the mouse AP

Similarly to what observed in the hypothalamus, CNTF administration induced STAT3, STAT1 and STAT5 phosphorylation also in the AP, where the nucleus of numerous cells was strongly stained (**Fig. 11A-C**). On the contrary, in the brainstem parenchyma of vehicle-treated mice, cells exhibiting nuclear P-STAT3, P-STAT1 or P-STAT5 were few, sparse and weakly labelled. Double-staining experiments, performed at different rostrocaudal levels, showed that P-STAT3 staining was ubiquitous and was also detected in the border of the AP, the funiculus separans, whereas immunoreactivity for P-STAT1 and P-STAT5 involved a smaller number of cells, mainly located in the central portion of the AP (**Fig. 11D-F**).

In addition, the majority of CNTF-responding cells were positive for at least two STAT isoform. In particular, 76.91% $\pm$ 3.20 (n=3) of STAT3-reactive cells were also positive for P-STAT1 (**Fig. 11G-I**), and 77.86% $\pm$ 1.31 (n=3) of STAT3-positive cells were also positive for P-STAT5 (**Fig. 11J-L**). Conversely, all P-STAT1- and P-STAT5-positive cells were also positive for P-STAT3. Therefore, STAT3 is the main transduction factor activated by CNTF in the mouse AP, and several CNTF-responding cells activate STAT3 as well as STAT1 and/or STAT5.

To characterize the phenotype of CNTF-responsive cells in the AP, double-immunostaining and confocal microscopy experiments were performed using mature and immature neuronal and glial markers (Tab. 1). Results showed that 14.27% $\pm$ 0.25 (n=3) of P-STAT3-positive cells were HuC/D-positive neurons (**Fig. 12A-C**), whereas 11.97% $\pm$ 0.11 (n=3) co-localized with nestin (**Fig. 12D-F**), 11.51% $\pm$ 1.42 (n=3) with vimentin (**Fig. 12G-I**), and 6.57% + 0.81 (n=3) with GFAP (**Fig. 12J-L**). Notably, since nestin, vimentin, and GFAP immunoreactivity was seen in cell processes rather than cell bodies, the extent of co-localization was probably underestimated. These data showed that circulating CNTF acts on both neurons and glial cells of the AP, where a considerable proportion of CNTF-responsive cells exhibit immaturity markers such as nestin and vimentin; this situation is highly reminiscent of the action of CNTF on the hypothalamic ME.

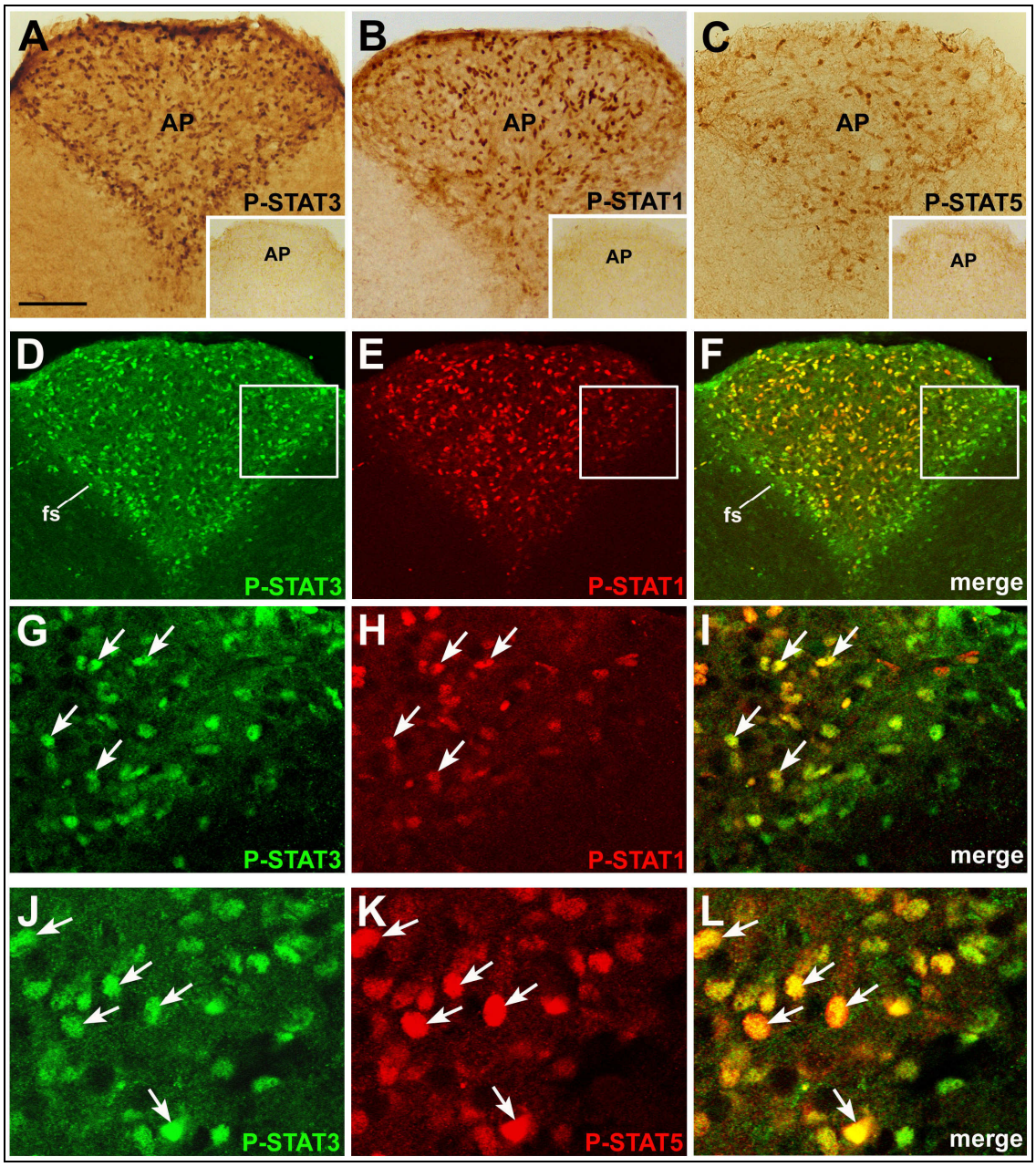

Fig. 11 – P-STAT immunohistochemistry in the mouse AP. After 45 min from CNTF treatment, P-STAT3 (A), P-STAT1 (B) and P-STAT5 (C) immunoreactivity was detected in the nucleus of many cells of the AP. The insets represented the AP of control mice processed for immunohistochemistry against the three P-STAT isoforms. Double-staining experiments in CNTF-treated mice showed that P-STAT3 staining was ubiquitous and is also found in the funiculus separans (fs), whereas P-STAT1 positivity is detected in a smaller number of cells, mainly in the central part of the AP (D-F). The majority of P-STAT3-positive cells also expressed P-STAT1 (G-I, arrows) or P-STAT5 (J-L, arrows). Panels (G-I) are enlargements of the areas framed in panels (D-F), respectively. Bar: A-F=120 µm; insets of A-C=300 µm; G-I=25 µm; J-L=18 µm.

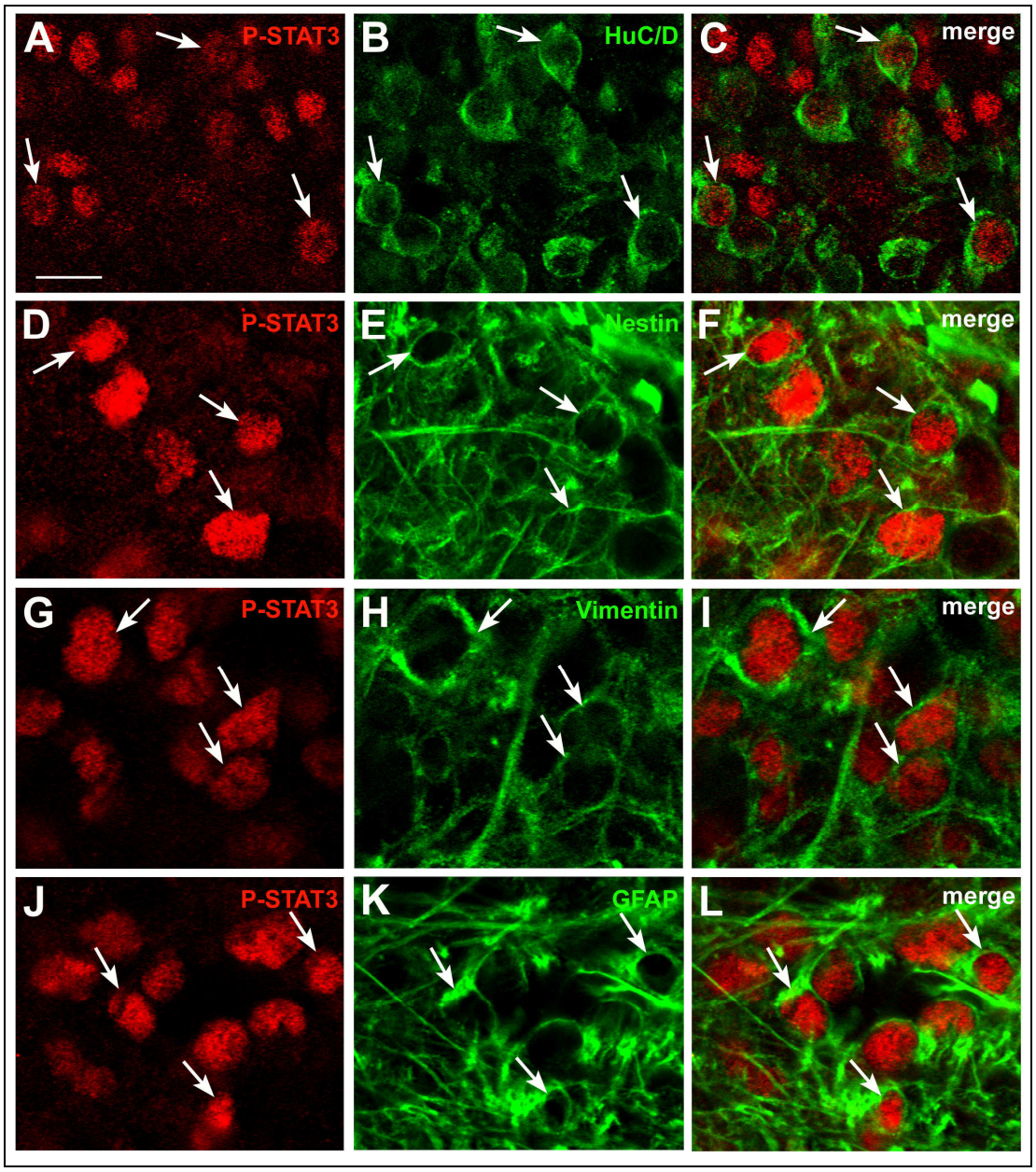

Fig.12 – Double staining and confocal microscopy on the AP of CNTF-treated mice. Some P-STAT3-positive cells co-localized with HuC/D (A-C, arrows), nestin (D-F, arrows), vimentin (G-I, arrows) or GFAP (J-L, arrows). Bar: A-C=12 µm; D-L=8 µm.

To investigate the nature of neuronal populations activated by circulating CNTF we performed double-immunostaining experiments with dopamine-beta-hydroxylase (DBH), tryptophan hydroxylase (TPH), glutamic acid decarboxylase 67 (GAD67) and choline acetyltransferase (ChAT), which are widely used markers for noradrenergic, serotoninergic, GABAergic and cholinergic neurons respectively (Tab.1). CNTF-responsive P-STAT3-positive neurons only very rarely expressed DBH (**Fig. 13A-C**) or TPH (**Fig. 13D-F**), whereas evidence of co-localization was never found for the GABAergic (**Fig. 13G-I**) or cholinergic (**Fig. 13J-L**) neurons. To gain insight into the effects of CNTF on the AP neurons we looked for co-localization of P-STAT3-HuC/D and c-Fos in sections from mice treated with CNTF for 80 min, a treatment duration that induces c-Fos transcription while maintaining the Jak-STAT pathway active (Hubschle *et al*., 2001). Interestingly, c-Fos positivity was found in nearly all non-neuronal CNTF-responsive cells, but not in CNTF-responsive HuC/D-positive neurons (**Fig. 13M-P**).

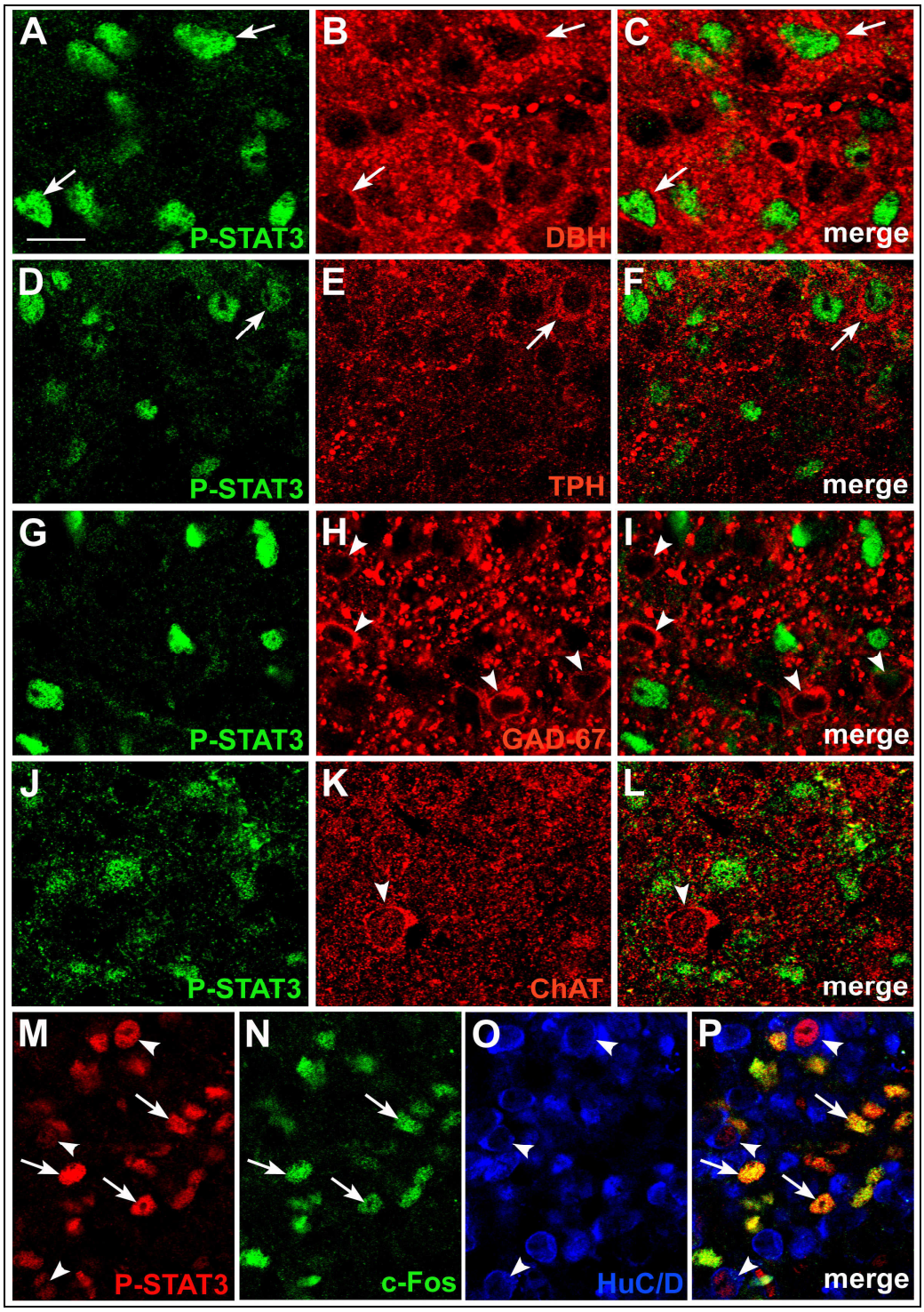

Fig. 13 – Phenotypic characterization of CNTF-responsive neurons in the mouse AP. Double-staining experiments showed that in mice treated with CNTF after 45 min, few P-STA3-positive cells were also positive for DBH (A-C, arrows)or TPH (D-F, arrows). In contrast, GAD67 (G-I, arrowheads) and ChAT (J-L, arrowheads) expressing neurons never co-localized with P-STAT3. In a mouse treated with CNTF after 80 min (M-P), some P-STAT3-positive cells also expressed c-Fos (arrows), whereas the P-STAT3 HuC/D-positive neurons did not (arrowheads). Bar: A-C=10 µm; D-L=12 µm; M-P=18 µm.

5.4. CNTF induces c-Fos expression in the mouse NTS

Further insights into the action of circulating CNTF on brainstem feeding centers were obtained by examining P-STATs and c-Fos expression and tissue distribution in mice treated with an intraperitoneal injection of vehicle or CNTF for 120 min, a time interval that is known to induce full protein expression of the gene (Sheng and Greenberg, 1990). P-STAT3 (**Fig. 14A-C**), P-STAT1, and P-STAT5 were again detected only in the AP of CNTF-treated mice but staining was reduced compared with mice treated for 45 min, suggesting that after 120 min Jak-STAT signaling had begun to abate. In these mice, STAT-positive nuclei were mainly detected in the GFAP-positive tanycyte-like cells forming the funiculus separans (insets of **Fig. 14A-C**), the glial structure on the ventrolateral border of the AP separating it from the adjacent nuclei (McKinley *et al*., 2003). As expected, c-Fos was barely detectable in vehicle-injected mice (insets of **Fig. 14D-E**) and it was strongly expressed in CNTF-treated mice, both in the AP and in the rostral and caudal NTS (**Fig**. **14D-E**). In the AP, very few neurons exhibited weak c-Fos expression (**Fig. 14F-H**), whereas in the NTS all the c-Fos immunoreactive cells were neurons, as demonstrated by their immunoreactivity for the neuronal marker HuC/D (**Fig. 14I-K**). In some sections, c-Fos positive neurons were also detected in the DMX (**Fig. 15A-C**). These neurons showed different size, and they were small and medium-size in the NTS, whereas they were larger in the DMX (**Fig. 15D-F**). Double-staining experiments with the cholinergic marker ChAT demonstrated that these neurons were mainly cholinergic (**Fig. 15G-I**).

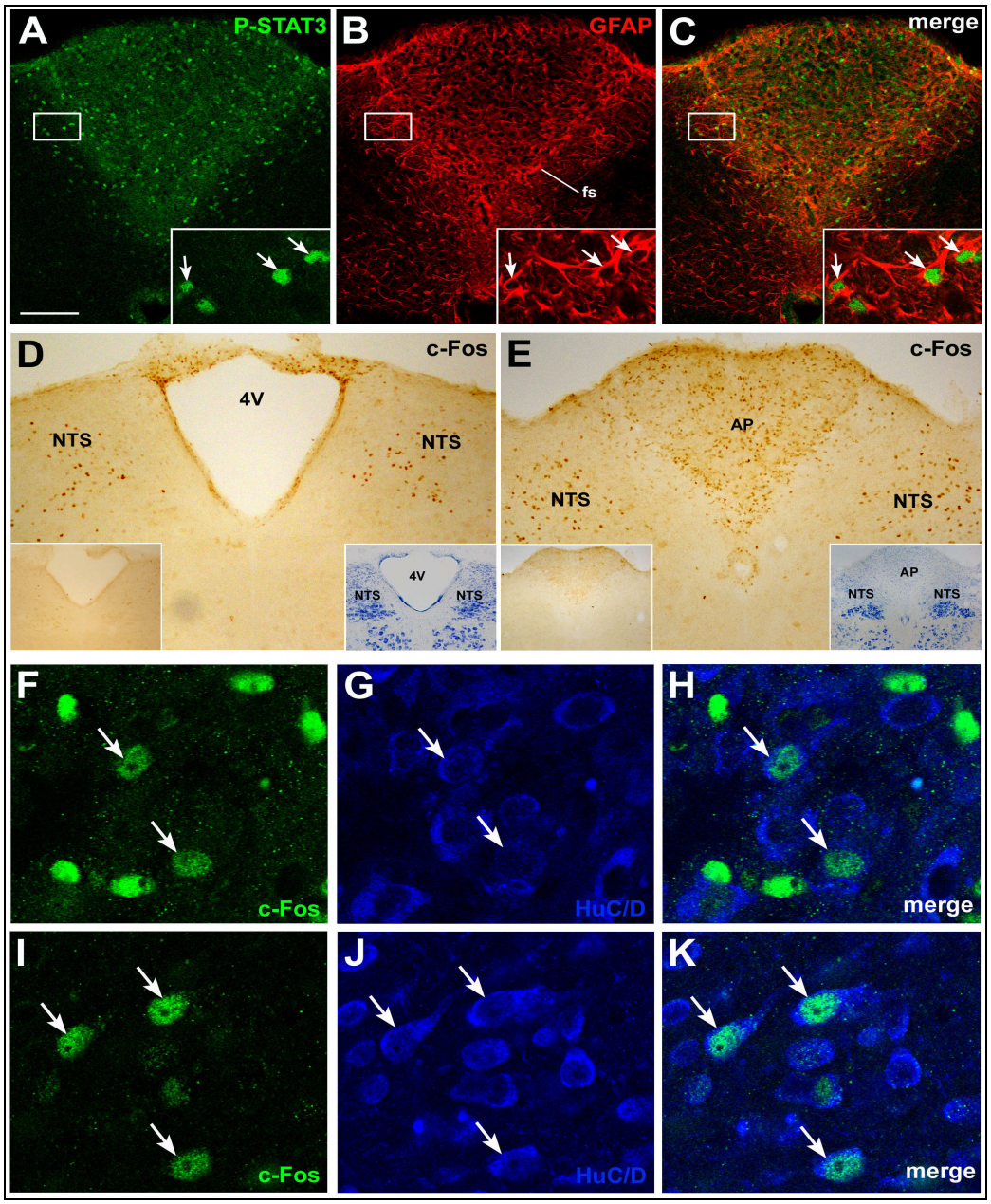

Fig.14 – P-STAT3 and c-Fos immunohistochemistry in coronal brainstem sections from mice treated with CNTF for 120 min. P-STAT3-positive cells in the AP (A-C), including the funiculus separans (fs), co-localized with GFAP (insets, arrows). Immunoperoxidase histochemistry showed nuclear c-Fos expression in numerous cells in the rostral (D) and caudal (E) portions of the NTS and in the AP. 4V, fourth ventricle. Double-staining experiments demonstrated that in the AP (F-H) only few HuC/D-positive neurons expressed weak c-Fos staining (arrows), whereas in the NTS (I-K) all c-Fos positive cells were HuC/D-positive neurons. Inset of A-C are enlargement of the corresponding framed areas. In D and E, insets on the left represent corresponding structures from control mice processed for c-Fos immunohistochemistry, those on the right show Nissl-stained sections. Bar: A-C=150 µm; insets of A-C=20 µm; D and E=150 µm; insets of D and E=400 µm; F-K=10 µm.

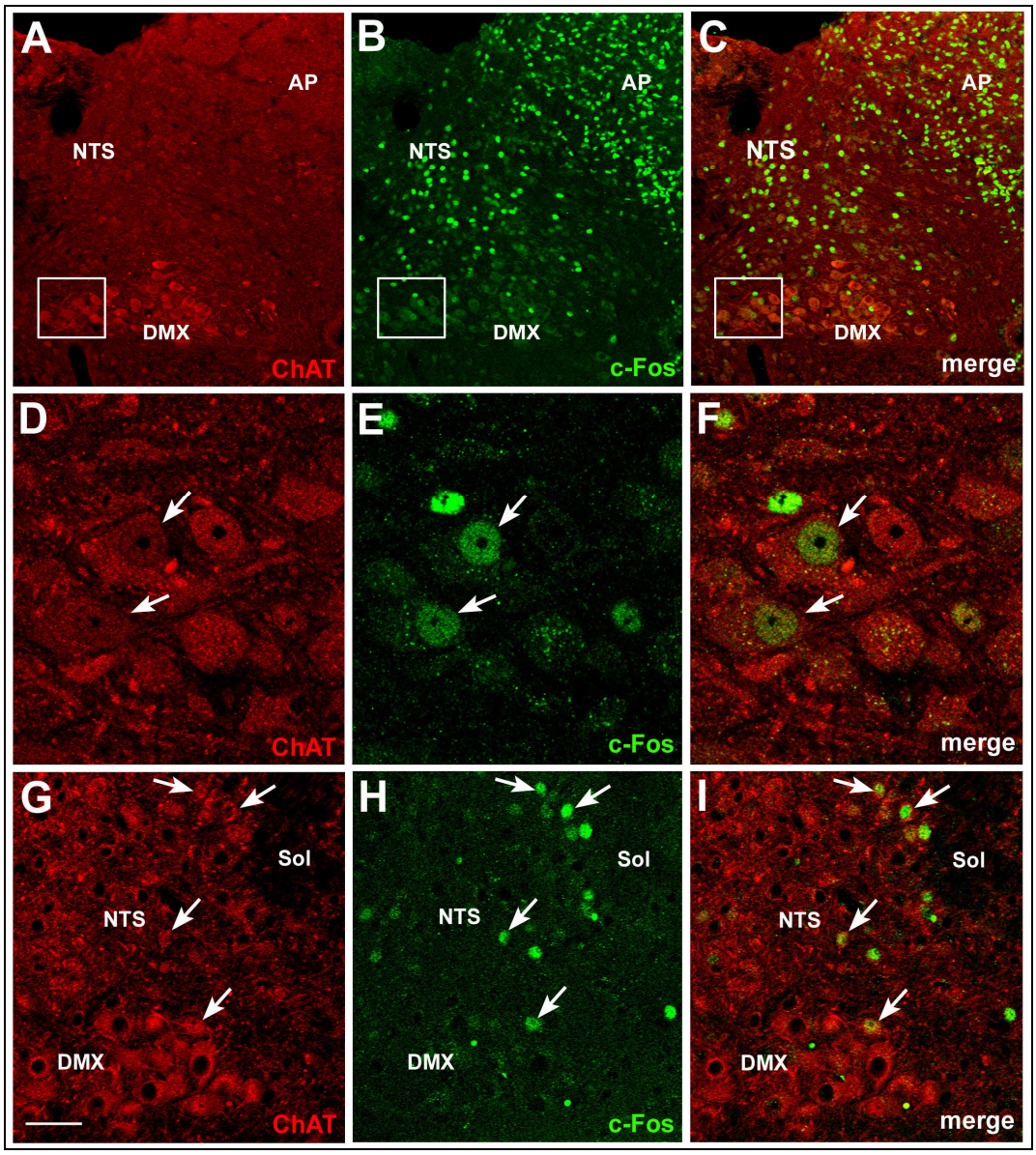

Fig.15 – c- Fos activation in brainstem cholinergic neurons by CNTF. After 120 min from CNTF treatment many cells of the AP, NTS, DMX showed c-Fos expression (A); some of these cells co-localized with ChAT (B-C), the marker of cholinergic pre-ganglionic vagal neurons. Panels D-F are enlargement of the corresponding areas framed in A-C, showing two large DMX cholinergic neurons expressing c-Fos (arrows). Some cholinergic neurons of the NTS were also positive for c-Fos (G-I, arrows). Bar: A-C=50 µm; D-F=10 µm; G-I=25 µm.

5.5. The action of CNTF on the mouse brainstem is not dependent on leptin but engages leptin-responsive NTS neurons

To assess whether the effects of CNTF on brainstem centers depend on leptin, the expression and distribution of P-STATs and c-Fos were examined in brainstem of genetically obese *ob/ob* and *db/db* mice, which lack leptin and the long form of the leptin receptor, respectively. Results showed STAT3, STAT1 and STAT5 activation in the AP of *ob/ob* (**Fig. 16A-C**) and *db/db* (**Fig. 16E-G**) mice 45 min after CNTF treatment and c-Fos expression in the NTS after 120 min (**Fig. 16D** and **H**). Thus P-STATs and c-Fos expression and distribution didn't display obvious differences in wild-type and genetically obese models, suggesting that the action of CNTF on the brainstem is not dependent on leptin signaling. Circulating leptin acts as an anoressigenic factor not only on hypothalamic neuronal networks but also on the NTS, where it activates the Jak-STAT3 pathway (Hosoi *et al*., 2002; Hayes *et al*., 2010). Indeed, treating mice with leptin for as little as 25 min induced P-STAT3 phosphorylation in a discrete subset of neurons and projections in the caudal NTS (**Fig. 16I-J**); on the contrary, 25 min of treatment was not sufficient to induce c-Fos expression in leptin-responsive neurons (Hubschle *et al*., 2001; Frontini *et al*., 2008). Based on the above findings, mice were treated with CNTF for 120 min, to induce c-Fos expression, and with leptin for 25 min, to induce STAT3 phosphorylation: then, P-STAT3 and c-Fos co-localization experiments were performed. Results showed that 14.11% $\pm$ 2.85 (n=3) of c-Fos positive and CNTF-responsive neurons in the caudal NTS were also positive for P-STAT3 (**Fig. 16K-M**) suggesting that this neuronal population is responsive for both CNTF and leptin and possibly represents an extra-hypothalamic site where CNTF bypasses leptin resistance.

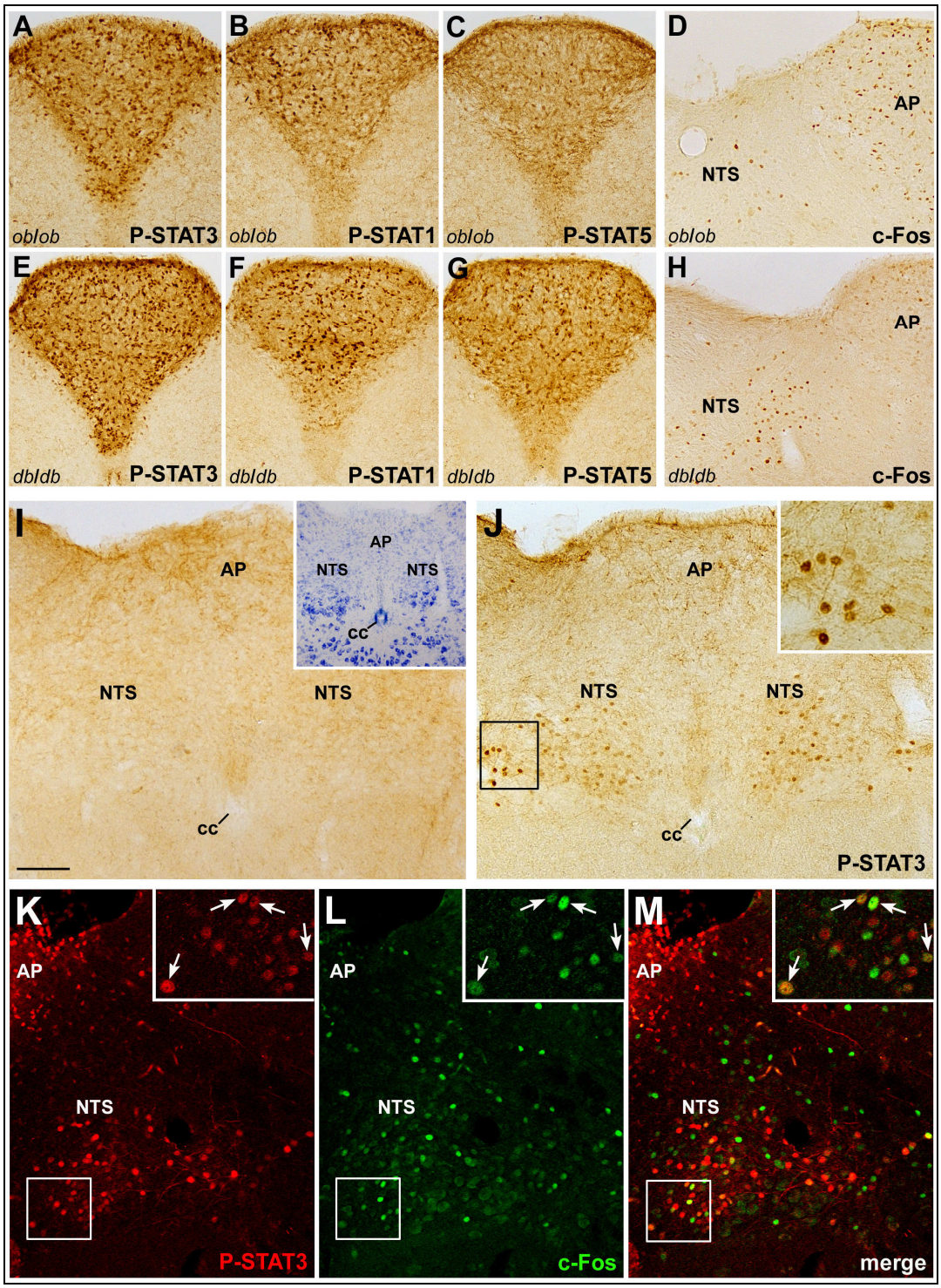

Fig.16 – Relationship between CNTF and leptin signalling in the mouse brainstem. CNTF treatment after 45 min induced STAT3 (A, E), STAT1 (B, F) and STAT5 (C, G) phosphorylation in the AP of *ob/ob* (A-D) and *db/db* (E-H) obese mice, and c-Fos expression (D-H) in the AP and NTS after 120 min of treatment. Treatment with leptin for 25 min (J) activated STAT3 signaling in neurons and fibers in the caudal portion of NTS (enlargement in the inset). Panel I represents corresponding structures from a control mouse processed for P-STAT3 immunohistochemistry. Inset of I shows Nissl-stained section. Double-staining experiments in coronal brainstem sections from a mouse

treated for 120 min with CNTF to induce c-Fos expression and with leptin for 25 min to induce STAT3 signaling (K-M) show some NTS neurons (enlargement in the insets, arrows) expressing both c-Fos and P-STAT3. Bar: A-C and E-G=150 µm; D, H-M=120 µm; insets of J-M=30 µm; inset of I=300 µm.

5.6. Mismatch of CNTF-responsive and CNTF-producing cells in the mouse AP

To identify possible endogenous CNTF sources in the AP, we performed CNTF immunohistochemistry: specific CNTF staining was detected throughout the rostrocaudal extent of the AP, where it was confined to thick projections and few, small cellular profiles that were mainly located close to the funiculus separans (**Fig. 17A**) and the meningeal sheet on the dorsolateral border of the AP, separating it from the subarachnoid space (**Fig. 17B**). Sometimes, long and strongly stained tanycyte-like projections branched from the funiculus separans or the meningeal sheet and penetrated into the brain parenchyma (left insets of **Fig.17A** and **B**). CNTF-knockout mice showed no specific staining at any of these sites (right insets of **Fig. 17A** and **B**). Double-labeling experiments demonstrated that CNTF positive cells were also positive for GFAP (**Fig. 17C-E**), suggesting that in the AP as in the hypothalamus and other brain regions CNTF is produced by glial cells. Overall, CNTF immunoreactivity was weak and sparse respect to the high responsiveness to exogenous CNTF seen in the AP cells.

RT-qPCR quantification of CNTF and CNTFα in micropunched brainstem tissue containing the AP was performed and compared to the amount found in micropunched hypothalamus tissue containing ME, where a distinctive spatial mismatch between the CNTF-producing and CNTF-responding cells was described (Severi *et al*., 2012). RT-qPCR showed that the hypothalamus tissue was enriched with POMC whereas the brainstem samples were enriched with vimentin (**Fig. 18**). CNTFRα expression was 264.94 times higher than CNTF expression in the hypothalamus samples, and 424.89 times higher in brainstem tissue (**Fig. 18**). Histological analysis confirmed that the micropunched slices contained neither the AP nor the mediobasal

hypothalamus (**Fig. 19**). These findings support the hypothesis that most CNTFRα-bearing cells of the mouse ME and AP are exposed to blood- or cerebrospinal fluid-derived CNTF more than to locally produced CNTF.

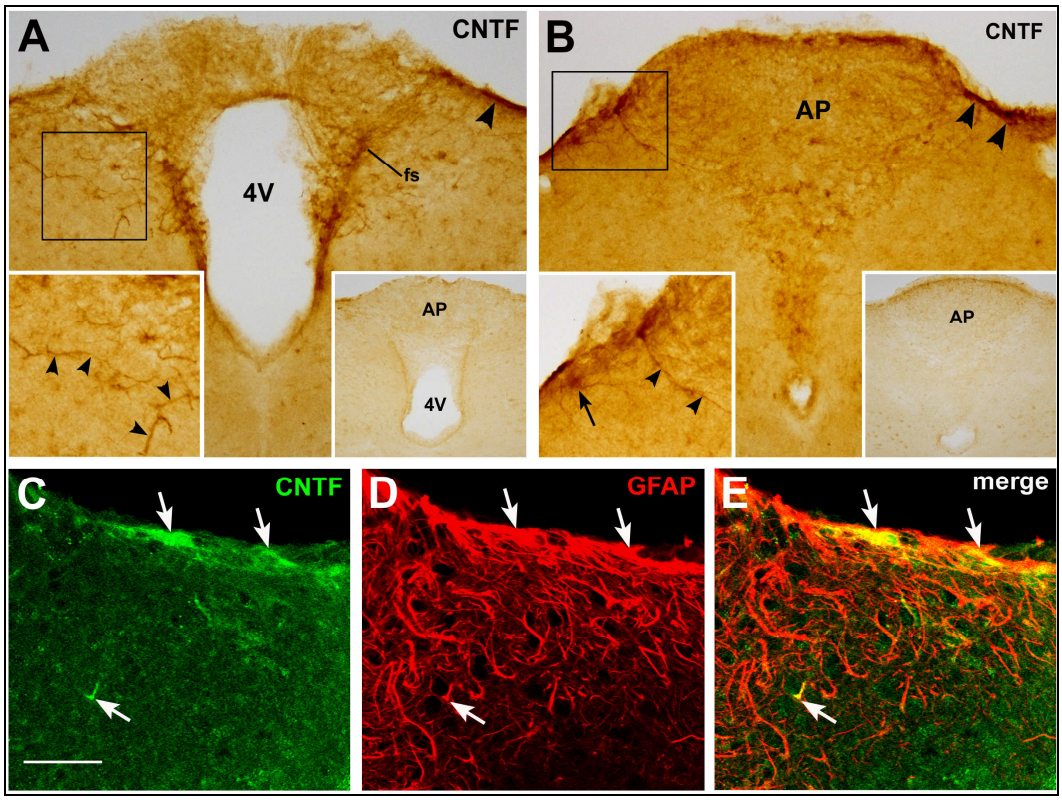

Fig. 17 – CNTF and CNTFRα expression in the mouse AP. CNTF-immunoreactivity was present in small portions of the funiculus separans (fs, A) and meninges (A-B, arrowheads); in particular, positivity was found in cellular projections (left-side insets, arrowheads) and rarely in cell somata (left-side inset in B, arrow). In A and B, insets on the left are the enlargements of the corresponding framed areas, whereas those on the right show the corresponding structures processed for CNTF immunohistochemistry from a CNTF-knockout mouse. Double-labeling experiments (C-E) showed that CNTF-positive structures were also positive for the GFAP (arrows). Bar: A-B=100 µm; left insets of A-B=60 µm; right insets of A-B=300 µm; C-E=50 µm.

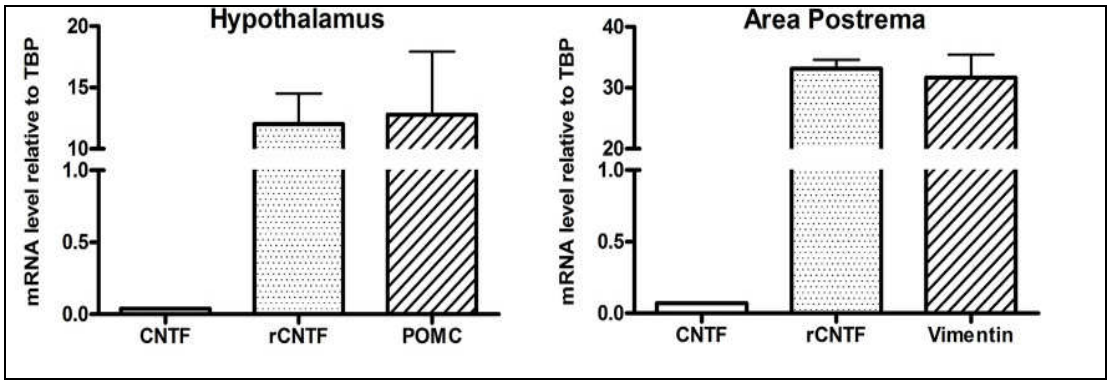

Fig. 18 - CNTF and CNTFRα expression. RT-PCR demonstrated that in the hypothalamus CNTFRα mRNA was 264.94 times higher than that of CNTF, whereas in the brainstem samples it was 424.89 times higher than that of its ligand.

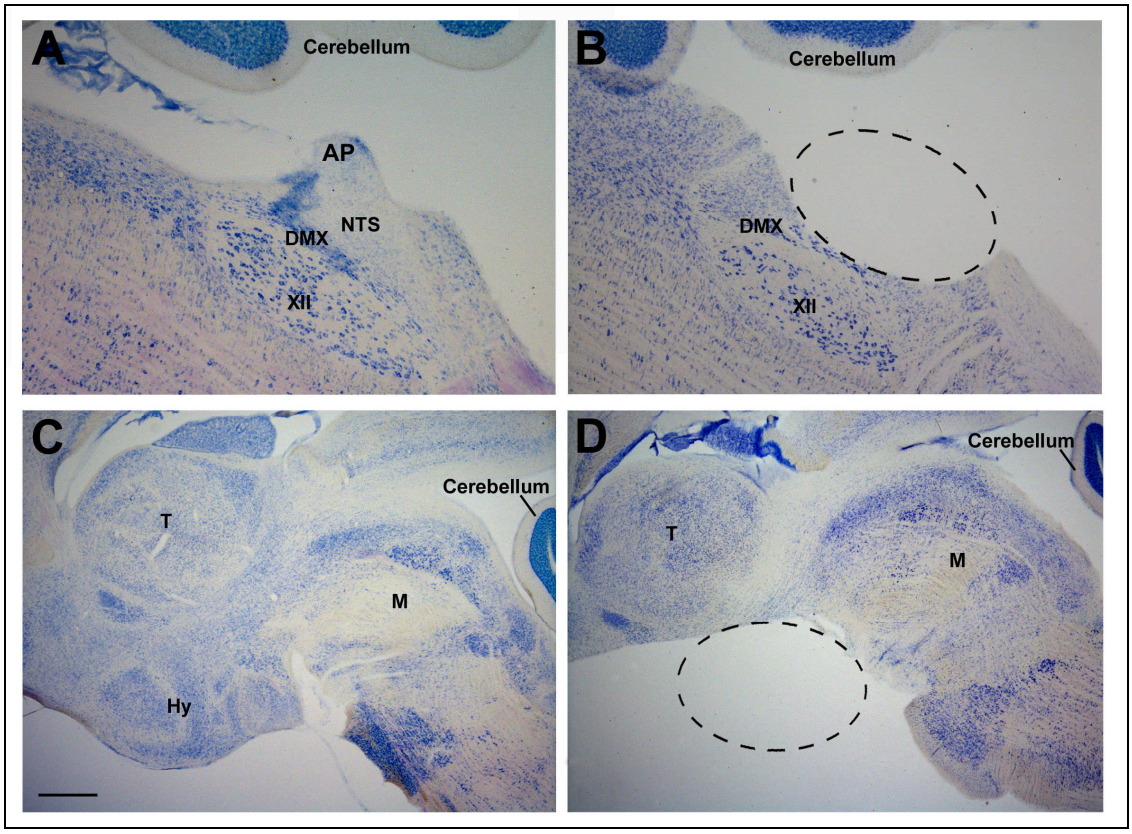

Fig.19 – Nissl-stained sagittal sections from control (A and C) and micropunched (B and D) slices at diencephalic (C-D) and brainstem (A-B) levels. The dotted areas depict the micropunched tissues containing the AP (B) and the mediobasal hypothalamus (Hy, D). NTS, nucleus of solitary tract. DMX dorsal motor nucleus of the vagus. XII, hypoglossal nerve. T, thalamus. M, mesencephalon. Bar. A-B=600 µm; C-D=1,2 mm.

6. DISCUSSION

CNTF was first purified approximately fifty years ago and it was identified as a survival factor for chick ciliary ganglion neurons (Adler *et al*., 1979). Ten years later, CNTF was purified and cloned from sciatic nerve (Lin *et al*., 1989) and it was shown that in addition to its pro-survival functions (Stöckli *et al*., 1989, Sendtner *et al*., 1992), it also promoted differentiation of sympathetic neurons (Saadat *et al*., 1989) and glial progenitors into astrocytes (Hughes *et al*., 1988; Lillien *et al*., 1988). Compared to other growth factors acting on the nervous system, CNTF has distinctive features. Its amino acid sequence lacks a signal peptide (Lin *et al*., 1989), and thus it may not be released by endocytic vescicles (Andrei *et al*., 1999). In addition, it is hardly detectable during embryo development and its synthesis increases greatly after birth reaching adult levels during the postnatal period (Stöckli *et al*., 1989, 1991). CNTF has been described as a lesion factor released from degenerating cells after an injury (Guthrie *et al*., 1997; Lee *et al*., 1997b) and it was tested for its role in nerve damage in numerous clinical trials. In one of these studies involving amyotrophic lateral sclerosis patients, CNTF was described as an anti-obesogenic factor (ALS CNTF Treatment Study Group, 1996). Obesity and type 2 diabetes are the most prevalent metabolic diseases in the western world; as a result, important efforts have been made to investigate the mechanisms involved in energy balance regulation and to identify new satiety factors in order to develop specific therapies (Wilding, 2002; Xu *et al*., 2016). CNTF acts both centrally and peripherally; in fact, it exerts an anorectic action on the hypothalamic neuronal networks involved in energy balance regulation, but it has also an important action on skeletal muscle, liver and adipose tissue, enhancing insulin sensitivity, thermogenesis, fat oxidation and energy expenditure (Matthews and Febbraio, 2008). Therefore, CNTF action consists not only in reducing food intake and losing weight, but also in improving hyperglycemia, hyperinsulinemia and hyperlipidemia, which are usually closely linked to obesity. CNTF treatment is effective also in high-fat diet obese animals, in *db/db* mice (Gloaguen *et al.*, 1997; Lambert *et al*., 2001) and in most cases of human obesity (Ettinger *et al*., 2003), where high levels of

circulating leptin do not result in reduced food intake or increased energy expenditure. Being leptin resistance a near-universal feature of human obesity, the ability of CNTF to bypass it, highlights its potential as an anti-obesity drug. The anorectic role of CNTF was first ascribed to its ability to activate Jak-STAT3 signaling in NPY and POMC neurons in the ARC (Lambert *et al*., 2001; Anderson *et al*., 2003); subsequently, it was shown that CNTF administration induces generation of new leptin-responsive neurons that may render, by integrating into the energy balance neurocircuits, the animals more leptin-sensitive (Kokoeva *et al*., 2005). Recent studies demonstrated that in mouse hypothalamus CNTF is constitutively expressed by ependymocytes and tanycytes of the ependymal layer of the third ventricle and, to a lesser extent, by grey matter and median eminence astrocytes. These CNTF-producing cells are in close proximity to CNTF-responsive ependymocytes and tanycytes, suggesting that the third ventricle ependyma can be involved in CNTF-mediated responses to physiological or pathological stimuli via autocrine and/or paracrine loops (Severi *et al*., 2012). Moreover, in the tuberal hypothalamus, CNTF expression and responsiveness significantly increase in mice rendered obese by HFD, while they significantly decrease in mice kept in CR conditions, supporting the notion that endogenous CNTF is a novel glial-derived modulator of the energy balance (Severi *et al*., 2013).

Our group demonstrated for the first time that CNTF, besides activating STAT3 in vivo, also activates the transcription factors STAT1 and STAT5 in ependymal and radial-glial cells of the ME, many of which are positive for immaturity and plasticity markers, such as nestin and vimentin. For this reason, these cells may be suitable targets for CNTF-dependent, structural, long-lasting hypothalamic neuronal and/or glial modifications. On the contrary, leptin does not act through STAT1 and STAT5 in the tuberal hypothalamus in normal conditions (McCowen *et al*., 1998; Rizk *et al*., 2001): these data highlight a marked difference between the signaling pathways engaged by leptin and CNTF. The Jak-STAT system is a signaling pathway mainly involved in the modulation of gene expression and it has an important role in neuronal growth and differentiation (Nicolas *et al*., 2013). Activation of the same STAT protein by different cytokines and activation of different

STATs by a single cytokine is a known mechanism that could contribute to redundancy and pleiotropy of cytokine action. It will be interesting to establish whether these signaling pathways are activated by CNTF also in peripheral organs, where additional CNTF-dependent metabolic effects could be demonstrated.

The ME is a circumventricular organ containing different cell types. It contains few neurons, whose phenotype and function are still unclear, numerous scattered radial glia-like cells and β-tanycytes located in the ependyma forming the roof of the ME. Tanycytes are highly specialized glial cells characterized by a cell body located in the ependymal layer and a single and long basal process that projects to discrete regions of hypothalamus (Langlet *et al*., 2013a). Recent studies described their role in the regulation of energy balance (Bolborea and Dale, 2013).

It has recently been shown that ME β-tanycytes and radial glia-like cells proliferate under normal conditions and generate newborn neurons that migrate to hypothalamic parenchyma, becoming sensitive to metabolic inputs such as leptin or fasting (Lee *et al*., 2012). Our findings documenting that in these cells CNTF activates STAT3, STAT1 and STAT5 strongly suggest that the stem or progenitor cells stimulated by CNTF (Kokoeva *et al*., 2005) are β-tanycytes and/or radial glia-like cells. β-tanycytes have an important role in energy balance homeostasis by sensing the levels of circulating metabolites and hormones and regulating the diffusion of these blood-borne molecules to cerebrospinal fluid and the adjacent ARC (Mullier *et al*., 2010; Levin *et al*., 2011). Interestingly, the fed or fasted state is able to plastically adjust the tanycyte-regulated diffusion of molecules to ARC neurons (Langlet *et al*., 2013a; Schaeffer *et al*., 2013) suggesting that β-tanycytes can undergo important morpho-functional changes in response to metabolic requirements. Since CNTF specifically targets β-tanycytes, it is possible that it has a role in affecting their structural and functional properties and consequently it could modulate the transport of metabolic hormones, such as leptin or insulin, to the adjacent ARC and/or other brain regions (Severi *et al*., 2015).

Brainstem is another important brain area containing three main structures involved in energy homeostasis: (i) the AP, the circumventricular organ

lacking the blood-brain barrier and sensing circulating factors, (ii) the NTS, the main sensory relay for the viscera and (iii) the DMX, which is the source of vagal efferents controlling gut motility and secretion (Young, 2012).

We demonstrated that CNTF also affects brainstem centers: in fact, we reported a strong Jak-STAT signaling activation in the AP and induction of strong c-Fos expression in the adjacent NTS following CNTF intraperitoneal administration. The AP is also a source of endogenous CNTF that is produced by glial cells, mainly located in the funiculus separans and in the meninges. CNTF action in *ob/ob* and *db/db* mice, is similar to that found in wild-type mice, suggesting that, as previously observed in the hypothalamus, also in brainstem centers the action of CNTF is leptin-independent. CNTF activates STAT3, STAT1 and STAT5 in nestin- and vimentin-positive AP glial cells: thus, CNTF-responsive cells in the AP are closely reminiscent of the profile described in the CNTF-responsive cells in the ME. Sensory circumventricular organs in adult rodents (Bennet *et al*., 2009) and humans (Sanin *et al*., 2013) have recently been shown to be a rich source of neural stem cells providing novel cells to adjacent brain regions. Two type of neural stem cells have been identified: nestin-positive tanycyte-like cells and nestin-positive astrocyte-like cells (Furube *et al*., 2015). Our data showing that CNTF activates multiple STAT isoforms in vimentin and nestin-positive glial cells strongly suggest that CNTF may promote neurogenesis and/or gliogenesis also in mouse brainstem. The action of CNTF on the AP could also be related to glio-vascular rearrangements resulting in greater vascular permeability and contact between circulating metabolites and adjacent brain areas. In fact it has been demonstrated that circumventricular organs, including AP (Morita *et al*., 2015) and ME (Langlet *et al*., 2013b), are characterized by a continuous vascular remodeling induced by vascular endothelial growth factor.

Since CNTF is a protective factor, it is upregulated in the brain after nerve tissue stress or injury (Lee *et al*., 1997a; Watt *et al*., 2006), inducing neurogenesis and gliogenesis in circumventricular organs (Lin *et al*., 2015; Sanin *et al*., 2013). In pathological conditions, it is still unclear whether endogenous CNTF is delivered in the AP from the ependyma through the cerebrospinal fluid (Severi *et al*., 2012), from the periphery through the

bloodstream (Guillet *et al*., 1995; Laaksovirta *et al*., 2008) or it is produced locally by funiculus separans glial and tanycyte-like cells (as demonstrated by our studies). Another possibility is that CNTF could be involved in the brain inflammatory response in the AP. In fact, Jak-STAT signaling in glial cells of circumventricular organs, including the AP, is related to neuroendocrine and behavioral host responses to peripheral inflammation (Roth *et al*., 2004).

CNTF activates the Jak-STAT3 pathway in a small percentage of AP neurons, which were not a homogeneous and distinctive population of cholinergic, GABAergic, dopaminergic or serotoninergic neurons, which are the most common neurochemical phenotypes found in the mouse AP (Armstrong *et al*., 1981; Miceli *et al*., 1987; Tago *et al*., 1989; Fong *et al*., 2005). CNTF-does not activate in these AP responsive-neurons c-Fos, a marker of functionally activated neurons, or do it very weakly, suggesting that CNTF induces the transcription of immediate early genes other than c-Fos (Kelly *et al*., 2004) or that it exerts an inhibitory effect on AP neurons. These findings underline a difference between the action of CNTF and other circulating factors, such as amylin and GLP-1, that typically act on the AP by inducing c-Fos in noradrenergic neurons projecting to the NTS (Yamamoto *et al*., 2003; Potes *et al*., 2010). Future electrophysiological studies will better clarify the action of CNTF on AP neurons.

CNTF administration also induces a widespread c-Fos activation of NTS and DMX neurons. NTS is an important integrative node for several cardiovascular, respiratory and metabolic responses (Ganchrow *et al*., 2014) and it is reciprocally connected with other brainstem centers, including the AP and DMX, and the hypothalamus, including the ARC and the PVN (van der Kooy and Koda 1983; Shapiro and Miselis 1985; Watson *et al*., 2012). The NTS contains neurochemically different neuronal populations, and non-activation of the Jak-STAT pathway in these neurons after CNTF treatment suggests that circulating CNTF lacks direct access to them and their activation is secondary to the action of CNTF on other centers, that are primarily targeted by it. Thus, our findings suggest that AP neurons may be primarily engaged by circulating CNTF, and that they secondarily stimulate NTS and DMX neurons. However, activation of NTS neurons in CNTF-treated mice may also depend

on descending projections of CNTF-activated hypothalamic neurons (Kelly *et al*., 2004; Purser *et al*., 2013) or on a still unappreciated action of CNTF on peripheral sensory nerve endings. To provide insights on the route by which CNTF activates NTS neurons, it will be interesting to evaluate the c-Fos activation in the NTS of CNTF-treated mice after surgical ablation of the AP or after deafferentation by systemic capsaicin treatment or subdiaphragmatic vagotomy (Senzacqua *et al*., 2016).

CNTF activation of NTS is distinctively different from that induced by circulating leptin, which activates Jak-STAT3 signaling in neurons found on the caudal half of the NTS (Hosoi *et al*., 2002; Hayes *et al*., 2010). On the contrary, both the rostral and the caudal portion of the NTS are engaged after CNTF administration, suggesting that in addition to food-related signals, CNTF could be involved in detection of gustatory and other sensory visceral stimuli. A small percentage of leptin-responsive neurons of the caudal NTS also respond to CNTF, and this can explain the satiety effect exerted by CNTF at the brainstem level also in conditions of leptin-resistance (Senzacqua *et al*., 2016).

In conclusion, these studies provide evidence that CNTF, like other satiety factors, not only affects the hypothalamic centers, but also exerts a redundant and parallel action on the brainstem centers involved in feeding and energy balance regulation. Furthermore, in the brainstem, as well as in the hypothalamus, the considerable mismatch between the site of CNTF production and its targets strongly suggests the interesting possibility that CNTF may be a circulating satiety factor.

The results obtained during the three year PhD program have been published on international, scientific, peer-reviewed journals:

- de Guglielmo G, Melis M, De Luca MA, Kallupi M, Li HW, Niswender K, Giordano A, **Senzacqua M**, Somaini L, Cippitelli A, Gaitanaris G, Demopulos G, Damadzic R, Tapocik J, Heilig M, Ciccocioppo R (2015) PPARγ activation attenuates opioid consumption and modulates mesolimbic dopamine transmission. *Neuropsychopharmacology* 40:927-37.

- Severi I, **Senzacqua M**, Mondini E, Fazioli F, Cinti S, Giordano A. (2015) Activation of transcription factors STAT1 and STAT5 in the mouse median eminence after systemic ciliary neurotrophic factor administration. *Brain Research* 1622: 217-29

- **Senzacqua M**, Severi I, Perugini J, Acciarini S, Cinti S, Giordano A. (2016) Action of Administered Ciliary Neurotrophic Factor on the Mouse Dorsal Vagal Complex. *Frontiers in Neuroscience* 10:289

7. REFERENCES

Adler R, Landa KB, Manthorpe M, Varon S (1979) Cholinergic neurotrophic factors: intraocular distribution of trophic activity for ciliary neurons. *Science* 204:1434-1436.

Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM, Bloom SR (1985) Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology* 89:1070-7.

ALS CNTF Treatment Study Group (1996) A doubleblind placebo-controlled clinical trial of subcutaneous recombinant human ciliary neurotrophic factor (rHCNTF) in amyotrophic lateral sclerosis. *Neurology* 46:1244-1249.

Anderson KD, Lambert PD, Corcoran TL, Murray JD, Thabet KE, Yancopoulos GD, Wiegand SJ (2003) Activation of the hypothalamic arcuate nucleus predicts the anorectic actions of ciliary neurotrophic factor and leptin in intact and gold thioglucose-lesioned mice. *J Neuroendocrinol* 15: 649-660.

Andrei C, Dazzi C, Lotti L, Torrisi MR, Chimini G, Rubartelli A (1999) The secretory route of the leaderless protein interleukin-1β involves exocytosis of endolysosome-treated vesicles. *Mol Biol Cell* 10:1463- 1475.

Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, *et al*. (2001) Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab* 86:4753-8.

Armstrong DM, Pickel VM, Joh TH, Reis DJ, Miller RJ (1981) Immunocytochemical localization of catecholamine synthesizing enzymes and neuropeptides in area postrema and medial nucleus tractus solitarius of rat brain. *J. Comp. Neurol.* 196; 505-517.

Balthasar N, Coppari R, McMinn J, Liu SM, Lee CE, Tang V *et al*. (2004) Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. *Neuron* 42: 983-991.

Barrachina MD, Martínez V, Wang L, Wei JY, Taché Y (1997) Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. *Proc Natl Acad Sci U S A* 94:10455-60.

Bauer S, Kerr BJ, Patterson PH (2007) The neuropoietic cytokine family in development, plasticity, disease and injury. *Nat Rev Neurosci* 8:221-232.

Bazan JF (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci U S A* 87:6934-6938.

Bennet L, Yang M, Enikolopov G, Iacovitti L (2009) Circumventricular organs: a novel site of neural stem cells in the adult brain. *Mol. Cell. Neurosci*. 41: 337-347.

Blevins JE, Chelikani PK, Haver AC, Reidelberger RD (2008) PYY(3-36) induces Fos in the arcuate nucleus and in both catecholaminergic and non-catecholaminergic neurons in the nucleus tractus solitarius of rats. *Peptides* 29:112-9.

Bolborea M and Dale N (2013) Hypothalamic tanycytes: potential roles in the control of feeding and energy balance. *Trend Neurosci*. 36: 91-100.

Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I, *et al*. (1997) Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. *Science* 278:477-483.

Borison HL and Wang SC (1953) Physiology and pharmacology of vomiting. *Pharmacol Rev* 5:193-230.

Broberger C (2005) Brain regulation of food intake and appetite: molecules and networks. *J Intern Med* 258:301-27.

Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, *et al*. (2000) A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* 141: 3518-3521.

Cattaneo E, Conti L, De-Fraja C (1999) Signalling through the JAK-STAT pathway in the developing brain. *Trends Neurosci* 22:365-369.

Challis BG, Pinnock SB, Coll AP, Carter RN, Dickson SL, O'Rahilly S (2003) Acute effects of PYY3-36 on food intake and hypothalamic neuropeptide expression in the mouse. *Biochem Biophys Res Commun* 311:915-9.

Ciriello J, McMurray JC, Babic T, de Oliveira CV (2003) Collateral axonal projections from hypothalamic hypocretin neurons to cardiovascular sites in nucleus ambiguus and nucleus tractus solitarius. *Brain Res* 991: 133-41.

Cone DR (2005) Anatomy and regulation of the central melanocortin system. *Nat Neurosci* 8: 571-578.

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, *et al.* (1996) Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 334:292-295.

Cowley MA, Pronchuk N, Fan W, Dinulescu DM, Colmers WF, Cone RD (1999) Integration of NPY, AGRP, and melanocortin signals in the hypothalamic paraventricular nucleus: evidence of a cellular basis for the adipostat. *Neuron* 24: 155-63.

Cowley MA, Smart JL, Rubinstein M, Cerdán MG, Diano S, Horvath TL, *et al*. (2001) Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411:480-484.

Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL *et al*. (2003) The distribution and mechanism of action of Gherlin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37:649-661.

Dallaporta M, Pecchi E, Pio J, Jean A, Horner KC, Troadec JD (2009) Expression of leptin receptor by glial cells of the nucleus tractus solitarius: possible involvement in energy homeostasis. *J Neuroendocrinol* 21:57-67.

Dale N (2011) Purinergic signaling in hypothalamic tanycytes: potential roles in chemosensing. *Semin Cell Dev Biol* 22: 237-244.

Dallner C, Woods AG, Deller T, Kirsch M, Hofmann H-D (2002) CNTF and CNTF receptor alpha are constitutively expressed by astrocytes in the mouse brain. *Glia* 37: 374-378.

DeChiara T M, Vejsada R, Poueymirou W T, Acheson A, Suri C, Conover JC, *et al*. (1995) Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound motor neuron deficits at birth. *Cell* 83: 313-322.

Derouet D, Rousseau F, Alfonsi F, Froger J, Hermann J, Barbier F, *et al*. (2004) Neuropoietin, a new IL-6-related cytokine signaling through the ciliary neurotrophic factor receptor. *Proc Natl Acad Sci U S A* 101:4827-4832.

Edwards GL and Ritter RC (1981) Ablation of the area postrema causes exaggerated consumption of preferred foods in the rat. *Brain Res* 216:265-276.

Emond M, Schwartz GJ, Ladenheim EE, Moran TH (1999) Central leptin modulates behavioral and neural responsivity to CCK. *Am J Physiol* 276:R1545-1549.

Ettinger MP, Littlejohn TW, Schwartz SL, Weiss SR, McIlwain HH, Heymsfield SB, et al. (2003) Recombinant variant of ciliary neurotrophic factor for weight loss in obese adults: a randomized, dose-ranging study. *JAMA* 289:1826-1832.

Fan W, Ellacott KL, Halatchev IG, Takahashi K, Yu P, Cone RD (2004) Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. *Nat Neurosci* 7:335-6.

Flynn FW and Grill HJ (1983) Insulin elicits ingestion in decerebrate rats. *Science* 221:188-190.

Fong AY, Stornetta RL, Foley CM, Potts JT (2005) Immunohistochemical localization of GAD67-expressing neurons and processes in the rat brainstem:

subregional distribution in the nucleus tractus solitarius. *J Comp Neurol* 493:274-290.

Frederich RC, Hamann A, Anderson S, Löllmann B, Lowell BB, Flier JS (1995) Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat Med* 1: 1311-4.

Frontini A, Bertolotti P, Tonello C, Valerio A, Nisoli E, Cinti S, Giordano A (2008) Leptin-dependent STAT3 phosphorylation in postnatal mouse hypothalamus. *Brain Res* 1215: 105-115.

Furube E, Morita M, Miyata S (2015) Characterization of neural stem cells and their progeny in the sensory circumventricular organs of adult mouse. *Cell Tissue Res*. 362: 347-365.

Gibbs J and Smith GP (1977) Cholecystokinin and satiety in rats and rhesus monkeys. *Am J Clin Nutr* 30:758-61.

Gil K, Bugajski A, Thor P (2011) Electrical vagus nerve stimulation decreases food consumption and weight gain in rats fed a high-fat diet. *J Physiol Pharmacol* 62:637-646.

Gloaguen I, Costa P, Demartis A, Lazzaro D, Di Marco A, Graziani R *et al*. (1997) Ciliary neurotrophic factor corrects obesity and diabetes associated with leptin deficiency and resistance. *Proc Natl Acad Sci U S A* 94:6456-6461.

Ganchrow D, Ganchrow JR, Cicchini V, Bartel DL, Kaufman D, Girard D, Whitehead MC (2014) Nucleus of the solitary tract in the C57BL/6J mouse: Subnuclear parcellation, chorda tympani nerve projections, and brainstem connections. *J Comp Neurol* 522:1565-96.

Grill HJ and Norgren R (1978a) The taste reactivity test. I. Mimetic responses to gustatory stimuli in neurologically normal rats. *Brain Res* 143:263-279.

Grill HJ and Norgren R (1978b) The taste reactivity test. II. Mimetic responses to gustatory stimuli in chronic thalamic and chronic decerebrate rats. *Brain Res* 143:281-297.

Grill HJ and Smith GP (1988) Cholecystokinin decreases sucrose intake in chronic decerebrate rats. *Am J Physiol* 254:R853-856.

Grill HJ, Ginsberg AB, Seeley RJ, Kaplan JM (1998) Brainstem application of melanocortin receptor ligands produces long-lasting effects on feeding and body weight. *J Neurosci* 18:10128-35.

Gropp E, Shanabrough M, Borok E, Xu AW, Janoschek R, Buch T, *et al*. (2005) Agouti-related peptide-expressing neurons are mandatory for feeding. *Nat Neurosci* 8: 1289-1291.

Guillet C, Fourcin M, Chevalier S, Pouplard A, Gascan H (1995) ELISA detection of circulating levels of LIF, OSM, and CNTF in septic shock. *Ann N Y Acad Sci* 762:407-9.

Guthrie KM, Woods AG, Nguyen T, Gall CM (1997) Astroglial ciliary neurotrophic factor mRNA expression is increased in fields of axonal sprouting in deafferented hippocampus. *J Comp Neurol* 386: 137-148.

Hahn TM, Breininger JF, Baskin DG, Schwartz MW (1998) Co-expression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nat Neurosci* 1:271-2.

Hayes MR, Skibicka KP, Leichner TM, Guarnieri, DJ, DiLeone RJ, Bence KK *et al*. (2010). Endogenous leptin signaling in the caudal nucleus tractus solitarius and area postrema is required for energy balance regulation. *Cell Metab* 11: 77-83.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 374:1-20.

Heinrich PC, Behrmann I, Müller-Newen G, Schaper F, Graeve L (1998) Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J* 334:297-314.

Henderson JT, Seniuk NA, Richardson PM, Gauldie J, Roder JC (1994) Systemic administration of ciliary neurotrophic factor induces cachexia in rodents. *J Clin Invest* 93:2632-8.

Hetherington AW and Ranson SW (1939) Experimental Hypothalamico-Hypophyseal Obesity in the Rat. *Proc Soc Exp Biol Med* 41: 465-466.

Hetherington AW and Ranson SW (1940) Hypothalamic lesions and adiposity in the rat. *Anat Rec* (Hoboken) 78: 149-172.

Hetherington AW and Ranson SW (1942a) The relation of various hypothalamic lesions to adiposity in the rat. *J Comp Neurol* 76: 475-499.

Hetherington AW and Ranson SW (1942b) The spontaneous activity and food intake of rats with hypothalamic lesions. *Am J of Physiol* 136: 609-617.

Hornby PJ (2001) Central neurocircuitry associated with emesis. *Am J Med* 8A:106S-112S.

Horsburgh A and Massoud TF (2013) The circumventricular organs of the brain: conspicuity on clinical 3T MRI and a review of functional anatomy. *Surg Radiol Anat* 35:343-349.

Hosoi T, Kawagishi T, Okuma Y, Tanaka J, Nomura Y (2002). Brain stem is a direct target for leptin's action in the central nervous system. *Endocrinology* 143: 3498-3504

Hoyda TD, Smith PM, Ferguson AV (2009) Gastrointestinal hormone actions in the central regulation of energy metabolism: potential sensory roles for the circumventricular organs. *Int J Obes (Lond)* 1:S16-21.

Hubschle T, Thom E, Watson A, Roth J, Klaus S., Meyerhof W (2001) Leptin-induced nuclear translocation of STAT3 immunoreactivity in hypothalamic nuclei involved in body weight regulation. *J. Neurosci* 21: 2413-2424.

Hughes SM, Lillien LE, Raff MC, Rohrer H, Sendtner M (1988) Ciliary neurotrophic factor induces type-2 astrocyte differentiation in culture. *Nature* 335:70-3.

Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR *et al.* (1997) Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88: 131-141.

Hyde TM and Miselis RR (1983) Effects of area postrema/caudal medial nucleus of solitary tract lesions on food intake and body weight. *Am J Physiol* 244:R577-87.

Ip NY, McClain J, Barrezueta NX, Aldrich TH, Pan L, Li Y, *et al*. (1993) The alpha component of the CNTF receptor is required for signaling and defines potential CNTF targets in the adult and during development. *Neuron* 10:89-102.

Johnson AK and Gross PM (1993) Sensory circumventricular organs and brain homeostatic pathways. *FASEB J* 7: 678–686.

Kalia M (1981). Brain stem localization of vagal preganglionic neurons. *J Auton Nerv Syst* 3: 451–481.

Kelly JF, Elias CF, Lee CE, Ahima RS, Seeley RJ, Bjørbaek C, *et al*. (2004) Ciliary neurotrophic factor and leptin induce distinct patterns of immediate early gene expression in the brain. *Diabetes* 53:911-920.

Kenny PJ (2011) Reward mechanisms in obesity: new insights and future directions. *Neuron* 69:664-79.

Kirchgessner AL and Sclafani A (1988) PVN-hindbrain pathway involved in the hypothalamic hyperphagia-obesity syndrome. *Physiol Behav* 42:517-28.

Kishi T, Aschkenasi CJ, Lee CE, Mountjoy KG, Saper CB, Elmquist JK (2003) Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. *J Comp Neurol* 457: 213-35.

Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW (2002) Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene* 20:1-24.

Kissileff HR, Pi-Sunyer FX, Thornton J, Smith GP (1981) C-terminal octapeptide of cholecystokinin decreases food intake in man. *Am J Clin Nutr* 34:154-60.

Koda S, Date Y, Murakami N, Shimbara T, Hanada T, Toshinai K *et al*. (2005) The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats.*Endocrinology* 146:2369-75.

Kokoeva MV, Yin H, Flier JS (2005) Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. *Science* 310:679-683.

Konner AC, Janoschek R, Plum L, Jordan SD, Rother E, Ma X (2007) Insulin action in AgRP-expressing neurons is required for suppression of hepatic glucose production. *Cell Metab* 5:438-449.

Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS *et al*. (1998) Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* 393: 72-6.

Ladyman SR, Fieldwick DM, Grattan DR (2012) Suppression of leptin-induced hypothalamic JAK/STAT signalling and feeding response during pregnancy in the mouse. *Reproduction* 144: 83-90.

Laaksovirta H, Soinila S, Hukkanen V, Röyttä M, Soilu-Hänninen M (2008) Serum level of CNTF is elevated in patients with amyotrophic lateral sclerosis and correlates with site of disease onset. *Eur J Neurol.* 15:355-9.

Lambert PD, Anderson KD, Sleeman MW, Wong V, Tan J, Hijarunguru A, *et al*. (2001) Ciliary neurotrophic factor activates leptin-like pathways and reduces body fat, without cachexia or rebound weight gain, even in leptin-resistant obesity. *Proc Natl Acad Sci USA* 98: 4652-4657.

Langlet F, Mullier A, Bouret SG, Prevot V, Dehouck B (2013a) Tanycyte-like cells form a blood-cerebrospinal fluid barrier in the circumventricular organs of the mouse brain. *J Comp Neurol* 521: 3389-405.

Langlet F, Levin BE, Luquet S, Mazzone M, Messina A, Dunn-Meynell AA, *et al*. (2013b). Tanycytic VEGF-A boosts blood-hypothalamus barrier plasticity and access of metabolic signals to the arcuate nucleus in response to fasting. *Cell Metab* 17:607-617.

Langlet F (2014) Tanycytes: a gateway to the metabolic hypothalamus. *J Neuroendocrinol* 26: 753-60.

Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI *et al*. (1996) Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379:632-635.

Lee J, Martin E, Paulino G, de Lartigue G, Raybould HE (2011) Effect of ghrelin receptor antagonist on meal patterns in cholecystokinin type 1 receptor null mice. *Physiol Behav* 103:181-7.

Lee MY, Hofmann HD, Kirsch M (1997a) Expression of ciliary neurotrophic factor receptor-alpha messenger RNA in neonatal and adult rat brain: an in situ hybridization study. *Neuroscience* 77:233-46.

Lee MY, Deller T, Kirsch M, Frotscher M, Hofmann HD (1997b) Differential regulation of ciliary neurotrophic factor (CNTF) and CNTF receptor alpha expression in astrocytes and neurons of the fascia dentata after entorhinal cortex lesion. *J Neurosci* 17:1137-46.

Lee DA, Bedont JL, Pak T, Wang H, Song J, Miranda-Angulo A *et al.* (2012) Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche. *Nat. Neurosci* 15: 700-702.

Lerant A, Kanyicska B, Freeman ME (2001) Nuclear translocation of STAT5 and increased expression of Fos related antigens (FRAs) in hypothalamic dopaminergic neurons after prolactin administration. *Brain Res*. 904: 259-269.

Levin BE, Magnan C, Dunn-Meynell A, Le Foll C (2011) Metabolic sensing and the brain: who, what, where, and how? *Endocrinology* 152: 2552-2557.

Lillien LE, Sendtner M, Rohrer H, Hughes SM, Raff MC (1988) Type-2 astrocyte development in rat brain cultures is initiated by a CNTF-like protein produced by type-1 astrocytes. *Neuron* 1:485-94.

Lin LF, Mismer D, Lile JD, Armes LG, Butler ET, Vannice JL, *et al*. (1989) Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). *Science* 246:1023-1025.

Lin R, Cai J, Nathan C, Wei X, Schleidt S, Rosenwasser R, *et al.* (2015). Neurogenesis is enhanced by stroke in multiple new stem cell niches along the ventricular system at sites of high BBB permeability. *Neurobiol. Dis.* 74: 229-239.

Liu QS, Wang QJ, Du GH, Zhu SY, Gao M, Zhang L, *et al.* (2007) Recombinant human ciliary neurotrophic factor reduces weight partly by regulating nuclear respiratory factor 1 and mitochondrial transcription factor *A. Eur J Pharmacol* 563:77-82.

Luquet S, Perez FA, Hnasko TS, Palmiter RD (2005) NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* 310:683-5.

Lutz TA, Del Prete E, Scharrer E (1994) Reduction of food intake in rats by intraperitoneal injection of low doses of amylin. *Physiol Behav* 55:891-895.

Lutz TA, Mollet A, Rushing PA, Riediger T, Scharrer E (2001) The anorectic effect of a chronic peripheral infusion of amylin is abolished in area postrema/nucleus of the solitary tract (AP/NTS) lesioned rats. *Int J Obes Relat Metab Disord* 25:1005-11.

Lutz TA (2012) Control of energy homeostasis by amylin. *Cell Mol Life Sci* 69:1947-1965.

MacLennan AJ, Neitzel KL, Devlin BK, Garcia J, Hauptman GA, Gloaguen I, *et al*. (2000) In vivo localization and characterization of functional ciliary neurotrophic factor receptors which utilize JAK-STAT signaling. *Neuroscience* 99: 761-772.

Manthorpe M, Skaper SD, Williams LR, Varon S (1986) Purification of adult rat sciatic nerve ciliary neuronotrophic factor. *Brain Res* 367:282-6.

Marks JL, Porte D Jr, Stahl WL, Baskin DG (1990) Localization of insulin receptor mRNA in rat brain by in situ hybridization. *Endocrinology* 127:3234-6.

Masu Y, Wolf E, Holtmann B, Sendtner M, Brem G, Thoenen H (1993) Disruption of the CNTF gene results in motor neuron degeneration. *Nature* 365:27-32.

Matthews VB and Febbraio MA (2008) CNTF: a target therapeutic for obesity-related metabolic disease? *J Mol Med* 86:353-361.

McCowen KC, Chow JC, Smith RJ (1998) Leptin signaling in the hypothalamus of normal rats in vivo. *Endocrinology* 139: 4442-4447

McKinley MJ, McAllen RM, Davern P, Giles ME, Penschow J, Sunn N, *et al*. (2003). The sensory circumventricular organs of the mammalian brain. *Adv. Anat. Embryol. Cell Biol.* 172, III-XII, 1-122, back cover.

Merchenthaler I, Lane M, Shughrue P (1999) Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. *J Comp Neurol* 403:261-80.

Miceli MO, Post CA, van der Kooy D (1987) Catecholamine and serotonin colocalization in projection neurons of the area postrema. *Brain Res.* 412: 381-385.

Miller RG, Petajan JH, Bryan WW, Armon C, Barohn RJ, Goodpasture JC, *et al*. (1996) A placebo-controlled trial of recombinant human ciliary

neurotrophic (rhCNTF) factor in amyotrophic lateral sclerosis. rhCNTF ALS Study Group. Ann. *Neurology* 39: 256-260.

Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ *et al*. (1997) *Nature* 387:903-8. Congenital leptin deficiency is associated with severe early-onset obesity in humans.

Moran TH, Baldessarini AR, Salorio CF, Lowery T, Schwartz GJ (1997) Vagal afferent and efferent contributions to the inhibition of food intake by cholecystokinin. *Am J Physiol* 272:R1245-51.

Morita S and Miyata S (2012) Different vascular permeability between the sensory and secretory circumventricular organs of adult mouse brain. *Cell Tissue Res* 349:589-603.

Morita S, Furube E, Mannari T, Okuda H, Tatsumi K, Wanaka A, *et al*. (2015) Vascular endothelial growth factor-dependent angiogenesis and dynamic vascular plasticity in the sensory circumventricular organs of adult mouse brain. *Cell Tissue Res.* 359: 865-884.

Morton GJ, Cummings DE, Baskin DG, Barsh G.S, Schwartz MW (2006) Central nervous system control of food intake and body weight. *Nature* 443:289-95.

Morton GJ, Meek TH, Schwartz MW (2014) Neurobiology of food intake in health and disease. *Nat Rev Neurosci* 15: 367-78.

Mullier A, Bouret SG, Prevot V, Dehouck B (2010) Differential distribution of tight junction proteins suggests a role for tanycytes in blood-hypothalamus barrier regulation in the adult mouse brain. *J Comp Neurol* 518: 943-962.
Mussa BM and Verberne AJ (2013) The dorsal motor nucleus of the vagus and regulation of pancreatic secretory function. *Exp Physiol* 98: 25–37.

Mutze J, Roth J, Gerstberger R, Hubschle T (2007) Nuclear translocation of the transcription factor STAT5 in the rat brain after systemic leptin administration. *Neurosci. Lett*. 417: 286-291.

Nicolas CS, Amici M, Bortolotto ZA, Doherty A, Csaba Z, Fafouri A et al. (2013) The role of JAK-STAT signalling whithin the CNS. *JAK-STAT* 2:1.

Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers MG Jr, Schwartz MW (2001) Intracellular signalling. Key enzyme in leptin-induced anorexia. *Nature* 413:794-795.

Ott V, Fasshauer M, Dalski A, Klein HH, Klein J (2002) Direct effects of ciliary neurotrophic factor on brown adipocytes: evidence for a role in peripheral regulation of energy homeostasis. *J Endocrinol* 173:R1-R8.

Pardini AW, Nguyen HT, Figlewicz DP, Baskin DG, Williams DL, Kim F *et al*. (2006) Distribution of insulin receptor substrate-2 in brain areas involved in energy homeostasis. *Brain Res* 1112:169-78.

Parise EM, Lilly N, Kay K, Dossat AM, Seth R, Overton JM *et al*. (2011) Evidence for the role of hindbrain orexin-1 receptors in the control of meal size. *Am J Physiol Regul Integr Comp Physiol* 30: R1692-9.

Paxinos G, Franklin KBJ (2001) The mouse brain in stereotaxic coordinates. Second Edition. Academic Press, San Diego.

Pócsai K and Kálmán M (2014) Extracellular matrix components mark the territories of circumventricular organs. *Neurosci Lett* 566:36-41.

Potes CS, Turek VF, Cole RL, Vu C, Roland BL, Roth JD *et al*. (2010) Noradrenergic neurons of the area postrema mediate amylin's hypophagic action. *Am J Physiol Regul Integr Comp Physiol* 299:R623-31.

Price CJ, Hoyda TD, Ferguson AV (2008) The area postrema: a brain monitor and integrator of systemic autonomic state. *Neuroscientist* 14:182-194.

Purser MJ, Dalvi PS, Wang ZC, Belsham DD (2013) The cytokine ciliary neurotrophic factor (CNTF) activates hypothalamic urocortin-expressing neurons both in vitro and in vivo. *PLoSONE* 8:e61616.

Raposinho PD, Pierroz DD, Broqua P, White RB, Pedrazzini T, Aubert ML (2001) Chronic administration of neuropeptide Y into the lateral ventricle of C57BL/6J male mice produces an obesity syndrome including hyperphagia, hyperleptinemia, insulin resistance, and hypogonadism. *Mol Cell Endocrinol* 185: 195-204.

Riediger T (2012) The receptive function of hypothalamic and brainstem centres to hormonal and nutrient signals affecting energy balance. *Proc Nutr Soc* 71:463-77.

Riediger T, Schmid HA, Lutz T, Simon E (2001) Amylin potently activates AP neurons possibly via formation of the excitatory second messenger cGMP. *Am J Physiol Regul Integr Comp Physiol* 281:R1833-1843.

Riediger T, Schmid HA, Lutz T, Simon E (2002) Amylin and glucose co-activate area postrema neurons of the rat. *Neurosci Lett* 328:121-124.

Rizk NM, Stammsen D, Preibisch G, Eckel J (2001) Leptin and tumor necrosis factor-α induce the tyrosine phosphorylation of signal transducer and activator of transcription proteins in the hypothalamus of normal rats in vivo. *Endocrinology* 142: 3027-3032.

Rodríguez EM, Blázquez JL, Pastor FE, Peláez B, Pena P, Peruzzo B, et al. (2005) Hypothalamic tanycytes: a key component of brain-endocrine interaction. *Int Rev Cytol* 247: 89-164.

Roth J, Harré EM, Rummel C, Gerstberger R, Hubschle T (2004) Signaling the brain in systemic inflammation: role of sensory circumventricular organs. *Front. Biosci.* 9:290-300

Rudge JS, Morrissey D, Lindsay RM, Pasnikowski EM (1994) Regulation of ciliary neurotrophic factor in cultured rat hippocampal astrocytes. *Eur J Neurosci* 6:218-229.

Saadat S, Sendtner M, Rohrer H (1989) Ciliary neurotrophic factor induces cholinergic differentiation in rat sympathetic neurons in culture. *J Cell Biol* 108: 1807-1816.

Sanin V, Heeß C, Kretzschmar HA, Schüller U (2013) Recruitment of neural precursor cells from circumventricular organs of patients with cerebral ischaemia. *Neuropathol Appl Neurobiol* 39: 510-518.

Schaeffer M, Langlet F, Lafont C, Molino F, Hodson DJ, Roux T *et al*. (2013) Rapid sensing of circulating ghrelin by hypothalamic appetite-modifying neurons. *Proc Natl Acad Sci USA* 110: 1512-1517.

Schneeberger M, Gomis R, Claret M (2014) Hypothalamic and brainstem neuronal circuits controlling homeostatic energy balance. *J Endocrinol* 220:T25-46.

Schwartz GJ, Salorio CF, Skoglund C, Moran TH (1999) Gut vagal afferent lesions increase meal size but do not block gastric preload-induced feeding suppression. *Am J Physiol* 276: R1623-9.

Schwartz GJ (2006) Integrative capacity of the caudal brainstem in the control of food intake. *Philos Trans R Soc Lond B Biol Sci* 361: 1275-1280.

Schwartz GJ (2009) Brainstem integrative function in the central nervous system control of food intake. *Forum Nutr* 63:141-51.

Schwartz MW, Peskind E, Raskind M, Boyko EJ, Porte DJ (1996) Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. *Nature Med* 2: 589-593.

Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P *et al*. (1997) Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* 46: 2119-23.

Schwartz MW, Woods SC, Porte DJ, Seeley RJ, Baskin DG (2000) Central nervous system control of food intake. *Nature* 404:661-671.

Seeley RJ, van Dijk G, Campfield LA, Smith FJ, Burn P, Nelligan JA *et al*. (1996) Intraventricular leptin reduces food intake and body weight of lean rats but not obese Zucker rats. *Horm Metab Res* 28: 664-8.

Seeley RJ and Woods SC (2003) Monitoring of stored and available fuel by the CNS: implications for obesity. *Nat Rev Neurosci* 4:901-9.

Sendtner M, Carroll P, Holtmann B, Hughes RA, Thoenen H (1994) Ciliary neurotrophic factor. *J Neurobiol* 1994 Nov;25(11):1436-53.

Sendtner M, Kreutzbergt GW, Thoenen H (1990) Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature 345:440-441.

Sendtner M, Schmalbruch H, Stöckli KA, Carroll P, Kreutzberg GW, Thoenen H (1992) Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. *Nature* 358:502-504.

Senzacqua M, Severi I, Perugini J, Acciarini S, Cinti S, Giordano A (2016) Action of Administered Ciliary Neurotrophic Factor on the Mouse Dorsal Vagal Complex. *Front Neurosci.* 10:289.

Severi I, Carradori MR, Lorenzi T, Amici A, Cinti S, Giordano A (2012) Constitutive expression of ciliary neurotrophic factor in mouse hypothalamus. *J. Anat* 220: 622-631.

Severi I, Perugini J, Mondini E, Smorlesi A, Frontini A, Cinti S, Giordano A (2013) Opposite effects of a high-fat diet and calorie restriction on ciliary neurotrophic factor signaling in the mouse hypothalamus. *Front Neurosci*.7:263.

Severi I, Senzacqua M, Mondini E, Fazioli F, Cinti S, Giordano A (2015) Activation of transcription factors STAT1 and STAT5 in the mouse median eminence after systemic ciliary neurotrophic factor administration. *Brain Res* 1622:217-29.

Shapiro RE and Miselis RR (1985) The central neural connections of the area postrema of the rat. *J Comp Neurol* 234:344-64.

Sheng M and Greenberg ME (1990) The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* 4:477-85.

Simi A and Ibanez CF (2010) Assembly and activation of neurotrophic factor receptor complexes. *Dev Neurobiol* 70, 523–531.

Sipols AJ, Baskin DG, Schwartz MW (1995) Effect of intracerebroventricular insulin infusion on diabetic hyperphagia and hypothalamic neuropeptide gene expression. *Diabetes* 44: 147-51.

Sleeman MW, Anderson KD, Lambert PD, Yancopoulos GD, Wiegand SJ (2000) The ciliary neurotrophic factor and its receptor, CNTFR alpha. *Pharm Acta Helv* 74:265-272.

Sleeman MW, Garcia K, Liu R, Murray JD, Malinova L, Moncrieffe M, *et al*. (2003) Ciliary neurotrophic factor improves diabetic parameters and hepatic steatosis and increases basal metabolic rate in db/db mice. *Proc Natl Acad Sci U S A* 100:14297-14302.

Stefater MA, MacLennan AJ, Lee N, Patterson CM, Haller A, Sorrell J, *et al*. (2012) The anorectic effect of CNTF does not require action in leptin-responsive neurons. *Endocrinology* 153:2647-2654.

Stöckli KA, Lillien LE, Naher-Noe´ M, Breitfeld G, Hughes RA, Raff MC, *et al*. (1991) Regional distribution, developmental changes, and cellular localization of CNTF-mRNA and protein in the rat brain. *J Cell Biol* 115: 447-459.

Stöckli KA, Lottspeich F, Sendtner M, Masiakowski P, Carroll P, Götz R, *et al*. (1989) Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. *Nature* 342:920-923.

Tago H, McGeer PL, McGeer EG, Akiyama H, Hersh LB (1989) Distribution of choline acetyltransferase immunopositive structures in the rat brainstem. *Brain Res.* 495: 271-297.

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, *et al.* (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83:1263-1271.

Travagli RA, Hermann GE, Browning KN, Rogers RC (2006) Brainstem circuits regulating gastric function. *Annu Rev Physiol* 68:279-305.

Travers JB, Travers SP, Norgren R (1987) Gustatory neural processing in the hindbrain. *Annu Rev Neurosci* 10: 595-632.

van der Kooy D and Koda LY (1983) Organization of the projections of a circumventricular organ: the area postrema in the rat. *J Comp Neurol* 219:328-38.

Wang QP, Guan JL, Pan W, Kastin AJ, Shioda S (2008) A diffusion barrier between the area postrema and nucleus tractus solitarius. *Neurochem Res* 33:2035-2043.

Watson C, Paxinos G, Puelles L (2012) The Mouse Nervous System. *Amsterdam: Academic Press.*

Watt MJ, Dzamko N, Thomas WG, Rose-John S, Ernst M, Carling D, *et al*. (2006) CNTF reverses obesity-induced insulin resistance by activating skeletal muscle AMPK. *Nat Med* 12:541-548.

Wen R, Tao W, Li Y, Sieving PA (2012) CNTF and retina. *Prog Retin Eye Res*. 31: 136–151.

Wilding JP (2002) Neuropeptides and appetite control. *Diabet Med* 19:619-27.

Woods SC, Lotter EC, McKay LD, Porte DJ (1979) Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature* 282:503-505.

Xu B, Dube MG, Kalra PS, Farmerie WG, Kaibara A, Moldawer LL, *et al*. (1998) Anorectic effects of the cytokine, ciliary neurotropic factor, are mediated by hypothalamic neuropeptide Y: comparison with leptin. *Endocrinology* 139:466-473.

Xu B and Xie X (2016) Neurotrophic factor control of satiety and body weight. *Nat Rev Neurosci*. 17:282-92.

Yamamoto H, Kishi T, Lee CE, Choi BJ, Fang H, Hollenberg AN *et al.* (2003). Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites. *J Neurosci* 23: 2939-2946.

Young A (2005) Inhibition of gastric emptying. *Adv Pharmacol* 52:99-121.

Young AA (2012) Brainstem sensing of meal-related signals in energy homeostasis. *Neuropharmacology* 63:31-45.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* 372: 425-432.

Zigman JM and Elmquist JK (2003) Minireview: From anorexia to obesity - the yin and yang of body weight control. *Endocrynology* 144: 3749-3756.

Zvonic S, Cornelius P, Stewart WC, Mynatt RL, Stephens JM (2003) The regulation and activation of ciliary neurotrophic factor signaling proteins in adipocytes. *J Biol Chem* 278:2228-2235.