Endocrine Reviews

Central regulation of pituitary-thyroid axis under physiological and pathophysiological conditions
--Manuscript Draft--

Manuscript Number:	ER-13-1087R1
Full Title:	Central regulation of pituitary-thyroid axis under physiological and pathophysiological conditions
Short Title:	Regulation of hypophysiotropic TRH neurons
Article Type:	Review
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Central regulation of the hypothalamic-pituitary-thyroid axis under physiological and

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[1]Department of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary.

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Abbreviate title: Regulation of hypophysiotropic TRH neurons

Key terms: hypophysiotropic TRH neurons, negative feedback regulation, fasting, infection

Word count: 14803

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XI. Acknowledgement

I. Introduction

The hypothalamic-pituitary-thyroid (HPT) axis primarily functions to maintain normal, circulating levels of thyroid hormone that is essential for the biologic function of all tissues including brain development, regulation of cardiovascular, bone and liver function, food intake and energy expenditure among many others (1). Key to this regulatory system are a group of neurons that reside in the hypothalamic paraventricular nucleus (PVN), produce thyrotropin-releasing hormone (TRH) and integrate a wide variety of humoral and neuronal signals to regulate the HPT axis. In the present review, we will summarize current knowledge about the anatomy and physiology of these so called "hypophysiotropic" TRH neurons involved in the central regulation of the HPT axis under physiological and specific, pathophysiological conditions.

II. Organization of the central machinery regulating the hypothalamic-pituitary-thyroid axis

a. Thyrotropin-releasing hormone (TRH) as a central regulator of the hypothalamic-pituitary-thyroid axis

TRH is a tripeptide amide (pGlu-His-ProNH2) (2) discovered simultaneously by the groups of Schally and Guilleman in 1969 (3, 4). In these pioneering studies, extracts from more than 250,000 porcine or sheep hypothalami containing only a few milligrams of TRH was shown to have TSH releasing activity. The extracted material contained only three amino acids: glutamic acid, histidine and proline (2), and subsequently was shown to require cyclization of the glutamyl residue and amidation of the proline residue to achieve TSH releasing activity (2).

TRH regulates the synthesis, release and biological activity of TSH (5-7). This effect is mediated *via* the type 1 TRH receptor (TRHR1) (8). At first, TRH stimulates the release of

presynthesized TSH (6), and then increases the synthesis of both TSH subunits, the α-glycoprotein hormone subunit (α-GSU), common to all three glycoprotein hormones of the anterior pituitary, and the TSH-specific β subunit (5). Binding of TRH to TRHR1 results in activation of phospholipase C, calcium mobilization and activation of protein kinase C. This cascade leads to the synthesis of α-GSU through effects on the pituitary LIM homeodomain factor (P-LIM), cAMP response element binding protein (CREB) and CREB binding protein (CBP) transcription factors (5). In contrast, the synthesis of the TSH-β subunit is mediated by the pituitary specific transcription factor-1 (Pit-1) and CBP transcription factors (5). TRH also has important role in regulating the glycosylation of TSH by altering the oligosaccharide composition and structure of its 3,N-linked carbohydrate chains, important for the folding, assembly, secretion, metabolic clearance and ultimately increasing the biological activity of TSH (6, 9-11). Indeed, TRH deficiency in both mouse models and man results in decreased TSH bioactivity and low peripheral thyroid hormone levels (12, 13).

b. *Molecular characterization of the TRH gene*

The first partial sequence of the preproTRH gene was cloned from frog skin by Richter et al. (14), and the nearly full length cDNA of preproTRH isolated from rat hypothalamic λgt11 library (15). A single copy of the preproTRH gene is present in the rat (chromosome 4), mouse (chromosome 6) and human (chromosome 3) genomes (16-19). The structure of the rat preproTRH gene is summarized in Figure 1. In all species, the gene contains three exons and two introns (16). In rats, the size of the introns are 750 and 450 base pairs, respectively (16). The first exon encodes the 5' untranslated region of the mRNA, and the second exon encodes the signal peptide and a portion of the amino terminus of the proTRH peptide. The third exon encodes the remaining part of the amino-terminal peptide, five copies of the TRH sequence separated by non-TRH peptide sequences and the carboxyl terminal peptide

followed by the 3' untranslated region of the mRNA (16). The promoter of the gene contains a TATA box 28 base pairs upstream of the transcriptional initiation site (16). In addition, a series of regulatory elements has been identified in the proximal promoter of the gene. The human preproTRH gene contains three, negative, thyroid hormone response elements (TRE) (20). The so called "site 4" is located -55 to -60 base pairs from the transcriptional initiation site. This thyroid hormone receptor (TR) binding half site binds both thyroid hormone receptor (TR) homodimers and TR-retinoid X receptor (RXR) heterodimers (20-22). Two other functional TREs are present in the first exon between +14 - +19 and +37 - + 42, respectively (20). These sites can only bind TR monomers, but are also necessary for the regulation of the gene by thyroid hormone (20). Site 4 of the human preproTRH gene is also thought to function as a cAMP response element (CRE), resulting in competition between cAMP and TR for binding (23, 24). In the rat preproTRH gene, however, site 4 does not bind cAMP and functions exclusively as a TRE (21). A CRE-2 site identified 5' to site 4 at -101 to -94 seems to function as the primary cAMP response element in this gene (21). Glucocorticoid response element (GRE), activator protein 1 (AP-1), Krüppel-like factor 4 (Klf4), SP1 and STAT3 binding sites have also been identified in the proximal promoter of the preproTRH gene (21, 24, 25).

c. Processing of preproTRH

The 26 kDa rat preproTRH protein is composed of 255 amino acids (26). The N-terminal 25 amino acids comprise the signal peptide that directs the newly synthesized protein into the lumen of the rough endoplasmic reticulum (RER) after its synthesis on ribosomes (26). This sequence is cleaved during the passage into the RER (27), leaving a proTRH protein that contains 5 copies of TRH progenitor sequences, Gln-His-Pro-Gly, four non-TRH peptides located between the TRH progenitor sequences, and C-terminal and N-terminal flanking

peptides (27). The proTRH protein of other species also contains multiple copies of TRH progenitor sequences (27), 6 copies in the human and 7 copies in the frog (27). TRH progenitor peptides are flanked by paired basic amino acids, Lys-Arg or Arg-Arg, that serve as signals for endoprotease enzymes (27). The N-terminal flanking peptide is further cleaved at the pair of Arg amino acids located in the 51-52 position of the preproTRH molecule. Therefore, the processing of the rat proTRH results in 5 copies of TRH and 7 non-TRH peptides (27). Since the non-TRH peptide located between the 4th and 5th TRH progenitors can be further cleaved, and incomplete processing at Arg-Arg residues following the 3rd and 4th TRH progenitor sequences can lead to C-terminally extended TRH peptides (27, 28), the actual number of peptides derived from proTRH processing can be even higher. In addition to mature TRH, other proTRH derived peptides such as proTRH 160-169 and proTRH 177-199, also have biological activity (27). Therefore, the large number of peptides derived from proTRH and evidence for differential processing of proTRH in different regions of the brain may serve to increase the diversity of how TRH neurons influence their targets.

The cleavage of proTRH at paired basic amino acid residues is primarily performed by two enzymes, prohormone convertase 1/3 (PC1/3) and prohormone convertase 2 (PC2) (27). However, both PC enzymes can cleave proTRH at multiple sites. In PC1/3 KO mice, the concentration of the TRH tripeptide is reduced by almost 80%, but only by 44% in PC2 KO mice. Therefore, PC1/3 may be more important in the processing of TRH (29). The lack of PC2 has more profound effects on the concentration of some of the non-TRH proTRH peptides (29). After cleavage, the basic amino acid residues are removed by carboxipeptidase E (CPE) (27). The TRH progenitor, Gln-His-Pro-Gly, is then amidated by peptidylglycine alpha amidating monooxygenase (PAM) using the C-terminal glycine as amide donor (27). Finally, the N-terminal glycine is cyclized to pyroglutamate (27), catalyzed by N-glutaminyl cyclase (30), resulting in the mature form of TRH.

The processing of the proTRH protein takes place in the trans-Golgi network (TGN) and in the regulated secretory pathway (RSP) (27). The first cleavage of proTRH occurs in the TGN (27) where PC1 processes the prohormone at the second or third TRH precursor resulting in a 9.5 kDa or 15 kDa N-terminal peptide and a 16.5 kDa or 10 kDa C-terminal, intermediate peptide (27). This initial cleavage is also critical for the targeting of proTRH-derived peptides into the regulated secretory pathway and the appropriate sorting of these peptides into secretory vesicles (31). After completion of the initial cleavage, the N- and C-terminal peptides of proTRH are sorted into different vesicles of the regulated secretory pathway (31). Prevention of the initial cleavage by mutation of the paired basic residues, however, directs proTRH protein into the constitutive secretory pathway (31). The C-terminal intermediate protein is further cleaved in the TGN at residues 201-202, but all other cleavage steps and the maturation of the TRH precursor takes place in the immature and mature secretory vesicles (31).

d. Inactivation of TRH

Inactivation of secreted TRH in the brain is primarily catalyzed by a membrane-bound ectoenzyme, pyroglutamyl peptidase II (PPII) (32-34). PPII is a type II integral membrane protein comprised of a small, N-terminal, intracellular region and a large, extracellular domain containing the active site of the enzyme (32). PPII has stringent substrate specificity as it can only degrade peptides that are no longer than 4 amino acids with a pGlu-His-X structure, where X can be Pro, Ala, Trp, Pro-NH_2, Pro-Gly or Pro-β-NA (32). PPII produces the dipeptide, His-ProNH_2, from TRH, which is further degraded by dipeptidyl aminopeptidase IV, or spontaneously cyclizes to His-Pro-diketopiperazine (32). PPII activity can be detected in most brain regions where the axons of TRH neurons terminate, but some mismatch is observed (32). PPII is primarily synthesized by neurons, but it is also produced

by tanycytes, a specialized glial cell type, in the hypothalamus (32, 35). Inhibition of PPII activity markedly increases the amount of TRH released from brain tissue slices, supporting the importance of this peptidase in the metabolism of TRH (32).

In serum, TRH is degraded by a soluble enzyme that was formerly called thyroliberinase (35), but subsequently shown to be a product of the PPII gene produced in the liver by proteolytic cleavage of membrane bound PPII (36). Two, broad specificity cytosolic peptidases, pyroglutamyl peptidase I and prolyl endopeptidase, can also degrade TRH. However, as there is no evidence for the presence of these enzymes in the extracellular space and only a small proportion of the extracellular TRH is internalized, these enzymes do not play a major role in the inactivation of released TRH (32).

e. Anatomical characteristics of hypophysiotropic TRH neurons

TRH-synthesizing neurons are present in several brain regions, but only hypophysiotropic TRH neurons located in the PVN are involved in the central regulation of the HPT axis (37). This nucleus is a critical vegetative center of the hypothalamus and located symmetrically at the upper third of the third ventricle.

The PVN contains a magnocellular and a parvocellular division. The magnocellular division houses oxytocin and vasopressin neurons that project to the posterior pituitary. The parvocellular division is further divided into anterior, periventricular, medial, ventral, dorsal and lateral parvocellular subdivisions (38). In rats, TRH neurons are found in all parvocellular subdivisions (Fig. 2 A-C) (39), but the hypophysiotropic TRH neurons are located only in the medial and periventricular subdivisions at the mid and caudal levels of the PVN (Figs. 2 D-F, 3 A-F) (40-42). In mice, hypophysiotropic TRH neurons are located only at the mid level of the PVN (Fig. 3 G-L), intermingled with the magnocellular neurons (43, 44). The periventricular subdivision of the PVN does not contain TRH neurons, and the

medial parvocellular subdivision at the caudal levels of the PVN houses non-hypophysiotropic TRH neurons (43, 44). In humans, the PVN also contains a large population of TRH neurons especially in its medial part, but the location of hypophysiotropic TRH neurons is not yet known (45, 46).

Hypophysiotropic TRH neurons are functionally different from the non-hypophysiotropic TRH neurons in the PVN. Only hypophysiotropic TRH neurons project to the external zone of the median eminence (Fig. 4) where their axon terminals release TRH into the extracellular space of this blood-brain barrier-free circumventricular organ (37). TRH is then conveyed to the anterior pituitary *via* the hypophysial portal circulation where TRH regulates the secretion of TSH from thyrotrophs and prolactin from lactotrophs (37, 47). In addition to TRH, hypophysiotropic neurons also express a second neuropeptide, cocaine- and amphetamine regulated transcript (CART) (42, 48). CART is simultaneously released into the hypophysial portal circulation and has been shown to inhibit the effect of TRH on prolactin secretion, but has no effect on TRH-induced release of TSH (49). Hypophysiotropic TRH neurons also express the vesicular glutamate transporter 2 (VGLUT2), establishing the glutamatergic phenotype of these cells (50). Since TRH axon terminals in the median eminence contain a large number of small, clear vesicles (51), it is likely that glutamate is co-released with TRH, but its physiological significance in the median eminence is currently unknown.

In contrast to the hypophysiotropic TRH neurons, non-hypophysiotropic TRH synthesizing neurons are widely distributed in the central nervous system (39). Currently relatively little information is known about the projection fields and function of these neuronal groups, but some may be involved in the regulation of energy homeostasis. Non-hypophysiotropic TRH neurons in the anterior parvocellular subdivision of the PVN, for example, are densely innervated by inputs containing feeding-related peptides including axons containing AGRP, α-MSH, CART, galanin and GALP (42, 52, 53). In addition, these cells project to feeding-

related nuclei such as the arcuate and dorsomedial nuclei and the amygdala (54). In the perifornical region, TRH is co-synthesized with another anorexigenic peptide, urocortin 3 (UCN3) (55). These neurons have a prominent projection field to the hypothalamic ventromedial nucleus (54, 55), known to be involved in the regulation of food intake (56). TRH neurons in the preoptic area influence energy homeostasis by regulating thermogenesis (57).

f. Neuronal inputs of hypophysiotropic TRH neurons

Hypophysiotropic TRH neurons are embedded in a dense network of neuronal axons in the PVN. These axons form numerous synaptic associations on the surface of TRH neurons and modulate the activity of these cells (58). Integration of these inputs together with humoral signals that can reach TRH neurons through the rich vascular supply of the median eminence or the PVN (59, 60), ensures fine tuning of the activity of the HPT axis and its adaptation to changing environmental conditions. Currently, three, main, neuronal groups are known to send synaptic inputs to the hypophysiotropic TRH neurons: the hypothalamic arcuate nucleus, the hypothalamic dorsomedial nucleus (DMN), and catecholamine-producing neurons in the brainstem (Fig. 5, 6) (58, 61).

The hypothalamic arcuate nucleus plays key role in the regulation of energy homeostasis by relaying humoral signals to second order neuronal groups in the brain (56). Two, major, feeding-related neuronal populations are involved, the medially-located, orexigenic neurons that synthesize neuropeptide Y (NPY), agouti related protein (AGRP) and gamma-amino butyric acid (GABA), and laterally located anorexigenic neurons that produce alpha-melanocyte-stimulating hormone (α-MSH) and CART (56). Both neuronal groups are responsive to peripheral, feeding-related signals such as changes in leptin, ghrelin, insulin and glucose (56), but are oppositely regulated (56).

Numerous AGRP- and NPY-immunoreactive (IR) axon varicosities form juxtaposition with virtually all TRH neurons in the PVN (Fig. 5 A) and establish a symmetric type of synaptic association with these cells, indicative of an inhibitory function (52, 62, 63). Since the neurons that synthesize AGRP are found only in the arcuate nucleus, the innervation of the TRH neurons by AGRP-IR axons must originate exclusively from this nucleus (63). NPY, however, is synthesized by a number of neuronal populations, but the two major sources for the NPY-IR innervation of the PVN are the arcuate nucleus and catecholaminergic neurons in the brainstem (64). The arcuate nucleus neurons provide approximately 75% of the NPY innervation to TRH neurons in the PVN (64, 65), while the adrenergic NPY neurons of the brainstem contribute the remaining 25% (64).

Anorexigenic α-MSH/CART neurons of the arcuate nucleus also innervate TRH neurons in the PVN (Fig. 5 A) (58), but establish fewer synapses than observed for axons containing AGRP/NPY (52). α-MSH is synthesized in two brain regions, the hypothalamic arcuate nucleus and the nucleus tractus solitarius (NTS) of the brainstem. Only α-MSH-containing neurons in the arcuate nucleus co-express CART (42), and therefore, the co-localization of these two peptides can be used as a marker for α-MSH axons originating from the arcuate nucleus. As all α-MSH-IR axon varicosities on the surface of the TRH neurons contain CART, the arcuate nucleus is the exclusive source of the α-MSH-IR innervation to TRH neurons in the PVN (42). Not all CART-IR axons in juxtaposition to the TRH neurons contain α-MSH, however, indicating that the arcuate nucleus is not the only source for the CART-IR innervation of these cells (42). Indeed, only a relatively small portion of the CART-IR varicosities on the surface of TRH neurons derive from the arcuate nucleus. CART-synthesizing neurons that innervate the PVN can also be found in the lateral hypothalamus, perifornical area, zona incerta, C1-3 adrenergic neuronal groups and the medial subnucleus of the NTS (66). As unilateral transection of the ascending brainstem

pathways to the PVN results in an approximately 60% reduction of the CART innervation of hypophysiotropic TRH neurons, it would appear that the brainstem gives rise the majority of the CART input to these cells (67).

TRH neurons in the PVN of the human hypothalamus also receive inputs from the infundibular nucleus, the analogue of the rodent arcuate nucleus (46). Similar to the rodent, the human infundibular nucleus contains separate populations of AGRP/NPY and α-MSH-synthesizing neurons (46). In addition, TRH neurons in the human PVN are densely innervated by axons containing these peptides (46), suggesting that the arcuato (infundibulo)-paraventricular pathway is evolutionarily conserved and thereby, of importance in the regulation of hypophysiotropic TRH neurons (46). There is a major difference between the human and the rodent pathways, however. While CART and α-MSH are co-expressed by the arcuate nucleus neurons in the rodent, CART is not present in α-MSH neurons of the human infundibulum (68). Moreover, CART can be detected in approximately 30% of AGRP/NPY neurons (68), indicating that CART may have a somewhat different role in the regulation of the thyroid axis in humans.

The DMN also plays important role in the regulation of energy homeostasis and vegetative functions (69). Like the feeding-related neurons of the arcuate nucleus, DMN neurons sense circulating energy homeostasis-related hormones such as leptin (70), but these signals also influence the DMN indirectly *via* the arcuate nucleus (71). In addition, the DMN is a critical node in the circuit regulating food-entrainable circadian rhythms and the stress response (69, 72), integrating these signals and relaying the information to other neuronal populations such as the sympathetic nervous system and the PVN (69). The DMN also contributes to the regulation of the hypothalamic-pituitary-thyroid axis, as bilateral destruction of the DMN increases 24h release of T3 (73), suggesting a net inhibitory effect. Indeed, anterograde tract tracing studies have demonstrated (Fig. 5 B) that the vast majority of the TRH neurons in the

PVN receive input from the DMN, primarily establishing symmetric type synaptic associations with the TRH neurons characteristic of an inhibitory function (74). Little is known about how the DMN regulates hypophysiotropic TRH neurons, however, but it has been hypothesized that it may be involved in circadian regulation of the hypophysiotropic TRH neurons (58).

Brainstem catecholaminergic cells groups are involved in the regulation of a wide variety of physiological functions including attention, sleep ∕ wakefulness, learning, memory, emotion, reproduction, neuroendocrine processes and central responses to stress (75). These neurons can be subdivided into adrenergic and noradrenergic subtypes based on their transmitter content. Both neuronal populations produce dopamine-beta hydroxylase (DBH), the noradrenaline-synthesizing enzyme, but only adrenergic neurons express phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts noradrenaline to adrenaline (76).

Hypophysiotropic TRH neurons receive a dense catecholaminergic innervation (77), perhaps comprising the largest input to these neurons. Catecholaminergic axons establish asymmetric type synaptic specializations on the surface of TRH neurons (77), indicative of an activating effect and consistent with the observation that noradrenaline stimulates the transcription of TRH gene (78). It has been shown by triple-labeling immunofluorescence (Fig. 5 C) that approximately two thirds of the catecholaminergic innervation of TRH neurons originate from adrenergic neurons, while the noradrenergic neuronal groups give rise to the remaining one third (61). Adrenergic neurons are located exclusively in the C1-3 regions of the medulla, and since the axons of all three adrenergic regions have highly similar distribution patterns in the PVN (79), it is likely that each adrenergic group contributes to the innervation of hypophysiotropic TRH neurons (61).

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In addition to the classical transmitters, subpopulations of the adrenergic neurons that innervate the TRH neurons in the PVN also synthesize peptidergic transmitters. Approximately, 50% of the adrenergic innervation to TRH neurons co-contain CART (80), and more than 70% co-contain NPY (64). A large proportion of these adrenergic terminals contain pituitary adenylate cyclase-activating polypeptide (PACAP) (81). Currently, it is unknown how the co-release of multiple transmitters modulates the effect of adrenergic neurons on their targets, but it is likely that the capacity to release a large array of transmitters from the same terminals provides substantial flexibility for the ability of adrenergic neurons to differentially respond to diverse physiological and pathophysiological conditions.

The brainstem has six noradrenergic cells groups (A1-6), but only the A1, A2 and A6 noradrenergic cell groups project to the PVN (82). The A1 noradrenergic cell group innervates primarily the magnocellular part of the PVN in rats (83), suggesting that this cell group may be only a minor source of the noradrenergic innervation to TRH neurons. The A2 and A6 noradrenergic cells groups, however, densely innervate the periventricular and medial parvocellular subdivisions of the PVN where the hypophysiotropic TRH neurons reside (83), making it likely that these two noradrenergic cell populations are the primary sources of the innervation of hypophysiotropic TRH neurons.

In addition to the above mentioned inputs, the TRH neurons in the PVN receive galanin-, TRH-, corticotrophin-releasing hormone (CRH)-, somatostatin- and endocannabinoid receptor-containing inputs (50, 53, 81, 84-87). Non-synaptic contacts between serotoninergic axons and the TRH neurons have also been described (88). However, very little is known about the involvement of these inputs in the regulation of hypophysiotropic TRH neurons. The known inputs of the hypophysiotropic TRH neurons are summarized in Figure 6.

g. Tanycytes as regulators of the hypothalamic-pituitary-thyroid axis

Tanycytes are specialized glial cells lining the ventrolateral walls and the floor of the third ventricle between the rostral and caudal limits of the median eminence (Fig. 7 A, B) (89). Characteristic of these cells are a small cell body located in the ependymal layer, and a long, basal process that projects either into to the median eminence or the arcuate, ventromedial or dorsomedial nuclei (90). Based on their location, morphology, cytochemistry and ultrastructure, tanycytes can be classified into four subtypes, α1 and α2 tanycytes that line the ventrolateral walls of the third ventricle, and β1 and β2 tanycytes that line the floor and lateral extensions of the third ventricle (Fig. 7 B) (89, 90). Alpha 1 tanycytes are located approximately in the middle third of the ventricular wall and project into the ventromedial and dorsomedial nuclei where their end feet processes terminate on neurons (89, 90). Alpha 2 tanycytes are located ventral to α1 tanycytes and project their processes into the arcuate nucleus, terminating on neurons and around capillaries. The most ventrally located α2 tanycytes, however, send their processes to the most lateral portion of the tuberoinfundibular sulcus (89, 90).

Beta 1 tanycytes line the lateral invaginations of the infundibular recess, and their processes arch toward the tuberoinfundibular sulcus and terminate on the surface of the pars tuberalis of the pituitary (89, 90). The β2 tanycytes line the floor of the infundibular recess, and their processes travel through the median eminence to terminate around the portal capillaries in the external zone of the median eminence (89, 90).

In addition to their distinct anatomical locations, the various tanycyte subtypes also differ in their chemical signatures, suggesting that they have independent functions. Alpha and β1 tanycytes express the glucose transporter-1, a blood-brain barrier (BBB) marker (91). This observation is in keeping with the morphological observations that α tanycytes surround capillaries in the arcuate and ventromedial nuclei and β1 tanycytes create the barrier that

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separates median eminence from the mediobasal hypothalamus (91). However, α but not β tanycytes express somatostatin sst2a receptors, whereas β1 tanycytes are reactive for N-cadherin, while only α1 tanycytes express adenosine triphosphatase enzyme (90). In addition, β2 tanycytes lack the glucose transporter-1 but express Rab 4, a protein involved in vesicular transport (90, 91).

At the ultrastructural level, a common characteristic of tanycytes is the presence of both early and late endosomes near the apical surface of the cell bodies, suggesting that all types of tanycytes actively incorporate substances from the CSF (90). However, as tight junctions are absent between α and β1 tanycytes (90), substances from the CSF can also enter the neuropil directly through the ventrolateral portions of the third ventricular wall. In contrast, β2 tanycytes are bound together by both zonula adherens and tight junctions, forming an impermeable barrier between the CSF and the median eminence (90).

In addition to functioning as barrier cells, it is becoming increasingly clear that tanycytes are involved in neuroendocrine regulation (90). The role of tanycytes in the regulation of the hypothalamic-pituitary-gonadal axis has been long known (90). Changes in estrogen levels induce cytoskeletal remodeling of tanycytes resulting in retraction of tanycyte end processes from the capillaries during the GnRH surge, allowing GnRH axons to secrete their products into the portal circulation (92). The role of tanycytes in the regulation of the HPT axis has been more recently recognized (89). Tanycytes express thyroid hormone receptors (Fig. 7 C, D), and changes in circulating thyroid hormone levels result in plastic remodeling of tanycyte end feet processes (28, 93), perhaps also to regulate the entry of TRH released by the hypophysiotropic terminals into the portal circulation (93). In addition, the tanycytes express the TRH degrading enzyme, pyroglutamyl peptidase II (PPII, Fig. 7 H) (35), which is regulated in parallel to circulating thyroid hormone levels (35). Thus, hyperthyroidism results in upregulation of tanycyte PPII, contributing to inhibition of TSH secretion by reducing the

amount of TRH reaching the portal system. In support of this hypothesis, inhibition of PPII in the median eminence of hyperthyroid animals significantly increases the amount of secreted TRH (35).

Tanycytes are also thought to be involved in feedback regulation of the HPT axis through their expression of type 2 iodothyronine deiodinase (D2, Fig. 7 E-G), an enzyme that catalyzes 5' deiodination of T4 resulting in the generation of the active form of thyroid hormone, triiodothyronine (T3) (94). In most regions of the brain, D2 is expressed by astrocytes, but in the hypothalamus, D2 is primarily expressed by tanycytes (95-97). Most regions of the hypothalamus including the PVN are devoid of D2 activity (98).

Tanycyte D2 activity is precisely regulated at transcriptional and posttranslational levels (58, 99). Tanycytes synthesize ubiquitine ligase and deubiquitinase enzymes, WSB1 and USP33, respectively, that can quickly and reversibly regulate the activity of D2 (100). In addition, tanycytes are richly replete in the thyroid hormone transporters, MCT8 and OATP1C1 (Fig. 7 C, D) (51, 101, 102), that facilitate the entry of T4 from the circulation or CSF, and release generated T3 into the neuropil or CSF. Under special conditions, tanycytes also express the thyroid hormone degrading enzyme, type 3 iododthyronine deiodinase enzyme (D3) (103). Precise transcriptional and posttranslational regulation of D2 and D3 in tanycytes provides for a powerful mechanism to tightly control hypothalamic T3 availability that may contribute to regulation of the HPT axis.

h. Involvement of the autonomic nervous system in the regulation of the hypothalamic-pituitary-thyroid axis

In addition to the stimulation of TSH secretion of the anterior pituitary by TRH, the central nervous system can also regulate thyroid function *via* the autonomic nervous system. The thyroid gland is innervated by both adrenergic nerve fibers of the sympathetic nervous system and the cholinergic axons originating from the vagus nerve (104, 105). Both sympathetic and

parasympathetic nerves densely innervate the blood vessels of the thyroid gland, but axon terminals of these autonomic systems can also be found around the thyroid follicles (104, 105), indicating that not only the blood flow, but also the activity of thyroid follicles could be under direct control of autonomic inputs. Retrograde, virus mediated tract tracing studies has verified the existence of both the sympathetic and parasympathetic innervation of the thyroid gland, showing that two days after the injection of pseudorabies virus directly into the thyroid gland, sympathetic preautonomic neurons in the intermediolateral column of the spinal cord and the parasympathetic preautonomic neurons in the dorsal vagal complex were retrogradely labeled (106).

….Relatively little data is available about how the autonomic inputs to the thyroid gland regulate thyroid function. However, the sympathetic input seems to have an inhibitory action as electrical stimulation of the cervical sympathetic trunk decreases thyroid blood flow (107). Noradrenaline also inhibits the stimulatory effect of TSH on the thyroid cells *in vitro* (108) and decreases thyroid hormone secretion *in vivo* (109).

In contrast, electric stimulation of thyroid nerve, that carries parasympathetic inputs to the thyroid gland, results in increased thyroid blood flow that can be prevented by atropine pre-treatment (107). In addition, transection of inferior laryngeal nerve that also carries parasympathetic input to the thyroid gland results in a fall of circulating T4 levels, supporting the stimulatory effect of the parasympathetic inputs on the activity of thyroid gland (110).

In addition to the classical transmitters, the neuropeptides, NPY and vasoactive intestinal peptide (VIP), are also present in axons innervating the thyroid gland (107). NPY is present in the sympathetic innervation of the thyroid gland and similar to norepinephrine, inhibits thyroidal blood flow (111). In contrast, VIP increases the thyroid blood flow and thyroid hormone secretion (107).

In addition to the primary preautonomic neuronal groups, multisynaptic connection of the the suprachiasmatic nucleus and energy homeostasis related neuronal groups of the hypothalamus including the PVN and the arcuate nucleus with the thyroid gland have also been demonstrated (106), suggesting that these hypothalamic cell groups may also be involved in the autonomic regulation of thyroid gland. As cenral melanocortin and NPY signaling contributes to the metabolism of thyroid hormone in the liver by regulating sulfotransferases (112), it is conceivable that the autonomic nervous system is also involved in regulating the peripheral metabolism of thyroid hormones in addition to their synthesis, although the mechanism remains uncertain.

III. Negative feedback regulation of hypophysiotropic TRH neurons

a. Classical view of negative feedback regulation

Negative feedback regulation of hypophysiotropic TRH neurons is an important regulatory mechanism to ensure stability of circulating thyroid hormone levels (113). When circulating thyroid hormone levels are increased, TRH gene expression is decreased in hypophysiotropic neurons, whereas the converse is true in association with hypothyroidism (Fig. 8) (58). Regulation of TRH transcription by thyroid hormone is relatively rapid, as the exogenous administration of thyroid hormone can suppress transcription of the TRH gene in the PVN within 5 h (114). This regulatory mechanism is a unique feature of hypophysiotropic TRH neurons, as thyroid hormone does not regulate TRH gene expression in non-hypophysiotropic TRH neurons (113). Thyroid hormone is sensed directly by the hypophysiotropic TRH neurons since implantation of crystalline T3 immediately adjacent to the PVN in hypothyroid animals results in marked inhibition of TRH mRNA on that side, but has no effect on TRH neurons on the contralateral side (115).

Hypophysiotropic TRH neurons express the TRα1, TRβ1 and TRβ2 isoforms of the thyroid hormone receptors, although the TRβ1 isoform is present in relatively low abundance (116). The presence of all three thyroid hormone receptor isoforms in the same cell type may seem to be redundant. However, all isoforms have different roles in the regulation TRH gene expression in the hypophysiotropic neurons. In TRβ knock out mice, in which TRα1 is the only functional thyroid hormone receptor, T3 treatment results in a significant increase of TRH gene expression in the PVN, while the lack of TRα1 in TRα knock out mice enhances the T3-induced decrease in TRH mRNA in the PVN (117). These data suggest that T3 positively regulates the TRH gene *via* TRα1. This view is also supported by the stimulatory effect of T3 on the TRH gene expression in cell cultures of developing hypothalamic cells at the 12 DIV stage when the TRα1 is the predominant TR isoform (118). This TRα1 mediated stimulatory effect of T3 can be overridden by the TRβ-mediated inhibitory effect of T3 in wild type mice and also in later stages of cultured hypothalamic cells (117, 118). Since a positive TRE has not been identified in the promoter of TRH gene, it is not clear whether the TRα1-mediated stimulatory effect exerted directly on the TRH promoter or through indirect effects (118). In contrast to the TRα knock out mice, the T3 induced inhibition of TRH gene expression is completely absent in the PVN of both TRβ and TRβ2 knock out mice (117, 119). Therefore, it has been suggested that negative feedback regulation of the TRH gene is mediated exclusively by the TRβ2 isoform (119). This view was challenged by Guissouma et al. (120) using siRNA-mediated knock down of the different TRβ isoforms in the hypothalamus of mouse pups. The results of these experiments revealed that siRNA-mediated knock down of either TRβ2 or TRβ1 prevents the T3 dependent inhibition of the activity of a TRH-luciferase construct transfected into the hypothalamus, suggesting that both TRβ1 and TRβ2 contribute to negative feedback regulation of the TRH gene. It is not clear, however, how the two TRβ isoforms interact in this regulatory process.

The two TRβ isoforms, however, unequivocally play different roles in ligand-independent stimulation of the TRH gene (117, 120). While the TRH mRNA level is markedly increased in the PVN of TRβ2 knock out mice independent of thyroid status (119), TRH expression is significantly decreased in both hypo- and euthyroid TRβ knock out mice (117). These data suggest that the TRβ1 isoform is critical for ligand-independent stimulation of the TRH promoter and supported by evidence that siRNA-mediated knock down of the TRβ1 isoform significantly decreases activity of the TRH promoter in the mouse hypothalamus (120).

In addition to the regulation of TRH at the transcriptional level, thyroid hormone also influences posttranslational processing of proTRH. Increased levels of circulating thyroid hormone lead to the downregulation of two, major, proTRH processing enzymes, PC1/3 and PC2, but selective to the PVN, resulting in the accumulation of intermediate processing products (121). Thyroid hormone may also affect expression of neuropeptide receptors in hypophysiotropic TRH neurons, such as the melanocortin 4 receptor (122), influencing the sensitivity of TRH neurons to their excitatory and inhibitory inputs.

b. *Involvment of type 2 and type 3 deiodinases, thyroid hormone transporters and pyroglutamyl-peptidase II in negative feedback regulation of hypophysiotropic TRH neurons*

1. Role of deiodinases

The concept that the circulating level of T3 is solely responsible for negative feedback regulation of hypophysiotropic TRH by acting directly on these neurons was challenged by Kakucska et al. (123), showing that restoration of circulating levels of T3 to normal in hypothyroid rats without the administration of T4 does not normalize TRH gene expression in the PVN (Fig. 9). Only if very high, hyperthyroid levels of T3 were achieved in the circulating blood was it possible to decrease TRH mRNA levels in the PVN into the normal,

euthyroid range (123). These data indicate that in addition to T3, circulating T4 is also necessary for appropriate feedback control of hypohysiotropic TRH neurons. However, since T4 functions primarily as a prohormone, its conversion to T3 within the CNS must be an essential part of the feedback regulatory mechanism.

As noted previously, the conversion of T4 to T3 in the brain is catalyzed by D2 (94), and in the hypothalamus, this enzyme is expressed primarily in tanycytes (95-98, 124). Although D2 is also present in less abundance in astrocytes in the median eminence and arcuate nucleus region (95), selective ablation of D2 from astrocytes in transgenic mice has no significant effect on feedback regulation of the hypophysiotropic TRH neurons (125), indicating that astrocytes have little or no role in the regulation of this response. Presumably, therefore, tanycyte D2 is responsible for generating the additional T3 required to maintain normal homeostasis in the thyroid axis, although this has not yet been directly tested experimentally. Under certain conditions, the thyroid hormone inactivating enzyme, type 3 iodothyronine deiodinase (D3), is also present in tanycytes (126, 127). It is unknown, however, whether this enzyme contributes to the regulation of hypothalamic T3 concentrations.

In most brain regions, the primary role of D2 is to maintain local T3 concentrations if circulating levels of T4 and T3 decline (128). In the cortex, for example, hypothyroidism upregulates D2 activity to produce more T3, while hyperthyroidism downregulates D2 activity (94). Therefore, the local T3 concentration in the cortex is unchanged even if the circulating T4 concentrations vary over a relatively wide range (128). D2 is also regulated by thyroid hormone in tanycytes at the transcriptional level (97, 129), however, the increased gene expression is not accompanied by a rise of D2 activity in these cells (130). While hypothyroidism results in a more than 4-fold increase of D2 activity in the cortex (131), it has no effect on D2 activity in the mediobasal hypothalamus (130). Similarly, no increase in D2 activity has been observed in the mediobasal hypothalamus in association with iodine

deficiency, contrary to other regions in the brain (132). The posttranscriptional attenuation of thyroid hormone-induced regulation of D2 activity in the MBH suggests that the main role of D2 in this region is not to maintain a constant, local, T3 concentration, but rather to allow the hypothalamus to sense changes in peripheral thyroid hormone levels using T3 as a regulatory signal. This feature is important because stable hypothalamic T3 concentrations would reduce the sensitivity of feedback regulation mechanism of TRH neurons in the PVN.

Despite all the above mentioned data supporting the role of D2 expression of tanycytes in the feedback regulation of the hypophysiotropic TRH neurons, D2 KO mice are euthyroid and their HPT axis has seemingly intact negative feedback regulation (133). This data could question the role of D2 in the feedback regulation of the HPT axis. However, it is important to note that during the embryonic period, thyroid hormone levels have a major influence on the development of the setpoint for feedback regulation of thyroid hormone on the HPT axis (134). In D2 KO animals, D2 activity is already absent from the tanycytes during the embryonic period. Therefore, it is likely that during development, compensatory mechanisms are brought into play that allow feedback regulation of the HPT axis to proceed normally, even in the absence of the thyroid hormone activating capacity of tanycytes. This hypothesis is supported by the observation that thyroid hormone availability during the neonatal period can highly influence the setpoint for negative feedback regulation of the HPT axis (135). Namely, exogenous administration of T4 during the first 12 days after birth results in a permanent decrease in circulating TSH and thyroid hormone concentrations that persists into adulthood despite the normal TSH secretory capacity of animals in response to exogenous TRH administration (135). Were the setpoint of negative feedback regulation to be altered (lowered) during development by chronically low T3 levels in hypothalamus of D2 KO mice, the HPT axis of the adult D2 KO mice would be expected to function normally despite low T3 availability in the hypothalamus of adult D2 KO mice. However, it is likely that ablation of

D2 expression in adult animals would have far more profound impact on the parameters of the HPT axis.

Some hypophysiotropic axon terminals in the median eminence contain D3 (51), indicating that these neurons can regulate T3 availability intracellularly independent of the thyroid hormone concentration in the neuropil by degrading T3. Importantly, however, the vast majority of the TRH-containing hypophysiotropic terminals do not contain D3 and therefore, do not have an internal mechanism for degrading T3 once taken up into the neuron (51).

2. Role of thyroid hormone transporters

Despite their lipophylic nature, the transport of thyroid hormone through cell membranes requires active transport (136). Currently, two, main, thyroid hormone transporters are known to be involved in thyroid hormone transport in the brain, OATP1C1 and MCT8, members of the organic anion transporting polypeptide (OATP) and the monocarboxylate transporter (MCT) families, respectively (136). OATP1C1 has a similar high affinity for T4 and T3 and is abundantly expressed in endothelial cells of brain blood vessels, the choroid plexus and tanycytes (102, 137). The activity of the HPT axis is not affected by the lack of OATP1C1 in knockout mice (138), however, suggesting that this transporter does not play a crucial role in feedback regulation of TRH neurons. In contrast, the MCT8 transporter, which is preferentially expressed in neurons including hypophysiotropic TRH neurons (51, 139), and tanycytes (139), has preferential affinity for T3 (139). In MCT8 knockout mice, TRH gene expression increases in the PVN (140).

The location of tanycytes at the blood-brain and CSF-brain barriers, and their high expression of thyroid hormone transporters, place them in a strategic position to extract T4 from the bloodstream or CSF. The former could be accomplished through their end feet processes terminating on portal capillaries or on blood vessels in the arcuate nucleus, and the

latter *via* apical specializations after T4 has traversed the choroid plexus (101, 102, 141). While increased circulating levels of T3 in MCT8 KO mice, and the high expression of this transporter in tanycytes implicate MCT8 as an essential component of the feedback regulation mechanism on hypophysiotropic TRH neurons, the considerable distance between tanycytes and the cell bodies of hypophysiotropic TRH neurons in the PVN raises questions as to how locally synthesized T3 can be transported from tanycytes to the perikarya of TRH neurons. It was initially hypothesized that T3 released from tanycytes into the CSF or neuropil can reach TRH neurons by diffusion through the brain extracellular space (97). This hypothesis, however, does not address how it is possible that only hypophysiotropic TRH neurons are regulated by the changes of peripheral thyroid hormone levels, whereas non-hypophysiotropic TRH neurons in the hypothalamus (including the PVN) that also express thyroid hormone receptors, are not affected (116).

Despite the relatively large distance between the tanycytes and the perikarya of the hypophysiotropic TRH neurons, there is one location where the two cell types are closely associated. This is the external zone of the median eminence where the axon terminals of hypophysiotropic TRH neurons are intertwined with end-feet processes of β2 tanycytes (142). MCT8 is present on the surface of practically all axon terminals in the external zone of the median eminence, including the terminals of hypophysiotropic TRH neurons (51). This observation makes it likely that T3 secreted from the tanycytes can be taken up by hypophysiotropic TRH terminals *via* MCT8 and then transported to the perikarya of TRH neurons by retrograde, axonal transport. In support of this hypothesis, early studies by Dratman et al. (143) presumed axonal transport of T3, based on the presence of radiolabeled T3 in neuronal processes and migration of autoradiographic signal among brain areas during the first 48h after iv administration of radiolabeled T3. In addition, rapid retrograde axonal transport of other bioactive molecules such as neurotrophins, has already been proven (144,

145). To demonstrate that retrograde transport of T3 from the median eminence actually occurs, and changes of peripheral thyroid hormone levels results in different intranuclear T3 concentration in the hypophysiotropic and non-hypophysiotropic TRH neurons, however, will require further study.

The median eminence is a unique brain region, as its T3 content derives from at least two, different sources. Being outside the blood brain barrier (90), T3 circulating in the peripheral blood can readily enter the extracellular space of the median eminence through fenestrated portal capillaries without the need for a specific transport mechanism. However, tanycytes may also contribute to the T3 content in the median eminence given their high concentration of D2 (90) and hence, ability to convert T4 to T3. As nearly a twice normal circulating level of T3 is required to normalize the TRH mRNA level in the PVN if only T3 is administered to rats after inhibition of thyroid hormone synthesis (123), the contribution of T3 derived from tanycytes may contribute substantially to the T3 content of the median eminence in euthyroid animals. Since only hypophyiotropic TRH neurons project to the median eminence, this hypothesis would also provide an explanation for why hypophysiotropic and non-hypophysiotropic TRH neurons are differentially regulated by thyroid hormone. However, further studies are needed to demonstrate that the T3 content of hypophysiotropic and non-hypophysiotropic TRH neurons is differentially regulated by changes of peripheral thyroid hormone levels.

3. Role of PPII

In addition to modulating feedback regulation of TRH neurons by influencing T3 availability in the median eminence, tanycytes can also influence of the amount of TRH that reaches portal capillaries in the median eminence. Tanycytes synthesize the TRH degrading enzyme, PPII, which is highly regulated by circulating levels of thyroid hormone (35).

Peripheral administration of T4 markedly increases PPII mRNA synthesis and activity in tanycytes (35). Tanycyte end-feet processes surround the hypophysiotropic terminals in the external zone of the media eminence and PPII is a membrane bound protein with a large extracellular C-terminal region that contains the exopeptidase and catalytic motifs (146). Therefore, PPII synthesized by tanycytes is in anatomical position to degrade secreted TRH from TRH-containing axon terminals in the extracellular space of the median eminence (35). In support of this hypothesis, inhibition of PPII by *Hermodice carunculata* protease inhibitor (HcPI) increases TRH release from median eminence explants, whereas *in vivo*, a single peripheral injection of HcPI results in a significant increase in circulating TSH levels in animals exposed to a cold environment compared to the vehicle injected animals (35).

A summary of the mechanisms contributing to feedback regulation of hypophysiotropic TRH neurons is illustrated in Figure 10.

IV. Central regulation of the hypothalamic-pituitary-thyroid axis during fasting

The HPT axis plays critical role in the regulation of energy expenditure by affecting basal metabolic rate and through the actions of thyroid hormone to stimulate mitochondrial oxygen consumption and increase thermogenesis (147). It is not surprising, therefore, that alternations in energy availability would be intimately linked to control of the HPT axis. During fasting, for example, circulating thyroid hormone levels decline associated with low or normal TSH levels and inhibition of TRH gene expression in the PVN characteristic of central hypothyroidism (Fig. 11 A, B) (148-150). Presumably, fasting-induced hypothyroidism is a homeostatic mechanism to conserve energy stores until food is once again available. The mechanism by which hypophysiotropic TRH neurons sense alterations in peripheral energy stores is orchestrated by leptin, a white adipose tissue-derived circulating hormone, (148-150), as fasting-induced central hypothyroidism can be completely prevented by the

exogenous administration of leptin (Fig. 11 C) (148). The primary target site for leptin that

mediates its effect on the HPT axis is the arcuate nucleus, because ablation of this nucleus

abolishes both fasting- and leptin-induced regulation of the HPT axis (151). The circuitry and

peptide mediators involved are discussed in detail below and illustrated in Figure 12.

   a. Role of the arcuato-paraventricular pathway in the regulation of the HPT axis during

     fasting

Two antagonistic, neuronal populations in the arcuate nucleus, the orexigenic

NPY/AGRP/GABA neurons and the anorexigenic α-MSH/CART neurons, are primarily

responsible for sensing and relaying information to hypophysiotropic TRH neurons about the

concentration of leptin in the bloodstream (56). Both leptin-sensitive neuronal groups directly

target hypophysiotropic TRH neurons and establish synaptic specializations with their

perikarya and dendrites in the PVN (58). Similar to leptin, central administration of α-MSH

or CART to fasted animals completely prevents fasting-induced inhibition of TRH gene

expression in the PVN (Fig. 13) (42, 52), and can increase TRH release from hypothalamic

explants (52, 152). α-MSH has also been shown to depolarize TRH neurons in the PVN and

increase their firing rate (153). In contrast, central administration of NPY or AGRP to fed

animals induces a state of central hypothyroidism similar to that observed in fasted animals

(Fig. 14), despite that the animals markedly increase their food intake secondary to the potent

orexigenic effect of NPY (152, 154, 155). NPY has also been shown to hyperpolarize the

TRH neurons in the PVN and decrease the firing rate of these neurons (153).

The α-MSH/CART and NPY/AGRP/GABA arcuate neurons interact at multiple levels to

regulate hypophysiotropic TRH neurons. First, both α-MSH and AGRP are ligands of the

melanocortin 3 and 4 receptors (MC3R and MC4R) (156), with α-MSH functioning as an

agonist and AGRP as a high affinity antagonist (156). The MC4R is expressed by

hypophysiotropic TRH neurons (24), and as the inhibitory effect of AGRP on TRH gene expression is abolished in MC4R KO mice (157), melanocortin signaling to TRH neurons must be mediated primarily by the MC4R. All TRH neurons that are innervated by α-MSH-containing axon varicosities are also innervated by AGRP-containing terminals (Fig. 5 A) (52), indicating direct, functional interactions between these two peptides. The interaction of the two peptides in the regulation of the TRH neurons is further supported by *in vitro* studies, demonstrating that AGRP prevents the stimulatory effect of α-MSH on TRH release (152).

A second mechanism may involve post-receptor effects arising from the interaction between α-MSH and NPY. The MC4R is coupled to G_s proteins and when activated by α-MSH, induces the adenylyl cyclase–protein kinase A cascade (158). The resultant increase in cAMP and hence, CREB phosphorylation, regulates TRH gene expression by the binding of phosphoCREB (pCREB) to cAMP response elements (CREs) present in the TRH gene (21, 159). NPY also regulates cAMP synthesis, but as NPY receptors couple to G_i or G_0 proteins, NPY inhibits adenylate cyclase, reducing cAMP accumulation (160). In this manner, NPY could inhibit α-MSH-induced CREB phosphorylation in hypophysiotropic TRH neurons by reducing the intracellular cAMP concentration. In fact, pretreating animals with centrally administered NPY markedly reduces the ability of icv injected α-MSH to induce CREB phosphorylation in TRH neurons (161).

The inhibitory effects of NPY on hypophysiotropic TRH neurons are mediated primarily by Y_1 and Y_5 receptors, as the central administration of either selective Y1 or Y5 receptor agonists are equally effective in inhibiting hypophysiotropic TRH gene expression and the HPT axis (162). In addition, Y1 receptor mRNA is expressed in TRH neurons in the PVN (163), and the Y5 receptor has been localized to the PVN (164), although its specific expression in hypophysiotropic TRH neurons has not been studied. Since the fasting-induced fall of leptin stimulates NPY/AGRP neurons and inhibits α-MSH/CART neurons (56), the

increased inhibitory tone of orexigenic peptides and simultaneous decrease in the stimulatory input from anorexigenic neurons of arcuate nucleus origin play a critical role in the development of fasting-induced central hypothyroidism. This hypothesis is supported by evidence showing that fasting-induced central hypothyroidism is prevented in mice lacking both NPY and the MC4R (112).

Currently, little is known about the interactions between CART and melanocortins or NPY, partly hindered by the fact that the CART receptor(s) has not yet been identified. Central administration of CART increases CREB phosphorylation at least in the CRH neurons in the PVN, but pCREB is absent from hypophysiotropic TRH neurons after this treatment (165). Therefore, it is unknown whether the effect of CART on the hypophysiotropic TRH neurons involves the interaction with the NPY and AGRP signaling at the level of second messengers, or only at the level of the TRH promoter.

In addition to leptin, insulin and glucose also have anorexigenic effects when administered centrally (56). Circulating levels of these substances decrease during fasting and are sensed by the feeding-related neurons of the arcuate nucleus (56), raising the possibility that similar to leptin, insulin and glucose are also involved in fasting-induced regulation of hypophysiotropic TRH neurons. However, while central administration of leptin completely prevents fasting-induced inhibition of the TRH gene expression, central administration of insulin or glucose has no effect on TRH gene expression in fasted rats (166). These observations suggest that changes in insulin and glucose levels do not have a critical role in the fasting-induced regulation of the HPT axis, and indicate that leptin, insulin and glucose have different effects on feeding-related neuronal groups in the arcuate nucleus. Indeed, while leptin can completely reverse fasting-induced changes in NPY, AGRP, POMC and CART gene expression in arcuate nucleus neurons, centrally administered insulin only affects NPY and POMC gene expression, and glucose only affects NPY gene expression (166).

Therefore, it would appear that all four feeding-related peptides of arcuate nucleus origin are necessary to appropriately regulate hypophysiotropic TRH neurons during fasting through the arcuato-paraventricular pathway (166).

Similar to the rodent arcuate nucleus, the human infundibular nucleus also contains neurons that co-express AGRP and NPY, and neurons that synthesize α-MSH (46). These neuronal populations give rise to axonal projections that directly innervate TRH neurons in the PVN (46). In contrast to the rodent, however, CART is not co-synthesized with α-MSH, but is present in a subpopulation of the AGRP- and NPY-synthesizing neurons in the infundibular nucleus (68). The significance of this difference is uncertain, but raises the possibility that CART may have a different role in the regulation of the HPT axis in the human brain compared to that observed in rodents (68).

b. *Direct action of leptin on hypophysiotropic TRH neurons*

Despite the observation that regulation of the hypophysiotropic TRH neurons by fasting and leptin is lost in arcuate nucleus ablated animals (151), there has been an ongoing debate about whether leptin can exert a direct effect on hypophysiotropic TRH neurons (24, 37, 58, 153, 167). The presence of the leptin receptor in TRH neurons was shown in primary cultures of fetal rat hypothalamic cells (167), but the approach used did not differentiate between hypophysiotropic TRH neurons and the many other TRH-synthesizing neuronal populations present in the hypothalamus. In addition, it is not clear whether the expression of the leptin receptor observed in fetal cultures is sustained in adult animals or only exists transiently during development. Harris et al. (24), however, have convincingly demonstrated that leptin administration induces the expression of suppressor of cytokine signaling 3 (SOCS3), an accepted marker of leptin receptor signaling, in a small subset (10%) of TRH neurons in the PVN. Furthermore, signal transducers and activators of transcription-3 (STAT3), a second

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messenger utilized by the leptin receptor, was shown to directly bind to the TRH promoter in mouse hypothalamus after peripheral administration of leptin (24). These data support the notion that a subgroup of hypophysiotropic TRH neurons in the PVN can be directly targeted by leptin, a view further supported by *in vitro* patch clamp electrophysiology data, demonstrating a direct excitatory effect of leptin on TRH neurons in the PVN (153).

However, it remains uncertain under what physiologic conditions direct *vs* indirect leptin signaling to TRH neurons is utilized physiologically. During fasting, it seems clear that the arcuato-paraventricular pathway is most important pathway that mediates the effect of leptin on hypophysiotropic TRH neurons. This is based on the observations that arcuate nucleus ablation prevents the effect of fasting and leptin administration on TRH gene expression in the PVN (148). In addition, while central administration of leptin results in both STAT3 and CREB phosphorylation in TRH neurons of the PVN (168), the administration of the melanocortin antagonist, SHU9119, completely prevents the stimulatory effect of centrally administered leptin on TRH release and TSH secretion (168), indicating that leptin influences hypophysiotropic TRH neurons primarily *via* the melanocortin system. Furthermore, in NPY/MC4R double KO mice lacking both NPY and melanocortin signaling, fasting-induced suppression of the HPT axis is completely prevented (112). These data, however, do not exclude the possibility that a direct effect of leptin on hypophysiotropic TRH neurons may be of secondary importance by increasing the expression of MC4R in these neurons (169) and hence, their melanocortin sensitivity. In this manner, a direct effect of leptin may sensitize hypophysiotropic TRH neurons to the arcuate nucleus inputs, rather than directly influence the activity in these cells. A direct effect of leptin on hypophysiotropic TRH neurons, however, may be of importance in maintaining the activity of the HPT axis in association with obesity (170), and is discussed in greater detail in section VI.

c. Involvement of tanycytes in the regulation of the HPT axis during fasting

In addition to neuronal groups projecting onto hypophysiotropic TRH neurons and a direct action of leptin, hypothalamic tanycytes may also participate in the central regulation of the HPT axis during fasting. Fasting results in an approximately 2-fold increase in D2 hybridization signal in the mediobasal hypothalamus (130) and a 1.6-fold increase of D2 activity (130). These changes are independent of the fasting-induced fall in peripheral thyroid hormone levels (130), as hypothyroidism has a relatively minimal effect on D2 activity in the MBH, and T4 treatment does not prevent the fasting-induced increase in MBH D2 activity (130). Changes of leptin or corticosterone levels, alone, have no effect on D2 activity in the MBH of fed animals (171), but either removal of the adrenal glands or preventing leptin levels from falling during fasting prevents the increase in D2 activity in the MBH in fasting animals (171). It is hypothesized, therefore, that the fall in leptin levels in fasted animals has a permissive role to enable the stimulatory effect of corticosterone on D2 activity (171). It has been further suggested that the fasting-induced increase of D2 activity in the MBH may contribute to inhibition of the HPT axis by increasing local tissue levels of thyroid hormone (172). This hypothesis is supported by a report that third ventricular administration of a deiodinase inhibitor, iopanic acid, prevents the fasting-induced increase in hypothalamic T3 concentrations and blunts inhibition of TRH gene expression in the PVN (172). However, iopanoic acid has broad target specificity, inhibiting all deiodinase enzymes and blocking thyroid hormone transporters (173, 174). Therefore, it cannot be unequivocally stated that the observed effect of iopanoic acid treatment is due to inhibition of D2 activity in the MBH, especially because the fasting-induced fall in thyroid hormone levels is very similar in wild type and D2 KO mice (175). An alternative hypothesis is that increased T3 production by tanycytes stimulates NPY neurons in the arcuate nucleus (175), indirectly inhibiting TRH neurons in the PVN. However, since thyroid hormone has no inhibitory effect on

hypophysiotropic TRH neurons in TRβ2 KO mice, but the fasting-induced suppression of TRH mRNA in the PVN is completely intact in these animals (119), tanycyte generated T3 during fasting likely has a minimal role in the regulation of the HPT axis. In addition, fasting inhibits TRH neurons in the PVN of Siberian hamsters regardless of whether they are kept under short or long day photoperiods, even though fasting has opposite effects on tanycyte D2 activity during long and short day photoperiods (176).

   d. Role of other neuronal pathways in the regulation of the HPT axis during fasting

In addition to the arcuate nucleus, TRH neurons also receive inputs from other neuronal groups known to be involved in the regulation of energy homeostasis including the hypothalamic dorsomedial nucleus and neuronal groups in the brainstem (58). Axons arising from all subdivisions of the DMN densely innervate the vast majority of TRH neurons in the PVN, (74). Approximately two thirds of the PVN projecting DMN neurons are located in the medial part of ventral subdivision of the DMN, however, with neurons in the compact subdivision contributing only approximately 10% (71).

The physiologic importance of the DMN in regulating hypophysiotropic TRH neurons remains uncertain, but may be implicit in the knowledge that the DMN also has an important role in the regulation of energy homeostasis (177). Electrolytic and chemical (ibotenic acid) lesions of the DMN results in hypophagia due to an altered setpoint of body weight regulation (177). The DMN likely has both orexigenic and anorexigenic neuronal populations, however, as DMN lesions also increase food intake during the first hour of refeeding after a period of fasting, and attenuate CCK-induced decrease in food intake (177).

As the DMN contains leptin-, insulin- and ghrelin-receptive and glucosensing neurons (70, 177-179), some of these neurons may function to transmit humoral signals to TRH neurons in the PVN. However, it is unlikely that the DMN functions independently of the arcuate

nucleus in the regulation of hypophysiotropic TRH neurons, at least during fasting, as animals deficient in arcuate nucleus NPY and melanocortin peptides lose the ability to induce central hypothyroidism in response to fasting (112, 151).

Neurons of the DMN, however, receive feeding-related inputs from the arcuate nucleus, and are densely innervated by both AGRP- and α-MSH-containing axons (71, 180). Furthermore, the majority of DMN neurons that project to the PVN receive melanocortin input from the arcuate nucleus (71). This circuitry indicates that the DMN may serve as a relay station from the arcuate nucleus to the PVN, contributing to the effects of melanocortin signaling on the hypophysiotropic TRH neurons (71).

In addition to the regulation of food intake, the DMN is also involved in circadian regulation of hypothalamic systems (181). The DMN receives both direct and indirect inputs from the SCN, and ablation of the DMN reduces the circadian rhythm of wakefulness, feeding, locomotor activity, and the hypothalamic-pituitary-adrenal (HPA) axis (181). The DMN, therefore, may integrate feeding and circadian rhythm-related signals involved in the regulation of the HPT axis. In fact, the DMN is known to be a critical regulator of food-entrainable circadian rhythm of several hypothalamic systems (182). Fasting blunts the nocturnal increase of TSH secretion in humans (183), but it is unclear yet whether the DMN is involved in this process.

Hypophysiotropic TRH neurons also receive dense input from the brainstem that contains feeding related peptides such as NPY and CART (64, 80). These inputs, however, do not seem to have a role in the fasting-induced regulation of TRH neurons, because transection of ascending brainstem pathways does not affect fasting-induced inhibition of TRH gene expression in hypophysiotropic neurons (unpublished personal observations).

V. Effects of dehydration-induced anorexia on the hypothalamic-pituitary-thyroid axis

Dehydration induced by replacement of drinking water with 2.5% NaCl, gradually decreases daily food intake by 80-90% and is associated with significant weight loss despite the fact that the animals have free access to food (184). This experimental model, commonly referred to as dehydration induced anorexia (DIA), has been used by researchers to explore central mechanisms involved in the regulation of anorexia (184). Of interest, while food restriction inhibits all components of the HPT axis, DIA is accompanied by an increase in TRH gene expression in the PVN, elevated circulating TSH levels, yet unaltered T4 and decreased T3 concentrations in the peripheral blood (185). The increase in TSH concentration is likely secondary to increased TRH release, while the low T3 level may be secondary to increased peripheral T3 metabolism or clearance.

The effect of DIA on TRH neurons in the PVN, however, is not uniform (186). Similar to fasting, TRH mRNA is decreased in the mid level of the PVN (186), raising the possibility that these neurons are regulated by the arcuato-paraventricular pathway. Hypophysiotropic TRH neurons in the caudal part of the PVN, however, are stimulated by DIA, while inhibited in food restricted animals (186), indicating that any inhibitory effect of the arcuato-paraventricular pathway is overridden by other factors. Since the TSH concentration is increased in DIA animals (185), it is likely that stimulation of the caudal TRH cell group and inhibition of the mid level TRH cell population in the PVN results in a net increase of TRH release into the hypophysial portal circulation.

One, major difference between the peptide expression profile of the food restricted and DIA animals is increased CRH expression in lateral hypothalamic neurons in DIA animals (184). These CRH neurons are known to project to the PVN (184) and may contribute to the innervation of TRH neurons in the PVN (86). In support of this hypothesis, CRH stimulates TRH biosynthesis, and administration of a CRH-R2 antagonist prevents DIA-induced effects on TRH gene expression in the PVN (187).

In addition to the caudal part of the PVN, DIA also stimulates TRH neurons in the anterior parvocellular subdivision of the PVN (186). These neurons are not involved in the regulation of the HPT axis (58). Nevertheless, since the central administration of TRH inhibits food intake (188) and TRH neurons in the anterior PVN project to brain nuclei that are involved in the regulation of food intake (54), it is possible that the increased activity of TRH neurons in the anterior PVN contributes to the anorexigenic effect of DIA.

VI. Regulation of the HPT axis in high fat diet-induced obese animals

Consumption of high fat diet (HFD) results in increased caloric intake, increased weight gain and also a stimulation of the HPT axis, characterized by increased TRH mRNA and protein levels in the PVN and TSH and thyroid hormone levels in the circulation (170). Since the HFD-induced obese (DIO) animals have elevated leptin levels, and the HFD does not stimulate the HPT axis in leptin deficient ob/ob mice (170), the effect of HFD on the HPT axis seems to be mediated by leptin (170). However, expression of the proopiomelanocortin (POMC, the precursor of α-MSH) gene in the arcuate nucleus of DIO rats remains unchanged (189), suggesting that HFD decreases leptin sensitivity of anorexigenic neurons in this nucleus. This concept is supported by decreased STAT3 phosphorylation and increased SOCS3 and PTP1B synthesis in the arcuate nucleus of HFD animals after leptin treatment (190, 191).

Therefore, in contrast to fasting, leptin would appear to regulate the HPT axis independently from POMC neurons. Since central leptin administration results in STAT3 phosphorylation in hypophysiotropic TRH neurons in DIO rats, it is hypothesized that the HPT axis is regulated by a direct action of leptin on these neurons (170). However, leptin-induced regulation of AGRP synthesis in the arcuate nucleus and the leptin sensitivity of neurons in the dorsomedial nucleus remain intact in DIO animals (189, 190), necessitating the

need for additional studies to exclude the possibility that the effect of leptin on hypophysiotropic TRH neurons in DIO animals is mediated indirectly by these neuronal groups.

VII. Central regulation of the HPT axis during infection and prolonged critical illness

Similar to fasting, infection and other acute and chronic disorders can lead to inhibition of the HPT axis. In man, this condition is commonly referred to as the non-thyroidal illness syndrome (192) and is discussed in further detail in section IX. Characteristic of this syndrome is a decline in circulating T3, and in more severe cases, a general inhibition of the HPT axis due to the development of central hypothyroidism (192-194).

a. Role of neuronal pathways in the regulation of hypophysiotropic TRH neurons during infection

A commonly used model for infection is the peripheral administration of bacterial lipopolysaccharide (LPS). However, while both fasting and LPS administration have a similar effect to induce central hypothyroidism, the mechanisms by which these two conditions influence the hypophysiotropic TRH neurons are different. As discussed previously, the fall in the circulating level of leptin orchestrates the inhibitory effect of fasting on hypophysiotropic TRH neurons *via* the arcuato-paraventricular pathway (37, 58). In contrast, LPS treatment increases circulating leptin levels (58, 195), stimulates POMC and CART gene expression in arcuate nucleus neurons, and does not increase NPY expression (195, 196), changes that would predict activation of hypophysiotropic TRH neurons rather than suppression as observed (37, 58, 194, 197). Presumably, therefore, a more potent, inhibitory mechanism becomes activated during infection that overrides any stimulatory action of leptin, α-MSH or CART on hypophysiotropic TRH neurons.

One possibility to explain the effect of LPS on hypophysiotropic TRH neurons is activation of adrenergic neurons in the medulla, as these neurons are known to mediate the effect of LPS on CRH neurons in the PVN and result in a marked increase in circulating levels of corticosterone (198). As described above, medullary adrenergic neurons directly innervate hypophysiotropic TRH neurons (77). Unilateral transection of ascending brainstem pathways or ablation of the catecholaminergic input of the PVN which markedly attenuates LPS-induced c-Fos expression in the PVN and the increase in CRH gene expression (198, 199), however, results in an even more profound inhibition of TRH gene expression in the PVN (199). Thus, brainstem inputs would appear to stimulate rather than inhibit hypophysiotropic TRH neurons during infection, and in keeping with the activating effects of catecholamines on hypophysiotropic TRH (78).

b. Tanycytes as key regulators of hypophysiotropic TRH neurons during infection

LPS has a potent effect on tanycyte D2 gene expression and enzymatic activity, resulting in an approximately four-fold increase of D2 mRNA level and activity in the MBH within 12 hours of administration (Fig. 15 A, B) (197, 200). It has been hypothesized, therefore, that LPS may inhibit hypophysiotropic TRH neurons by increasing local T3 levels in the median eminence, where the processes of β tanycytes and the axon terminals of the hypophysiotropic TRH neurons are closely associated (51, 99). In support of this hypothesis, LPS-induced inhibition of hypophysiotropic TRH neurons is abolished in the D2 knock out mice (201). While D2 activity rapidly increases in tanycytes and is maximal 9-12h after LPS treatment, D2 activity gradually increases in other responsive tissues such as the cortex and the anterior pituitary, and becomes maximal at approximately 24h (99, 202). This difference in the D2 response favors distinct, tissue-specific mechanisms for activation of D2. The increase in D2 activity in the cortex and anterior pituitary is inversely related to peripheral thyroid hormone

levels and can be prevented by clamping circulating T4 to euthyroid levels (99, 197). This indicates that in the cortex and anterior pituitary, D2 activation is secondary to LPS-induced reduction in peripheral thyroid hormone levels. The T4 clamp, however, has no effect on the LPS-induced increase in D2 activity in the tanycytes (197).

The mechanism by which LPS stimulates D2 activity in tanycytes remains uncertain. One attractive possibility includes a direct action of LPS on tanycytes *via* their expression of Toll-like receptor 4 (TLR4) (203), as lack of TLR4 in TLR4 knock out mice prevents the LPS induced non-thyroidal illness syndrome (204). The main second messenger utilized by TLR4 and many of the cytokine receptors is the NF-κB system (205), and, the promoter of the D2 gene is highly responsive to NF-κB (99, 206). The human D2 gene contains two, high affinity, NF-κB binding sites at positions -683bp (called no.2) and -198bp (no. 5), 5' to the transcriptional starting site (206). Although both sites have a similar binding affinity by EMSA assay, site-directed mutagenesis and promoter assay indicates that only site no. 5 possesses transactivation potency in the presence of the p65 subunit of NF-κB, increasing D2 promoter activity 150-fold (206). However, LPS-induced activation of IκBα mRNA, a marker of NF-κB signaling, is observed only in a subset of α tanycytes and only 12h after LPS administration (Fig. 15 C-E), significantly later than the peak of the LPS-induced increase in D2 mRNA in these cells (142, 202). Thus, LPS-induced NF-κB activation *via* TLR4 or other cytokine receptors seem less likely the initial stimulus that increases D2 activity in tanycytes in response to LPS treatment, and rather may be involved in the maintenance of increased D2 (142).

IκBα mRNA, however, markedly increases in the pars tuberalis of the pituitary gland 3h after LPS treatment (Fig. 15 D) (142), a portion of the pituitary that is immediately adjacent to tanycyte end-feet processes in the external zone of the median eminence and in the sulcus tuberoinfundibularis (207). The pars tuberalis contains a large number of TSH-producing

cells, but in contrast to thyrotropes in the anterior pituitary, TSH-producing cells of the pars tuberalis are not affected by TRH or thyroid hormone (207). Since the synthesis of TSHβ subunit increases significantly in these cells 9h after LPS treatment (Fig. 15 F, G) (142), and the TSH receptor is expressed in tanycytes (103), TSH secretion from the pars tuberalis may be involved in mediating the effects of LPS on the tanycytes. Indeed, TSH has been shown to increase D2 activity in these cells *via* activation of cAMP (208). In addition, CREB phosphorylation, a marker of increased cAMP production, is evident in tanycytes 9h after LPS administration (Fig. 15 H) when D2 synthesis peaks, but absent from tanycytes at 6h after LPS administration when D2 synthesis is already increased (142).

The effect of LPS on the tanycytes, therefore, may be mediated by at least three factors. The first factor is responsible for early increase in D2, but remains unknown. TSH released from the pars tuberalis may then contribute to enhancing D2 activation shortly thereafter, followed by other factors that maintain D2 activity by stimulating NF-κB signaling in tanycytes. While changes in circulating levels of corticosterone have been suggested to have a role in the stimulation of D2 activity in fasted animals (171), a corticosterone clamp in adrenalectomized animals does not prevent the LPS-induced increase of D2 activity (202) nor influence LPS-induced inhibition of TRH synthesis (194).

c. *Regulation of hypophysiotropic TRH neurons during prolonged critical illness*

The effect of prolonged critical illness on the HPT axis has been studied in a rabbit model using third-degree burn injury (209, 210). As in infection, prolonged critical illness results in low T4, T3 and TSH concentration in the circulation and decreased TRH mRNA level in the PVN (209, 210). In addition, it is associated with increased D2 gene expression in the MBH (210), indicating that similar to infection, burn injury may inhibit the HPT axis by inducing local hypothalamic hyperthyroidism caused by the increased thyroid hormone activating

capacity of tanycytes. Comparison of the T3 content in whole hypothalamic blocks of healthy and ill rabbits did not support this hypothesis, however, as T3 levels were not found to be higher in the experimental animals (210). Nevertheless, the approach did not take into account that burn-induced tissue hyperthyroidism may take place in only focal regions of the hypothalamus such as the median eminence and paraventricular nucleus, which may be sufficient to inhibit hypophysiotropic TRH neurons.

Prolonged illness is also accompanied by a nutritional deficit, raising the possibility that the decreased calorie intake also contributes to the development of central hypothyroidism during prolonged critical illness (209). Early parenteral nutrition, however, does not influence the illness-induced decrease in TRH gene expression and the decline in circulating level of T4 (209). The expected decline in the circulating level of T3 was prevented, however, suggesting that the nutritional deficit may have its greatest influence on thyroid hormone metabolism in peripheral tissues (209).

VIII. Regulation of hypophysiotropic TRH neurons by cold exposure and suckling

Thyroid hormone has a critical role in the regulation of both obligatory and adaptive thermogenesis (211). In the presence of hypothyroidism, obligatory thermogenesis, the heat dissipated due to the biochemical processes necessary for the maintenance of life, is reduced by approximately 30% (211). Hypothyroidism also interferes with adaptive thermogenesis, required to maintain normal body temperature in a cold environment (211). It is not surprising, therefore, that cold exposure serves as an important stimulus for TRH gene regulation in the PVN to increase thyroid hormone levels (212). The increase in TRH mRNA by cold exposure is transient, however, increasing by 100% within 1h of the cold exposure, followed by a decline to normal levels during the second hour (213). Peak TRH release from the median eminence also occurs 40 minutes after the cold exposure (213), and is facilitated

by a cold-induced increase in the synthesis of prohormone convertase enzymes, PC1/3 and PC2, involved in the processing of proTRH (214).

The physiological role of this early, transient increase in thyroid hormone levels is not completely clear. Cold exposure markedly increases D2 activity in BAT (94). As a result, thyroid hormone receptors are fully occupied by T3 in the BAT after 4h of cold exposure (211) when the thyroid hormone levels have already normalized after a transient increase. In addition, administration of low doses of T4 to thyroidectomized rats can completely normalize the thermogenesis without normalizing the thyroid hormone concentration in the circulation (215), suggesting that even low circulating levels of thyroid hormone are sufficient for normal thermogenesis. We hypothesize, therefore, that the cold-induced transient increase in thyroid hormone may be necessary to initiate thermogenesis in BAT before D2 activity is sufficiently increased to maintain BAT thermogenesis, even without increased thyroid hormone levels in the circulation. This hypothesis, however, requires further clarification.

As central administration of TRH stimulates thermogenesis in BAT *via* the activation of the sympathetic nervous system (57), the cold-induced increase in TRH gene expression in the PVN might be hypothesized to increase heat production in BAT simultaneously with stimulation of the HPT axis. Indeed, TRH has been shown to stimulate the BAT after its administration into the DMN, preoptic area, hypothalamic ventromedial nucleus (VMN), anterior hypothalamus or hindbrain (57, 216). However, as none of these brain regions are innervated by hypophysiotropic TRH neurons (unpublished observations), the effect of TRH on BAT is likely mediated by other groups of TRH-synthesizing neurons.

The effect of cold on the activity of hypophysiotropic TRH neurons is thought to be mediated primarily by the brainstem catecholaminergic neurons, because noradrenaline stimulates TRH synthesis (78) and inhibition of noradrenaline prevents cold-induced activation of the HPT axis (217). In addition, the HPT axis does not respond to cold during

the first, ten, postnatal days in rodents during which time catecholaminergic axons are still migrating to TRH neurons in the PVN (1). Furthermore, inhibition of adrenergic receptors blocks cold-induced TRH synthesis and release (214, 218).

Cold stimulates TRH gene expression, biosynthesis and proteolytic processing *via* β adrenergic receptors, and by increasing cAMP production (214). Both adrenergic and noradrenergic brainstem neurons densely innervate hypophysiotropic TRH neurons in the PVN (61), but the A2 noradrenergic cell group is the only catecholaminergic population in which cold-induced c-Fos activation can be observed. The release of TRH is also stimulated by cold *via* α adrenergic receptors (214, 218). This effect is independent of the regulation of TRH synthesis (214, 218).

Similar to cold exposure, suckling also stimulates TRH gene expression (213, 219). TRH mRNA is increased in the PVN during the first days of lactation, but then declines after the 8th day (219). Acute suckling also results in a 2-fold increase in TRH mRNA in the PVN after 30 min of suckling, that gradually decreases to presuckling levels after 6h (213, 219). This response is associated with increased prolactin levels in the circulation, but surprisingly, with unaltered TSH and thyroid hormone levels (219, 220). The mechanism for suckling-induced activation of hypophysiotropic TRH neurons is currently unclear, but the differential response of the anterior pituitary to TRH is likely mediated by several factors. Corticosterone may be important in the prevention of TRH-induced TSH secretion, as corticosterone-clamped, adrenalectomized animals increase TSH in response to suckling (219). A decline in dopamine release in response to suckling may also contribute to the increased sensitivity of lactortophs to TRH (47). CART, co-synthesized in hypophysiotropic TRH neurons, has been shown to modulate the effect of TRH on lactortophs, preventing TRH from releasing prolactin without influencing TSH release during cold exposure and in association with hypothyroidism (220, 221). As opposed to cold exposure and hypothyroidism when CART is increased in

hypophysiotropic neurons (220), CART remains unchanged during suckling in hypophysiotropic neurons, potentially favoring the release of prolactin (220).

IX. Translational ramifications

Because of the similarities between the anatomical organization of the hypophysiotropic TRH-synthesizing neuronal system in experimental animals and man (see above), it is reasonable to assume that regulation of the HPT axis in man may have similarities to that observed in rodents. In support of this hypothesis, the important role of TRH in the regulation of anterior pituitary TSH secretion in man is suggested by the presence of central hypothyroidism associated with mutations in the TRH receptor gene. Namely, individuals homozygous for TRH receptor mutations or compound heterozygotes demonstrate low levels of total and free T4, and have inappropriately low or normal TSH levels (222, 223). In addition, their TSH secretion is not influenced by exogenous administration of TRH (222, 223).. Hence, although these individuals are able to produce TSH, the biological activity of their TSH is decreased, and they regulate TSH secretion at a lower set point, presumably due to deficiency in TRH signaling, resulting in lower than normal circulating levels of T4 (222, 223).

As in rodents, feedback regulation of the HPT axis in man would also appear to require the MCT8 thyroid hormone transporter. This is apparent in the Allan-Herndon-Dudley syndrome, secondary to mutations in MCT8, characterized by elevations in T3, a low T4 level and a circulating TSH level in the upper normal range (224, 225). Although one might have anticipated a greater elevation in TSH if MCT8 has an important role in the transport of thyroid hormone to hypophysiotropic TRH neurons in man, these patients can have a blunted response to exogenously administered TRH (226), indicating that anterior pituitary thyrotropes may still retain the ability to transport T3 through other mechanisms, diminishing

the direct stimulatory effect of TRH. Negative feedback inhibition of the HPT axis by thyroid hormone would also appear to be primarily mediated by TRβ in man, as individuals with mutations in TRβ show major abnormalities in circulating thyroid hormone levels and have inappropriately elevated TSH level (227) In contrast, whereas mutation of TRα is associated with clinical signs of severe hypothyroidism, it results in slightly low circulating levels of T4 and normal levels of T3 and TSH (228). This observation is also consistent with the concept that TRα1 may have an activating effect on the synthesis of TRH in hypophysiotropic TRH neurons (see above, Section III)

Leptin is also likely to have an important role in the regulation of the thyroid axis in man, as suggested by the close correlation between the circadian rhythm of leptin secretion and TSH (229). Circulating levels of both hormones are the highest between midnight and early morning and lowest in the early to mid-afternoon (229). In addition, subjects with congenital leptin deficiency, due to mutations in the leptin gene, have impaired thyroid function characterized by low levels of T4, inappropriately normal levels of TSH, disregulation of TSH pulsatility and circadian rhythms, and/or an exaggerated response to exogenously administered TRH that is a characteristic feature of central hypothyroidism (229, 230). The thyroid phenotype, however, tends to be heterogeneous among the various families described (229, 230). In the Pakistani family with leptin deficiency reported by Montague et al (231), leptin replacement therapy significantly increased their T3 and T4 levels that were maintained at these higher levels with continued leptin treatment (232). Leptin also appears to orchestrate the suppression in circulating thyroid hormone levels during fasting and other disorders associated with a reduction in caloric intake in man such as hypothalamic amenorrhea and eating disorders. Leptin administration to normal individuals undergoing a 3 day fast restored fasting-induced effects on TSH pulsatility and increased T4 levels (233). In addition, leptin administration to individuals undergoing a more prolonged period of a less severe caloric

restriction prevented the fall in T3 and T4 levels (234), although a similar response was not observed in a separate study working with obese patients (235). Leptin and thyroid hormone levels also tend to be low in individuals with hypothalamic amenorrhea induced by strenuous exercise and eating disorders (236). These alterations can be reversed with leptin replacement (237). Although there has been a reluctance to use leptin to treat patients with anorexia nervosa due to its suppressive effects on appetite and to increase energy expenditure, a correlation between low thyroid hormone and leptin levels is also well described in this disorder (238). Presumably, as in rodents, this response is homeostatic to reduce energy expenditure. Whether leptin is also responsible for increases in thyroid hormone levels observed in obese individuals is uncertain, but a correlation between increased leptin levels and higher T3 and TSH levels has been observed in obese patients (239).

A somewhat more complex issue is whether leptin is involved in the development of the so-called "nonthyroidal illness syndrome" or "euthyroid sick syndrome", observed in approximately 40-70% of patients with severe illness in intensive care settings (240, 241). Low circulating levels of thyroid hormones but seemingly inappropriately low or normal TSH levels, diminished TSH pulsatility and decreased TRH gene expression in the paraventricular nucleus are observed, suggesting the presence of central hypothyroidism, but the changes are completely reversible when the patient recovers (193, 240, 241). The decrease in thyroid hormone levels may be a homeostatic response to conserve energy stores under these adverse conditions and consistent with the physiology described above in experimental animals and human subjects associated with fasting. In support of this hypothesis, recent studies by Casaer et al (242) and Langouche et al (243) have demonstrated that early nutrient restoration to patients with critical illness in an intensive care unit setting is associated with greater complications and a more prolonged hospitalization. A very low T4 level, however, is predictive of poor outcomes in the intensive care unit setting, and perhaps an even better

indicator of the severity of illness than the APACHE II score (244, 245). When T4 falls to less than 4 μg/dl, the risk of death rises to ~50%, and when T4 falls to <2 μg/dl, mortality increases even more to ~80% (246-248). Thus, when T4 begins to decline, it remains unclear whether this is the progression of an adaptive response and does not require therapy, or whether it is indicative of a maladaptive response and should be vigorously treated to restore circulating thyroid hormone levels to normal. Evidence that the mechanisms for central hypothyroidism associated with infection is vastly different than that elucidated associated with fasting, cited in section VII, would give credence to the concern that the nonthyroidal illness syndrome associated with severe illness may not be a homeostatic mechanism mediated by leptin but, in fact, maladaptive and pathophysiologic (246). Namely, central hypothyroidism may be an epiphenomenon of infection-induced upregulation of type D2 deiodinase in tanycytes causing hypothalamic hyperthyroidism and not part of an intended, homeostatic, physiologic response to reduce metabolic rate and energy expenditure to promote survival.

A number of small clinical trials have attempted to determine whether thyroid hormone replacement in intensive care unit patients has any beneficial or detrimental effects on overall outcomes (246, 247, 249). Most of these studies have shown T4 or T3 to be safe and well tolerated (250, 251). Improvement in cardiac hemodynamic parameters including cardiac output, end diastolic volume and stroke volume, and a reduction in peripheral arterial resistance have been observed in patients receiving T3 following coronary artery bypass surgery and/or in patients with dilated cardiomyopathy (249, 252-255), but at the expense of further suppression in circulating levels of TSH. However, other studies have not found a benefit as summarized by Kaptein et al (256). It could be argued, however, that because of the associated rise in D1 and D3 in peripheral tissues in these patients (257), neither T4 nor T3 is appropriate therapy due to the effects of these enzymes to increase the conversion of T4

to reverse T3 (rather than T3) or degrade T3, respectively (246). The appropriate studies to determine whether correcting the HPT axis to normal in patients with severe illness, therefore, have not been conducted. A novel approach for the treatment of the nonthyroidal illness syndrome in critically ill patients has recently been suggested by Van den Berghe et al (258, 259). Using a continuous infusion of TRH together with a growth hormone secretagogue restored thyroid hormone levels and TSH pulsatility in these patients, and also improved catabolic parameters. However, carefully controlled studies are needed to determine whether this approach is associated with improved outcomes and reduced mortality.

X. Conclusion

Maintenance of circulating levels of thyroid hormones is controlled by what is classically called a "simple" negative feedback mechanism exerted on hypophysiotropic TRH neurons in the PVN. This regulatory mechanism, however, is vastly more complex, being modulated by glial-neuronal and neural-neural interactions as well as other circulating, humoral substances such as leptin. Given that hypophysiotropic TRH neurons are innervated by axons containing multiple peptides whose origin and physiological significance has not yet been determined (see Fig. 6), it is likely that the regulatory mechanisms involved are even more complex. Presumably, this finely tuned machinery provides flexibility to the HPT axis, enabling continuous adaptation to the constantly changing internal and external milieu. In most situations, alterations in the setpoint for negative feedback regulation would appear to be adaptive, contributing to the optimization of energy homeostasis. Elucidation of the basic mechanisms involved in central regulation of the thyroid axis, however, raise questions as to whether under certain circumstances, such as prolonged critical illness, regulation of the HPT axis may be maladaptive. Further research to elucidate the neurobiology involved in central regulation of hypophysiotropic TRH neurons will be instrumental in understanding how these

disorders give rise to alterations in the HPT axis and to facilitate the appropriate approach to therapy.

XI. Acknowledgement

Figure Legends

Figure 1. Schematic illustration of the organization of the rat preproTRH gene.

Figure 2. Distribution of TRH-synthesizing neurons in the rat PVN. (A-C) Low power micrographs illustrate the TRH neurons at three rostrocaudal levels of the PVN. Schematic drawings (D-F) illustrate the subdivisions of the PVN where hypophysiotropic TRH neurons are localized (gray). AP, anterior parvocellular subdivision; DP, dorsal parvocellular subdivision; LP, lateral parvocellular subdivision; MN, Magnocellular part of PVN; MP, medial parvocellular subdivision, PV, periventricular parvocellular subdivision, III, third ventricle (From Fekete and Lechan (58), copyright 2007, with permission from Elsevier.)

Figure 3. Darkfield photomicrographs showing proTRH mRNA expression in the anterior-, mid- and posterior levels of the PVN in (A-C) control and (D-F) hypothyroid rats, and (G-I) control and (J-L) hypothyroid mice. Note dramatic increase of in silver grains denoting proTRH mRNA in the mid and caudal level of the hypothyroid rat PVN (E, F), while hypothyroidism increases proTRH mRNA only in mid-level neurons in mice (K). III, third ventricle. (G-L are from Kádár et al. (44), copyright 2010, with permission from Wiley-Liss Inc.)

Figure 4. Distribution of TRH-immunoreactive (IR) terminals in the mouse median eminence. TRH-IR axons densely innervate the external zone of the median eminence. III, third ventricle; ME, median eminence

Figure 5. Innervation of the TRH neurons in the rat PVN from neurons originating in the (A) arcuate nucleus , (B) DMN and (C) catecholaminergic neurons in the brainstem. In (A) TRH

neurons (blue) are contacted by axon terminals containing α-MSH (*red; arrowhead*) and AGRP (*green; arrows*). In (B), axon varicosities containing the anterogradely transported marker protein, PHA-L (black) are juxtaposed to TRH-synthesizing neurons (brown) after iontophoretic administration of the tracer into the DMN. In (C), both noradrenergic- (red, open arrows) and adrenrenergic-containing (yellow, white arrows) axons establish contacts with the TRH neurons. (Modified from Fekete et al. (52), Mihaly et al. (74), copyright 2001, with permission from Elsevier; and Fuzesi et al. (61), copyright 2009, with permission from Elsevier)

Figure 6. Schematic drawing summarizing known inputs to TRH neurons in the PVN. The inputs with identified origins are depicted by a neuron sending its axon to the TRH neuron. Inputs with currently unknown origins are labeled with axon terminals on the surface of the TRH neuron.

Figure 7. (A,B) Organization of tanycyte subtypes in the mediobasal hypothalamus. (A) Vimentin-immunolabeled (red) coronal section with DAPI counterstaining (blue) shows the distribution of tanycytes and their processes. The schematic diagram (B) illustrates the location of tanycyte subtypes in the wall and floor of the third ventricle. All tanycyte subtypes synthesize (C) MCT8, and (D) OATP1C1 thyroid hormone transporters. In (E) silver grains denoting type 2 deiodinase mRNA are accumulated over the cells lining the wall of the third ventricle, the tuberoinfundibular sulci (arrow heads) and around blood vessels in the arcuate nucleus (arrows). Higher power micrographs shows the association of D2 mRNA with (F) the tuberoinfundibular sulcus (arrow heads) and a blood vessel (arrows) in the arcuate nucleus, and (G) in the external zone of the median eminence. (H) Tanycyte expression of pyroglytamyl peptidase II (PPII) mRNA. III, third ventricle; Arc, arcuate

nucleus, DMN, hypothalamic dorsomedial nucleus; ME, median eminence; VMN, ventromedial nucleus. (Modified from Sanchez et al. (35); Kalló et al. (51); Fekete et al. (124), copyright 2000, with permission from Elsevier; (E) courtesy of Dr. Gábor Wittmann)

Figure 8. *In situ* hybridization autoradiograms showing the effect of hypo- and hyperthyroidism on proTRH mRNA level in the medial parvocellular subdivision of the PVN. A substantial increase in silver grain accumulation is observed in the (A) hypothyroid animal compared to the (B) fed control. In contrast, hyperthyroidism results in a marked reduction of proTRH mRNA level in the PVN (C). (From Dyess et al (115), and from Fekete and Lechan (58), copyright 2007, with permission from Elsevier.))

Figure 9. *In situ* hybridization autoradiographs of proTRH mRNA in the paraventricular nucleus (PVN) of (A) hypothyroid, (B) euthyroid and (C,D) hypothyroid animals receiving a constant infusion of (C) 0.5 μg or (D) 0.75 μg of T3/100 gm/bw/d. Mean plasma triiodothyronine (T3) levels (±SEM) are shown for each group at the bottom of the photomicrographs. Note that only the higher dose of T3 that raised plasma T3 levels into the supranormal range was capable of suppressing proTRH mRNA to euthyroid levels. (E) Regression analysis of above experiment. Interrupted line represents the mean ln(proTRH mRNA) for euthyroid animals, and its intercept with the regression line estimates the plasma T3 concentration required to suppress proTRH mRNA to euthyroid levels. Ninety-five percent confidence intervals for each intercept are bracketed. Open dots denote values for hypothyroid animals and hypothyroid animals infused with graded doses of T3. Closed dots denote values for euthyroid controls. (Modified from Kakucska et al (123), and from Fekete and Lechan (58), copyright 2007, with permission from Elsevier.))

Figure 10. Schematic illustration of the machinery involved in negative feedback regulation of the HPT axis by thyroid hormone.

Figure 11. Darkfield illumination photomicrographs of proTRH mRNA in the hypothalamic PVN in (A) fed, (B) fasted, and (C) fasted animals receiving leptin. Note the marked reduction in *silver grains* over neurons in the PVN in the fasted animals but restoration to normal in the fasted animals receiving leptin. III, third ventricle (From Fekete et al (155), and from Fekete and Lechan (58), copyright 2007, with permission from Elsevier.).)

Figure 12. Schematic drawing summarizing the regulation of TRH neurons by fasting.

Figure 13. Darkfield illumination micrographs of proTRH mRNA in the medial and periventricular parvocellular subdivisions of the hypothalamic PVN in (A, D) fed, (B, E) fasted and (C,F) fasted animals receiving an intracerebroventricular infusion of either (C) α-MSH or (F) CART every 6 hr for 64 hr. Note the reduction in the accumulation of silver grains over the PVN in fasted animals compared with the fed controls. Both α-MSH and CART administration prevent the fasting-induced fall of proTRH mRNA . III, Third ventricle (From Fekete et al (42, 52), and from Fekete and Lechan (58), copyright 2007, with permission from Elsevier.)

Figure 14. Darkfield illumination photomicrographs of proTRH mRNA in the medial parvocellular subdivision of the PVN in (A) control, (B) AGRP- , and (C) NPY-treated animals. Note marked reduction in silver grains over neurons in the PVN in both the AGRP- and NPY-infused groups. III, Third ventricle (From Fekete et al (155), and from Fekete and Lechan (58), copyright 2007, with permission from Elsevier.)

Figure 15. (A,B) Regulation of type 2 deiodinase gene expression in tanycytes by LPS. Low-power darkfield micrographs of the caudal part of the hypothalamus showing the D2

expression in (A) control animals and (B) 12h after LPS administration. Note marked increase in the density of silver grains, particularly in the external zone of the median eminence. (C-E) Darkfield illumination photomicrographs of Iκ-Bα mRNA expression in the MBH in animals receiving (C) saline or LPS (D) 3 h, or (E) 12 h before sacrifice. Note the marked accumulation of silver grains over the pars tuberalis (PT) in (D) 3 h after the administration of LPS. Iκ-Bα mRNA is only seen in a subset of α tanycytes 12 h after LPS administration (*arrows* in E). (F, G, *arrows*) Expression of TSHβ mRNA in the pars tuberalis (PT) of animals receiving (F) saline or (G) LPS 9h before sacrifice. Note increased expression of TSHβ mRNA in the PT after LPS administration. Ventricular borders are demarcated with *dotted lines*. Double-labeled fluorescent image (H) illustrates the presence of phospho-CREB (green, yellow, arrows) in the nucleus of tanycytes (red) 9h after administration of LPS. III, Third ventricle; ME, median eminence; PT, pars tuberalis (Modified from fekete et al. (99); and Sanchez et al. (142).)

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Figure 1
Click here to download high resolution image

AP1
-218 to -213
-204 to -198

CRE-2/AP-1
- 101 to -94

Klf4
-92 to -88

TATA box

exon 1

exon 2

exon 3

28bp

intron 1

intron 2

PolyA

GRE
-210 to -205

STAT3
-142 to -133

SP1
-125 to -119

Site 4
TRE/Cre-1/AP1
-59 to -52 bp
(Cre-1 is functional only
in the human promoter)

TREs
+14 to +19
+37 to +42

5' untranslated region

sequence coding signal peptide

sequences coding non-TRH peptides

sequences coding TRH

3' untranslated region
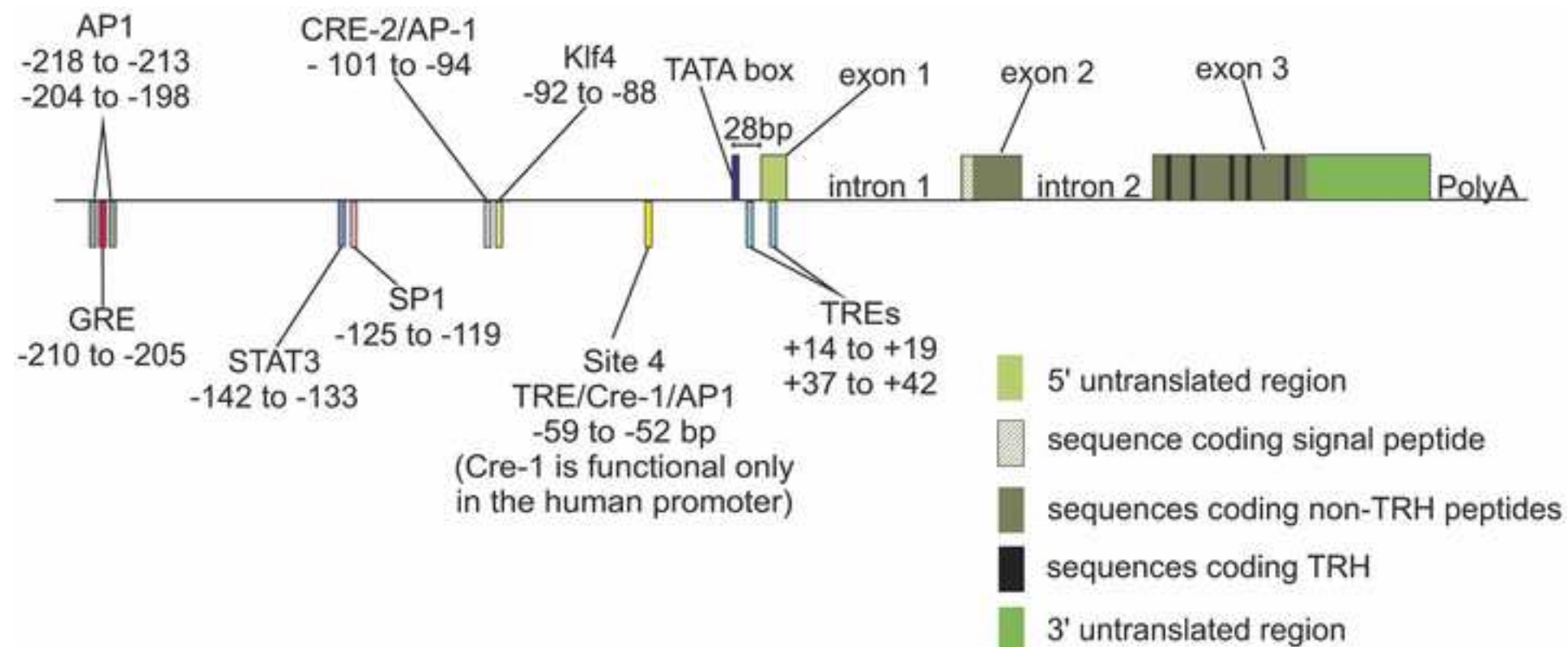
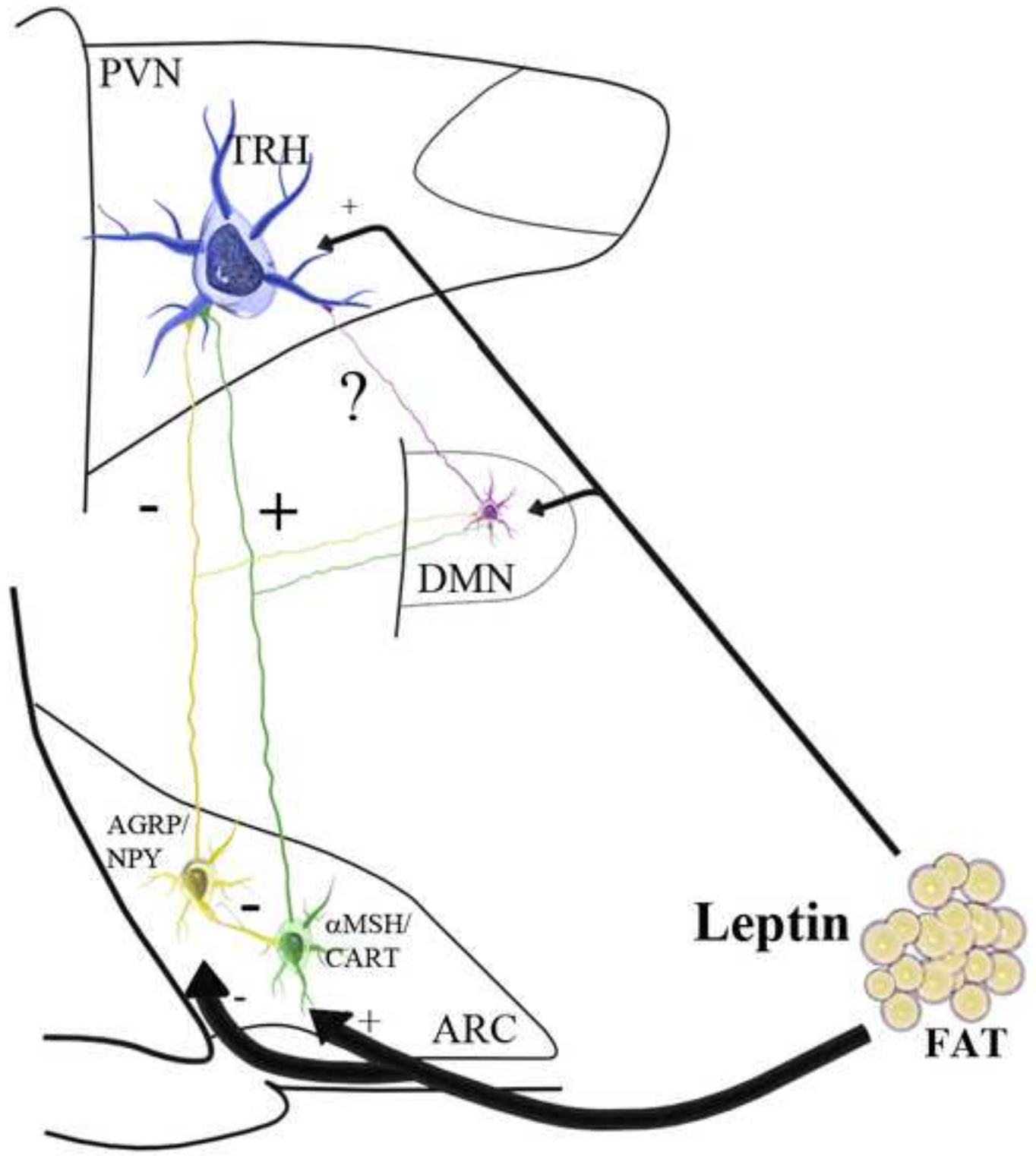Figure 2
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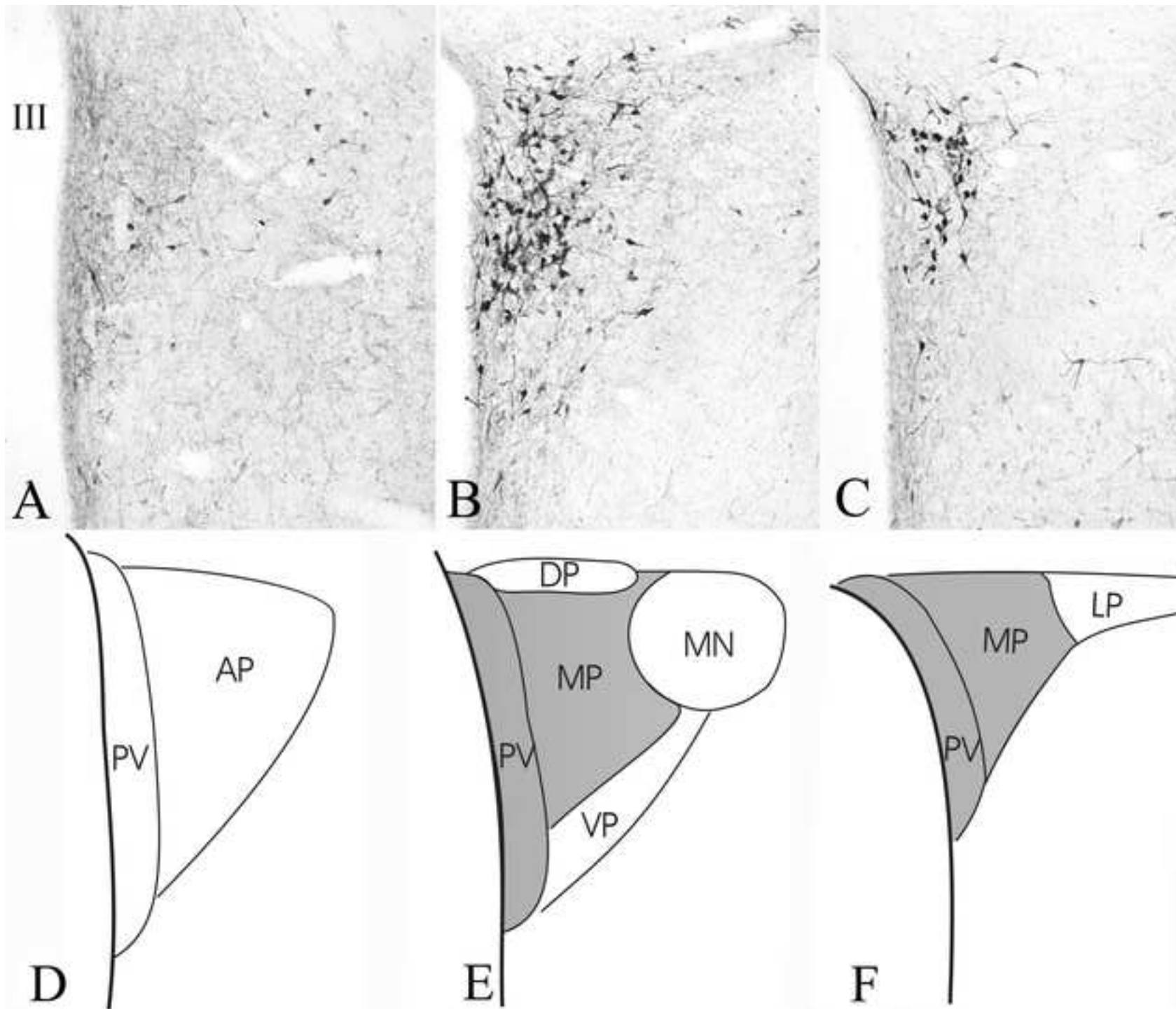
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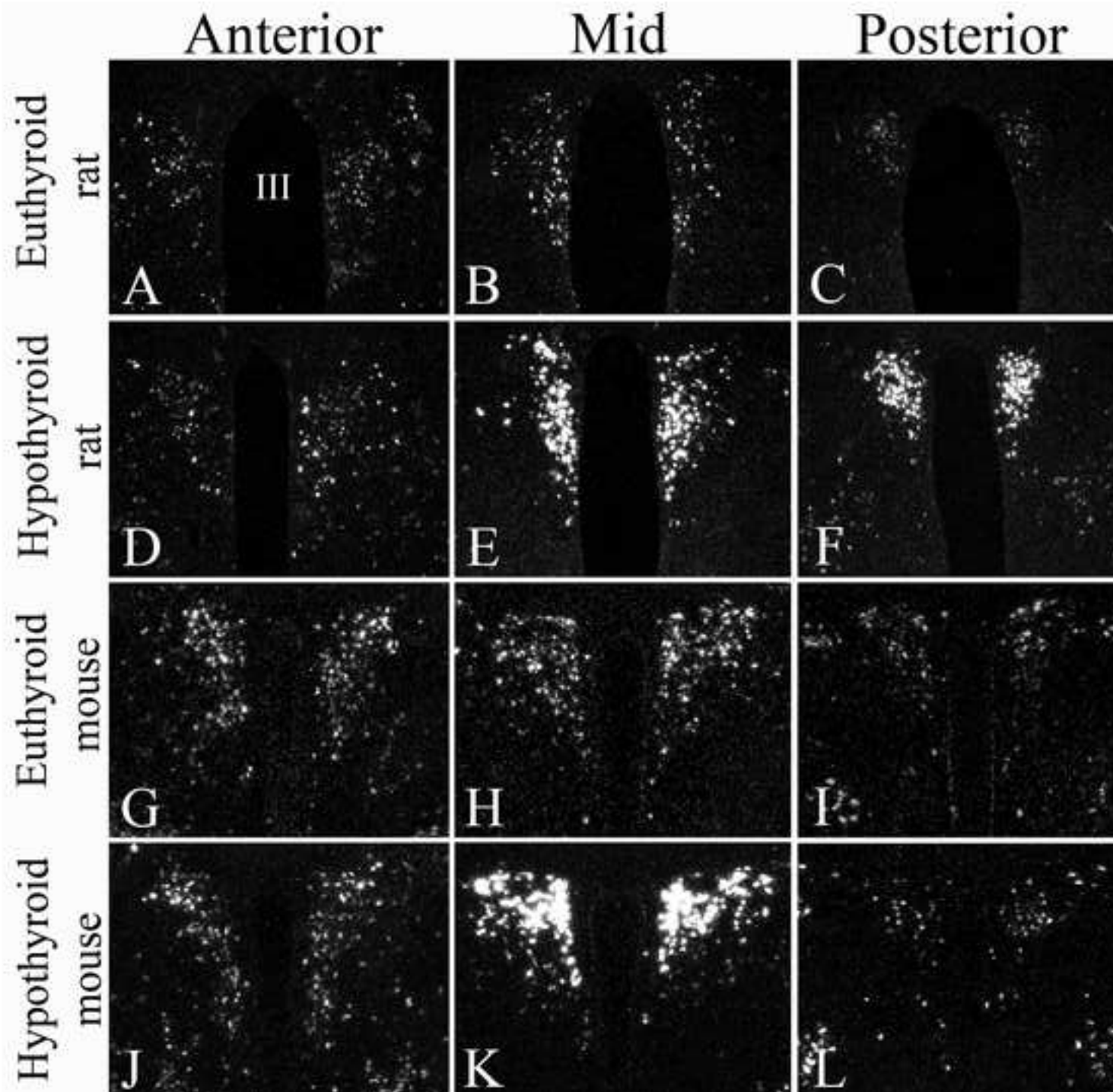
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III.

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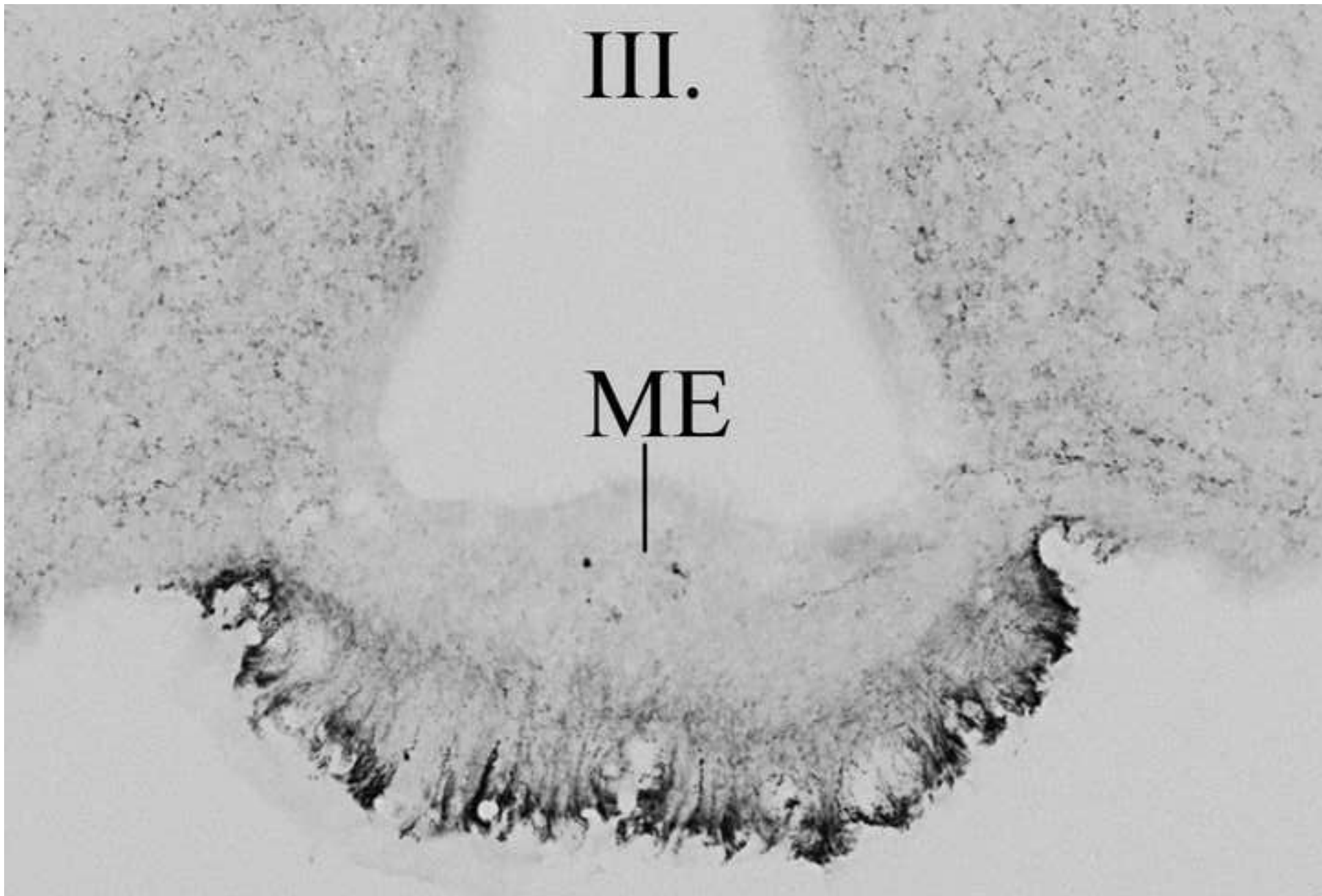
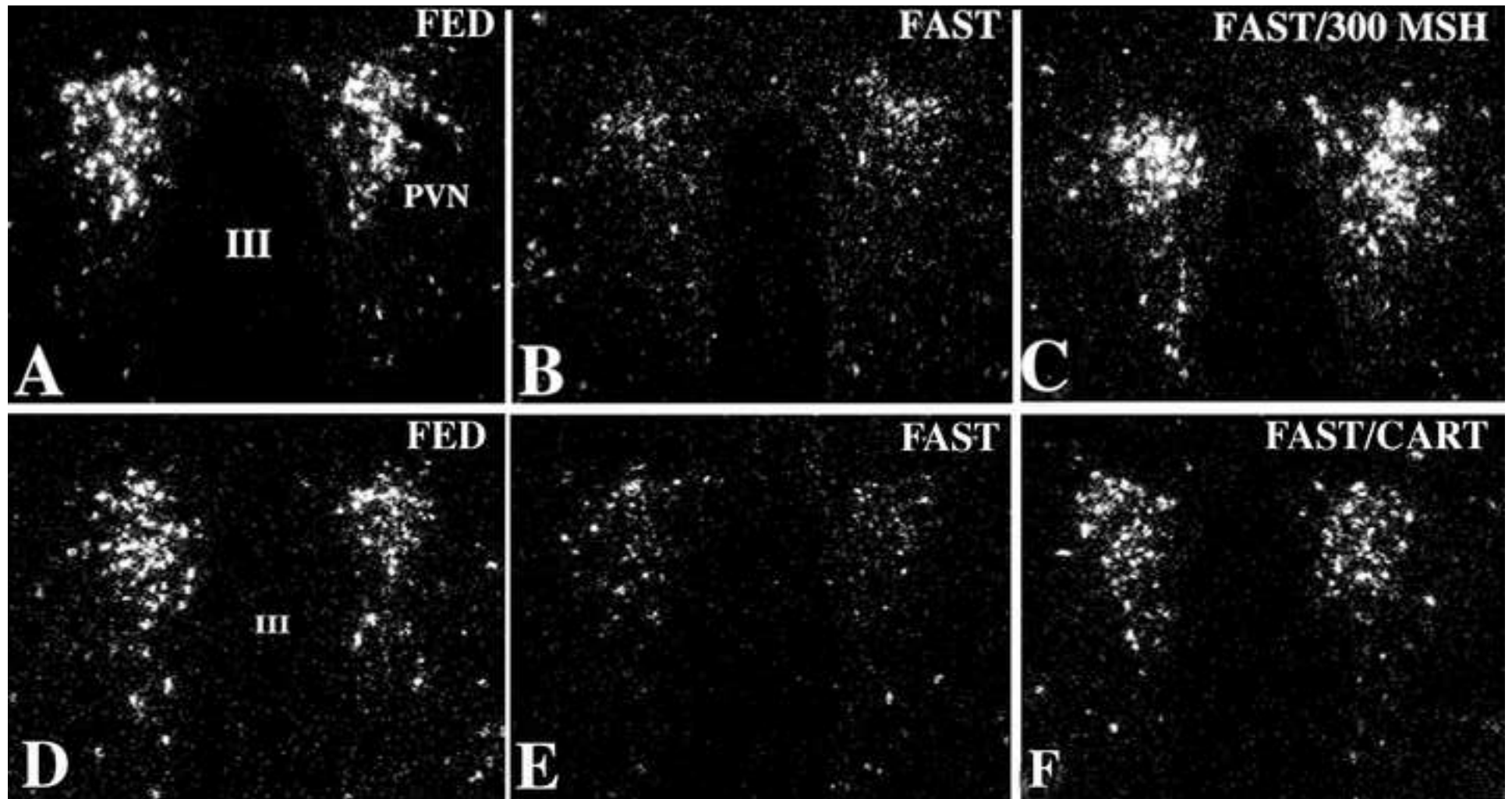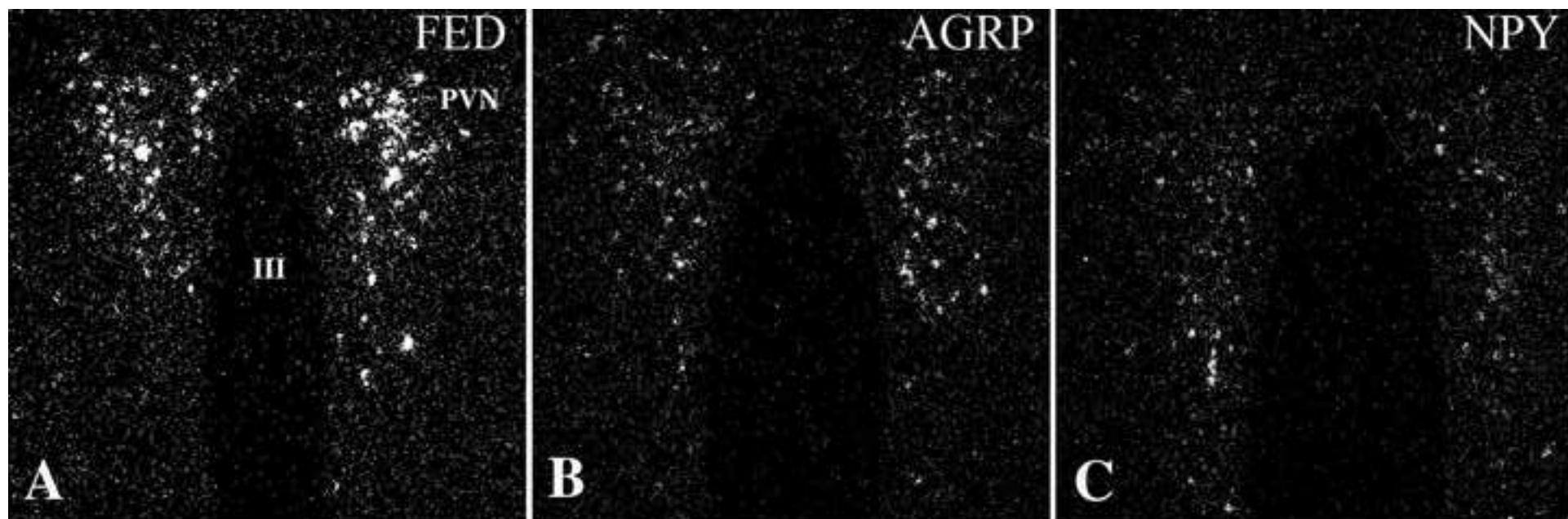Figure 5
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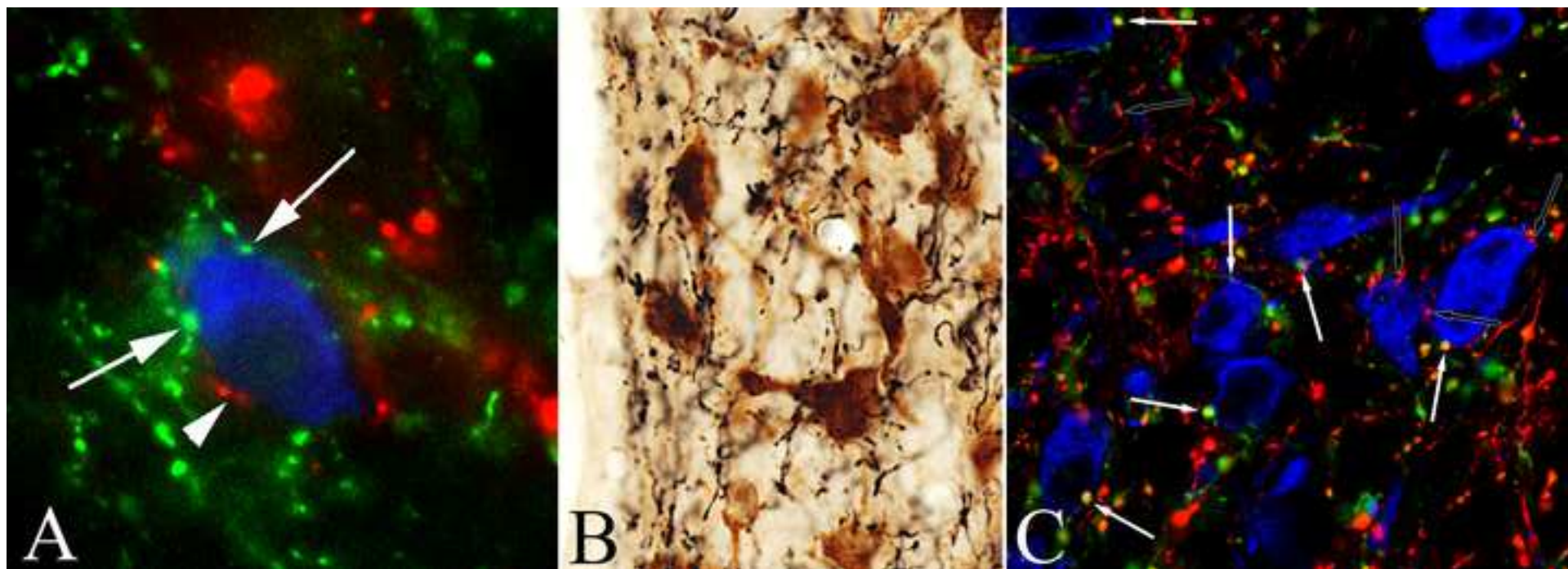
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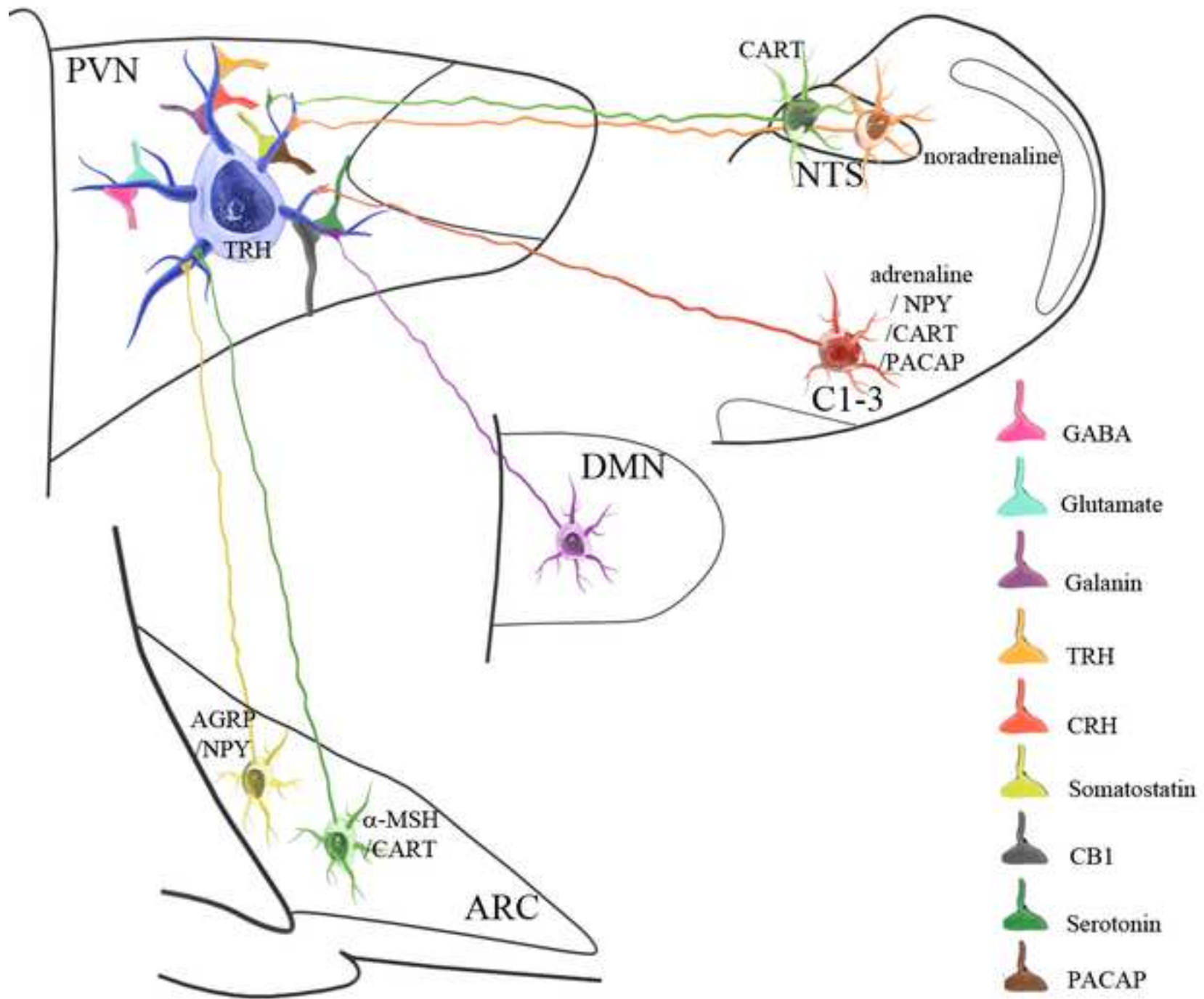
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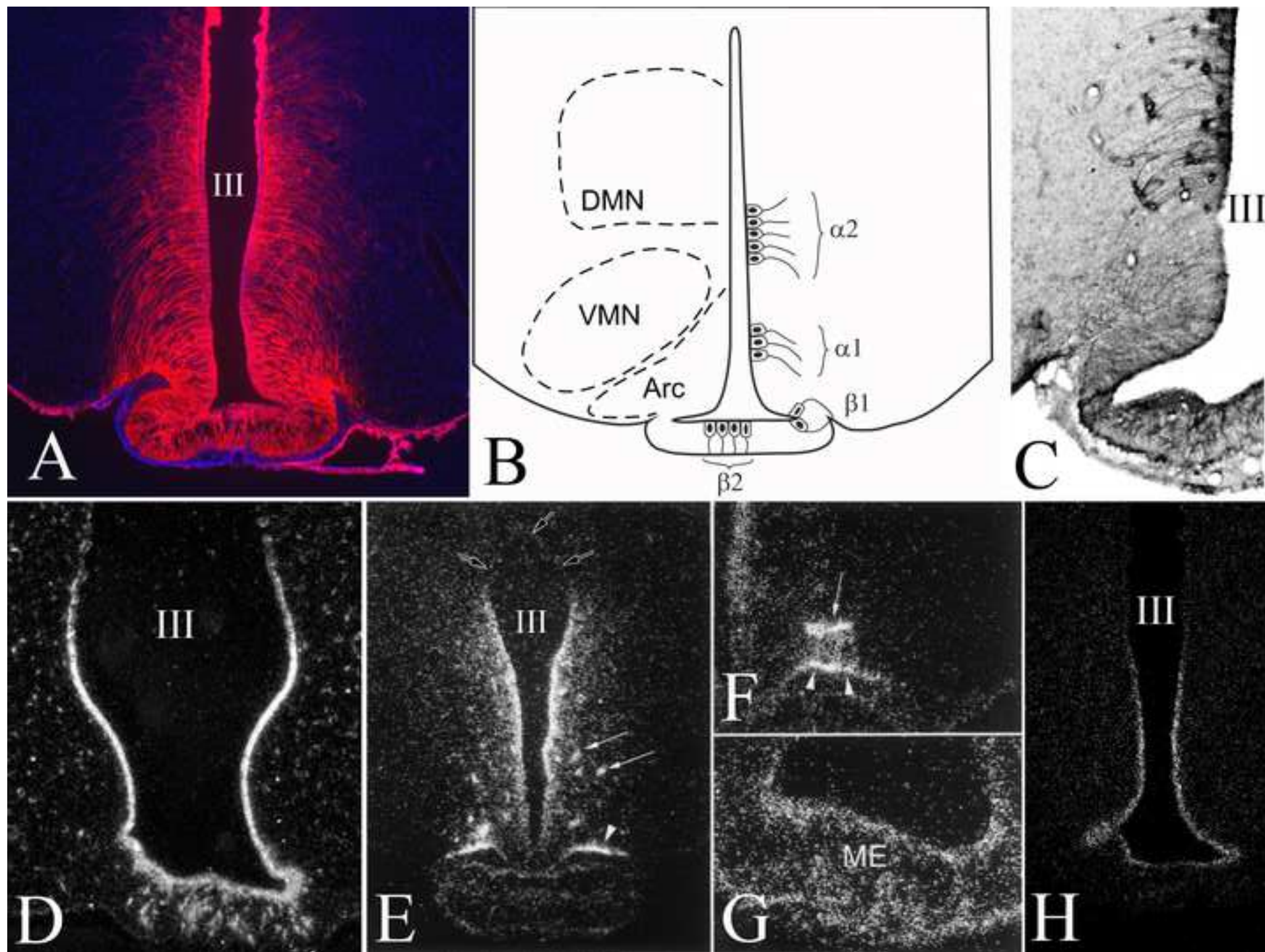
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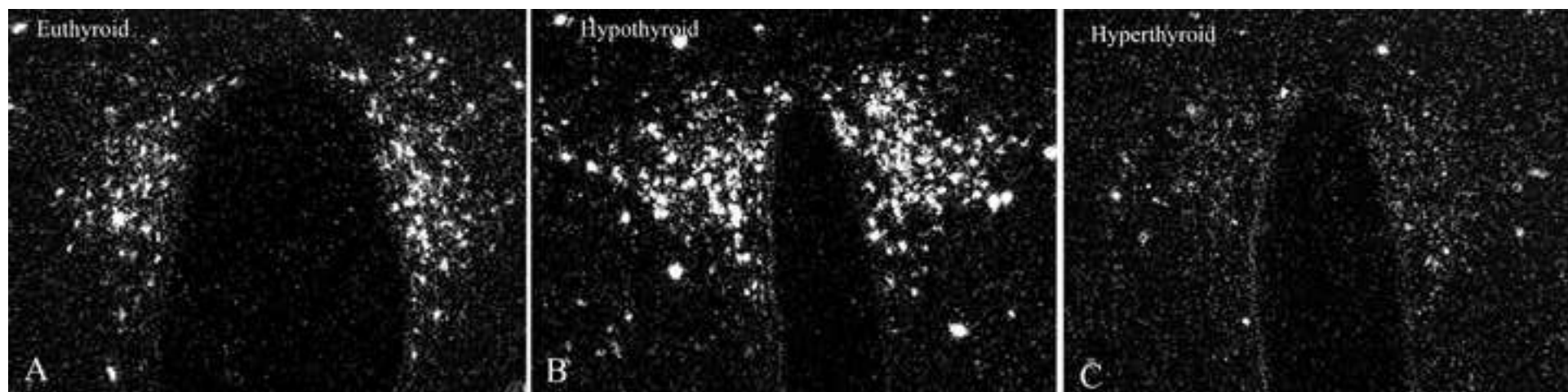
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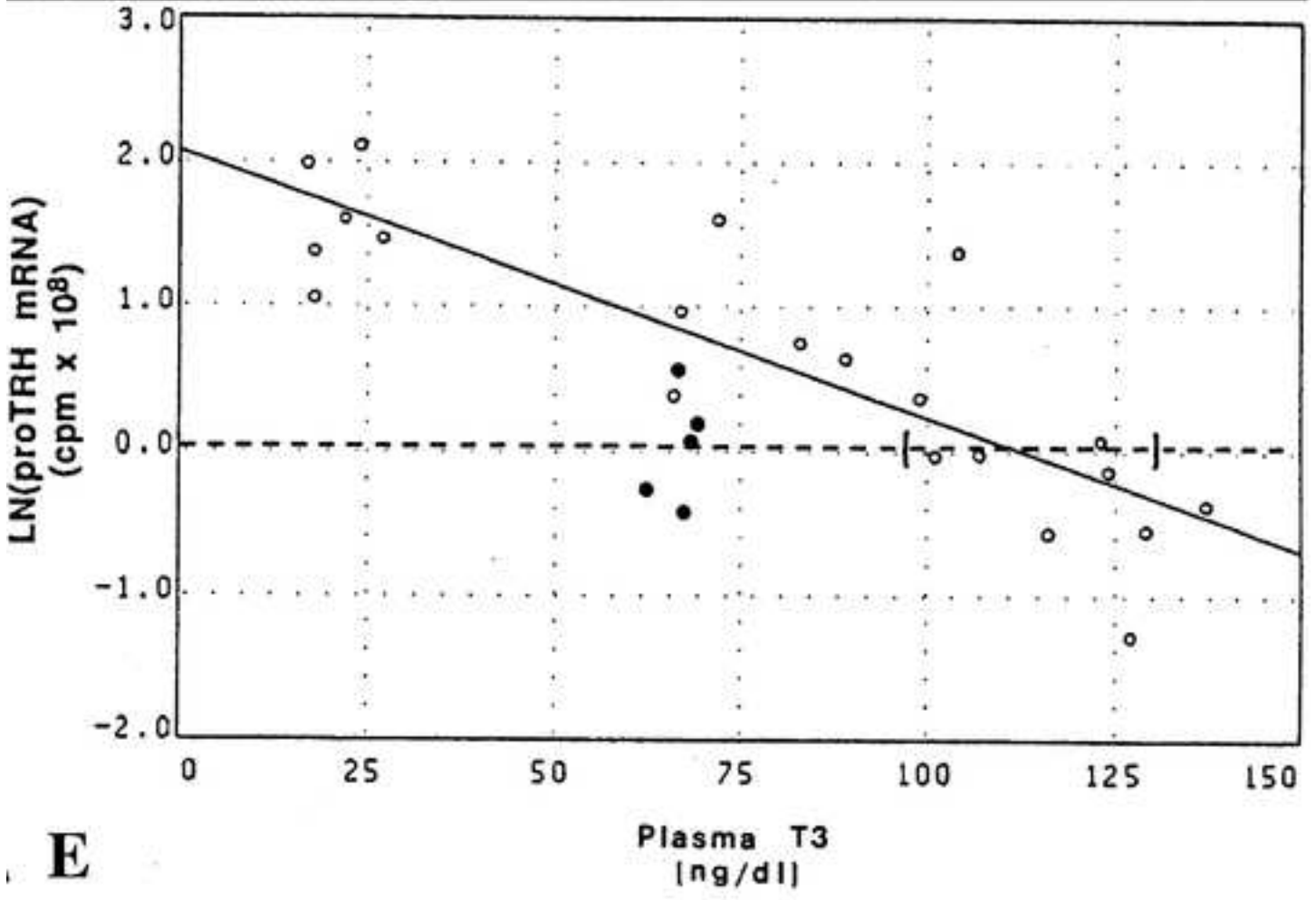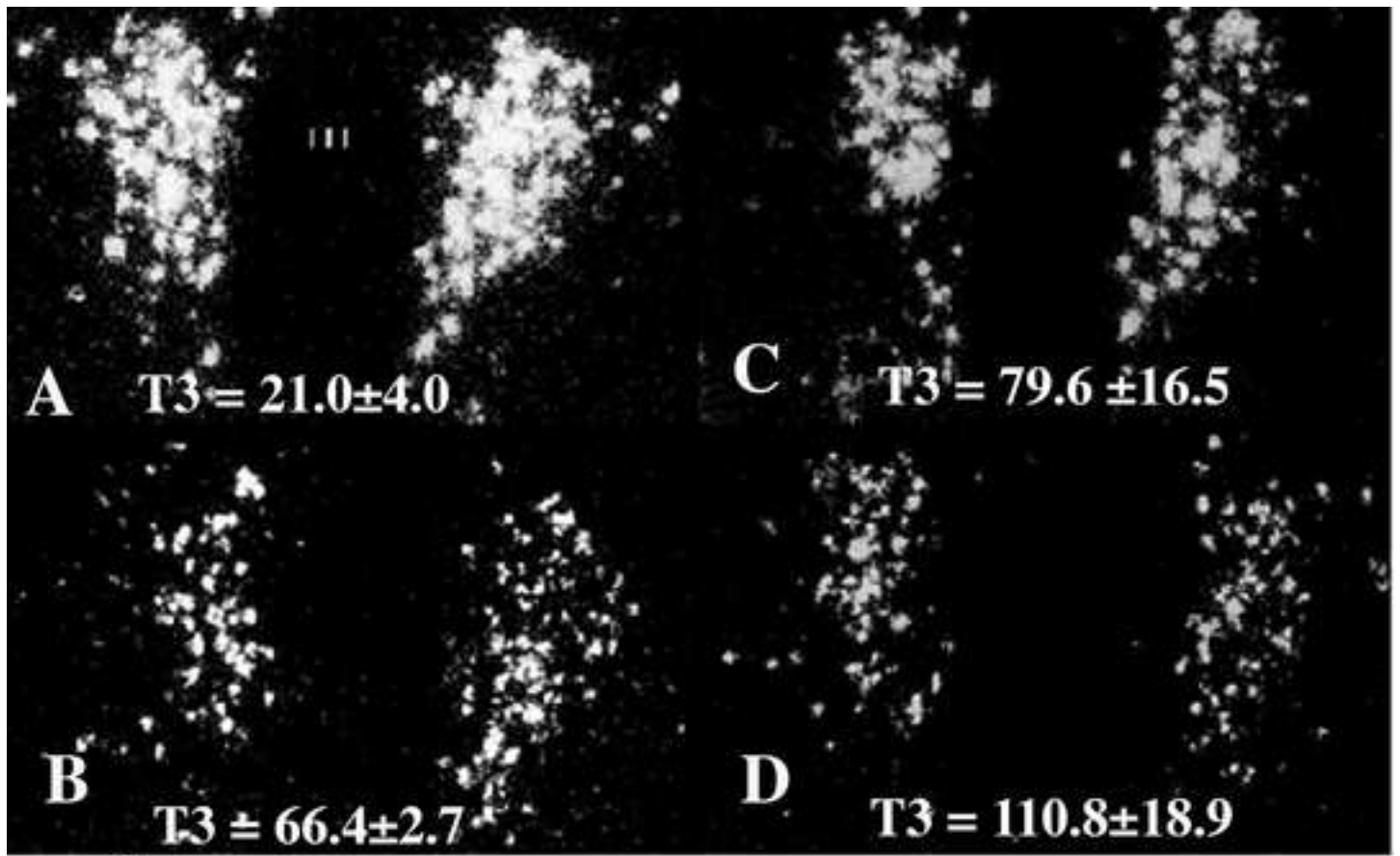
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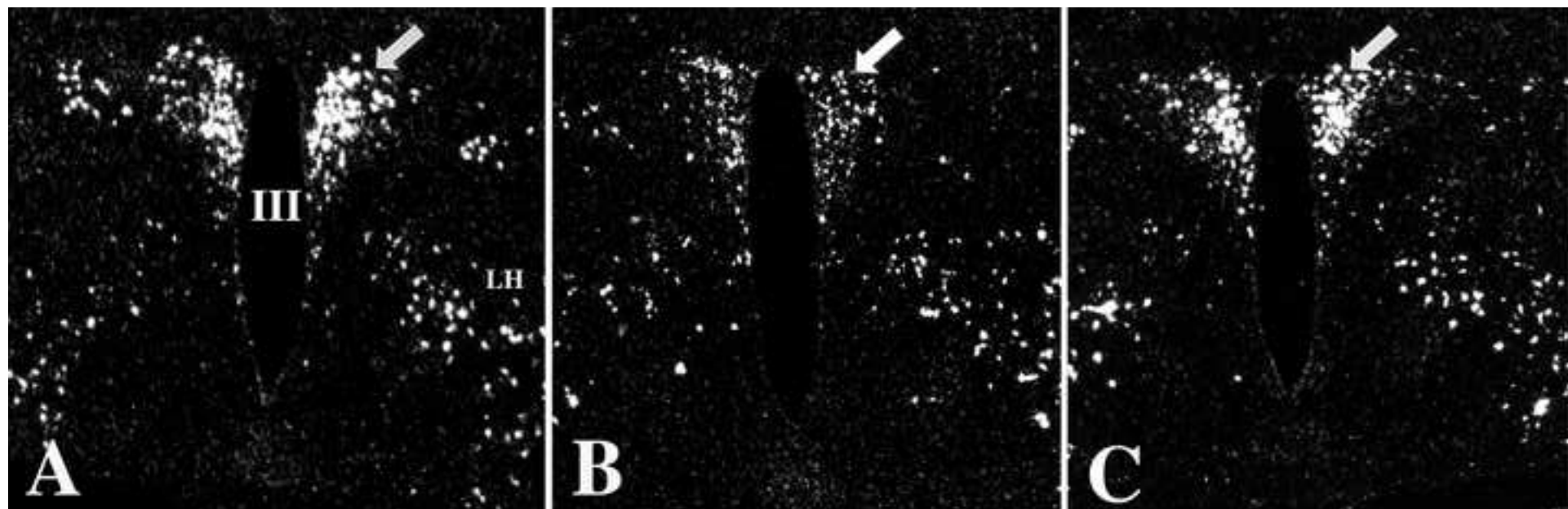
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PVN

TRH

+
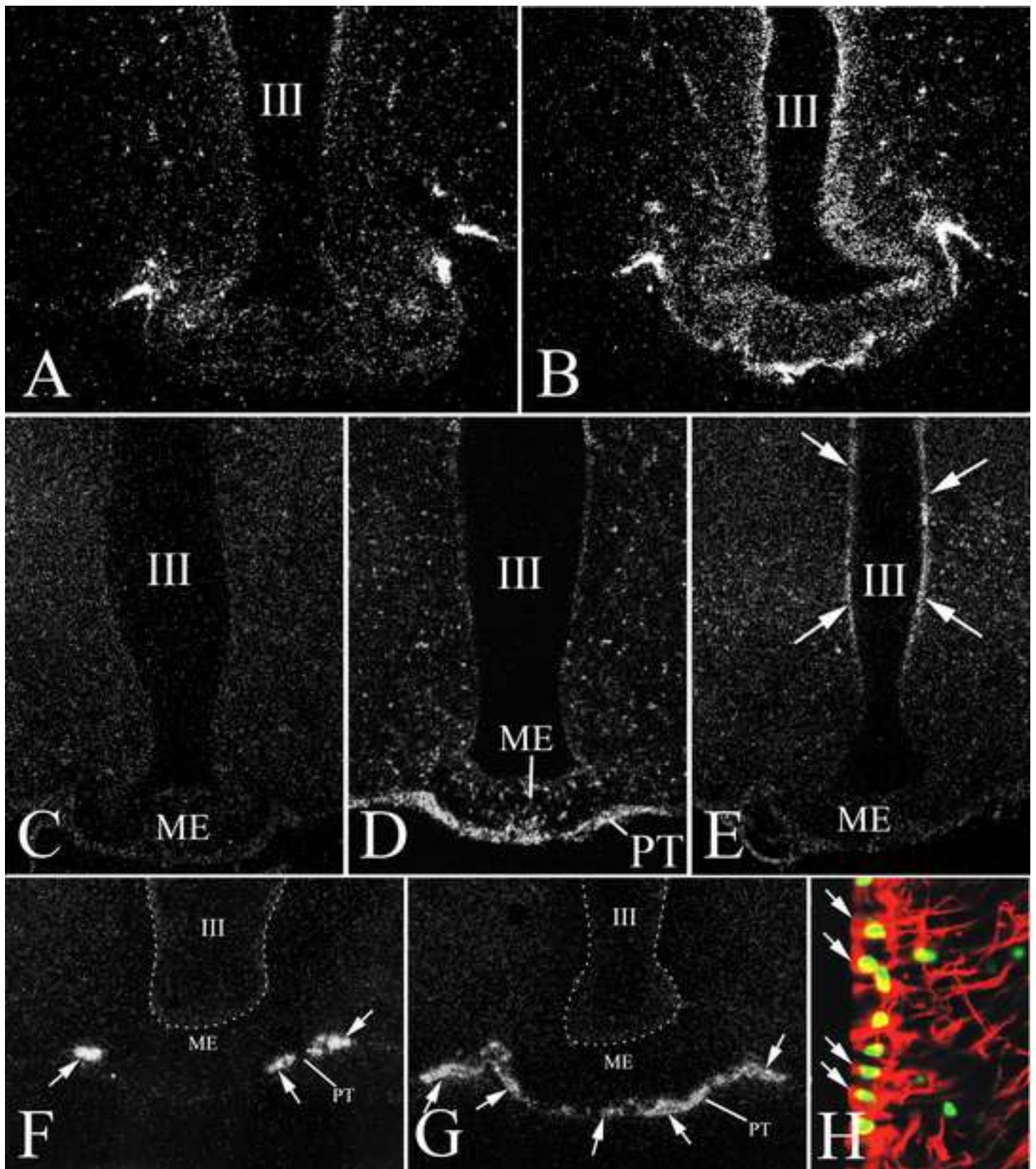
?

-   +

DMN

AGRP/
NPY

-

αMSH/
CART

-

+   ARC

Leptin

FAT

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Figure 14

Figure 15
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III ventricle

PVN

TRH

T3

T4

Tc

T4

Tc

T3

Pc

Pit

TSH

Pit   Pituitary
Pc   Portal capillary
Tc   Tanycyte
o   MCT8, OAPT1c1
↓   T4
↓   T3
↓   TRH
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Expected publication date	Jan 2014
Estimated size of new article (number of pages)	45
Elsevier VAT number	GB 494 6272 12
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