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Cholinergic and peptidergic neurotransmission in the adrenal medulla: a dynamic control of stimulus-secretion coupling

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Running head: Remodeling of adrenal medulla neurotransmission

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Abbreviations: ACh, acetylcholine; ATP, adenosine triphosphate; CGRP, calcitonin gene-related peptide; E, epinephrine; GABA, γ -aminobutyric acid; NPY, neuropeptide Y; NE, norepinephrine; nAChRs, nicotinic acetylcholine receptors; PACAP, pituitary adenylate cyclase-activating polypeptide; PNMT, phenylethanolamine N-methyltransferase; ROS,

reactive oxygen species; sEPSCs, spontaneous excitatory post-synaptic currents; SP, substance P

Abstract

Synaptic neurotransmission at the splanchnic nerve-chromaffin cell synapse is a chief element of the stimulus-secretion coupling in the adrenal medullary tissue, managing and regulating the secretion of catecholamines. Making the state of play more intricate than initially envisioned, the synaptic vesicles of nerve terminals innervating the medulla contain various compounds, including various neurotransmitters and neuropeptides. Under basal conditions associated with a low splanchnic nerve discharge rate, neurotransmission is ensured by the synaptic release of the primary neurotransmitter acetylcholine (ACh). Under sustained and repetitive stimulations of the splanchnic nerve, as triggered in response to stressors, the synaptic release of neuropeptides, such as the pituitary adenylate cyclase-activating polypeptide PACAP, supplants ACh release. The anatomical and functional changes that occur presynaptically at the preganglionic splanchnic nerve, combined with changes occurring postsynaptically at nicotinic acetylcholine receptors (nAChRs), confer the adrenomedullary synapses a solid and persistent aptitude to functional remodeling, from birth to aging. The present review focuses on the composite cholinergic and non-cholinergic nature of neurotransmission occurring at the splanchnic nerve-chromaffin cell synapse and its remodeling in response to physiological or pathological stimuli.

INTRODUCTION

Catecholamine secretion from the adrenal medullary tissue is a key adaptive response triggered by an organism to cope with stressful situations. Epinephrine (E) and norepinephrine (NE) secretion is ensured by chromaffin cells, the neuroendocrine cell population of the adrenal medulla. A peculiarity to the adrenal medulla, as compared to other endocrine/neuroendocrine glands, is its functional similarity to a sympathetic ganglion. The medulla receives a sympathetic innervation arising from the intermediolateral cell column of thoracic spinal segments (1). The axons pass through the sympathetic chain, exit as the greater and the lesser splanchnic nerves and directly synapse onto adrenal chromaffin cells. Taking the rat as an example, about 500-700 axon terminals innervate the medulla (2, 3) and each chromaffin cell receives 1-4 presynaptic boutons (4, 5). Beyond the sympathetic innervation, the adrenal medulla also receives a parasympathetic and a sensory innervation (6, 7).

The chromaffin cell population of the adrenal neuroendocrine tissue is composed of both E-secreting cells and NE-secreting cells, depending on the presence of the phenylethanolamine N-methyltransferase (PNMT), the biosynthetic enzyme which converts NE into E. The two chromaffin cell populations are innervated by functionally distinct sympathetic preganglionic neurons (8, 9). Before innervating the adrenal gland, the splanchnic nerve bifurcates into two divisions (anterior and posterior branches) (10), but whether the two divisions of the splanchnic nerve specifically innervate E- or NE-secreting chromaffin cells remains to be elucidated (11).

The splanchnic nerve-chromaffin cell synapse is a privileged regulatory site of stimulus-secretion coupling. Indeed, catecholamine release is chiefly driven by the electrical activity of the splanchnic nerve (12). It is of note that a local gap junction-mediated communication between chromaffin also regulates hormone secretion (13-17). Under basal ('unstressed') conditions, the sympathetic nervous system discharges at a moderate firing rate (<1 Hz (18)),

contributing to a normal blood pressure and to the homeostatic 'rest and digest' status of energy storage. Accordingly, adrenal chromaffin cells release small amounts of catecholamines into the circulation. Contrasting with this 'resting' state, a stressful situation (induced for example by a systemic stress, a psychological stress, a physical injury,...) strongly activates the sympathetic nervous system, leading to drastically increased splanchnic nerve firing discharge (several Hz). This event is part of the 'fight or flight' response and the contribution of the adrenal medullary tissue to this primarily consists of a surge of catecholamine secretion. From a mechanistic point of view, the synapse formed between the preganglionic sympathetic splanchnic nerve terminals and chromaffin cells is excitatory and uses acetylcholine (ACh) as primary neurotransmitter (19, 12). But interestingly, as early as in the 80's, after the review published by Marley and Livett (20), the question of '*whether, in addition to acetylcholine, some other excitatory substance(s) is released from splanchnic nerve...*' (21) has emerged. This envisioned hypothesis has been further reinforced by the presence of biologically active substances, such as peptides (22, 23) in the splanchnic nerve fibers, thus designating the splanchnic nerve-chromaffin cell synapse as a putative site of a non-cholinergic neurotransmission. In this regard, PACAP is undoubtedly the best example, with its established involvement in the stress response (see (24) for a recent review). The present review brings into focus both the cholinergic and the non-cholinergic (peptidergic) nature of the neurotransmission taking place at the splanchnic nerve synapse and its plasticity under physiological or pathological conditions.

1- Cholinergic neurotransmission at the splanchnic nerve-chromaffin cell synapse: acetylcholine as the 'resting' primary neurotransmitter

Since the pioneer work of Felberg and colleagues (25), it is known that ACh mediates the discharge of adrenaline from the adrenal gland. As asserted by WW Douglas in the "First

Gaddum Memorial Lecture" at Cambridge in 1967 and then published in 1968 (19), "*ACh acts at the medullary synapse, where its function is to induce secretion*". At the cholinergic synapse, the respective contribution of nicotinic (nAChRs) and muscarinic receptors (mAChRs) to the secretory function of the chromaffin tissue differs between species (26-28), though nAChRs appear to have a major involvement (29-31).

The anatomico-functional properties of cholinergic neurotransmission occurring at the splanchnic nerve-chromaffin cell synapse became more easily studied with the development of *ex vivo* adrenal slice preparations, where the architectural organisation of native tissues (cortex and medulla) is maintained and electrophysiological/optical recordings of synaptic events are feasible. In an early study published in 1992, Iijima and colleagues (32) combined electrical stimulation of acute adrenal slices to the use of a voltage-sensitive dye and an array of photosensitive diodes to investigate synaptic transmission at the splanchnic nerve-chromaffin junction in the rat. From an anatomical point of view, this study supports the notion that one nerve fiber innervates a cluster of chromaffin cells and that each cluster is innervated by approximately four nerve fibers. Regarding functional attributes of the synaptic transmission, the authors showed that a presynaptic spike induces a spike in the postsynaptic chromaffin cell, resulting from a trans-synaptic activation of nChRs. Later on, it was shown in guinea-pig bisected adrenal glands that the excitation of a single preganglionic fiber released sufficient amounts of ACh to evoke a sustained chromaffin cell membrane depolarization (33).

The first description of the biophysical properties of synaptic events taking place at the splanchnic nerve-chromaffin cell synapse comes from the work performed by Barbara and Takeda in rat acute adrenal slices (34). They recorded both spontaneous and local field stimulation-evoked excitatory post-synaptic currents (EPSCs) and proposed the first quantal model for ACh release onto chromaffin cells (34, 35). This allowed quantal size to be estimated at 20-25 pA, a value then confirmed by others studies (5, 36). Note that a similar value can be

calculated from the previous work published by Holman and colleagues in guinea-pig bisected adrenal glands (33). More recently, an *in vivo* study documented for the first time spontaneous and splanchnic nerve-evoked electrical activity in the medulla of anaesthetized mice (16).

Consistent with a dominant implication of nAChRs in catecholamine secretion, nicotinic receptors robustly contribute to post-synaptic excitatory events, as reported *ex vivo* in acute slices in rat (34, 37, 36) and *in vivo* in mouse (16). Adrenal chromaffin cells express various nAChR subtypes, including $\alpha 3$ -, $\alpha 6$ -, $\alpha 7$ - and $\alpha 9/\alpha 10$ -built receptors, depending on species (see (38-40) for recent reviews). Each nAChR subtype can individually contribute to post-synaptic events as long as it is located at the synapse. In adult rat and mouse, pharmacological experiments using the acetylcholine nicotinic receptor antagonist hexamethonium pointed to a major contribution of $\alpha 3$ -containing nAChRs to transynaptic signal transduction (34, 37, 36, 16) (Figure 1A). $\alpha 7$ -built and $\alpha 9/\alpha 10$ -containing nAChRs are also activated at the splanchnic nerve-chromaffin cell synapse, although to a lesser extent (41, 37, 36). Further complicating this scheme is the unsolved issue of nAChR subtype expression in either E-secreting and/or NE-secreting chromaffin cells. In adult mouse, $\alpha 7$ nAChRs appear to have a prominent expression in a subset of NE-secreting cells located near the edge of the cortex (42).

2- Non-cholinergic neurotransmission at the splanchnic nerve-chromaffin cell synapse: special focus on PACAP, a major autonomic stress neurotransmitter

As mentioned in the introduction, the presence of biologically active compounds, other than ACh, in the nerve fibers innervating the adrenal medullary tissue was highlighted early on. This finding supported the hypothesis that non-cholinergic neurotransmission could take place at the splanchnic nerve-chromaffin cell synapse (20). However, as compared to cholinergic synaptic transmission, the current knowledge of the non-cholinergic transmission is scarce, especially regarding its physiological/pathological relevance. Rather than a long and fastidious

description of peptides found in nerve fibers innervating the medulla, a table summarizing data collected for many decades was preferred (Table 1). The presence of a broad repertoire of neuropeptides is a strong indication for conceding a significant contribution of peptidergic neurotransmission to the adrenal gland function, envisioning intricate and unanticipated roles in the modulation of hormone secretion. Regarding this, the best documented example deals undoubtedly with PACAP. The adrenal PACAP history begins in the 90's with the finding of PACAP receptors in the medulla (43) and the presence of PACAP-immunoreactive nerve fibers innervating chromaffin cells (44) (Figure 2). The next noticeable milestone in the PACAP chronicle was the finding that PACAP acts as a neurotransmitter at the splanchnic nerve-chromaffin cell (45). Supporting this, i) PACAP is present in cell bodies of neurons located in the intermediolateral cell column of the thoracic spinal cord, from which the nerve fibers innervating the adrenal medulla originate (45), ii) PACAP co-localizes with the vesicular ACh transporter, a cholinergic marker, in the adrenal presynaptic nerve terminals (45), iii) the sustained and prolonged period of catecholamine secretion in response to a metabolic stress (hypoglycemia, which triggers nerve activation) is impaired in PACAP-deficient mice (45) and iv) the increased tyrosine hydroxylase activity, which usually follows the activation of sympathetic neurotransmission (46) does not occur in PACAP-deficient mice (45). As anticipated from iii) and iv), PACAP appears to have a significant contribution during stress episodes. This will be developed in more detail in the next chapter.

3- Physiological/pathological remodeling of the splanchnic nerve-chromaffin cell synapse

Unlike the extensive description of plasticity mechanisms at brain synapses, plasticity in autonomic peripheral neurons is poorly understood, particularly in sympathetic neurons innervating the adrenal gland. This chapter reports physiological and pathological changes that

can occur at pre- and post-synaptic sites of the adrenomedullary synapses, under physiological and pathological conditions.

- during postnatal development of the adrenal medulla

Synaptic contacts are already present in chromaffin cells at E15.5 (47). Although all determinants involved in adrenal stimulus-secretion coupling are present at the embryonic stage, innervation of the medullary tissue is not competent at birth, but fully matures during the first postnatal weeks, in rodents (48, 49). At the embryonic stage E14-E16, nerve fibers in the adrenal medulla contain either no or very few clear vesicles, while at E18-E20 numerous clear vesicles and a few dense-cored vesicles are visible (50). Maturation of the medullary tissue is associated with the acquisition of the neurogenic control of catecholamine secretion and the postnatal period thus critically shapes functional synaptic cholinergic transmission at the splanchnic nerve-chromaffin cell synapse. Postnatal remodeling of adrenal gland innervation is associated with an increased density of nerve fibers investing the adrenal medulla (increased number of neurofilament NF-10-immunopositive bundles) and with an increased number and staining intensity of acetylcholinesterase-immunoreactive nerve fibers (23). As a functional consequence, it is expected that the excitatory cholinergic synaptic events also undergo a postnatal remodeling. An example of the postnatal maturation of cholinergic sEPSCs in rat chromaffin cells is illustrated in figure 3. At birth, only small amplitude sEPSCs are found (37, 51). The amplitude and frequency of synaptic events gradually increases with age, reaching values close to those found in adult around postnatal day 11. This developmental remodeling is achieved, at least in part, through the action of agrin, a synaptic organizing protein initially described for its effects at the neuromuscular junction. At the adrenomedullary cholinergic synapse of neonate rats, agrin promotes an increase in sEPSC amplitude without significantly affecting their frequency (51). Consistent with agrin acting postsynaptically, chromaffin cell

responses to nicotine in neonates are reshaped by agrin (Figure 4). At P0, nicotine evokes a small membrane depolarization, supported by a low-amplitude inward current (Figure 4A, black traces), when compared to responses found in adults. However, when neonate chromaffin cells are exposed to agrin, the amplitude of nicotine-evoked depolarization drastically increases, resembling that observed in adults, also consistent with an increased amplitude in nicotine-activated currents (Figure 4A, red traces). Supporting a specific role of agrin in the developing adrenal medullary tissue, agrin does not have any effect in adult animals (Figure 4B).

Another crucial determinant of stimulus-secretion coupling are post-synaptic cholinergic (nicotinic and muscarinic) receptors expressed by chromaffin cells. The study of the developmental maturation of nicotinic and muscarinic AChRs unveiled a sequential expression of the two receptor types (50). The nicotinic receptor-mediated signaling pathway appears first in development (around E19.5), coinciding with a period of synaptogenesis and further development of neurotransmission. The signaling pathway triggered by the activation of muscarinic receptors takes place later, around P0. Regarding nAChR subtypes, their respective contribution to sEPSCs changes during the acquisition of a fully mature synaptic transmission, and the agrin protein contributes to the nAChR repertoire activated during the synaptic transmission (51). At birth, $\alpha 3$ -containing nAChRs primarily contribute to synaptic events, as evidenced by the complete blockade of sEPSCs by hexamethonium. Alpha-bungarotoxin-sensitive nAChRs (*i.e.* $\alpha 7$ - and/or $\alpha 9/\alpha 10$ -built receptors) are next recruited at the synapse, and together with $\alpha 3$ nAChRs, shape the adult nAChR patterning involved in the cholinergic synaptic transmission at the rat splanchnic nerve-chromaffin cell junction (51, 36, 52). Suggesting the recruitment of pre-existing alpha-bungarotoxin-sensitive nAChRs rather than an effect on gene expression is the finding that $\alpha 7$ nAChRs are expressed from E14.5 to the first postnatal week in the developing mouse adrenal gland (42).

Concurring with the postnatal maturation of the adrenal medullary tissue innervation and acquisition of the cholinergic neurogenic control of the stimulus-secretion coupling, the peptidergic neurotransmission is also postnatally remodeled, as shown by changes in peptide expression in nerve fibers surrounding chromaffin cells. In particular, enkephalin-immunopositive nerve fibers increase in number and staining intensity during the postnatal period, while calcitonin gene-related peptide (CGRP)- and galanin-expressing fibers are almost fully grown at P2 (23). Similarly, the abundance of substance P (SP)-immunoreactive fibers is low at birth, and gradually increases during the first postnatal weeks to reach a density similar to that found in adults at postnatal week 3 (53). Regarding PACAP, a few weakly immunopositive nerve fibers are detected in the medulla of newborn rats, contrasting with the dense network of PACAP-immunoreactive fibers in adult animals (44). Along similar lines, it is noteworthy that the postnatal peptide expression in nerve fibers invading the adrenal medulla is under regulation of the sympathetic preganglionic splanchnic nerve innervation. As illustrated by the work of Holgert and colleagues (54), a sympathectomy performed at P2 leads to a decreased number of enkephalin-immunopositive fibers, while leaving CGRP-like immunoreactivity in nerve fibers unchanged.

Collectively, all these data designate the postnatal development of the adrenal medullary tissue as a decisive period for the establishment of a functional stimulus-secretion coupling, shaping both cholinergic and peptidergic neurotransmission.

- in response to stressful situations

Activation of the sympatho-adrenal axis is a common attribute to stressors and one of the primary mechanisms allowing vertebrates to cope with stress. In this mechanism, adrenal chromaffin cells constitute the neuroendocrine arm of the sympathetic nervous system. These

cells thus play a major role and the stimulus-secretion coupling that leads to catecholamine release is obviously critical.

In response to a 5-day cold exposure stress, the adrenal medullary tissue undergoes a marked remodeling that enhances the stimulus-secretion coupling efficiency to respond to an increased catecholamine demand. Both pre- and post-synaptic elements of the neurotransmission at the splanchnic nerve-chromaffin cell synapse are affected, impacting on stimulus-secretion coupling (Figure 1B). First, the density of nerve fibers infiltrating the medulla is enhanced, as evidenced by an increased immunostaining of neurofilaments (55). Second, cold stress redefines chromaffin cell nAChR subtypes involved in the nicotinic response, by promoting a switch from a dominant contribution of $\alpha 3$ -containing nAChRs in control rats to a dominant contribution of $\alpha 9$ - and/or $\alpha 9/\alpha 10$ -built nAChRs in stressed rats (36, 56). Also supporting this switch are the findings that the transcript encoding $\alpha 9$ nAChR is upregulated in cold-stressed rats and that $\alpha 9$ nAChRs preferentially distribute at synaptic sites in stressed animals (36). Interestingly, similar pre-and post-synaptic adaptations of the adrenal stimulus-secretion coupling have been recently described in a rat model of neuropathic pain (57). Compared to controls, the pre-synaptic changes consist of a higher density of cholinergic nerve terminals synapsing onto chromaffin cells and an increased sEPSC frequency. Post-synaptically, chromaffin cells also undergo functional remodeling, as evidenced by i) a less negative resting potential associated with more frequent action potential discharges, ii) a greater fraction of acetylcholine-evoked currents linked to activation of $\alpha 9/\alpha 10$ -built nAChRs, instead of a primary $\alpha 3$ component in control animals and iii) an increased exocytosis evoked by voltage-dependent Ca^{2+} entry.

Another notable remodeling taking place in response to a metabolic stress or to sustained splanchnic nerve firing is the substitution of the classical cholinergic neurotransmission by PACAP-mediated neurotransmission (45, 58, 24, 59). The switch toward "*an emergency*

neurotransmitter" (45) in response to an elevated secretory demand makes sense as i) endogenous PACAP can be released when the sympathoadrenal system is highly activated, as observed during severe hypotension (60), ii) the adrenal medullary tissue responsiveness to PACAP is significantly enhanced during stress only when the sympathoadrenal system is activated (61), iii) PACAP specifically triggers catecholamine release under elevated splanchnic nerve firing activity (62), iv) after an immediate effect on catecholamine secretion, PACAP induces a long-lasting secretory response, which can last hours, as does prolonged or repetitive stress (63), v) PACAP secrete mostly E (E/NE ratio of 7 for PACAP *versus* 4 for ACh) (22) and E is the main stress hormone, vi) PACAP enhances gap junction-mediated electrical coupling, favouring the spread of electrical activation between chromaffin cells (64) and vii) gap junctional coupling between chromaffin cells contribute to catecholamine secretion (13-16) and is enhanced in stressed animals (55, 65, 16). From all these data, it is clear that the stress response upgrades PACAP as a pivotal neurotransmitter to optimize the secretion of catecholamines. In addition to PACAP, it seems that SP might exert a similar role at the sensory nerve terminals innervating the medulla (66).

Hypoxia, together with hypoglycemia and glycopenia, is the first stressful situation encountered by a mammal, during the transition of intra-uterine to air-breathing life. To cope with this stressor, an appropriate release of catecholamine consisting in a huge secretion peak must occur. As mentioned above, the neurogenic control of catecholamine secretion is not fully mature at birth, and, in the absence of functional synapses at the splanchnic nerve-chromaffin cell junction, regulation of hormone secretion is handled directly by chromaffin cells, which are most sensitive to hypoxia in the perinatal period ((67) for a review). Innervation of the adrenal medullary tissue and chromaffin cell sensitivity to oxygen are tightly correlated, as shown by i) prolonged impairment of the neurogenic control of catecholamine secretion, as long as hypoxia persists (68), ii) postnatal loss of sensitivity to hypoxia, rendering juvenile

chromaffin cells insensitive to hypoxia as compared to neonate cells (69), iii) parallel regression of direct chromaffin cell chemosensitivity with the acquisition of functional cholinergic innervation (70) and iv) the resurgence of chromaffin cell sensitivity to hypoxia after adrenal gland denervation (71). Of particular interest is the finding that neurotransmitters released by splanchnic nerve terminals during innervation contribute to the direct loss of chromaffin cell chemosensitivity to hypoxia (reviewed in (72)). This is demonstrated for cholinergic neurotransmission, by results showing a role of $\alpha 7$ -built AChRs activation (73, 74). The opioidergic signaling through μ and/or δ opioid receptors also participates in the suppression of chromaffin cell sensitivity to hypoxia (72).

Although less well documented than hypoxia, other stressors can also exert their effects through a remodeling of the synaptic transmission. This is in particular the case for food deprivation-induced stress response. The counter-regulatory response triggered to cope with fasting or insulin-induced hypoglycemia requires activation of the sympatho-adrenal axis and subsequent catecholamine secretion, to further increase hepatic glucose production while suppressing insulin and potentiating glucagon secretion from pancreatic endocrine cells. A recently published study elegantly showed the aptitude of presynaptic nerve terminals innervating chromaffin cells to display plasticity mechanisms during fasting (75). Food deprivation, through the involvement of neuropeptide Y (NPY), leads to a strengthening of cholinergic adrenomedullary neurotransmission. This results from the presynaptic modulation of transmitter release probability, as shown by a diminution of the paired-pulse ratio of evoked synaptic currents. To my knowledge, this is the first report of a synaptic strength change at the splanchnic nerve-chromaffin cell synapse, followed later on by the demonstration by the same group that agouti-related peptide (AgRP), by its antagonist action at melanocortin receptors located in the presynaptic splanchnic nerve terminals, can act as a brake on synaptic strength at

the adrenomedullary synapse during fasting, limiting an unrestrained and potentially deleterious elevation in sympathetic activity (76).

In certain disease states, the synaptic neurotransmission in autonomic ganglia is depressed. This pathological situation is found at the splanchnic nerve-chromaffin cell synapse in response to chronic hyperglycemia, indicating that the sympathoadrenal system is highly vulnerable to hyperglycemia/diabetes (77). The underlying mechanisms involve the production of reactive oxygen species (ROS), which then bind to post-synaptic nAChRs, rundown subsequent nicotinic currents, and depress neurotransmission. As a functional consequence, this defect in splanchnic nerve-evoked synaptic transmission in diabetic animals leads to a decrease in circulating catecholamines (especially E), impacting the organism's ability to trigger an appropriate counter-regulatory response to cope with diabetes-related pathological processes.

- by neurotransmitters and neuromodulators: focus on synaptically released factors

Although both factors released at the synapse and factors released by chromaffin cells are qualified candidates to regulate adrenomedullary synaptic transmission, I decided to concentrate the discussion on factors released by the presynaptic nerve terminals. At the synapse, both pre-synaptic and post-synaptic sites are potential targets of synaptic remodeling. Changes in transmitter release probability at the presynapse as well as effects on post-synaptic nAChRs engaged in the synaptic transmission (as exemplified above with ROS production) are proficient mechanisms to alter trans-synaptic signaling. In this context, as exemplified below, the allosteric modulation of nAChRs by synaptically released factors at the preganglionic splanchnic nerve terminals plays an important role (78, 79).

The neurotransmitter GABA has been reported to regulate nAChR-mediated cholinergic neurotransmission between splanchnic nerve endings and chromaffin cells. In addition to the presence of GABAergic nerve fibers in the medulla (80) (Table 1), rat chromaffin cells express

an intrinsic GABAergic system (81). An intriguing finding is the facilitating effect of the GABA_A receptor antagonist bicuculline on the release of catecholamine elicited by nicotinic receptor activation (81), indicating that i) catecholamine secretion mediated by activation of nAChRs is under tonic inhibitory control of GABA and ii) GABA signaling may modulate rat chromaffin cell responsiveness to cholinergic synaptic inputs. In the same line, the blockade of presynaptic GABA_A receptors by bicuculline enhances spontaneous synaptic current amplitude and frequency, a result that is indicative of the relief of spontaneous cholinergic neurotransmission (82, 83).

Since ACh released at the splanchnic nerve-chromaffin cell can activate both nAChRs and mAChRs, it is of interest to examine here the effects of mAChR activation. Activation of chromaffin cell mAChRs by muscarinic agonists leads to a drastic inhibition of nicotine-evoked currents associated with a substantial blockade of the quantal release of catecholamines (84). Although speculative, it is reasonable to propose that such a muscarinic inhibition of nicotinic signaling may occur during the adrenomedullary synaptic transmission and may contribute to remodel the synaptic transmission.

As mentioned above, neuropeptides also regulate the splanchnic nerve-chromaffin cell synaptic transmission. We intuitively first think of peptides that are localized in the synaptic nerve terminals investing the medullary tissue. In this regard, CGRP, which is present in nerve fibers innervating the medulla (85) (Table 1), has been reported to downregulate nAChR function through a phosphorylation-dependent mechanism (86). Because the phosphorylation state of nAChRs is known to control their ability to recover from desensitization, CGRP-induced inhibition of nAChR function can be assumed to have a significant role in modulating the cholinergic transmission. Similarly, SP through its interaction with a regulatory site distinct from the ACh binding region on nAChR, modulates nAChR function, resulting in an inhibition of ACh-induced membrane currents (87-89). SP both increases nicotinic receptor

desensitization (by stabilizing nAChR in its desensitized state) and blocks nAChR channel to indirectly enhance desensitization (89, 90). In this regard, it is noteworthy that the nAChR $\beta 4$ subunit exhibits a high affinity for SP (91), arguing for a significant contribution of SP to the modulation of chromaffin cell $\alpha 3\beta 4$ -containing nAChR function. Enkephalin and somatostatin, two other neuropeptides present in nerve fibers innervating the adrenal medulla (Table 1), can disable nAChR function, also likely through an inhibitory action at nicotinic receptor sites (87, 92, 93).

Another compound which may regulate synaptic transmission at the splanchnic nerve-chromaffin cell synapses is adenosine triphosphate (ATP). ATP is a constituent of cholinergic synaptic vesicles (94) and it is co-released with ACh at cholinergic synapses (95). In the adrenal medulla, most of the studies dealing with ATP, if not all, have focused on ATP in chromaffin cell secretory granules and its co-secretion with catecholamines (96). To date, the presence of ATP in cholinergic synaptic vesicles at the splanchnic nerve terminals still remains to be unambiguously documented. It is however highly plausible that ATP-mediated signaling can affect, in one way or another, synaptic transmission. Indeed, the preganglionic nerve fibers innervating the chromaffin tissue display an immunoreactivity to the purinergic P2X1 and P2Y2 receptors (97, 98), suggesting that ATP may have effects on the adrenal gland function by influencing chromaffin cell innervation.

4- Reciprocal interaction between splanchnic nerve electrical activity and neuropeptide expression by chromaffin cells: other forms of synaptic neurotransmission-linked plasticity

Not only splanchnic nerve fibers, but also chromaffin cells, synthesize neuropeptides. The firing activity of the splanchnic nerve can influence expression of these neuropeptides and this contributes to the regulation of the adrenal medulla functioning. An elevated splanchnic nerve

activity, as observed in response to a stressful situations (such as insulin-evoked hypoglycemia), leads to an increased content of neurotensin, SP and galanin in chromaffin cells (99) and to a reduced adrenal store of NPY (100). Along the same line, short-term fasting increases AgRP expression in chromaffin cells (76). When splanchnic neurotransmission is interrupted (immunological sympathectomy of ACh-containing preganglionic neurons by injection of antibodies against acetylcholinesterase), the chromaffin cell content in enkephalin, CGRP, SP, galanin, neurotensin and somatostatin is increased, while no change is found for NPY (101, 102). Note that the immunoreactivity of nerve fibers innervating the medulla is also remodeled by the impairment of cholinergic neurotransmission, resulting in the disappearance of enkephalin-immunoreactive nerve fibers. No change is observed for CGRP, galanin, somatostatin, SP and NPY immunoreactivity (101, 102).

Reciprocally, neuropeptides present in chromaffin cells also are qualified to remodel synaptic transmission, as recently reported for the requirement of NPY in increasing the splanchnic nerve-chromaffin cell synaptic strength in response to fasting (75).

CONCLUSION AND OPEN ISSUES

The splanchnic nerve-chromaffin cell synapse is a highly dynamic site, which gives rise to many of the regulatory mechanisms underlying adrenal stimulus-secretion coupling. From birth to aging, as well as under physiological or pathological conditions, both pre- and post-synaptic elements undergo remodeling, to share the same goal, *i.e.* ensure an optimized secretion of catecholamines. It is however interesting to keep in mind that among all the determinants involved in the adrenal secretion-stimulus coupling, the synaptic neurotransmission is the least studied. This is particularly apparent for non-cholinergic transmission, with many mechanisms still unresolved. Indeed, although the presence of peptides in nerve endings has been

documented many decades ago, their functional contribution to synaptic neurotransmission modulation, and more generally to impact on the adrenal gland functioning, remains to be characterized. The only exception relates to PACAP, for which a clear role in the stress response is ascertained. For the other peptides, the only data available until now reports their effects on the adrenal nicotinic response, showing alterations in nicotine-evoked membrane currents, but whether those changes can occur during the cholinergic synaptic transmission is unknown. By unveiling the mechanisms by which the peptidergic transmission at the splanchnic nerve-chromaffin cell synapse influences the chromaffin tissue and its ability to release catecholamines (or other biologically active compounds), substantial progress will be accomplished. This will likely disclose unanticipated and unexpected findings linking the adrenal peptidergic transmission to stress or stress-related pathologies such as arterial hypertension, obesity or diabetes will complete the knowledges on the role of the adrenal gland in body homeostasis, as recently reported for fasting (75).

Another open issue comes from the fact that most of the studies do not discriminate between E- and NE-secreting chromaffin cells. Although difficult to implement, this would be of great interest, particularly in the field of innervation. Indeed, the two populations of secreting cells display different numbers of synapses (32, 5), with more cholinergic (ACh esterase-positive) fibers contacting NE cells than E cells, in rat (103), in hamster (104) and in mouse (49). Along the same line, the activation of E and NE cells is stressor-specific (9). Regarding the functionality of synaptic transmission, the biophysical properties of spontaneous and evoked EPSCs at NE and E cells remain to be deciphered.

Another intriguing issue refers to the relationship between splanchnic innervation and regulation of adrenal cortical responses. Although the adrenal cortex and the adrenal medulla originate from distinct embryonic sheets, the two tissues are not secluded within the gland, and instead many functional interactions occur. Consistent with this, it is not surprising that the

preganglionic sympathetic innervation may influence cortical responses. This is well-illustrated by three studies conducted by Engeland and colleagues. The authors showed that the cortical response to a dehydration stress (105), the diurnal rhythm of plasma corticosterone (106) and the adrenal cortex regeneration (107) are splanchnic nerve-dependent.

Collectively, these findings and future prospects not only open novel perspectives of research for neurobiologist engaged in the study of hormone secretion, but also clearly indicate that the behavior of the adrenal medulla is yet not fully elucidated. In my opinion, understanding peptidergic neurotransmission and the functional interaction between the medulla and the cortex will refine our current knowledge of adrenal gland function.

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FIGURE LEGENDS

Figure 1: Schematic illustration of functional remodeling taking place at the splanchnic nerve-chromaffin cell synapse. A. In unstressed animals, the synaptic neurotransmission is cholinergic in nature, and synaptically released ACh primarily activates chromaffin cell α 3-containing nAChRs. Note a modest contribution of α 7- and α 9/ α 10-built receptors. B. In response to a stressful situation (a cold exposure for example), which is associated with an increase in the splanchnic nerve electrical discharge and an increase in the secretion of catecholamines, the synaptic transmission is profoundly remodeled, as shown by i) an increased number of nerve fibers innervating the medulla, ii) a switch from a cholinergic to a non-cholinergic neurotransmission, such that PACAP supplants ACh as the main neurotransmitter released at the synapse, iii) a change in chromaffin cell nAChR subtype activated by ACh, with a dominant contribution of α 9/ α 10-built receptors. Original data collected from (45, 37, 51, 55, 62, 36).

Figure 2: Historical overview of the main milestones regarding PACAP in the adrenal medullary tissue: focus on its role as a neurotransmitter. The adrenal PACAP history begins in the nineteen's, with the description of a PACAP immunoreactivity in nerve fibers invading the medulla and the first report of its secretagogue function on catecholamine secretion. From the 2000's, the secretory role of PACAP is periodically implemented and as such PACAP is now recognize as the main adrenomedullary neurotransmitter in the stress response.

Figure 3: Electrophysiological recordings of development-related spontaneous excitatory post-synaptic currents (sEPSCs) in the rat. Synaptic events were recorded in chromaffin cells voltage-clamped at -80 mV in acute adrenal slices from newborn (P0, postnatal day 0), juvenile (P5 and P11) and adult (>12 postnatal weeks) female Wistar rats. Charts recordings clearly show an increase in sEPSC frequency and amplitude during the post-natal period. Unpublished data.

Figure 4: Agrin-dependent maturation of the chromaffin cell electrical response to nicotine. Acute adrenal slices from newborn and adult female Wistar rats were treated (red traces) with a prolonged exposure to the extracellular matrix protein agrin (rat COOH-terminal agrin, 50 ng/ml, 4-5 hours, as reported in (51)), and the electrophysiological responses to nicotine recorded in current-clamp and voltage-clamp conditions were compared to the responses elicited in control adrenal slices (black traces). Nicotine (100 μ M, 100 ms, arrowhead) was pressure-ejected in the vicinity of the recorded cell. A. In newborn rats (P0), agrin substantially remodels the nicotinic response, as evidenced by a robust membrane depolarization compared to untreated slices (left traces), which associated with an increased amplitude of the nicotine-evoked macroscopic current (right traces). B. Conversely to what is

found in neonates, agrin does not modify the electrophysiological response to nicotine in adult rat. Unpublished data.

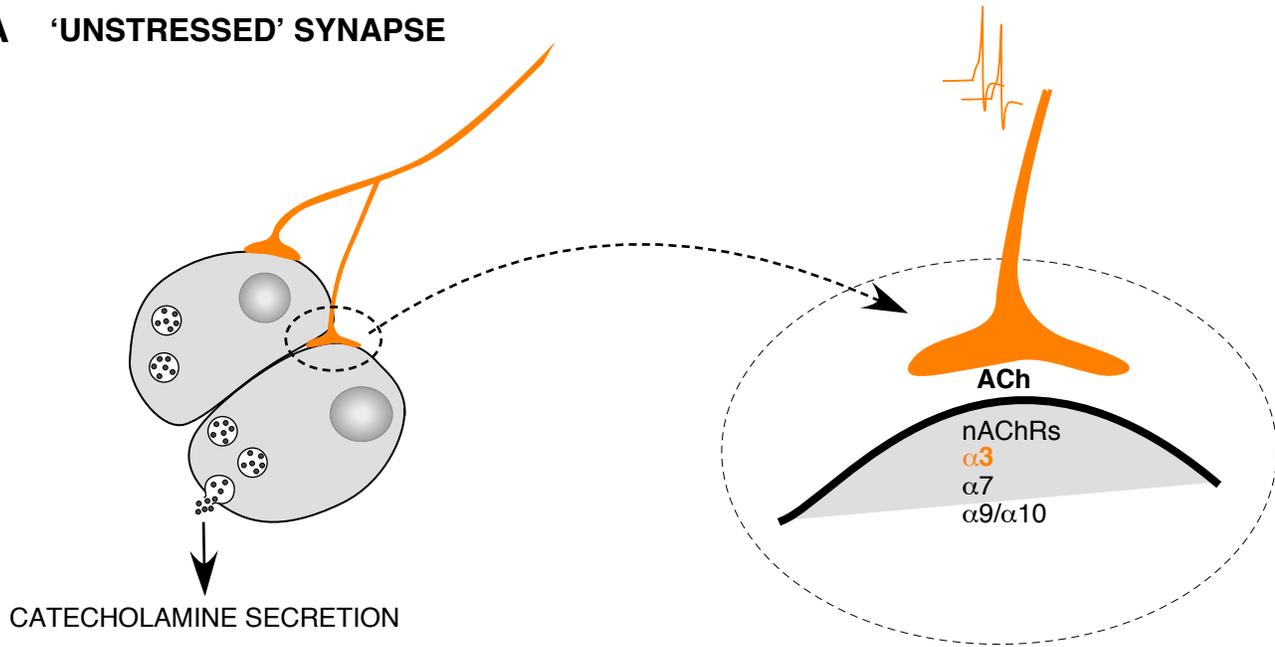
TABLE 1: Peptide expression in nerve terminals innervating the adrenal medullary tissue

<i>Compounds</i>	<i>Species</i>	<i>Remarks</i>	<i>References</i>
Angiotensin II	calf	angiotensin II-binding sites found in low density over nerve tracts in the medulla	{Marley, 1989 #1319}
Calcitonin gene-related peptide	rat	- CGRP immunoreactive fibers among and within small clusters of chromaffin cells - co-expression with substance P	{Kuramoto, 1987 #1320} {Ulrich-Lai, 2003 #1321} {Murabayashi, 2007 #1298}
	guinea-pig	nerve fibers expressing CGRP alone, or colocalization with substance P	{Heym, 1995 #1322}
Cholecystokinin	guinea-pig	colocalization in the nerve fibers with substance P	{Heym, 1995 #1322}
Enkephalin	rat	- expression in preganglionic sympathetic neurons innervating the adrenal medulla - control of a sub-population of chromaffin cells, most likely adrenergic chromaffin cells	{Schultzberg, 1978 #1323} {Holets, 1982 #1324} {Pelto-Huikko, 1985 #1325} {Kumar, 2010 #1326}
	cat	dense network of enkephalin immunoreactive nerves among adrenergic chromaffin cells	{Schultzberg, 1978 #1323} {Pelto-Huikko, 1987 #1327}
	kitten		{Holets, 1983 #1328}
	guinea-pig	moderate number of enkephalin immunopositive nerve fibers in the medulla	{Schultzberg, 1978 #1323}
Other opioids	rat	predominant non-cholinergic component at low splanchnic nerve neuronal activity	{Malhotra, 1987 #271}
GABA	rat	expression in preganglionic sympathetic neurons innervating the adrenal medulla	{Bacon, 1988 #1295}
Galanin	guinea-pig, duck, rat, chicken, pigeon, hamster	- distinct occurrence and distribution of galanin-positive fibers between species - presence of galanin immunoreactive fibers in rat, pigeon, guinea-pig and chicken	{Zentel, 1990 #1329}
	frog		{Gasman, 1996 #1330}

Histamine	dog	suspected to act indirectly on chromaffin cells, through reflex pathways and nerve endings	{Staszewska-Barczak, 1965 #1331}
	rat	- requirement of an intact innervation for histamine effects - neurogenic and non-neurogenic control of catecholamines secretion by histamine	{Yoshizaki, 1973 #1332} {Khalil, 1987 #1333}{Khalil, 1987 #1334}
Neuropeptide Y	rat	- sparse distribution of NPY immunopositive nerve fibers in the medulla - plexus of NPY immunopositive fibers in the subcapsular region with an extension to the zona glomerulosa	{Kuramoto, 1986 #1334}
Neurophysin	rat	expression in preganglionic sympathetic neurons innervating the adrenal medulla	{Holets, 1982 #1324}
	kitten		{Holets, 1983 #1328}
Neurotensin	hamster		{Pelto-Huikko, 1985 #1335}
	cat	dense network of neurotensin-immunoreactive nerves restricted among noradrenergic chromaffin cells	{Pelto-Huikko, 1987 #1327}
Oxytocin	rat	expression in preganglionic sympathetic neurons innervating the adrenal medulla	{Holets, 1982 #1324}
	kitten		{Holets, 1983 #1328}
PACAP		cf a dedicated chapter of the review	
Secretoneurin	rat	robust surrounding of chromaffin cells by secretoneurin-immunoreactive fibers	{Dun, 1997 #1336}
Serotonin	rat	- expression in preganglionic sympathetic neurons innervating the adrenal medulla - co-localization with substance P	{Holets, 1982 #1324} {Bacon, 1988 #1295} {Appel, 1986 #1337}
	rabbit	inputs to sympathetic preganglionic neurons projecting to the adrenal medulla	{Jensen, 1995 #1338}
	kitten		{Holets, 1983 #1328}

Somatostatin	rat	expression in preganglionic sympathetic neurons innervating the adrenal medulla	{Holets, 1982 #1324}
	kitten		{Holets, 1983 #1328}
	cat	few somatostatin-immunoreactive nerve fibers in the adrenal medulla	{Vincent, 1987 #1339}
Substance P	rat	- expression in preganglionic sympathetic neurons innervating the adrenal medulla - colocalization with serotonin immunoreactivity - innervation shown to originate from a population of dorsal root ganglia - substance P-immunoreactive nerve fibers in contact with clusters of noradrenergic chromaffin cells - co-expression with CGRP, choline acetyltransferase, nitric oxide synthase or PACAP	{Holets, 1982 #1324} {Bacon, 1988 #1295} {Appel, 1986 #1337} {Zhou, 1991 #1340} {Murabayashi, 2007 #1298}
	kitten		{Holets, 1983 #1328}
	guinea-pig	colocalization in the nerve fibers with cholecystokinin or calcitonin gene-related peptide	{Heym, 1995 #1322}
VIP	rat	- VIP immunoreactive fibers in both medulla and cortex, particularly in the zona glomerulosa - peptidergic nerve terminals found to innervate chromaffin cells - VIP as neurotransmitter in rat adrenal medulla - prominent VIP contribution at low neuronal activity	{Hokfelt, 1981 #1341} {Holzwarth, 1984 #1342} {Wakade, 1991 #267}

A 'UNSTRESSED' SYNAPSE



B 'STRESSED' SYNAPSE

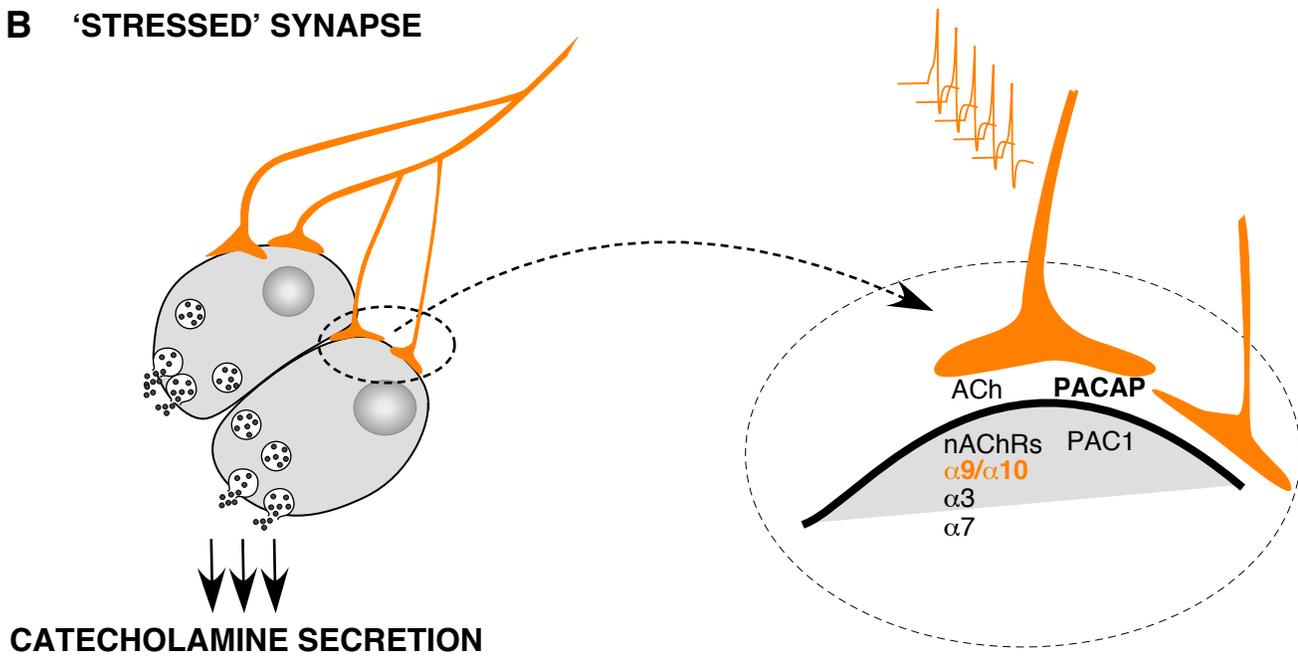


FIGURE 1

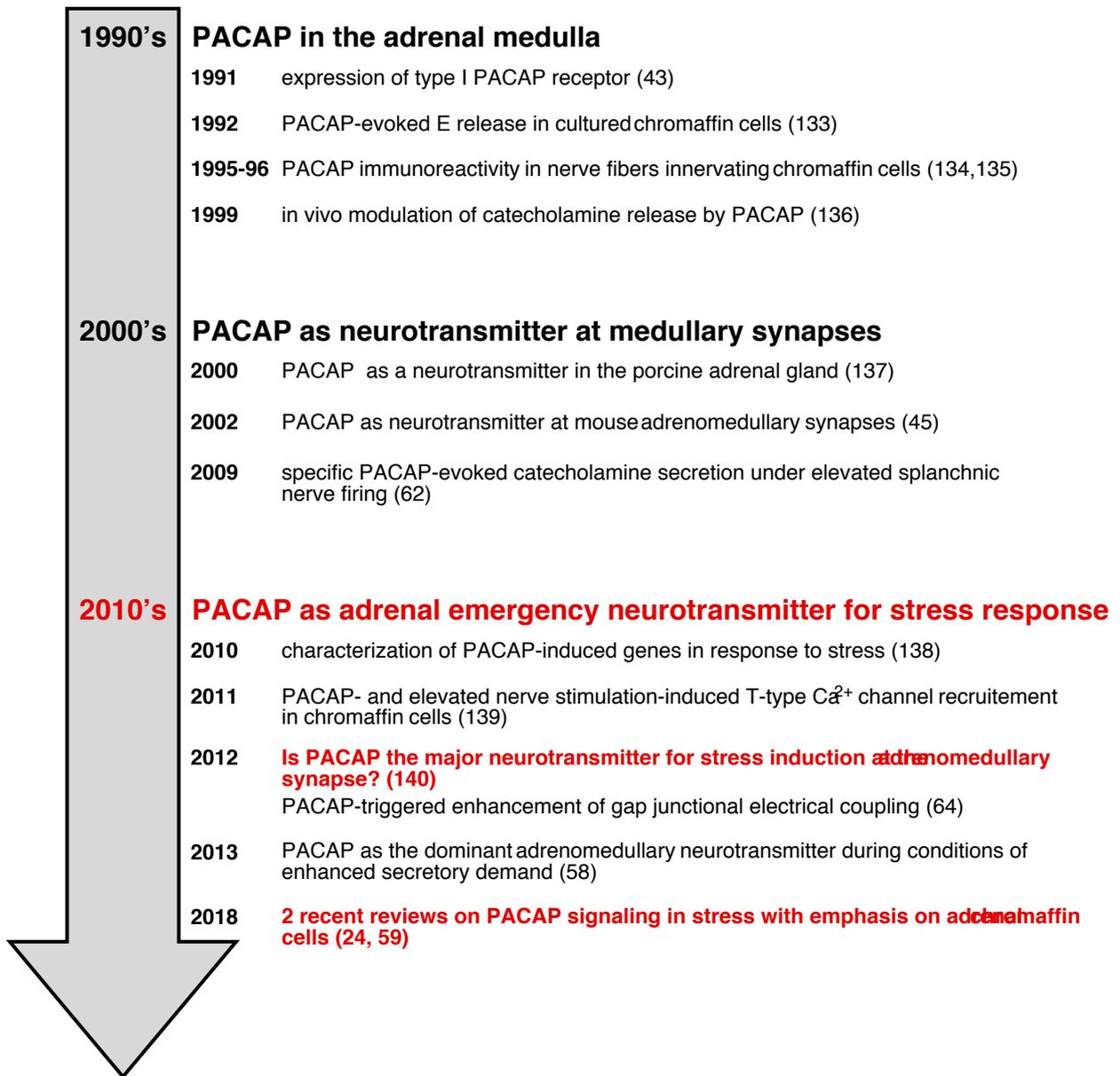


FIGURE 2

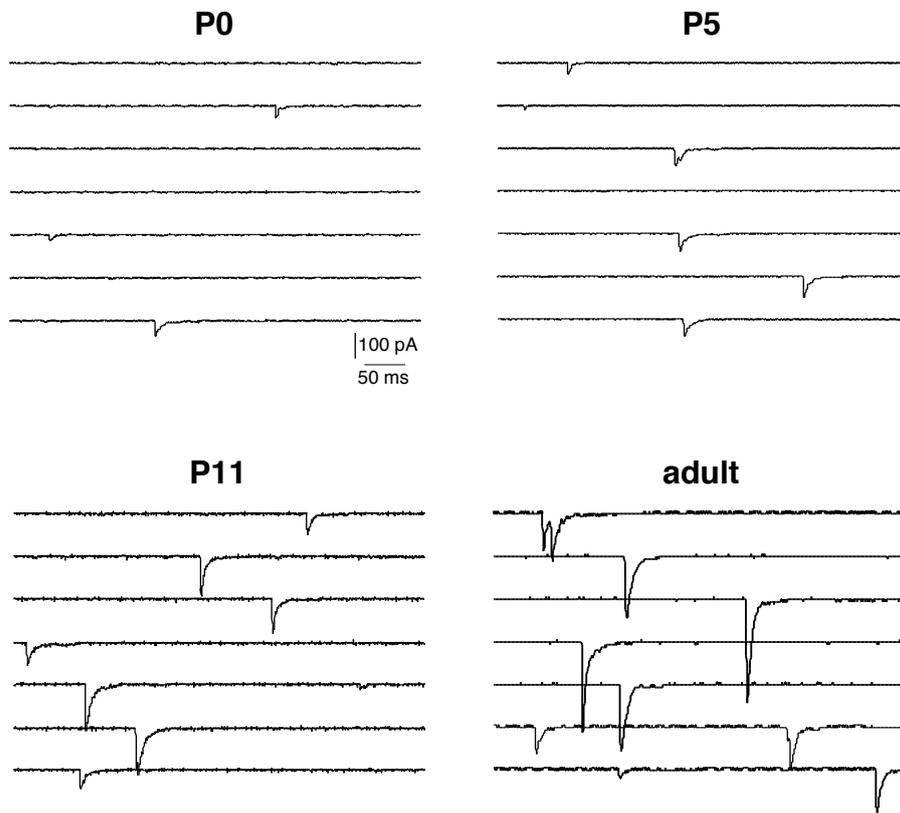


FIGURE 3

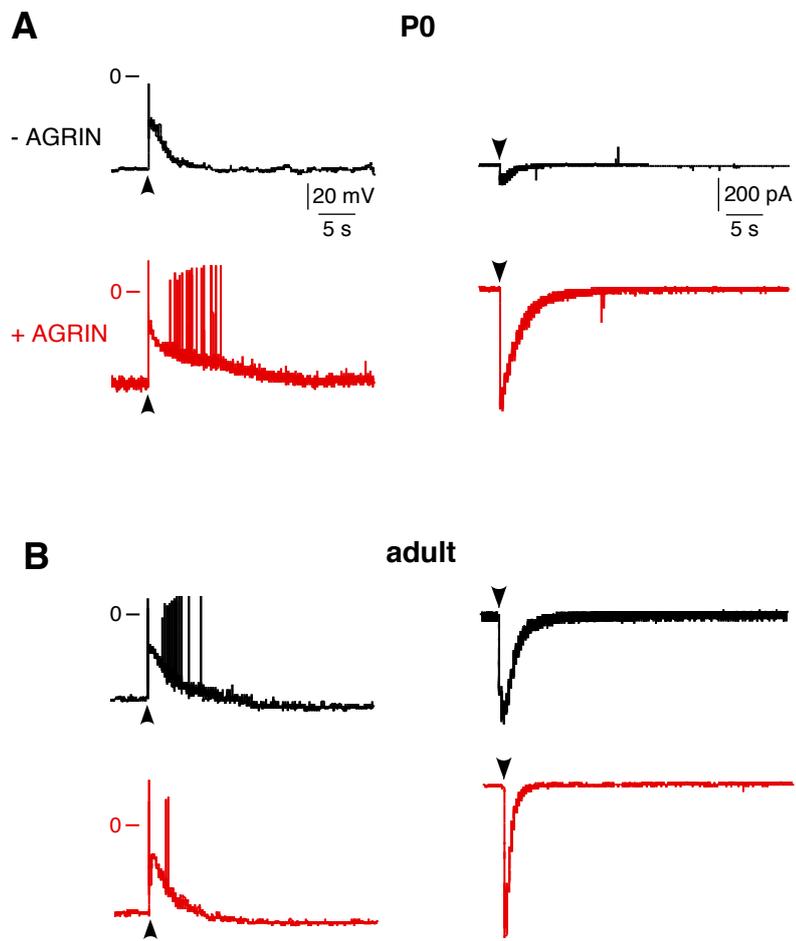


FIGURE 4