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**Gap junction-mediated intercellular communication in the adrenal medulla: an additional ingredient of stimulus-secretion coupling regulation**

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## **Abstract**

The traditional understanding of stimulus-secretion coupling in adrenal neuroendocrine chromaffin cells states that catecholamines are released upon trans-synaptic sympathetic stimulation mediated by acetylcholine released from the splanchnic nerve terminals. Although this statement remains largely true, it deserves to be tempered. In addition to its neurogenic control, catecholamine secretion also depends on a local gap junction-mediated communication between chromaffin cells. We review here the insights gained since the first description of gap junctions in the adrenal medullary tissue. Adrenal stimulus-secretion coupling now appears far more intricate than was previously envisioned and its deciphering represents a challenge for neurobiologists engaged in the study of the regulation of neuroendocrine secretion.

Keywords: gap junctional remodeling; adrenal medullary tissue; stimulus-secretion coupling; catecholamine release; stress; postnatal development; pathology

## **1. Introduction**

The presence of gap junctions in the adrenal gland was first reported in the early seventies. In their pioneer work, Friend and Gilula [1] described a wide distribution of gap junctional structures throughout the adrenal cortex. A preferential expression in the zona fasciculata and zona reticularis has been thereafter described [2]. The first study reporting the description of gap junctions in the adrenal medullary tissue was published a decade later [3]. Still today, most of the studies dealing with gap junctional communication in the adrenal gland focus on the adrenocortical zone ([4] for a recent review). Nevertheless, as the reader will see in this

review, gap junction-mediated cell-cell communication in the adrenal medulla significantly contributes to the physiological function of the medullary tissue.

## **2. Gap junctions in the adrenal medullary tissue: the milestones of three decades of research**

As a preamble, it is noteworthy that the presence and role of gap junctional communication in the adrenal medullary tissue have stayed poorly documented for long. As shown in figure 1, the story of adrenomedullary gap junctions begins in the eighties from observations of freeze-fractured specimens from several species [3]. Gap junction clusters have been found in mouse, guinea-pig, hamster and rabbit but surprisingly not in rat. Few years later, the existence of an “electrocoupling process” in the adrenal medulla has been proposed [5], and the authors made the assumption that a gap junction-mediated communication between adjacent chromaffin cells might contribute to the secretory process, in particular in stressful situations in which a massive and vigorous release of catecholamines is needed. It was then proposed that an electrocoupling process would act as an amplifying signal, thus enabling simultaneous catecholamine secretion from many chromaffin cells. This hypothesis will be confirmed only two decades later [6].

### *2.1. End 1980s-early 2000s: Suspicion and evidences for an electrotonic coupling between chromaffin cells*

The discovery of gap junction-mediated electrotonic coupling in the adrenal medulla has been a multistep process, starting at the end of the 1980s and ending at the beginning of the 2000s (figure 1). First suspicion of an electrical coupling in the adrenal medulla came from the comparison of the input resistance values measured in dissociated chromaffin cells and in

chromaffin cells *in situ* (table 1). The value of input resistance in chromaffin cells measured by sharp microelectrodes in isolated whole or hemisectioned glands is about 30-200 M $\Omega$  [7-9], while it is in the G $\Omega$  range in cultured isolated chromaffin cells [10-14]. In addition and consistent with an electrical coupling between chromaffin cells, the distribution of action potential amplitude recorded in chromaffin cells *in situ* shows a bimodal mode illustrating the presence of both small and large action potentials [8]. The authors proposed that the larger spikes originated from the impaled cells and the smaller spikes from neighboring electrically coupled cells, as reported before in pancreatic beta cells [15], known to be gap junction-coupled [16, 17]. All these findings prompted the authors to propose that chromaffin cells *in situ* behave as if they were electrically coupled.

It is only a decade later (end of the 1990s/beginning of the 2000s) that gap junctional communication between chromaffin cells returned to the spotlight, with experiments performed in acute adrenal tissue slices. The adrenal slice preparation is a suitable model for studying chromaffin cell behavior *in situ*, in respect with splanchnic nerve-mediated cholinergic innervation and cell-to-cell communication mechanisms [18, 19]. In the literature, the presence of a gap junction-built intercellular coupling in the adrenal medulla has been controversial. Indeed, two distinct studies published in 1996 and 1997 reported the absence of electrical coupling between chromaffin cells in rat adrenal slices [18, 20], whereas a low conductance (<1 nS) gap junction-mediated intercellular coupling was found in mouse adrenal slices [12]. By analyzing capacitative current kinetics, Moser [12] showed that chromaffin cells *in situ* display at least two current components with different kinetics: a fast component due to charging of the membrane capacitance of the patch-clamped cell and a slower component likely caused by charging membrane capacitances of adjacent coupled cells. In rats, the presence of gap junction-mediated intercellular communication between chromaffin cells has been ascertained for the first time by Martin and colleagues [6] who

demonstrated the transmission of electrical signals and associated calcium events between coupled chromaffin cells.

### *2.2. 1990s-2000s: Toward the identification of gap junction proteins in the adrenal medullary tissue*

Gap junctions consist of arrays of intercellular channels composed of head-to-head docking of hexameric assemblies (connexons) of tetraspan integral membrane proteins called connexin (Cx) in chordates [21]. Through a study dealing with the expression of gap junction connexins in endocrine and exocrine glands, Meda and colleagues [22] were the first to report the presence of immunoreactivity for Cx43 in the adrenal medullary tissue of adult rats. This result was further confirmed and extended by single cell RT-PCR identification of transcripts encoding Cx43 and Cx36 in rat chromaffin cells [6]. More recently, a third gap junction protein, Cx29, has been found to be expressed in S100-positive cells [23]. A more detailed chapter will be dedicated below to connexin expression with respect to adrenal medullary cell types.

### **3. Connexin expression in the adrenal medulla**

As illustrated in figure 2A, the adrenal medullary tissue encompasses several cell types. This includes mainly the neuroendocrine chromaffin cells, the non-endocrine sustentacular cells, the synaptic boutons arising from the splanchnic nerve and the blood vessels. This chapter reviews all the available data dealing with connexin expression in these different cell types (figure 2B).

To date, six connexins (Cx26, Cx29, Cx32, Cx36, Cx43 and Cx50) have been unambiguously found in the adrenal medullary tissue, with distinct cellular distribution and

depending also on the normal or tumoral state of the tissue (detailed below). As reported by Murray and colleagues [2], Cx31, Cx37, Cx40 and Cx46 proteins are expressed neither in rat, mouse, guinea-pig nor bovine adrenal medulla. Note that we recently identified Cx37 and Cx40 transcripts in the rat adrenal medulla (unpublished data). Table 2 summarizes the available data concerning the presence of connexin-related gap junctions in the adrenal medulla of different species.

### *3.1. Connexin expression in chromaffin cells*

Neuroendocrine chromaffin cells represent the dominant cell type of the adrenal medullary tissue, probably explaining why gap junctional communication is more documented in these cells. As illustrated in figure 2B, three connexins (Cx36, Cx43 and Cx50) can couple chromaffin cells. Rat chromaffin cells express both Cx43 [6, 22, 24] and Cx36 [6, 24]. Note that the transcripts encoding the two connexins can be simultaneously detected in the same chromaffin cell [6]. The dominant expression of Cx36 and Cx43 in chromaffin cells is noticeable but not surprising. First, chromaffin cells share their embryonic origin with neurons and Cx36 displays a preferential expression in cell types of neural origin [25, 26]. Second, Cx43 and Cx36 are two dominant connexins expressed in endocrine/neuroendocrine tissues [22, 27]. By contrast, mouse chromaffin cells dominantly exhibits a positive immunostaining for Cx36 ([28, 29] and our personal observations), and a very modest expression of Cx43 [2]. In human medulla, cells are coupled through Cx50 [30]. Unlike the expression of Cx36 and Cx43, which are detected in several species, the expression of Cx50 seems to be restricted to human.

It is noteworthy that the adrenergic or noradrenergic phenotype of gap junction-coupled chromaffin cells has not been addressed in any study. Yet, it is well established that the nature of the hormone secreted (*i.e.* noradrenaline and/or adrenaline) as well as the neuropeptides co-

stored with catecholamines in secretory granules, is stimulus-dependent. For example, adrenaline is preferentially released in response to muscarine [31]. On the other hand, splanchnic nerve stimulation at moderate frequency (5 Hz) induces a preferential increase in noradrenaline *versus* adrenaline in blood circulation, while higher frequencies (50 Hz) evoke a comparable increase of the two hormones and also trigger the release of neuropeptides [32]. The molecular and cellular mechanisms responsible for this selectivity are poorly understood. To date, we do not know whether gap junctions connect adrenaline-containing cells, noradrenaline-containing cells or both. Because i) these two populations of chromaffin cells are innervated and regulated by morphologically different nerve terminals originating from distinct spinal cord and brain regions [33-35] and ii) gap junctional coupling can be modulated by the cholinergic synaptic activity [36], addressing this question would help elucidating the mechanisms responsible for the selectivity of hormone release and understanding the role and contribution of gap junctional communication to the secretory process. Regarding this, it is tempting to speculate that cell-cell communication via gap junctions plays a role in harmonizing instructive signals within a specific chromaffin cell population. To address this question, a systematic study of the coupling (*i.e.* type of connexins, strength of coupling) should be performed in adrenergic *versus* noradrenergic cells. In rats, noradrenergic cells represent only 15-20% of the total cell number in the medulla [37] and are spread all over the tissue. It is at first glance difficult to conceive that gap junction communication could be a major pathway coordinating biological signals within this population. However, the cell location within the medulla was described using two-dimensional observation; a three-dimensional analysis might reveal an unsuspected organization, in continuous cell lobules for example, rendering a communication by gap junctions plausible.

### *3.2. Connexin expression in sustentacular cells*

The other main cell type present in the adrenal medullary tissue is composed of sustentacular cells, a non-neuroendocrine cell population. Sustentacular cells are homologous in nature with Schwann cells type and are immunoreactive for S100 protein [38, 39]. By using a transgenic mouse strain in which the coding region of Cx29 gene was replaced by the lacZ reporter gene, Eiberger and colleagues [23] found lacZ/Cx29 expression close to nerve fibers in S100-positive cells within the adrenal medulla (figure 2B). Based on the immunodetection of S100 protein and on their morphology, it is likely that Cx29 couples sustentacular cells. Note that LacZ signals also surrounds acetylcholine esterase-positive preganglionic sympathetic nerve fibers prior to their penetration into the adrenal capsula and inside the medulla. This finding extends previous reports describing the expression of Cx29 in glial cells [40]. In both neuronal and endocrine tissues, it has long been described that glial or glial-like cells (such as folliculostellate cells in the anterior pituitary) are highly coupled by gap junctions [41-43] and form a large-scale network. Based on common features between pituitary folliculostellate cells and adrenal sustentacular cells, we propose that gap junction-coupled sustentacular cells form a long-distance communication route, by which the sustentacular cell network may coordinate the exchange of instructive signals. Note that our assumption is consistent with a recent study suggesting that adrenal medulla sustentacular cells take an active part in Ca<sup>2+</sup> metabolism, regulating indirectly the synthesis and release of catecholamines from chromaffin cells [44]. Elucidating the function of gap junctions in sustentacular cells will await understanding the function of these cells.

### *3.3. Connexin expression in other adrenomedullary tissue components*

Beside chromaffin and sustentacular cells, the adrenal medullary tissues also contains ganglion cells, nerve fibers originated from preganglionic sympathetic axons, connective and

vascular tissue, small intensely fluorescent (SIF) cells and pluripotent stem cells [45-47]. Although the expression of gap junction proteins has not been reported in these cells yet, it is likely that they also express connexins. In particular, Cx37, Cx40 and Cx45 known to be expressed by the vasculature [48-50] are likely expressed in the adrenal, although Cx37 and Cx40 were detected neither in the rat, mouse, guinea-pig nor bovine adrenal glands [2].

It is noteworthy that the presence of cortical cells intermingled with chromaffin cells in the adrenal medulla [51] could introduce "artifactual" expression of connexins within the medulla, particularly when these are detected using biochemical approaches without visualization of their cellular localization. However, Cx43 being the major component of gap junctions in the adrenal cortex [2, 4], the presence of cortical cells in the adrenal medulla will thus affect only the distribution of this connexin.

As mentioned above, a gap junctional channel is formed by two apposed hemichannels, which are hexamers of connexins. Gap junctions can be homotypic (when formed by two identical hemichannels) or heterotypic (when formed by two different hemichannels). In turn, hemichannels of uniform connexin composition are called homomeric, while those with differing connexins are heteromeric. Because each connexin exhibits distinct biophysical properties [52-54], the composition of gap junction plaques significantly influences the subsequent behavior and functioning of coupled cell clusters. While homotypic or heterotypic gap junction channels are well documented between glial cells [43, 55, 56], no evidence for heterotypic channels has been reported yet in the adrenal medulla, but the enumeration of connexins expressed by the medullary tissue unlikely represents an exhaustive description. Since the cloning of the first members at the end of the 1980s, the connexin family has considerably expanded, bringing to about 20 the number of identified genes encoding connexins [57]. The expression of many connexins still remains to be examined in the adrenal medulla, we therefore cannot rule out the possibility that new gap junctional pathways

(connexin expression, connexon composition, permeability, regulation, ...) within the medullary tissue will be discovered in the future.

#### *3.4. Gap junctions in adrenal medullary tumors*

It is well known that gap junction-mediated cell-cell communication is altered in tumorigenesis [58-61]. In the adrenal medullary tissue, the more frequently encountered tumors are pheochromocytomas, which arise from catecholamine-secreting cells ([62-64], for three reviews). As illustrated in figure 3, connexin expression in the tumoral adrenal medulla remains poorly documented and to date, a unique study describing connexin expression in the human adrenal medulla has been published. In the rat tumoral chromaffin cell line PC-12, only Cx36 expression has been investigated and reported to be present [65]. Regarding human adrenal medulla, while Cx50 is the major connexin expressed in normal medulla, benign and malignant human pheochromocytomas appear also immunoreactive for Cx26 [30]. This result suggests that the acquisition of a tumoral phenotype in the human adrenal medulla might be accompanied by a change in connexin expression. More interestingly is the finding that expression of Cx50 in malignant human pheochromocytomas is significantly down-regulated in comparison to normal medulla and also in comparison to benign pheochromocytomas [30]. This later result could suggest the use of immunological testing for Cx50 to differentiate benign from malignant tumors, but the authors conclude their study by stating that immunological detection of connexin expression is not a reliable tool to distinguish between these two tumoral states [30]. Nevertheless, this work indicates that cancer cells exhibit a decrease of gap junctional intercellular communication and/or connexin expression. Consistent with a significant contribution of gap junctions in adrenal medulla tumorigenesis, gene deletion of Cx32 in mice can lead to the development of malignant adrenomedullary tumors [66]. Is a change in connexin expression related to enhanced catecholamine secretion

in pheochromocytomas? No experiment has been conducted yet to address this question. Nevertheless, it is noteworthy that Cx50 and Cx26 belong to distinct connexin families (alpha and beta group, respectively) [53, 67] and exhibit distinct biophysical properties (gating, phosphorylations, voltage sensitivity, ...). Consistently, gap junction-mediated regulation of stimulus-secretion coupling in chromaffin cells is likely modified in tumoral tissue. Accordingly, it is tempting to speculate that the switch in connexin expression pattern contributes to the increased catecholamine secretion observed in pheochromocytomas. In addition, gap junctional communications are strongly implicated in several cellular functions such as proliferation, mobility or adhesion, which are deregulated during tumor development. Indeed, numerous studies show evidence of gap junction-mediated intercellular coupling defect in tumorigenesis [58, 60] and several connexin genes are considered as members of a family of tumor-suppressor genes [59, 68, 69]. In summary, gap junction remodeling (change in connexin expression level and connexin isoform) during tumorigenesis in the adrenal medulla is ascertained but its contribution to tumorigenesis and catecholamine hypersecretion remains to be demonstrated. In particular, whether this change in connexin expression pattern originates or results from tumorigenesis is not yet known.

#### **4. Adrenal medullary hemichannels**

The assembly of gap junction channels results from the apposition of two hemichannels exported to the plasma membrane where they diffuse laterally into cell-contact regions to dock head-to-head with partner connexins present on the neighbouring cell [70, 71]. Hemichannels have long been thought only as structural precursors of gap junctions. However, they are also present in the non-junctional regions of the cell plasma membrane and can provide a direct communication pathway between the cytoplasm and the extracellular region.

Over the last decade, interest in the hemichannel research field increased so substantially that it is no longer tenable to review data on gap junctions without considering the role of hemichannels. Vertebrate hemichannels encompass two junctional protein families, connexins and pannexins [72, 73]. Pannexins display similar membrane topology to connexins but minimal amino acid homology. They are evolutionarily distinct from connexins, but form a single superfamily with invertebrate innexins [74, 75]. Pannexins and connexins mainly differ by the fact that pannexons do not assemble into gap junctions and form mainly hemichannels, while connexons rapidly assemble into gap junctions [76]. These two families also show distinct properties (unitary conductance, half-life, gating, ...) and have their own particular function [77] and sensitivity to extracellular calcium [76]. Compelling evidence suggests that hemichannels can open under certain conditions [78] and that ionic currents [79, 80] and small metabolites [81] can flow through hemichannels in the extracellular space. Connexons and pannexon, via complementary extracellular and direct cell-cell communication, may thus be involved in modulating cell activity and triggering long-range spread of signaling molecules and calcium waves [82]. To date, nothing is known about the expression and role of hemichannels in the adrenal gland. A unique study reporting a connexin hemichannel-mediated enhanced neurite outgrowth in transfected PC-12 cells has been published [83]. Nevertheless, based on i) the expression of Cx36 and Cx43 in chromaffin cells [6] and ii) the fact that Cx36 and Cx43 can form functional hemichannels [78, 84-87], it is quite likely that connexons are functional in the adrenal medullary. The pannexin gene family has been first cloned in 2003 [88]; three pannexin isoforms have been described in humans and rodents (Panx1-3). Since Panx1 and Panx2 expression has been reported in neurons [89, 90] and chromaffin cells share a common embryonic origin with neurons, it seems reasonable to hypothesize that pannexons are present in the adrenal medullary tissue. Very recently, mRNA and protein transcripts of Panx1 and Panx2, but not Panx3 have been found in the pituitary

gland, with a preferential distribution of Panx1 in the anterior lobe [91]. The presence of pannexins in the endocrine pituitary tissue reinforces the hypothesis that they could be expressed in the adrenal medulla. Additionally, pannexins are expressed in glial cells [92, 93], opening the possibility that pannexons may be present in sustentacular adrenal medullary cells. Accordingly, our preliminary data showing the presence of RNA transcripts encoding Panx1 and Panx2, but not Panx3, in the rat medulla are consistent with the hypothesis of pannexon expression in the adrenal gland (unpublished observations).

Because hemichannels (both connexons and pannexons) are described as a route for extracellular release of biologically relevant molecules such as ATP [86, 94, 95], glutamate [96], NAD<sup>+</sup> [97], glutathione [98] or prostaglandin E2 [99], we can reasonably propose that adrenal medullary hemichannels could have roles in autocrine or paracrine communication targeting chromaffin cells and several cell types. This could be of a particular physiological interest for at least two reasons. First, it is well established that ATP, one of the major components of chromaffin cell secretory granules, regulates chromaffin cells in an autocrine or paracrine manner via its action on specific purinergic P2 receptors. It exerts both positive [100] and negative effects on catecholamine secretion [101] and regulates the function of voltage-dependent calcium channels [102-106]. Although ATP is secreted from secretory granules in response to sympathetic nervous system stimulation, one cannot exclude that it may also be released via other mechanisms involving activation and opening of hemichannels. Second, evidence is increasing for a role of glutamate as an extracellular signal mediator in endocrine systems, in addition to its excitatory amino acid neurotransmitter role in the central nervous system. In the adrenal medullary tissue, chromaffin cells are able to release glutamate in response to depolarization, through both exocytotic and non-exocytotic pathways [107]. Although reversion of the electrogenic glutamate transporter is described to support the non-exocytotic pathway, it is tempting to propose that connexin-built

hemichannels also contribute to glutamate release. It is noteworthy that changes in membrane permeability via hemichannels can have positive consequences in some cells, whereas in others hemichannel activation can be detrimental, as reviewed [108].

Interestingly, many functions supported by hemichannels (*i.e.* release of signaling molecules such as ATP or arachidonic acid derivatives, channel opening in response to extracellular ATP via an interaction with the ionotropic P2X7 ATP receptor [90, 93, 109, 110]) could potentially modulate the stimulus-secretion coupling in chromaffin cells. Additionally, pannexins are involved in tumorigenesis [92, 111] and various pathological stimuli exert their deleterious effects through an activation of pannexons [112-115].

All these data strengthen the assumption that connexons and/or pannexons might be additional partners involved in the regulation of the adrenal medulla physiology and pathology.

## **5. Physiological relevance of gap junctions in the adrenal medulla: involvement in stimulus-secretion coupling**

Until the early 2000s, the current view regarding stimulus-secretion coupling in the adrenal medulla was that there is no need for a direct coupling between chromaffin cells to ensure hormone release since each chromaffin cell receives its own synaptic input. However, a series of recent data clearly indicate that the situation is far more intricate than previously envisioned.

### *5.1. Propagation of electrical signals between chromaffin cells*

Using dual patch-clamp recordings of cell pairs, Martin and colleagues [6] have recorded junctional currents between rat chromaffin cells in acute adrenal slices. This clearly shows

that electrical signals can propagate from one cell to an adjacent one through gap junction channels. In adult rat, the percentage of coupled cells significantly differs between females (~40%, [6, 12]) and males (~20%, [24]). A high percentage of coupled chromaffin cells (> 60%, [6, 12]) is also observed in the female mouse. By contrast, the coupling strength appears similar in male and female rats. Most of the coupled chromaffin cell pairs are weakly coupled, as evidenced by a low macroscopic junctional conductance (<0.5 nS in 75% and 100% of coupled cell pairs in female and male rats, respectively [6, 24]). A similar low conductance intercellular coupling (<1 nS) has been reported between mouse chromaffin cells [12]. Consistent with a weak electrical coupling, an action potential evoked in a single cell is distorted and leads to a small depolarization in the coupled cell. Although such a coupling is too low to support spreading of electrical activity between coupled cells, it could allow longer depolarization of one cell or simultaneous firing of several cells to trigger electrical activity in neighboring cells. As reported in excitable pituitary cells [116], even small amplitude (<10 mV) depolarization occurring near the resting potential can induce sustained increases in cytosolic calcium concentration. These  $[Ca^{2+}]_i$  rises might in turn control  $Ca^{2+}$ -dependent cell functions, other than exocytosis (secretory vesicle trafficking, gene expression, ...). Although a weak junctional coupling is mainly observed between chromaffin cells in resting conditions, some coupled cell pairs exhibit a more robust coupling (macroscopic conductance >1 nS), leading to the transmission of suprathreshold responses and action potential firing in the coupled cells.

### *5.2. Synchronized $Ca^{2+}$ transients between chromaffin cells*

Imaging spontaneous or action potential-evoked  $Ca^{2+}$  transient between chromaffin cells *in situ* revealed that synchronized  $Ca^{2+}$  signals frequently occur, both in rat [6] and mouse [117]. It is noteworthy that, at rest, the extent of simultaneous  $Ca^{2+}$  transients is restricted to only 2-3

cells within a cluster. A gap junction-built route between chromaffin cells likely contributes to synchronized  $\text{Ca}^{2+}$  transients since i) the cells exhibiting synchronized  $\text{Ca}^{2+}$  signals are gap junction-coupled, as evidenced by Lucifer yellow diffusion [6, 24], ii) the signal propagation is blocked by the uncoupling agent carbenoxolone, and iii) the concomitant  $[\text{Ca}^{2+}]_i$  rises between chromaffin cells occur with fast kinetics ( $\sim 500 \mu\text{m}/\text{sec}$ ) [6].

### 5.3. *Involvement in catecholamine release*

In endocrine glands in which secretagogues are delivered as a bolus from the blood circulation, it is well known that gap junctions are required to ensure fast synchronized hormone release [118, 119]. In the adrenal gland, the situation differs by the fact that the physiological stimulus for catecholamine secretion is delivered to each chromaffin cell individually, in the form of a synaptic release of acetylcholine at the splanchnic nerve terminal-chromaffin cell junction [120, 121]. However, even though the synaptic boutons present on each chromaffin cell by themselves represent an efficient process to induce fast release of catecholamines, the gap junction-delineated route is also used for chromaffin cell exocytosis. Using amperometric detection of catecholamine release in adrenal acute slices, Martin and colleagues [6] have shown that an iontophoretic application of nicotine on a single cell triggered catecholamine exocytosis from gap junction-coupled cells. The authors proposed that the functional coupling between adjacent chromaffin cells *in situ* represents an efficient complement to amplify catecholamine release after synaptic stimulation of a single excited chromaffin cell, thus validating a hypothesis raised two decades before [5].

Although indirect, another indication supporting the possible involvement of gap junctions in catecholamine release is provided by the convergence of data showing that i) adrenal medulla gap junctional communication is wider in females [6] versus males [24], and ii) sympathoadrenal activity and plasma catecholamine levels are also higher in females [122]. A

plausible explanation would be that connexins expression levels may be higher in female. Accordingly, the expression of connexins (Cx36 and Cx43 in particular) is regulated by steroid hormones in a variety of tissues, as shown in the rat myometrium [123] or in the suprachiasmatic nucleus of female rat [124]. Furthermore, Cx43 exhibits a sexually dimorphic hormonal regulation, as reported in hypothalamic astrocytes [125]. In addition to the fact that gap junctions between chromaffin cells are likely involved in regulating catecholamine release, direct cell-cell communication between other adrenal medullary cell types may also contribute to this regulation. For example, sustentacular cells, connected to each other by gap junctions as pituitary folliculostellate cells are [42], would form an extended network intermingled with the chromaffin cells. Gap junction-based communication within this network would be an efficient tool to coordinate the release of small signalling molecules within or between lobules and regulate catecholamine release from adjacent chromaffin cells. To definitively ascertain the contribution of gap junctions to catecholamine release, *in vivo* studies are now required.

An additional putative role for gap junctional communication in the adrenal medulla concerns the basal electrical activity of chromaffin cells and the regulation of the basal circulating catecholamine concentration. Indeed, in culture and slice preparations, a spontaneous action potential firing is observed. This may be in part due to spontaneous acetylcholine release from presynaptic boutons [18, 36] and/or to the presence of a pacemaker current underlying regular or phasic firing activities [126]. This spontaneous activity is modulated by several secretagogues and by  $K^+$  ions that are present in the blood and are regulated by physiological conditions known to affect the catecholamine secretion. As pointed out recently by Vandael and colleagues [126], it is highly plausible that the command brought by blood vessels spreads into the depth of cell lobules via direct cell-cell communication

involving gap junctions and also possibly via paracrine communication ensured by connexon- or pannexon-built hemichannels.

## **6. Gap junctional communication remodeling in the adrenal medulla**

An intriguing property of gap junctions in the adrenal medulla, which reinforces the hypothesis of their functional importance, is their capacity of acute or persistent remodeling along life and in response to specific physiological [127] or pathological [30] situations. The remodeling can occur at several levels including changes in connexin expression pattern, connexin expression level or in cell-cell communication behavior. Because we have already described the extreme changes in connexin expression pattern and level associated with tumorigenesis in the adrenal gland (see chapter 3.4), we will focus here on more subtle regulations of gap junctional communication occurring in physiological circumstances. As illustrated in figure 4, cholinergic synaptic transmission impairment [36], postnatal development [128], or stressful situations [24, 129] are particular conditions associated with a significant remodeling of gap junction-mediated chromaffin cell coupling. This plasticity encompasses both an increased number of electrically/dye-coupled cells (figure 4A) and an increased coupling strength (figure 4B).

### *6.1. Remodeling in the perinatal period: a role for coping with hypoxia?*

The main striking feature with respect to stimulus-secretion coupling in the perinatal adrenal medulla is the non-neurogenic control of catecholamine secretion. Indeed, at birth, synaptic transmission between splanchnic nerve terminals and chromaffin cells is non competent and it completely matures during the first postnatal week [130, 131]. Conversely, gap junctions between chromaffin cells are predominantly expressed in neonates when compared with

adults [36, 128] (figure 4), and they support the propagation of action potential-linked  $[Ca^{2+}]_i$  rises between cells [36]. We therefore propose that gap junctional communication might be one of the adrenal medullary determinants supporting the non-neurogenic control of catecholamine release. If so, one could expect that gap junctions play a crucial role in the hormone secretion that protects individuals against transient hypoxia at birth. From a metabolic and respiratory point of view, birth has to be considered as a traumatic experience for all mammals. Indeed, foetus expulsion is associated with major hypoxia that may have deleterious consequences. In human, most premature infants experience intermittent hypoxia as a consequence of recurrent apneas. Catecholamine secretion from the adrenal medulla is critical during these periods to stimulate the cardiovascular system and maintain homeostasis under hypoxic stress. Yet, the innervation of the adrenal medulla is immature at birth and the non-neurogenic catecholamine release constitutes the autonomous response from chromaffin cells to asphyxial stressors [132, 133].

It is now established that in perinatal adrenal chromaffin cells,  $K^+$  channels contribute to acute oxygen sensing [134-137]. Indeed, acute hypoxia directly stimulates catecholamine release from neonate chromaffin cells [131, 138] via  $K^+$  channel inhibition [139]. This results from inhibition of mitochondrial respiration and subsequent decrease in mitochondrial-derived reactive oxygen species (ROS) [137, 140, 141]. The hypoxic inhibition of  $K^+$  channels leads to depolarization [142-144], which in turn activates voltage-gated  $Ca^{2+}$  entry mainly through T-type and L-type  $Ca^{2+}$  channels and catecholamine secretion [142-144]. In the case of chronic intermittent hypoxia, it is well established that ROS levels are also modified, highlighting their crucial role in the non-neurogenic response of chromaffin cells to hypoxia [137]. All these findings raise the question of the physiological relevance of the predominant gap junctional communication between neonate chromaffin cells with respect to the hypoxic episode at birth. Although this issue has not been addressed directly yet, several

findings are consistent with an involvement of gap junctions. First, the non-neurogenic response to hypoxia involves  $\text{Ca}^{2+}$  ions and ROS, two messengers that are diffusible through gap junctions [145-149]. Second, only a proportion of neonate chromaffin cells are able to respond directly to hypoxia [150]. We propose that gap junctional communication facilitates the diffusion of hypoxic cell-derived signal (ROS would be one candidate) to the whole chromaffin cell population, thus helping to trigger a massive catecholamine release. Third, the direct sensitivity to hypoxia of neonatal chromaffin cells is greatly attenuated at 1-2 postnatal weeks [151], coinciding with a significant reduction of gap junctional communication and the complete maturation of splanchnic innervation. In addition, it has also been shown that in adult rats, the hypoxic sensitivity is regained when nerve traffic is interrupted for an extended period, indicating that prolonged deprivation of neural input leads to the re-emergence of non-neurogenic capabilities [151]. Interestingly, splanchnic denervation in the adult rat is also accompanied by an up-regulation of the gap junctional communication between chromaffin cells [36], reinforcing the correlation between the non-neurogenic response and gap junctional coupling.

#### *6.2. Remodeling during postnatal development: a role for establishing functional chemical synapses?*

As mentioned before, gap junctional communication between chromaffin cells is predominant in perinatal adrenal medullary tissue [36, 128] (figures 4 and 5). During postnatal development, the number of gap junctions in the adrenal medulla gradually decreases, coinciding with the establishment of functional cholinergic synapses between splanchnic nerve terminals and chromaffin cells. This finding is not restricted to the adrenal medullary tissue. Indeed, in the developing nervous system, gap junction-mediated electrotonic coupling is widespread among neurons [152-157], and gap junction-coupled neuronal assemblies often

precede the formation of synaptically connected neuronal network [158]. In addition, the number of gap junctions gradually decreases during postnatal development, coinciding with the establishment of functional chemical synapses [159]. A rapid developmental switch from electrical to chemical communication has also been reported during the construction of cortical columnar networks [160]. In the adrenal medulla, the gap junctional pathway between chromaffin cells decreases from 65% coupled cells in newborn rats to 40 and 20% in female and male adult rats, respectively [6, 24, 36]. In addition, the coupling strength also decreases during postnatal development. While 80% of coupled chromaffin cell pairs in neonates display a robust coupling (assessed by a macroscopic junctional conductance  $>1$  nS), only 20-25% of coupled cells in adults are robustly coupled [6, 128]. A not yet investigated issue concerns the expression pattern of connexins in neonate chromaffin cells. Does it differ from adults? Although this question remains to be experimentally addressed, the presence, both in neonates and in adults, of two coupled chromaffin cell populations (weakly and highly coupled) exhibiting similar macroscopic junctional conductance suggests that, at least, the same connexins might be expressed.

The regulatory mechanisms that underlie synaptogenic progression from electrical to chemical neurotransmission in the adrenal medulla are still poorly understood. As reported by Martin and colleagues [128, 161], the extracellular matrix protein agrin, when applied on acute neonate adrenal slices, renders neonate chromaffin cells readily responsive to synaptically-released neurotransmitters, while foregoing reducing information transfer through gap junctions. This likely contributes to promote acquisition of the neurogenic control of the stimulus-secretion coupling. To date, the involvement of other factors in the adrenal medulla developmental switch from electrical to chemical coupling has not been reported. Nevertheless, it is quite likely that other factors may contribute to the synaptogenic progression from electrical to chemical coupling, as reported in the developing central

nervous system regarding the developmental uncoupling of gap junctions via CREB-dependent downregulation of Cx36 [162].

### *6.3. Remodeling in response to synaptic impairment: an assistance to catecholamine release?*

As mentioned above, adrenal catecholamine secretion from chromaffin cells is under a dual control involving the synaptic cholinergic transmission and the gap junctional coupling between chromaffin cells, although to a lesser extent. This raises the question of functional links that may exist between these two forms of intercellular communication in the adrenal medulla. Interestingly, under experimental conditions associated with a cholinergic synaptic impairment (acute pharmacological blockade of postsynaptic nicotinic receptors or surgical adrenal denervation), gap junction-mediated coupling between chromaffin cells exhibits a significant up-regulation [36] (figures 6 and 7). Not only the percentage of coupled chromaffin cells is enhanced, but also the coupling extent. This upregulated gap junctional coupling is physiologically relevant, as evidenced by the increased extent of action potential-evoked synchronous multiple  $[Ca^{2+}]_i$  rises in chromaffin cell clusters [36]. Consistent with these data, enhanced synaptic activity (pharmacological stimulation of splanchnic nerve terminals by the  $Ca^{2+}$  ionophore ionomycin, figure 6A-C for original data) or persistent activation of nAChRs (acute bath-application of nicotine or acetylcholine) leads to a decrease in gap junctional coupling evidenced by Lucifer yellow diffusion between chromaffin cells (figure 6D).

All these findings indicate that i) the gap junctional coupling between chromaffin cells is subject to both acute (<1 hour) and persistent (many weeks) plasticity, ii) synaptic neurotransmission exerts a tonic inhibitory control on gap junctional cell-cell communication, and iii) the modulation of gap junction coupling is important under pathological conditions in

which synaptic transmission is reduced. By compensating for the loss/reduction in chemical neurotransmission, gap junction signaling could sustain catecholamine release by maintaining communication within the chromaffin cell network. Once again, this conclusion is not limited to the adrenal medullary tissue. In other neuronal structures, chemical neurotransmission has also been reported to modulate gap junctional communication [163] [164-166].

#### *6.4. Remodeling in response to stress: a contribution to enhanced catecholamine secretion?*

In stressful situations, the demand in catecholamines becomes higher, to help the organism to cope with stress [167]. Consistent with a crucial role of the adrenal gland in stress-induced catecholamine secretion, stimulus-secretion coupling is critically affected in “stressed” medulla [127]. Indeed, stress promotes a functional remodeling of both gap junctional coupling between chromaffin cells and cholinergic synaptic neurotransmission (figure 7). For instance, a 5-day cold stress induces a simultaneous up-regulation of both pathways [24, 129, 168]. Briefly, regarding synaptic transmission determinants, the “stressed” gland displays i) a higher density of nerve fibers innervating the medulla associated with an increased frequency of spontaneous post-synaptic currents [129] and ii) a dominant contribution of  $\alpha 9$ -containing nicotinic receptors to acetylcholine-evoked currents [168]. It is noteworthy that stress also affects chromaffin cells directly by increasing their excitability [129]. Gap junctional coupling is also remodeled in response to stressful situations. Both metabolic (evidenced by the passive diffusion of Lucifer yellow between gap junction-coupled chromaffin cells) and electrical (evidenced by dual patch-clamp recording of junctional currents in cell pairs) coupling between chromaffin cells are dramatically up-regulated in cold stressed rats [24]. In addition, a change in the electrical coupling strength is observed with the appearance of a robust coupling in ~50% of coupled cell pairs (macroscopic junctional conductance  $>1\text{nS}$ ) (figure 4).

Since it allows the transmission of action potentials between coupled cells, such a robust coupling is of a particular physiological relevance. Indeed, transmitted action potentials in coupled cells are efficient to trigger catecholamine release in these cells [6]. The enhancement of gap junctional coupling parallels an increase in expression levels of Cx36 and Cx43 proteins [24]. Interestingly, a similar correlation between an increased expression of junctional proteins and an increased insulin secretion has been also observed in cultured neonatal pancreatic islets [169, 170]. The current hypothesis is that gap junction-mediated communication between endocrine/neuroendocrine cells could be the main tool used by secretory tissues to dynamically adapt to an increased hormonal demand.

It is still unclear whether similar remodeling processes (change in connexin expression level, change in chromaffin cell-cell coupling strength) occur in response to other stressors. Beginning of an answer is provided by our recent data showing that the adrenal medullary tissue of rats exposed to a restraint stress is also remodeled. Both gap junctional communication (Lucifer yellow diffusion) and medulla innervation (neurofilaments immunolabeling) are up-regulated [129]. This suggests that the reshapes reported here might commonly occur in response to various stressors. However, because the response of the adrenal medulla is stressor specific [171], one could reasonably raise the hypothesis that other adaptive mechanisms take place in the adrenal medulla, also enabling the organism to cope with stress. In particular, adrenal medullary peptides (pituitary adenylate cyclase-activating polypeptide, substance P, neuropeptide Y, adrenomedullin, ...) which are co-secreted along with catecholamines in physiological and pathological conditions are good candidates. They have been reported to contribute to homeostatic regulations of the adrenal medulla and to play a role in the stress response and subsequent catecholamine secretion [172-174]. Another possible adaptive mechanism is the remodeling of gene expression, as exemplified by the contribution of glucocorticoids in the gene induction of catecholamine biosynthesis-related

enzymes (reviewed in [175]). Altogether, it is likely that a combination of all these changes contributes to optimize the stimulus-secretion coupling efficiency in the adrenal gland; a good adaptive response to stress needs the coordination of all of these molecular, cellular, and tissular mechanisms.

It is noteworthy that the “stressed” medulla represents an interesting model in which interactions between gap junction-mediated coupling and synaptic transmission are differently regulated from the “unstressed” medulla. In particular, the tonic inhibitory control exerted by synaptic neurotransmission on gap junctional communication that occurs in unstressed rats [36] is masked in stressed animals. The underlying mechanisms have not been investigated yet. One plausible possibility would be the synaptic release of non-cholinergic factors (ATP, nitric oxide, ...) or neurotransmitters (VIP, PACAP, ...) by presynaptic terminals [176-180]. Indeed, the relative amplitude of their release depends on the pattern of electrical activity in the incoming nerve [177, 181]. In addition, some of these transmitters are known to regulate gap junctional cell-cell communication [182-184]. Although not yet investigated in the adrenal medulla, one of their functions would be to counteract acetylcholine-mediated regulation of gap junctional communication between chromaffin cells.

## **7. Adrenomedullary gap junctions: possible repercussions on adrenocortical functions**

In mammals, adrenocortical and adrenomedullary tissues are interwoven to an astonishing degree with cortical cells located within the medulla and vice versa. This is functionally relevant since close interactions between adrenocortical cells and chromaffin cells are involved in both physiological and pathological processes (recently reviewed in [51, 185, 186]). In addition to well-described paracrine communications between chromaffin and cortical cells [2] and although no data ascertain this hypothesis yet, it is quite likely that

adrenomedullary gap junctional communication influences activities of cortical cells and further release of steroids and sex hormones. In particular, it has been shown that catecholamine release locally supports influence of chromaffin on neighboring cortical cells [187]. Thus, we propose that, by modulating catecholamine secretion, gap junctional coupling between chromaffin cells indirectly affects cortical cell function. Reciprocally, as reported for induction of catecholamine enzymes in chromaffin cells by glucocorticoids [188], steroids and sex hormones released from the cortex may also influence junctional coupling in the medulla.

## **8. Contribution of gap junctions to catecholamine release *in vivo*: a recurrent question mark**

The *in vivo* regulation of catecholamine secretion is likely a tightly regulated process, an excess or lack of catecholamines may have deleterious consequences. High circulating plasma catecholamine levels are associated with many multifactor diseases such as diabetes or dysfunction of the cardiovascular system (myocardial infarcts, hypertension) [189, 190]. Alterations of catecholaminergic systems may also be involved in various pathologies, as recently proposed for sudden infant death syndrome [191]. Conversely, low resting plasma adrenaline levels can be associated with an unfavorable survival rate in response to stressful situations [192]. Considering the proposed contribution of gap junctional communication to catecholamine release, it is likely that gap junctions play a role in altered catecholamine level-related pathologies.

To date, all findings strengthening the contribution of gap junction-mediated cell-cell communication to catecholamine secretion have been obtained *ex-vivo*, in acute adrenal slices. This raises the question of whether gap junction coupling also plays a role in hormone

secretion *in vivo*, in the intact gland. Ceña and collaborators [5] performed the first study raising the hypothesis of an involvement of a direct cell-to-cell communication between adrenal chromaffin cells, to amplify the secretory signal in the intact gland. From a morpho-functional point of view, it has long been known that the triggering of hormone release is chiefly achieved by synaptic neurotransmission at the splanchnic nerve-chromaffin cell contacts and that each chromaffin cell receives several synaptic inputs [193]. Consequently, it has been thought, if not still the case, that there was no need for an additional stimulatory signal exchanged between chromaffin cells, and indeed, the question of the involvement of adrenal medullary gap junctions to hormone release *in vivo* is recurrent and current views are controversial. To address this ultimate issue, it would be necessary to perform *in vivo* experiments in which the manipulation of gap junctions (pharmacological or immunological blockade, use of connexin-deficient mouse models,...) would be combined with both simultaneous splanchnic nerve stimulation and electrophysiological recordings of adrenal medullary cells and blood measurement of catecholamines. In a study published in 2004, Akiyama and colleagues [194] conducted *in vivo* measurements of catecholamine secretion in response to splanchnic nerve stimulation, but the contribution of gap junctions was not investigated. Also, to our knowledge, no information is available regarding plasma levels of adrenaline, which originates mainly from the adrenal medulla, in connexin knock-out mice.

## **9. Concluding remarks and future perspectives**

Compilation of all the data enclosed in this review shows that adrenal stimulus-secretion coupling is far more intricate than previously envisioned and introduces the gap junctional communication between chromaffin cells as an additional ingredient. Indeed, connexins are specifically expressed in various cell types in the adrenal medulla and their expression, as

well as the functionality of the connexin-based gap junctions, is tightly regulated during development and in response to physiological/pathological situations [127]. Keeping in mind that i) synaptic transmission arising from the splanchnic nerve is the major stimulus of catecholamine secretion and ii) each chromaffin cell receive several synaptic boutons, the added value of a gap junction-mediated communication between chromaffin cells remains questionable. Regarding the involvement of gap junctions in hormone release, our proposal is that gap junctional coupling acts as a modulator of synaptic transmission-dependent catecholamine secretion. By allowing cell-cell propagation of electrical and ensuing calcium signals, gap junctions are ideal candidates to complement the incoming nervous command from the splanchnic nerve and coordinate, enhance or limit, catecholamine secretion from chromaffin cells [195]. In case of reduced or impaired synaptic transmission, they would act in synergy to facilitate signal propagation and subsequent catecholamine release. Conversely, in response to a high nerve firing frequency, they would counteract synaptic transmission to impair signal propagation and avoid a huge release of potentially toxic molecules harmful for the organism or even lethal.

Many issues still remain unsolved. The most important concerns undeniably the role of gap junctions in the excitation-secretion coupling *in vivo*. Although the contribution of gap junctions to catecholamine release *in vivo* is not yet ascertained, numerous evidences arising from both *in vitro* and *ex-vivo* studies strongly support it. Moreover, it is likely that, beside their involvement in catecholamine secretion, adrenal medullary gap junctions coupling both endocrine and non-endocrine cell populations contribute to many other cell functions, such as growth, differentiation or apoptosis. This hidden face of adrenal medullary gap junctions still remains to be investigated.

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## **Figure legends**

### **Figure 1: Historical overview of the main findings regarding gap junctions in the adrenal medulla.**

The 1980s are marked by the first description of the presence of gap junctions in the adrenal medullary tissue. In the 1990s, a possible function for a gap junctional coupling between chromaffin cells is proposed. It was only during the 2000s that studies of this cell-to-cell communication mechanism are growing significantly. The references are indicated in square brackets.

### **Figure 2: Anatomical organization of the adrenal medulla: relationship between cell types and their intercellular gap junction-mediated connections.**

**A.** Schematic representation of an adrenal medullary lobule. Beside numerous chromaffin cells, a lobule mainly encompasses splanchnic nerve endings synapsing onto chromaffin cells (cholinergic synapse), the non-endocrine sustentacular cell network and blood vessels. **B.** Major connexins expressed in the mammalian adrenal medullary tissue. Note that chromaffin cells express several connexins, whereas sustentacular cells appear to be coupled by gap junctions formed by Cx29 only.

### **Figure 3: Summary of the connexin subtypes expressed in human pheochromocytomas and in the rat tumoral PC12 cell line.**

Note the significant differential expression of Cx50 between benign and malignant human pheochromocytomas. Except for Cx36 detected by PCR in PC12 cells, the identification of connexins was performed by immunohistological staining. The corresponding references are

indicated in square brackets. (-, not expressed; +/-, weakly expressed; +, moderately expressed; ++, highly expressed).

**Figure 4: Gap junctional remodeling in response to physiological/physiopathological conditions in which the stimulus-secretion coupling is affected: a study in rat**

**A.** Histogram illustrating the percentage of chromaffin cells exhibiting a dye (Lucifer yellow) coupling. Note the significant difference observed between male and female in control conditions. To help comparison, the sex of rats used in each study is indicated in parentheses. The reference is indicated in square brackets below each histogram bar. **B.** Histogram illustrating the distribution of weak and robust coupling in the same conditions as in A. In control rats, chromaffin cells are weakly coupled, whilst the percentage of cells exhibiting a robust electrical coupling dramatically increases in response to decreased cholinergic activity, in neonates and in stressed rats.

**Figure 5: Adrenal medullary tissue remodeling during postnatal development.**

In the adrenal medulla, postnatal development is associated with the establishment of the neurogenic control of catecholamine secretion by chromaffin cells. This is achieved by a switch from a predominant gap junctional coupling to a fully mature synaptic transmission. By sequentially decreasing gap junctional signaling and increasing cholinergic synaptic transmission, the proteoglycan agrin plays a crucial role in promoting the neurogenic control of catecholamine secretion. Note that the possible developmental remodeling of gap junctional coupling between sustentacular cells remains to be investigated.

**Figure 6: Cholinergic activity-dependent gap junctional plasticity in the rat adrenal medulla: a bidirectional modulation**

**A-C.** Effect of bath-applied ionomycin on spontaneous excitatory postsynaptic currents (EPSCs). **A.** Representative chart recordings of spontaneous EPSCs recorded in a chromaffin cell voltage-clamped at -80 mV, before (left panel) and after (right panel) bath-applied 2.5  $\mu$ M ionomycin. **B and C.** Analysis of ionomycin-induced changes in synaptic current frequency (**B**) and amplitude (**C**). Note that the spontaneous EPSC frequency, but not the amplitude, is modified in response to ionomycin, indicating a presynaptic site of action. **D.** Histogram showing that conditions associated with a reduced or impaired cholinergic activity (black bars) lead to a significant up-regulation of the coupling probability (evidenced by Lucifer yellow diffusion). Hexamethonium and  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) were used to antagonize postsynaptic nicotinic acetylcholine (ACh) receptors. Denervation consisted in a surgical unilateral splanchnectomy. These two latter sets of data come from reference [36]. Conversely, in response to an enhanced cholinergic activity (gray bars), the coupling probability decreases (unpublished personal data). Ionomycin (2.5  $\mu$ M) was used to increase the frequency of synaptic events (as illustrated in **B**); sustained stimulation of postsynaptic nAChRs was achieved by a prolonged bath-application of nicotine (200 nM) or ACh (200 nM).

**Figure 7: Schemes illustrating gap junctional communication plasticity occurring between chromaffin cells in response to a variety of physiopathological conditions.**

The upper part illustrates the gap junctional coupling and cholinergic synaptic neurotransmission in a control adult rat. The two physiopathological conditions illustrated in this figure (synaptic impairment and stress) are associated with an increased gap junction-mediated intercellular communication between chromaffin cells. Note also that the possible

remodeling of gap junctional coupling between sustentacular cells in response to synaptic impairment or stress still remains to be investigated.

**Table 1: Input resistance values: comparison between dissociated chromaffin cells and chromaffin cells in situ**

species	dissociated chromaffin cells	technical approach	chromaffin cells <i>in situ</i>	technical approach	references
rat	>400 M $\Omega$	intracellular			[10]
rat			~30-35 M $\Omega$ (isolated gland)	intracellular	[7]
bovine	5-10 G $\Omega$	patch-clamp			[11]
mouse			~100 M $\Omega$ (hemisectioned gland)	intracellular	[8]
guinea-pig			~200 M $\Omega$ (hemisectioned gland)	intracellular	[9]
rat			5-10 G $\Omega$ (acute slices)	patch-clamp	[18]
rat			1-2 G $\Omega$ (acute slices)	patch-clamp	[20]
mouse	~10 G $\Omega$	patch-clamp	~3 G $\Omega$ (acute slices)	patch-clamp	[12]
rat			800-900 M $\Omega$ range: 130 M $\Omega$ -3 G $\Omega$ (acute slices)	patch-clamp	[6]
rat	~2 G $\Omega$	perforated patch-clamp			[13]
mouse	>2 G $\Omega$	perforated patch-clamp			[14]

**Table 2: Summary of the connexin protein subtypes expressed in normal adrenal medulla of various species**

Data collected from the combination of western blot (WB), immunostaining (IS), single-cell PCR (PCR), real-time PCR (qPCR) and  $\beta$ -galactosidase assay ( $\beta$ -gal) techniques. Corresponding references are indicated in square brackets.

	rat	mouse	guinea-pig	human
<b>Cx26</b>	- IS [2]	n.i.	n.i.	- IS [30]
<b>Cx29</b>	n.i.	++ $\beta$ -gal [23]	n.i.	n.i.
<b>Cx31</b>	- IS [2]	- IS [2]	- IS [2]	n.i.
<b>Cx32</b>	- IS [2]	- IS [2]	- IS [2]	- IS [30]
<b>Cx36</b>	+ IS, PCR, WB, qPCR [6, 24]	++ $\beta$ -gal, WB, IS [28, 29]	n.i.	n.i.
<b>Cx37</b>	- IS [2]	+ IS [2]	- IS [2]	n.i.
<b>Cx40</b>	- IS [2]	+ IS [2]	- IS [2]	n.i.
<b>Cx43</b>	++ IS, PCR, WB, qPCR [6, 22, 24]	+/- IS [2]	+/- IS [2]	- IS [30]
<b>Cx46</b>	- IS [2]	- IS [2]	- IS [2]	n.i.
<b>Cx50</b>	n.i.	n.i.	n.i.	++ IS [30]

(-, not expressed; +/-, weakly expressed; +, moderately expressed; ++, highly expressed; n.i., not investigated)

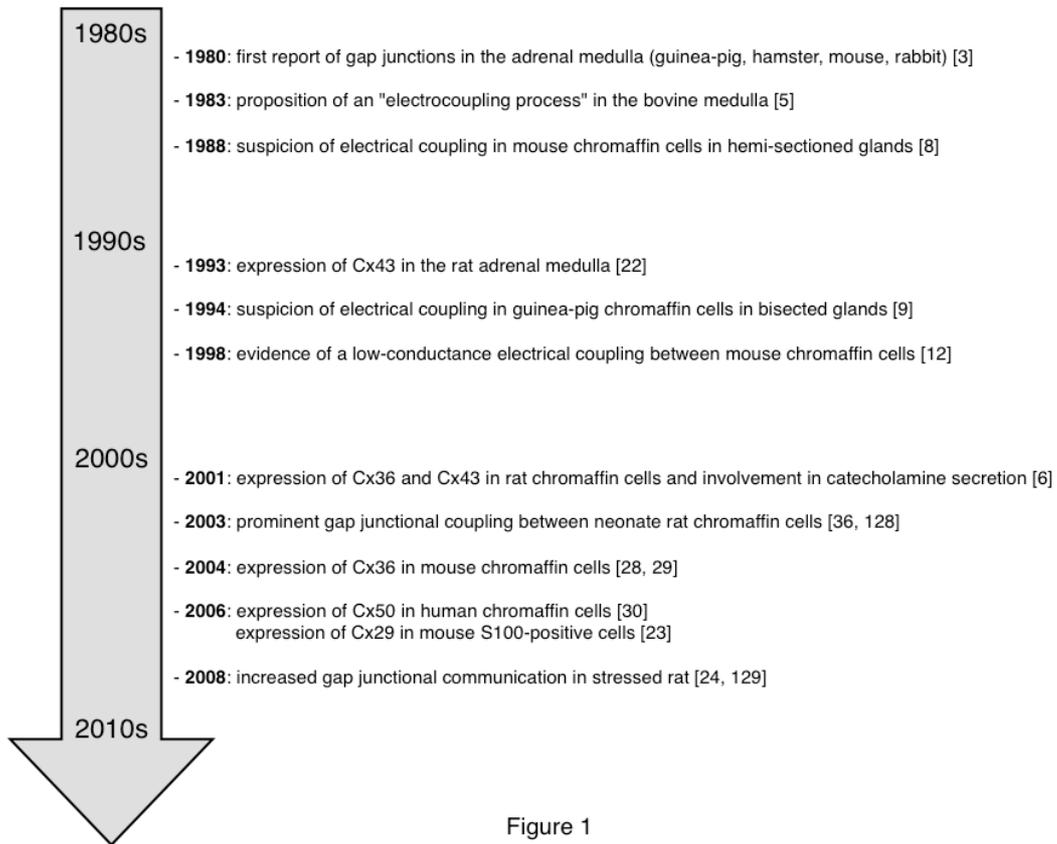
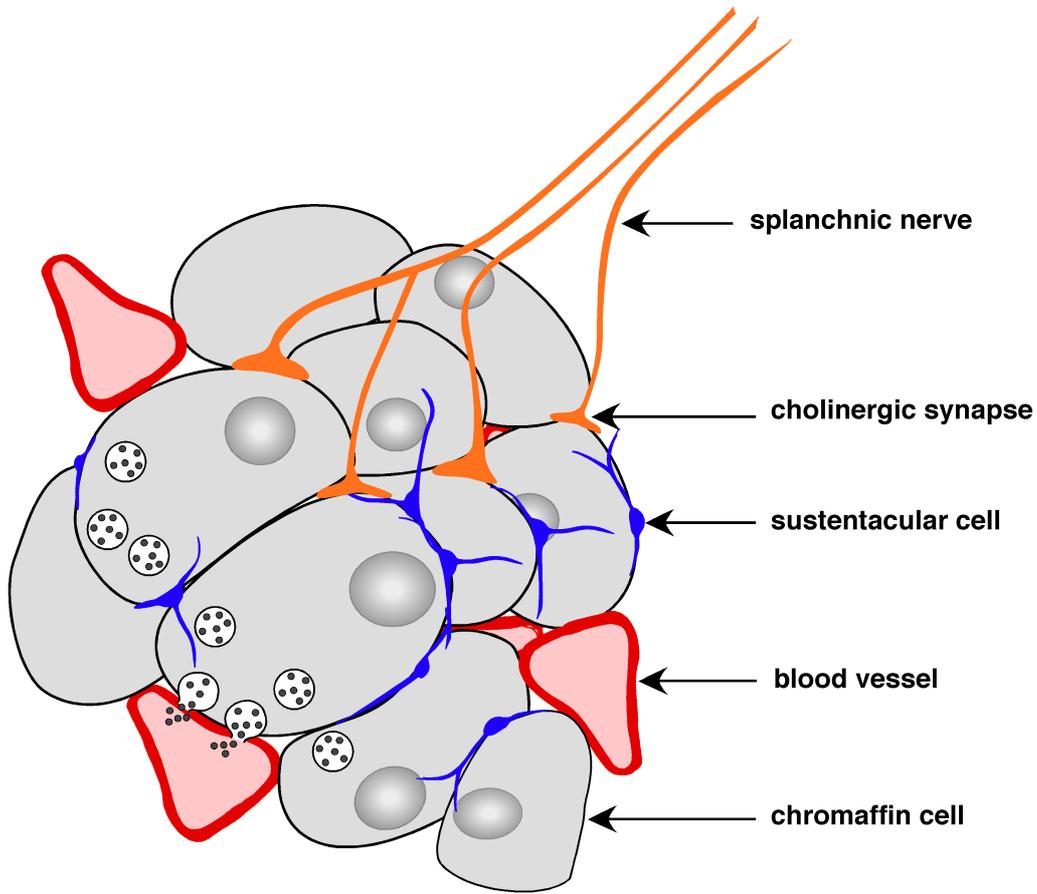


Figure 1

A



B

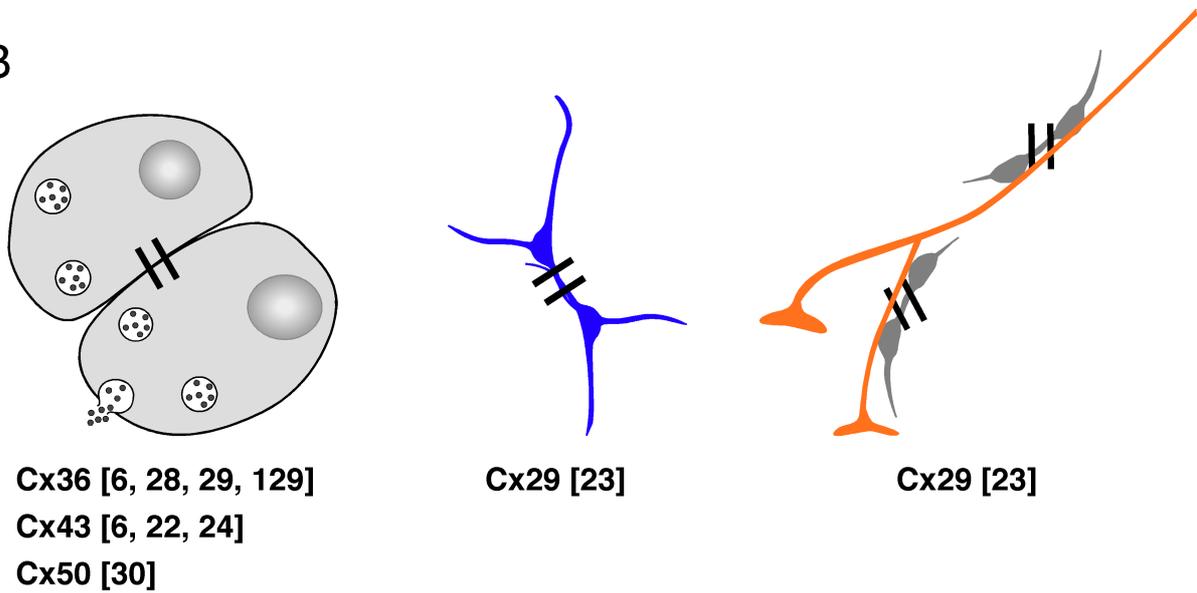


Figure 2

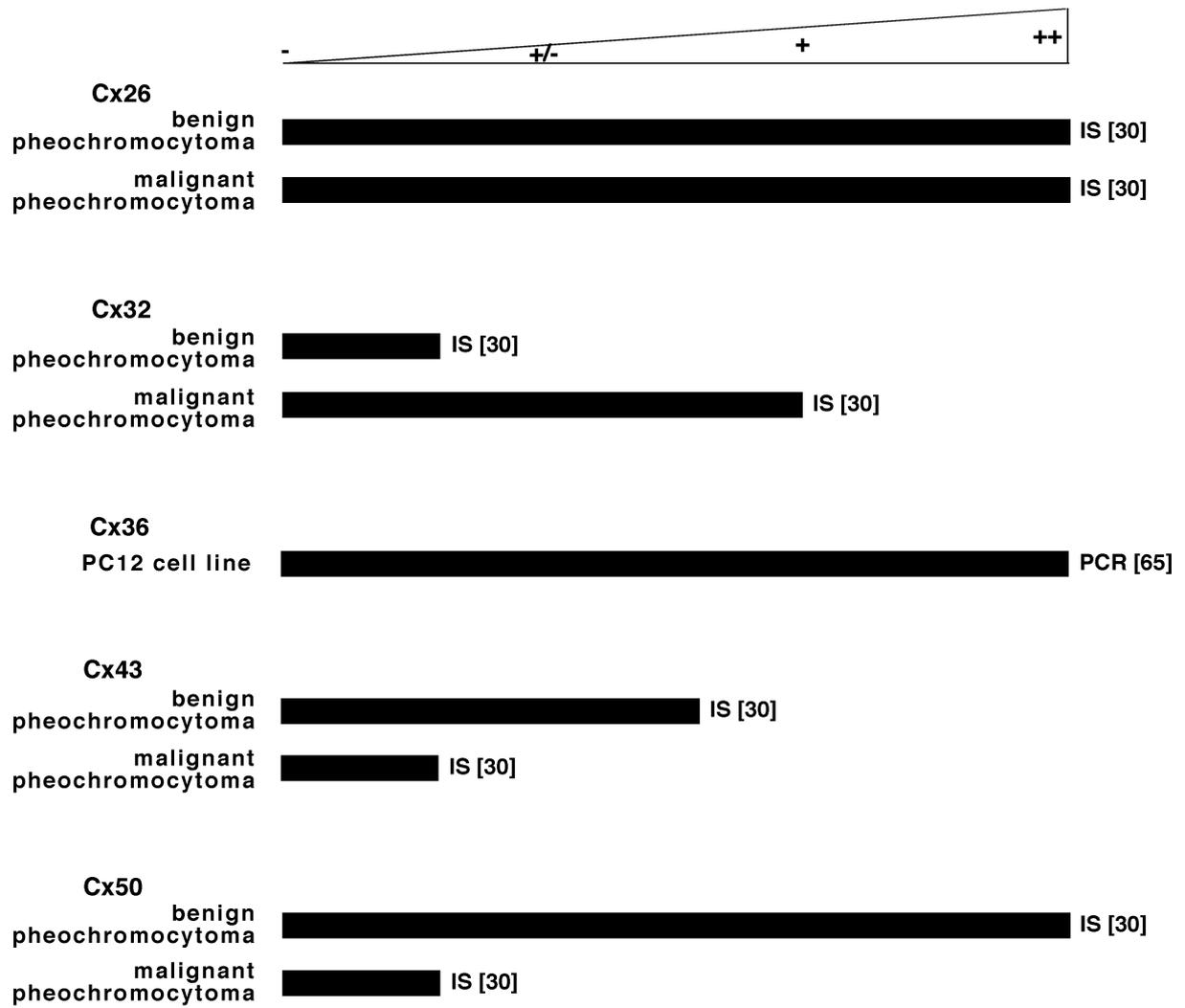


Figure 3

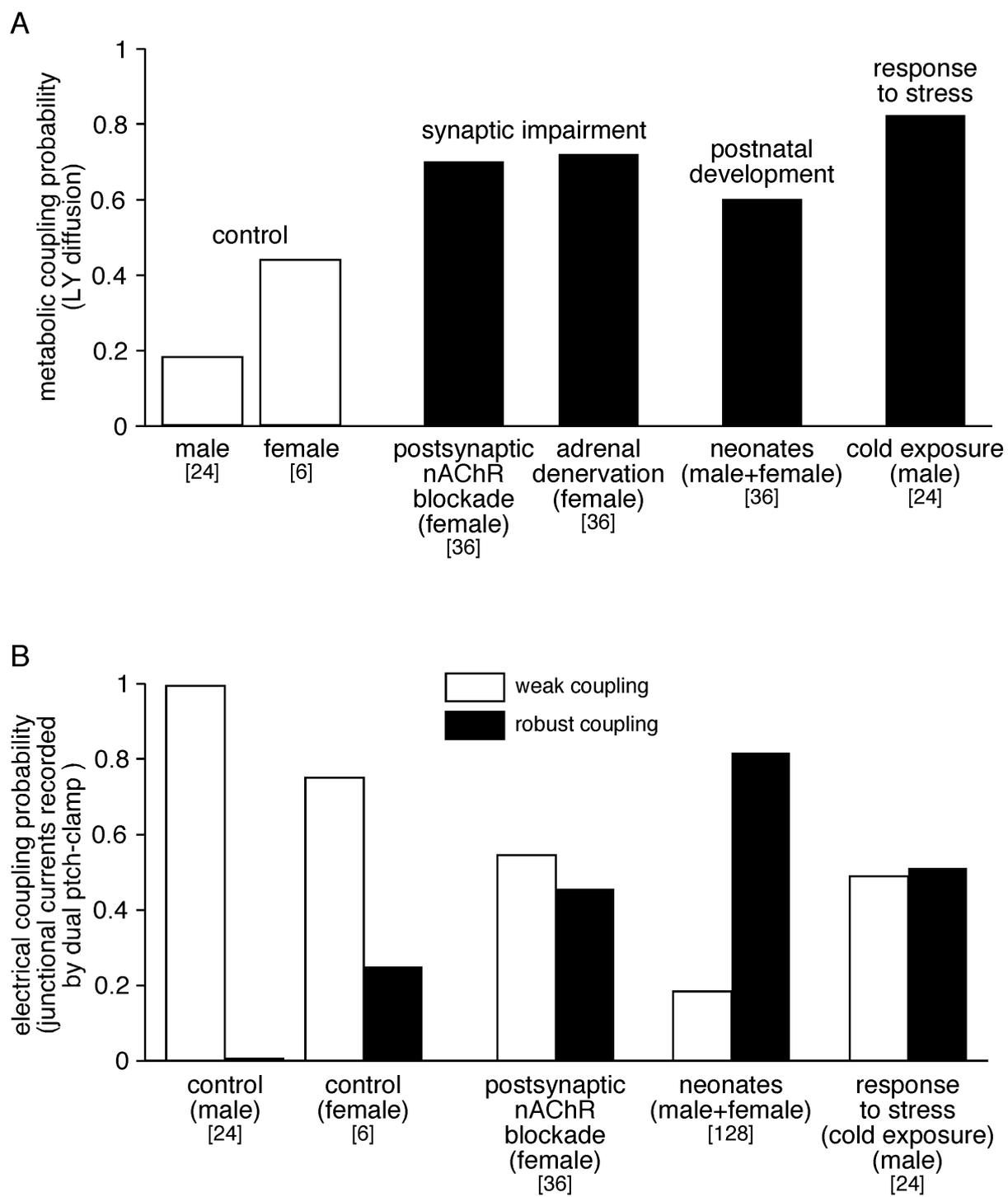


Figure 4

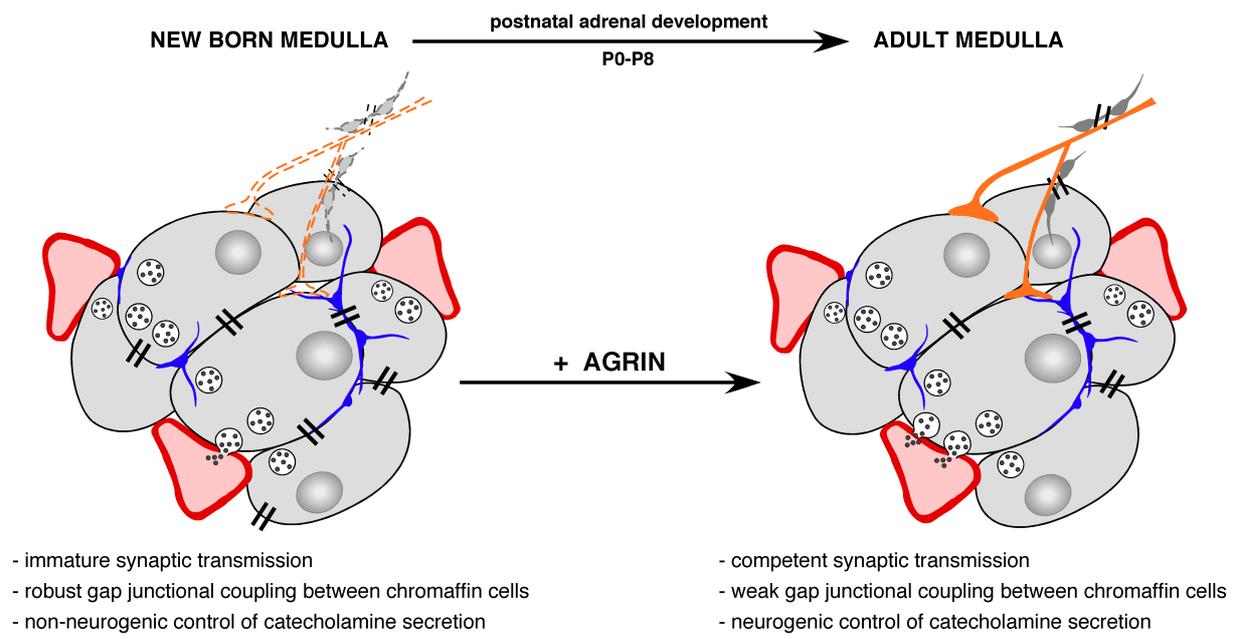


Figure 5

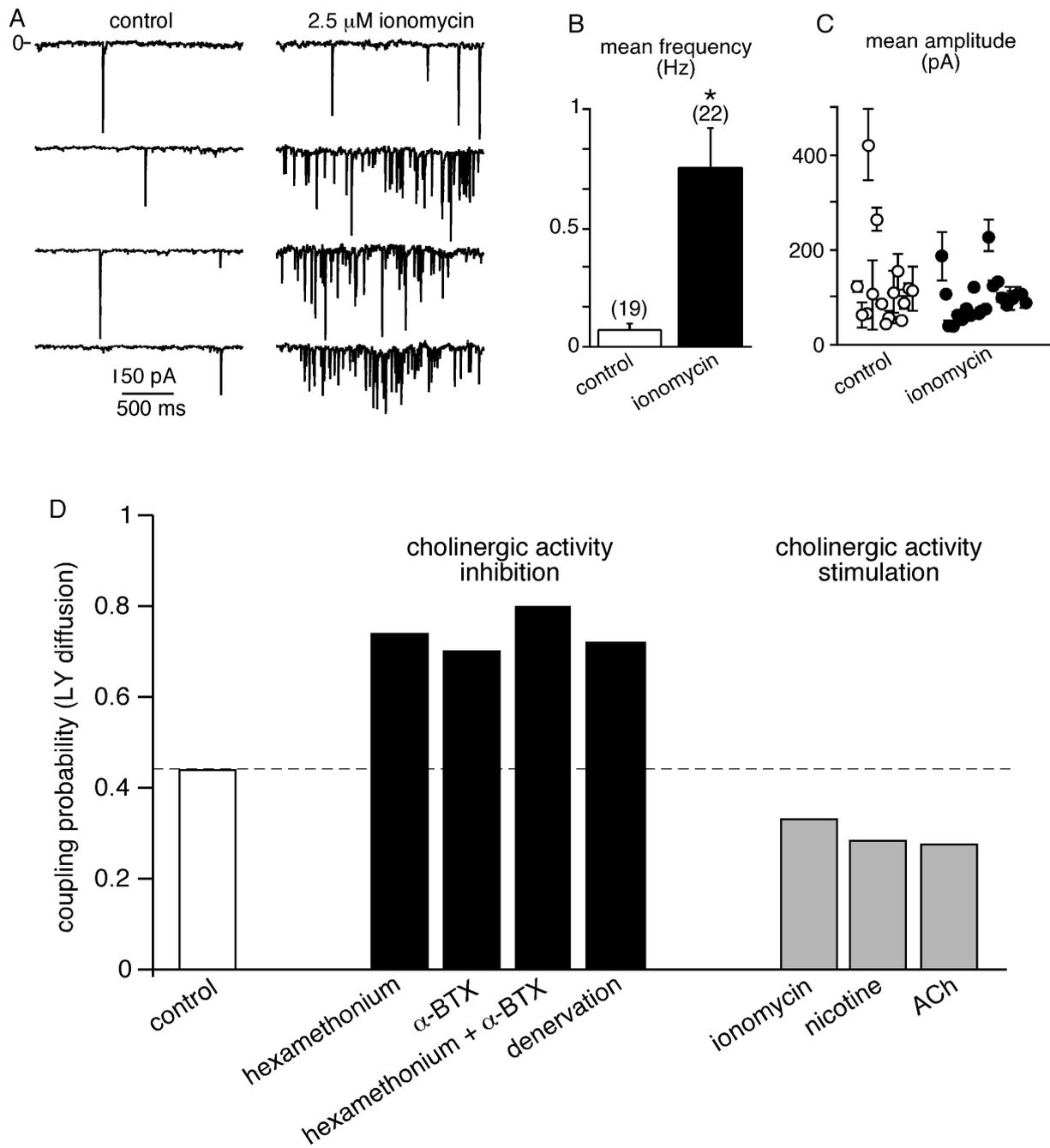


Figure 6

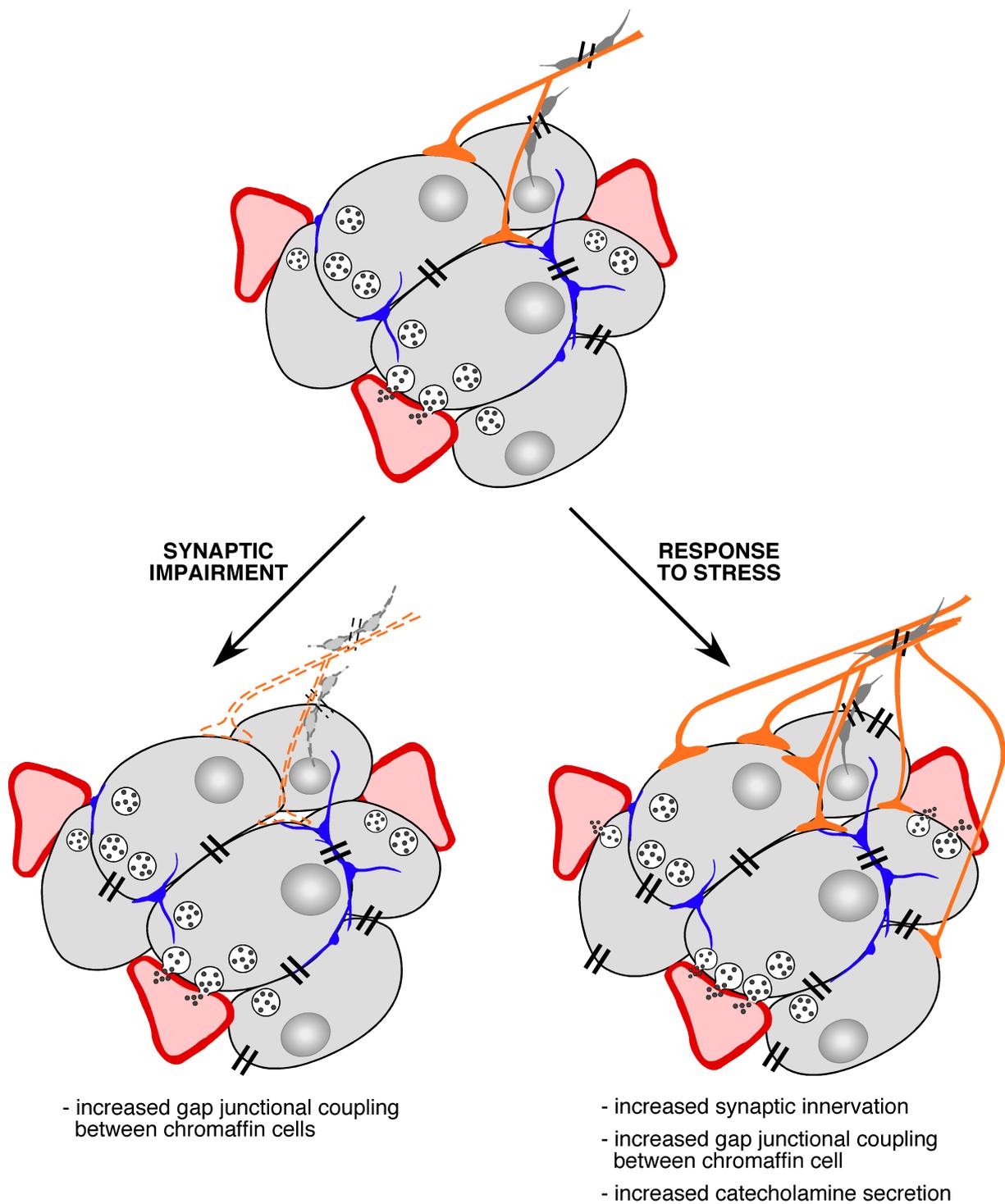


Figure 7