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Adaptive remodeling of the stimulus-secretion coupling: lessons from the 'stressed' adrenal medulla

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1 **Abstract**

2 Stress is part of our daily lives and good health in the modern world is offset by unhealthy
3 lifestyle factors, including the deleterious consequences of stress and associated pathologies.
4 Repeated and/or prolonged stress may disrupt the body homeostasis and thus threatens our
5 lives. Adaptive processes that allow the organism to adapt to new environmental conditions and
6 maintain its homeostasis are therefore crucial. The adrenal glands are major
7 endocrine/neuroendocrine organs involved in the adaptive response of the body facing stressful
8 situations. Upon stress episodes and in response to activation of the sympathetic nervous
9 system, the first adrenal cells to be activated are the neuroendocrine chromaffin cells located in
10 the medullary tissue of the adrenal gland. By releasing catecholamines (mainly epinephrine and
11 to a lesser extent norepinephrine), adrenal chromaffin cells actively contribute to the
12 development of adaptive mechanisms, in particular targeting the cardiovascular system and
13 leading to appropriate adjustments of blood pressure and heart rate, as well as energy
14 metabolism. Specifically, this chapter covers the current knowledge as to how the adrenal
15 medullary tissue remodels in response to stress episodes, with special attention paid to
16 chromaffin cell stimulus-secretion coupling. Adrenal stimulus-secretion coupling encompasses
17 various elements taking place at both the molecular/cellular and tissular levels. Here, I focus
18 on stress-driven changes in catecholamine biosynthesis, chromaffin cell excitability, synaptic
19 neurotransmission and gap junctional communication. These signaling pathways undergo a
20 collective and finely-tuned remodeling, contributing to appropriate catecholamine secretion and
21 maintenance of body homeostasis in response to stress.

22

23 **Keywords:** stimulus-secretion coupling; splanchnic nerve; stress; remodeling of
24 adrenomedullary tissue; chromaffin cells; synaptic transmission; gap junctions; secretory
25 granules; peptides; catecholamine secretion

26 **1. Introduction**

27 Our organism is daily subjected to environmental changes that challenge body homeostasis.
28 These changes are often perceived as stressors, with which the body copes *via* physiological
29 and metabolic adaptive responses. Activation of the 'stress system' leads to behavioral and
30 hormonal changes that improve the ability of the organism to adjust a state of 'threatened'
31 homeostasis and increase its chances of survival. Among the endocrine/neuroendocrine tissues
32 involved in the homeostatic response to stress, the adrenal glands are probably the most
33 emblematic and best known to the general public. Who does not know the word 'adrenaline'?
34 Who has never experienced an adrenaline rush during a stress episode? Behind stress-induced
35 adrenaline release are intricate and complex regulatory mechanisms that prepare the body to
36 fight or flight.

37 Let us begin this review with a brief introduction to the history of the biological concept
38 of stress. A recognized founding father of the concept of stress is Hans Selye with his
39 description of the 'General Adaptation Syndrome' (GAS). However, the history of the biology
40 of stress begins earlier with the fundamental studies of James Reilly on the pathogenesis of
41 many diseases (Hopkin & Laplane, 1978). Reilly was the first in the 1930s to demonstrate
42 experimentally the unambiguous, non-specific response to infections of various origins, and the
43 primary role of the sympathetic nervous system. Unlike Selye, the international dissemination
44 of his work has been more modest. For the record, Reilly described intense reactions in response
45 to bacterial products in certain animals, leading to death without being able to speak of an
46 infection, and showed that the primary damage to the organism does not take place where the
47 organic disorders appear, but targets the autonomic nervous system. From this nervous
48 pathway, the damage is transmitted to more or less distant tissues/organs, where it is occurred
49 secondarily. From this finding, James Reilly described the 'simple disease state syndrome', a
50 multivalent syndrome occurring in any disease, better known as Reilly's syndrome. At the same

51 time, Hans Selye works on rats exposed to noxious stimuli, and in a short note published in
52 Nature in 1936, he established the concept of GAS and gave a definition (Selye, 1936). In this
53 note, Selye wrote "*.... if the organism is severely damaged by acute non-specific noxious agents*
54 *such as, a typical syndrome appears, the symptoms of which are independent of the nature*
55 *of the damaging agent or the pharmacological type of the drug employed, and represent rather*
56 *a response to damage as such*". GAS consists in three sequential steps, the alarm, resistance
57 and exhaustion phases. In each of these, the adrenal glands, and the adrenomedullary tissue in
58 particular, are critically involved.

59 The adrenal glands are composed of a double endocrine/neuroendocrine tissue, which
60 encompasses the cortical and medullary zones. The adrenal cortex is activated by the
61 hypothalamo-pituitary-adrenocortical axis and releases corticosteroids (mainly
62 glucocorticoids). Increased levels of glucocorticoids induce anabolism processes that maintain
63 or increase glycemia. Interestingly, adrenocortical secretions also impact the secretory activity
64 of adrenomedullary chromaffin cells. The adrenomedullary tissue, which is mostly composed
65 of chromaffin cells, contributes to maintain body homeostasis in reaction to stressful
66 environmental changes *via* the release of catecholamines (mainly epinephrine) into the blood
67 circulation in response to splanchnic nerve activation. By far, the largest reservoir of
68 epinephrine in the body comes from adrenal chromaffin cells. Catecholamine secretion from
69 adrenal chromaffin cells occurs *via* a mechanism initially described by W.W. Douglas
70 (Douglas, 1968) and called 'stimulus-secretion coupling'. The initial stimulus comes from the
71 sympathetic nervous system that releases acetylcholine at splanchnic nerve terminals synapsing
72 onto chromaffin cells ((Douglas, 1968; Wakade, 1981) and reviewed in (Carbone, Borges,
73 Eiden, Garcia, & Hernandez-Cruz, 2019; Guerineau, 2020)). This traditional view of stimulus-
74 secretion coupling, with electrical discharges invading the splanchnic nerve endings as the only
75 physiological stimulus triggering catecholamine release *in vivo*, prevailed during many

76 decades. It was expanded in the early 2000s when a gap junction-mediated coupling between
77 chromaffin cells entered the fray as a novel protagonist involved in catecholamine release
78 (Martin, Mathieu, Chevillard, & Guerinéau, 2001). Indeed, studies performed both in acute
79 adrenal slices and in anaesthetized rodents revealed that the local communication mediated by
80 gap junctions between chromaffin cells represents a functional route by which biological
81 signals (electrical activity, second messengers...) propagate between adjacent cells and
82 subsequently generate instructive signals to trigger hormone secretion (Desarménien et al.,
83 2013; Martin, Mathieu, Chevillard, & Guerinéau, 2001).

84 At rest, these two signalling pathways are in equilibrium and the adrenal secretion of
85 catecholamines is minimal. Stress challenges this homeostasis, either briefly for an acute stress
86 or more robustly for a chronic stress, but in all cases, stress triggers a huge secretion of
87 catecholamines. This cannot be properly achieved without the remodeling of the
88 adrenomedullary stimulus-secretion coupling. Here, I propose to review the adaptive
89 mechanisms that take place in the stressed adrenal medulla, but because the elementary
90 components of the stimulation-secretion coupling are quite a few, the choice has been made to
91 focus on only some of them. The author apologizes for those that are not covered in this chapter.
92 I also apologize to those authors contributing to this field, whose articles were not cited because
93 of space limitations.

94

95 **2. Adrenomedullary stimulus-secretion coupling: a multi-faceted mechanism** 96 **for a continuous adaptation to stress-induced homeostasis changes**

97 The concept of stimulus-secretion is born in the sixties from the works of Douglas, Rubin and
98 Poisner (Douglas, 1968). Through a series of inaugural articles, the authors deciphered the
99 mechanisms by which acetylcholine stimulates catecholamine secretion (Douglas, Kanno, &
100 Sampson, 1967a, 1967b; Douglas & Poisner, 1966; Douglas & Rubin, 1961a, 1961b, 1963;

101 Poisner & Douglas, 1966). Since these pioneer studies, numerous publications have added to
102 our knowledge of the adrenal stimulus-secretion coupling.

103 Stimulus-secretion coupling relies on various mechanisms, which take place both at
104 cellular (*i.e.* chromaffin cells) and tissular (*i.e.* medulla) levels ((Guerineau, Campos, Le
105 Tissier, Hodson, & Mollard, 2022) for a recent review). Within the adrenomedullary tissue,
106 chromaffin cells are arranged in lobules surrounded by connective tissue. Chromaffin cells are
107 the neurosecretory unit of the stimulus-secretion coupling and are responsible for the secretion
108 of catecholamines (epinephrine (E) and norepinephrine (NE)) and others compounds such as
109 chromogranins and peptides. The adrenal medulla secretion is chiefly activated by a neuronal
110 pathway originated from the thoracic spinal cord and driven by the splanchnic nerve fibers that
111 synapse onto chromaffin cells (Fig. 1). The number of synaptic boutons that innervate a single
112 chromaffin cell depends on animal species; it is, for example, between 1 and 4 in the rat
113 (Kajiwara, Sand, Kidokoro, Barish, & Iijima, 1997). Electrical activity of the splanchnic nerve
114 patterns the synaptic release of acetylcholine (ACh) and consequently the cholinergic
115 stimulation of chromaffin cells (Fig.1, black pathway). Although ACh is the primary
116 neurotransmitter engaged at the splanchnic nerve-chromaffin cell synapse, other molecules
117 such as neuropeptides can take over (Guerineau, 2020 for a review), in particular under stress
118 conditions (Eiden, Emery, Zhang, & Smith, 2018) (see below). In addition to the neuronal
119 command, a local route delineated by gap junctions between chromaffin cells contributes also
120 to the secretory process (Colomer, Desarmenien, & Guerineau, 2009; Colomer, Martin,
121 Desarmenien, & Guerineau, 2012; Desarmenien et al., 2013; Martin, Mathieu, Chevillard, &
122 Guerineau, 2001) (Fig. 1, red pathway). In response to an increased demand for catecholamines
123 (during a stress episode for example), these two pathways remodel to achieve optimal secretion.
124 This functional adaptation, associated with the intrinsic remodeling of chromaffin cells (see

125 below), is not only the pivotal event of the 'fight-or-flight' response, but also contributes to the
126 body's adaptation to stressors such as cold, hypoglycemia, exercise or hypotension.

127

128 [Insert Figure 1 here]

129 **Fig. 1: Schematic representation of the adrenal medulla stimulus-secretion coupling: two**
130 **regulatory levels for an accurate control of basal and stress-triggered catecholamine**
131 **secretion.** The neuroendocrine chromaffin cells (grey-filled cells) are under the dual control of
132 synaptic inputs arising from the splanchnic nerve terminals (in black) and gap junctions (in
133 red). The primary neurotransmitter released by splanchnic nerve terminals is acetylcholine
134 (ACh), but under stress conditions, neuropeptides (such as PACAP, see below) are released
135 concomitantly to ACh. In addition to the synaptic transmission, a gap junction-driven route
136 promotes diffusion of biologically active molecules between coupled cells and as such actively
137 contributes to the secretory process.

138

139

140 **2.1 The neural command**

141 The perception of a stress activates the sympathetic nervous system and triggers an acute
142 response in which the splanchnic nerve and the adrenal chromaffin cells are two crucial players
143 (Fig. 2A). The splanchnic nerve encodes the stress signal by varying its electrical discharge
144 frequency and the encoded signal is then transduced to the adrenal medulla/chromaffin cells.
145 At rest (corresponding to homeostatic physiological conditions), the splanchnic nerve fires at a
146 moderate rate ($< 1\text{Hz}$) (Fulop, Radabaugh, & Smith, 2005; Holman, Coleman, Tonta, &
147 Parkington, 1994; Wakade, 1981; Wolf, Zarkua, Chan, Sridhar, & Smith, 2016). This basal
148 sympathetic tone allows maintaining the basal body homeostasis in terms of heart rate, blood
149 pressure, energy consumption/expenditure. Under these resting conditions, adrenal chromaffin

150 cells secrete modest amounts of catecholamines into the blood circulation. This contrasts with
151 stress conditions in which the sympathetic tone drastically increases and the frequency of
152 splanchnic nerve action potential discharges reaches several Hz (Fulop, Radabaugh, & Smith,
153 2005; Holman, Coleman, Tonta, & Parkington, 1994; Wakade, 1981; Wolf, Zarkua, Chan,
154 Sridhar, & Smith, 2016). As a result, chromaffin cells release a surge of catecholamines, NE
155 and/or E, in the blood circulation.

156

157 [Insert Figure 2 here]

158 **Fig. 2: Splanchnic nerve and chromaffin cells: two key players of the adrenal stimulus-**
159 **secretion coupling.** (A) Within the adrenal medullary tissue, chromaffin cell is the pivotal
160 neurosecretory unit connecting the electrical activity of the splanchnic nerve to the release of
161 catecholamines into the blood circulation (left panel). Hidden under a single classification,
162 chromaffin cells actually exhibit two distinct phenotypes (right panel). Depending on the
163 presence of phenylethanolamine N-methyltransferase (PNMT), enzyme converting
164 norepinephrine (NE) into epinephrine (E), a 'chromaffin cell' can be noradrenergic and secretes
165 NE or adrenergic and secretes E. Note that the two first enzymes involved in catecholamine
166 biosynthesis (TH and DbH) are common whatever the secretory phenotype. (B) Chromaffin
167 cells are excitable cells, exhibiting action potentials which can fire either regularly (left chart)
168 or in bursts (right chart). The recordings plotted here correspond to spontaneous electrical
169 activity recorded in mouse chromaffin cells in acute adrenal slices.

170

171 In addition to the nervous command mediated by the sympathetic endings of the
172 splanchnic nerve, the adrenal vascularization also significantly contributes to the stimulus-
173 secretion coupling, at two levels. First, the affluent blood supply carries secretagogues (such as
174 histamine, bradykinin, or angiotensin...) to the medulla, where they can exert their

175 activating/inhibiting action on catecholamine secretion. Second, the medulla vasculature has a
176 dedicated function of release of hormones in the general blood circulation. The adrenal blood
177 supply depicts a centripetal circulation. The medulla receives blood both from the cortex
178 through an arteriolar plexus and from a direct supply through medullary arteries (Coupland &
179 Selby, 1976). They penetrate the medulla and end in capillaries. The effluent blood containing
180 the adrenomedullary secretory products (hormones, peptides, chromogranins and derived-
181 factors...) is collected into a central vein, which projects into the renal vein for the left gland
182 while the venous blood flowing from the right gland is directly drained into the inferior *vena*
183 *cava*. It is also interesting to note that neural and vascular commands interact with each other,
184 as illustrated by the fact that the splanchnic nerve stimulation (or stressors as hemorrhage,
185 hypoxia, hypotension) raises the rate of blood flow throughout the adrenal gland (Breslow,
186 1992; Breslow, Jordan, Thellman, & Traystman, 1987; Edwards, 1982; Hinson, Cameron,
187 Purbrick, & Kapas, 1994; Kennedy, Breslow, Tobin, & Traystman, 1991). Reciprocally,
188 adrenal denervation leads to an overall decrease in blood flow (Breslow, Jordan, Thellman, &
189 Traystman, 1987).

190

191 **2.2 The nature of catecholamines secreted**

192 Behind the common classification of 'chromaffin' cells, there are actually two cell types
193 with distinct phenotypes. The standard and simplistic view is that depending on the presence of
194 the enzyme phenylethanolamine N-methyltransferase (PNMT), which mediates the
195 methylation of NE to form E, a 'chromaffin' cell can be noradrenergic and releases NE or
196 adrenergic and releases E (Fig. 2A, right panel). A recent article published by Borges' lab
197 (Gonzalez-Santana et al., 2020) argues for a more complex picture. In the absence of E (in
198 PNMT-deficient animals for example), a chromaffin cell can retain its phenotype of an
199 'adrenergic' cell (*i.e.* structural characteristics such as shape and electron density of granules).

200 'Adrenergic' granules can store NE (instead of E), but at lower efficiency. The authors end their
201 article with an exciting, emerging and somewhat puzzling concept, namely "*an adrenergic cell*
202 *is not adrenergic because it contains EPI (= E) but rather, it contains EPI because it is an*
203 *adrenergic cell*". The discussion is open....

204 Within the adrenal medullary tissue, the proportion of NE- and E-secreting chromaffin
205 cells differs between species. E cells are more numerous than NE cells in the rat, mouse, cat,
206 dog, hamster, ox, rabbit, chipmunk and humans (Edwards, Anderson, Southwell, & McAllen,
207 1996; Lundberg et al., 1979; Lundberg et al., 1986; Suzuki & Kachi, 1996), with a ratio E:NE
208 cells of 4:1 in the rat (Tomlinson, Durbin, & Coupland, 1987; Verhofstad, Coupland, Parker,
209 & Goldstein, 1985) and mouse (Iwasa, Oomori, & Tanaka, 1999). In the rabbit and chipmunk,
210 NE-containing cells are scarcely observed (Suzuki & Kachi, 1996). Conversely, NE cells are
211 the more abundant chromaffin cell type expressed in the adrenal medulla of the pig (Suzuki &
212 Kachi, 1996) and japanese quails (El-Desoky & Mustafa, 2021).

213 Whether a 'stressed' adrenal gland releases NE, E or NE+E in response to stress depends
214 on the nature of the stress (Pacak et al., 1995). The variability in the E:NE release ratio also
215 implies that E- and NE-secreting chromaffin cells can be differentially stimulated. Cold stress
216 leads to a selective secretion of NE (Vollmer, 1996). E is the main catecholamine secreted in
217 response to haemorrhage (Glaviano, Bass, & Nykiel, 1960) and insulin-induced hypoglycemic
218 stress (Vollmer, Balcita, Sved, & Edwards, 1997; Vollmer et al., 1992), acute immobilization
219 or restrain stress (Jeong et al., 2000). Repeated immobilization stress and hypoxia mobilize
220 both E and NE secretions (Jeong et al., 2000; Kumar et al., 2006).

221

222 **2.3 Intrinsic electrical activity of chromaffin cells**

223 In addition to stressor-specific NE and E release, NE and E secretion display a specific
224 frequency dependence (Damase-Michel et al., 1993). Chromaffin cells (NE-secreting and E-

225 secreting cells) are excitable cells and exhibit action potentials that fire spontaneously or in
226 response to a depolarizing stimulus (Guerineau, 2023). Interestingly, the spiking activity pattern
227 can be either regular with action potentials discharging with a constant time interval between
228 spikes, or in burst alternating between firing and silent periods (Fig. 2B). Bursts were clearly
229 identified in mouse and rat chromaffin cells, both *in vitro* in cultured isolated cells (Marcantoni
230 et al., 2023; Marcantoni et al., 2010; Vandael et al., 2015), *ex vivo* in acute adrenal slices
231 (Milman et al., 2021) and *in vivo* in anaesthetized animals (Desarmenien et al., 2013). In human,
232 chromaffin cells spike irregularly alternating between firing and silence periods, but burst
233 discharges were not clearly identified (Hernandez-Vivanco et al., 2017). Although not
234 investigated in detail in all species, distinct firing patterns impact the secretory function of
235 chromaffin cells and as such, is of great physiological interest. Indeed, the bursting mode has
236 been reported to efficiently stimulate catecholamine secretion (Duan, Yu, Zhang, & Zhou,
237 2003; Vandael et al., 2015). It is noteworthy that bursts, if not triggered spontaneously as
238 observed in mouse chromaffin cells, can be elicited by various physiological or
239 pharmacological stimuli. Neurotransmitters ((Carbone, Borges, Eiden, Garcia, & Hernandez-
240 Cruz, 2019) for a review) or changes in the expression and availability of ion channels involved
241 in action potential generation (Lingle, Martinez-Espinosa, Guarina, & Carbone, 2018;
242 Marcantoni et al., 2010; Martinez-Espinosa, Yang, Gonzalez-Perez, Xia, & Lingle, 2014;
243 Nassar-Gentina, Pollard, & Rojas, 1988; Vandael, Marcantoni, & Carbone, 2015; Vandael et
244 al., 2015) or extracellular pH (Guarina, Vandael, Carabelli, & Carbone, 2017) can generate a
245 bursting electrical activity. Ion channels operated near the resting membrane potential
246 (classified as 'leak' or 'background' channels), through their ability to set the membrane potential
247 close to the threshold value for triggering action potentials, can also contribute to generate
248 bursts of electrical activity in chromaffin cells (Guerineau, Monteil, & Lory, 2021; Milman et
249 al., 2021). Interestingly, the two electrical activity patterns spontaneously co-exist in a single

250 chromaffin cell (Milman et al., 2021), highlighting complex regulatory mechanisms of the
251 electrical activity and therefore of the secretory response at the level of a single cell. Keeping
252 in mind that the adrenal medulla is composed of thousands to millions chromaffin cells, this
253 renders the stimulus-secretion coupling at the level of the whole medulla much more intricate.

254 As such, from this introduction chapter, it clearly appears that the adrenal medulla
255 stimulus-secretion coupling is a multi-determinant process involving both extra- (splanchnic
256 nerve electrical activity) and intra-adrenal components (chromaffin cell excitability,
257 cholinergic/peptidergic neurotransmission, gap junctional communication, intra-adrenal
258 vasculature). It is the integration of the response to each determinant that will, *in fine*, pattern
259 the adrenomedullary secretory behavior. Taking the stress response as an example, the chapters
260 below illustrate the remarkable aptitude of the adrenal medullary tissue to remodel and generate
261 a secretory response that allows the body to cope with adverse situations and return to resting
262 homeostasis. This review is not intended to be exhaustive on every item but rather to present
263 an overview of the cellular/tissular mechanisms by which the adrenal medulla remodels under
264 stress conditions.

265

266 **3. The 'stressed' adrenal medulla**

267 **3.1 Stress-induced macroscopic changes in the adrenal gland**

268 An increase in the adrenal gland weight is a hallmark of the adrenal response to stress and has
269 long been used to assess stress levels in an animal. This parameter is reliable and does not
270 depend either on the species (rat, mouse), the strain, nor the stress paradigm (chronic cold
271 exposure, auditory stress, unpredictable chronic mild stress, restrain stress, chronic social
272 isolation, chronic social defeat stress) ((Almeida-Reis et al., 2010; Colomer, Olivos Ore, et al.,
273 2008; Jurtshuk, Weltman, & Sackler, 1959; Kaiser & Sachser, 1998; Welch & Welch, 1969)
274 for selected examples in rat, mouse and guinea-pig). As reported by Lemaire and colleagues,

275 the adrenal weight mostly depends on the activity of the hypothalamus-pituitary-adrenal axis
276 (Lemaire, Le Moal, & Mormede, 1993). The increased weight of the adrenal glands in stressed
277 animals likely reflects a weight gain in both the cortical and medullary tissues. Indeed, via their
278 respective hormone secretions (mainly glucocorticoids for the cortex and epinephrine for the
279 medulla), these two tissues are key players of the organism's response to stress. An increased
280 volume of the adrenal medulla has been reported, in particular in the mouse, in response to
281 unpredictable chronic stress (Santana et al., 2015), water-maze Morris' test (Tuma, Kolinko,
282 Jelinkova, Hilber, & Cendelin, 2017) or social stress (Rodriguez-Sureda, Lopez-Tejero,
283 Llobera, & Peinado-Onsurbe, 2007).

284

285 **3.2 Stress-induced remodeling of catecholamine-synthesizing enzymes**

286 Catecholamine biosynthesis requires several enzymes acting sequentially along the production
287 pathway. The first enzyme is tyrosine hydroxylase (TH), followed by dopamine beta
288 hydroxylase (DbH) and then the terminal enzyme phenylethanolamine-N-methyltransferase
289 (PNMT), which mediates the methylation of NE by S-adenosylmethionine to form E. For each
290 enzyme, the expression level of the transcript, the protein level and the enzyme activity can be
291 modified in response to stress ((Kvetnansky & Sabban, 1998) for a review, Table 1 for acute
292 stress and Table 2 for chronic stress). Note also that protein levels and enzyme activity are not
293 always commensurate with mRNA changes. This is particularly true for PNMT, as post-
294 transcriptional and post-translational steps are subject to additional regulatory controls.

295

296 [Insert Table 1 here]

297 **Table 1: Effect of various acute stress on gene expression, protein level and enzymatic**
298 **activity on catecholamine biosynthetic enzymes**

299

300

301 Although an individual analysis, enzyme by enzyme, stress by stress, species by species, shows
302 a certain degree of variability, the data collectively converge towards an increase in enzyme
303 expression and activity in both acutely and chronically stressed animals. An exception to this
304 conclusion is prenatal hypoxia. A few days exposure to low oxygen supply (10% O₂) during
305 gestation results in a decreased expression in catecholamine-synthesizing enzymes. This
306 indicates that hypoxic conditions trigger specific regulatory mechanisms. The timing of adrenal
307 gland sampling after an acute stress exposure (immediately or several hours after stress
308 cessation) is also a factor of variability. For example, the increase in DbH mRNA expression is
309 not detectable immediately in response to a single immobilization stress (Kvetnansky et al.,
310 2004; McMahon et al., 1992) but becomes significant several hours later (Kvetnansky et al.,
311 2008; Kvetnansky et al., 2004). Similarly, the increased expression in TH protein requires
312 several hours before being detected (Nankova et al., 1994). The same applies to TH activity
313 which remains unchanged immediately after stress (McMahon et al., 1992) but increases 3
314 hours later (Kvetnansky, Rusnak, Dronjak, Krizanova, & Sabban, 2003), or PNMT activity
315 which becomes elevated 6 hours after stress (Kvetnansky, Weise, & Kopin, 1970).

316

317 [Insert Table 2 here]

318 **Table 2: Effect of various chronic stress on gene expression, protein level and enzymatic**
319 **activity on catecholamine biosynthetic enzymes**

320

321

322 By which mechanisms are the adrenomedullary catecholamine-synthesizing enzymes
323 regulated in control and stressed animals? Consistent with the adrenal gland anatomy, both
324 neuronal (linked to splanchnic nerve activity) and humoral (linked to endocrine activity of the

325 adrenal cortex) mechanisms can be involved. Note that they are not mutually exclusive. I
326 summarize the principal knowledge here, detailed information can be found in many review
327 articles including (Kvetnansky, Lu, & Ziegler, 2013; Kvetnansky, Sabban, & Palkovits, 2009;
328 Nankova & Sabban, 1999; Sabban & Kvetnansky, 2001).

329 Splanchnic nerve electrical activity, which increases during stress episodes, leads to an
330 increased activity of several of the catecholamine biosynthetic enzymes. This regulatory
331 mechanism has been initially described by Axelrod and colleagues and named '*trans-synaptic*
332 *induction*' (Thoenen, Mueller, & Axelrod, 1969b). TH activity is induced by the activity of the
333 splanchnic nerve in rat, resulting in an increased rate of catecholamine biosynthesis.
334 Strengthening a neuronal induction of TH, treating animals with agents that interfere with the
335 post-ganglionic sympathetic transmission increases both *Th* mRNA levels (Dagerlind,
336 Schalling, Eneroth, Goldstein, & Hokfelt, 1990) and TH activity (Mueller, Thoenen, &
337 Axelrod, 1969a, 1969b; Patrick & Kirshner, 1971; Thoenen, Mueller, & Axelrod, 1969a;
338 Thoenen, Mueller, & Axelrod, 1969b). It is noteworthy that the trans-synaptic induction of TH
339 is a robust process as it occurs not only in rats, but also in mice, rabbits and guinea pigs
340 (Mueller, Thoenen, & Axelrod, 1969b). Regarding the other enzymes involved in
341 catecholamine biogenesis, an increased DbH activity is observed in response to splanchnic
342 nerve stimulation in goats (Yamada, Nakazato, Ito, Teraoka, & Ohga, 1988) and reserpine
343 treatment in rats (Molinoff, Brimijoin, Weinshilboum, & Axelrod, 1970; Patrick & Kirshner,
344 1971). While steady state levels of DbH are under hormonal control by the cortex
345 (Weinshilboum & Axelrod, 1970), an increase in DbH synthesis requires trans-synaptic
346 mechanisms (Ciaranello, Wooten, & Axelrod, 1975). For PNMT, the role of the splanchnic
347 nerve activity in its induction is less obvious and the pioneering studies of Axelrod and
348 colleagues identified the corticoid-mediated humoral pathway as the main mechanism for
349 induction of PNMT activity (Wurtman & Axelrod, 1965, 1966). A reserpine treatment induces

350 a decrease in *Pnmt* mRNA in the rat adrenal gland (Dagerlind, Schalling, Eneroth, Goldstein,
351 & Hokfelt 1990; Schalling et al., 1988), which is prevented by dexamethasone or ACTH
352 (Dagerlind, Schalling, Eneroth, Goldstein, & Hokfelt, 1990), indicating also the involvement
353 of a humoral component. A moderate but significant increase in PNMT activity have been also
354 reported in 6-hydroxydopamine-treated rats (Mueller, Thoenen, & Axelrod, 1969a; Thoenen,
355 Mueller, & Axelrod, 1970). The increase is abolished in denervated glands, indicating the
356 presence of a neuronal component in the induction of PNMT. Later on, Lee and colleagues
357 (Lee, Raia, Tonshoff, & Evinger, 1999) identified in *Pnmt* gene promoter sequences capable of
358 conveying responsiveness to neuronal stimuli. The current consensus is that PNMT is regulated
359 by both hormonal and neural stimuli. At steady state, the catalytic activity of PNMT is
360 predominantly glucocorticoid-regulated, orchestrated by the pituitary gland and the adrenal
361 cortex. Circulating glucocorticoids inducing *Pnmt* mRNA at maximal level, a further elevation
362 of PNMT level above the steroid-maintained plateau, requires other mechanisms. In this
363 context, splanchnic nerve impulses induce the synthesis of *de novo* PNMT molecules
364 (Ciaranello & Black, 1971; Wong, Bildstein, Siddall, Lesage, & Yoo, 1993).

365 In stressed animals, the mechanisms regulating the activity of catecholamine-
366 synthesizing enzymes are similar to those occurring in control animals. The rise in TH activity
367 observed in stressed rats (cold exposure or repeated immobilization paradigm) is abolished
368 when the splanchnic nerve is sectioned (Kvetnansky, Weise, & Kopin, 1970; Thoenen, 1970).
369 As previously reported in control animals, the regulation of PNMT activity by neuronal activity
370 is less obvious and could depend on the nature of the stress. In rats subjected to chronic social
371 stress, the increase in PNMT activity is fully abolished by splanchnicotomy (Lemaire, Le Moal,
372 & Mormede, 1993). By contrast, in repeatedly immobilized rats, despite PNMT activity is
373 somewhat decreased in the denervated gland when compared to the intact gland, the rise in the
374 enzyme activity persists in the denervated adrenal (Kvetnansky, Weise, & Kopin, 1970),

375 indicating that the neuronal pathway is not the main mechanism involved in the regulation of
376 PNMT activity. Thus, the two most important enzymes involved in catecholamine biosynthesis
377 (TH as the rate-limiting enzyme and PNMT as the enzyme converting NE into E) are
378 differentially regulated, the first being chiefly under a neuronal control and the second being
379 controlled by both humoral and neuronal factors, with a more important contribution of the
380 humoral (anterior pituitary-adrenocortical) system.

381 After identifying that catecholamine-synthesizing enzymes can be regulated through
382 two distinct pathways, let us then examine which factors are involved. At the adrenomedullary
383 synapses, under resting conditions, the splanchnic nerve releases ACh, while in stress
384 conditions, a composite cholinergic and peptidergic release takes place (see Guerineau, 2020).
385 As such, ACh and neuropeptides can potentially impact catecholamine biosynthetic enzymes.

386

387 *3.2.1. Neuronal cholinergic control: role of ACh and nAChR/mAChR activation*

388 Several lines of evidence collected from various animal species and various adrenal
389 preparations (chromaffin cell cultures, adrenal gland sections) argue for a crucial role played
390 by ACh through activation of nicotinic (nAChRs) and muscarinic (mAChRs) receptors.
391 Collectively, ACh and nAChR agonists positively regulate TH, as illustrated by these few
392 examples. i) Carbachol (which activates both nAChRs and mAChRs), but not muscarine
393 increases *Th* mRNA synthesis, TH protein level and activity in cultured bovine chromaffin cells
394 (Craviso, Hemelt, & Waymire, 1992). ii) The nAChR agonist 1,1-dimethyl-4-
395 phenylpiperazinium increases both *Th* gene transcription, *Th* mRNA level, TH protein level
396 and activity (Craviso, Hemelt, & Waymire, 1992). iii) nicotine elicits the induction of *Th*
397 mRNA, and increases the transcription rate of *Th* gene (Fossom, Carlson, & Tank, 1991) and
398 TH enzyme activity in the rat adrenal medulla (Fossom, Carlson, & Tank, 1991; Fossom,
399 Sterling, & Tank, 1991; Yoshimura, Xu, Sun, & Tank, 2004). Although less obvious and

400 consensus than the nicotinic regulation, a mAChR-mediated regulation of TH also occurs. In
401 the rat, the pharmacological inhibition of mAChRs by atropine increases TH activity (Mueller,
402 Thoenen, & Axelrod, 1970b). Chronic injections of bethanechol, a mAChR agonist, induce *Th*
403 mRNA, but do not change TH protein level or activity (Yoshimura, Xu, Sun, & Tank, 2004).
404 Mechanistically, the effect of ACh on TH activity is associated with protein phosphorylation,
405 one of the major mechanisms for controlling the activity of TH in sympathetic tissues, including
406 the adrenal medulla (Dunkley & Dickson, 2019). Upon cholinergic (nicotinic or muscarinic)
407 stimulation, TH protein is phosphorylated, mostly on Ser19 and Ser31 residues (Haycock &
408 Wakade, 1992).

409 Regarding DbH, the effects of cholinergic agonists and nAChR/mAChR activation have
410 been less investigated. At transcriptional level, nicotine stimulates *Dbh* gene expression in
411 PC12 cells (Gueorguiev, Zeman, Meyer, & Sabban, 2000), while in floating rat adrenal gland
412 sections, the expression of *Dbh* is not significantly induced by nicotine (Jahng, Houpt, Joh, &
413 Wessel, 1997). At functional level, an ACh-mediated increase in DbH activity has been
414 reported in goat adrenal glands (Yamada, Nakazato, Ito, Teraoka, & Ohga, 1988). The mAChR
415 agonist oxotremorine increase DbH activity in rat adrenal glands (Lima & Sourkes, 1986).

416 Like TH and DbH, PNMT is also regulated by cholinergic agonists (Wong, Anderson,
417 & Tai, 2002). In cultured bovine chromaffin cells, ACh, nicotine, carbachol and muscarine
418 increase the expression of *Pnmt* mRNA (Evinger, Ernsberger, Regunathan, Joh, & Reis, 1994;
419 Stachowiak, Hong, & Viveros, 1990). The effect is reduced when cells are bathed in presence
420 of hexamethonium or atropine, supporting thus a dual nicotinic and muscarinic component.
421 Nicotine, carbachol and muscarine activate *Pnmt* promoter (Morita & Wong, 1996; Wong,
422 Anderson, & Tai, 2002), through a cAMP/protein kinase A-dependent transduction pathway
423 (Wong, Anderson, & Tai, 2002). The effect of these cholinergic agonists on *Pnmt* promoter
424 activation is preceded by an increase in the mRNA level of the immediate early gene

425 transcription factor Early growth response (Egr)-1 (Morita, Bell, Siddall, & Wong, 1996;
426 Morita & Wong, 1996). Although this finding provides a strong support for the involvement of
427 Egr-1 in the neural regulation of *Pnmt* gene expression in the rat adrenal gland, other factors
428 such as the neural crest-associated developmental transcription factor Activating enhancer
429 binding Protein (AP)-2, the ubiquitous transcription factor Specificity protein (Sp)1, the
430 tumorigenic factor Myc-associated zinc-finger protein, (MAZ) and the glucocorticoid receptor
431 (GR) fuel the list of transcriptional activators of the *Pnmt* gene (Wong, 2003; Wong, Siddall,
432 Ebert, Bell, & Her, 1998). Interestingly, these factors can act cooperatively, leading to
433 synergistic activation of *Pnmt* promoter well beyond their independent contributions (Wong,
434 Siddall, Ebert, Bell, & Her, 1998).

435

436 *3.2.2. Neuronal peptidergic control: a major role for PACAP*

437 Under resting conditions, ACh is the primary neurotransmitter released at the splanchnic nerve-
438 chromaffin cell synapse. However, under stress conditions a peptidergic neurotransmission
439 mediated by the pituitary adenylate cyclase-activating polypeptide (PACAP) takes over (Eiden,
440 Emery, Zhang, & Smith, 2018; Smith & Eiden, 2012; Stroth, Holighaus, Ait-Ali, & Eiden,
441 2011), thus making PACAP as dormant regulator of catecholamine-synthesizing enzymes. The
442 effect of PACAP on PNMT, in particular on *Pnmt* promoter, is well described (Wong,
443 Anderson, & Tai, 2002). PACAP rapidly activates *Pnmt* promoter and contrasting with the
444 transient activation mediated by cholinergic agonists, the effect is long-lasting and no
445 desensitization is observed through 48 hours. This is consistent with the takeover of PACAP
446 over ACh and appears particularly relevant in the context of stress, in which the secretion of
447 catecholamines needs to be sustained, even prolonged. In bovine chromaffin cells, PACAP
448 elevates levels of *Th*, *DbH* and *Pnmt* mRNAs (Tonshoff, Hemmick, & Evinger, 1997) and
449 stimulates TH protein level and activity (Bobrovskaya, Gelain, Gilligan, Dickson, & Dunkley,

450 2007; Marley, Cheung, Thomson, & Murphy, 1996). In PC12 cells, PACAP stimulates a
451 transient increase in *Th* gene transcription and elevates *Th* mRNA expression (Corbitt, Hagerty,
452 Fernandez, Morgan, & Strong, 2002). Mechanistically, the effect of PACAP depends on
453 continuous activation of PAC1 receptors and subsequent formation of cAMP and activation of
454 PKA (Houchi et al., 1994; Marley, Cheung, Thomson, & Murphy, 1996; Wong, Anderson, &
455 Tai, 2002). This is followed by a sustained phosphorylation of TH protein, at Ser40 residue in
456 bovine chromaffin cells (Bobrovskaya, Gelain, Gilligan, Dickson, & Dunkley, 2007) and Ser31
457 and Ser40 in PC12 cells (Haycock, 1996).

458 PACAP and VIP (vasoactive intestinal peptide) are closely related neuropeptides. Not
459 only, they share common receptors (PAC1, VPAC1 and VPAC2 receptors), but VIP was early
460 considered as a sympathoadrenal neurotransmitter (Wakade, Blank, Malhotra, Pourcho, &
461 Wakade, 1991). Indeed, VIP-containing fibers innervate the medulla (Hokfelt, Lundberg,
462 Schultzberg, & Fahrenkrug, 1981) and like PACAP, VIP is released at the adrenomedullary
463 synapse (Gaspo, Yamaguchi, & de Champlain, 1995; Wakade, Blank, Malhotra, Pourcho, &
464 Wakade, 1991) and stimulates catecholamine secretion in various species (Anderova, Duchene,
465 Barbara, & Takeda, 1998; Cheung & Holzwarth, 1986; Malhotra & Wakade, 1987;
466 Misbahuddin, Houchi, Nakanishi, Morita, & Oka, 1986; Wilson, 1988). This logically raises
467 the issue of whether VIP regulates the catecholamine biosynthetic enzymes, as its 'cousin'
468 PACAP does? VIP increases TH activity in rat adrenal medulla, PC12 cells and cultured bovine
469 chromaffin cells (Tischler, Perlman, Costopoulos, & Horwitz, 1985; Waymire et al., 1991). At
470 transcriptional level, VIP transiently increases the *Th* gene promoter activity and produces an
471 increase in *Th* mRNA level (Corbitt, Hagerty, Fernandez, Morgan, & Strong, 2002; Wessels-
472 Reiker, Haycock, Howlett, & Strong, 1991). This effect is mediated *via* the cAMP second
473 messenger pathway (Waymire et al., 1991; Wessels-Reiker, Haycock, Howlett, & Strong,
474 1991), leading to a subsequent phosphorylation of TH (Waymire et al., 1991). Collectively, the

475 effects of PACAP and VIP on TH appear roughly similar (Haycock, 1996), although not
476 necessarily of the same extent. Interestingly, but rendering more intricate the role of VIP and
477 PACAP in the adrenomedullary function, PACAP upregulates the expression of *VIP* mRNA
478 and VIP protein (Lee, Hahm, Hsu, & Eiden, 1999). In addition, these neuropeptides both appear
479 to synergize with nicotine to enhance *Th* gene expression or TH activity (Olasmaa, Guidotti, &
480 Costa, 1992; Rius, Guidotti, & Costa, 1994).

481 As such, the catecholamine-synthesizing enzymes are under a dual neuronal control
482 exerted by both cholinergic and peptidergic neurotransmitters. Because ACh- and neuropeptide
483 (PACAP, VIP...)-containing vesicles require distinct afferent nerve activity to be released (Ip
484 & Zigmond, 1984), the frequency of splanchnic nerve discharges is of a great importance.
485 Consistent with the effects of ACh, PACAP or VIP on TH, stimulation of the splanchnic nerve
486 induces TH phosphorylation (Haycock & Wakade, 1992). More interestingly, depending on the
487 stimulation frequency, distinct serine residues (Ser19, Ser31, Ser40...) are phosphorylated,
488 sounding like the phosphorylation profile elicited by cholinergic agonists or neuropeptides.

489

490 3.2.3. Humoral control

491 The anatomical juxtaposition between the adrenal medulla and cortex acts as a hub for signal
492 exchanges between the two tissues and therefore offers propitious conditions for humoral
493 control. In this context, the cortex, which is the last link in the hypothalamus-pituitary-adrenal
494 (HPA) axis, provides high circulating levels of corticoids to the medulla. The two major HPA-
495 derived factors that can impact catecholamine biosynthesis are the adrenocorticotrophic hormone
496 (ACTH, released from the pituitary corticotrophs) and glucocorticoids (released from the *zona*
497 *fasciculata* of the cortex in response to ACTH stimulation). Note that the hypothalamic
498 corticotropin-releasing hormone (CRH), which is the main secretagogue for ACTH secretion,
499 also contributes to the regulation of catecholamine biosynthetic enzymes, by increasing DbH

500 and PNMT activity (Lima & Sourkes, 1987). In CRH receptor type 1-deficient mice, *Pnmt*
501 mRNA levels are drastically reduced, but can be partially restored after ACTH treatment
502 (Yoshida-Hiroi et al., 2002). The presence of CRH and its receptors in the adrenal medulla of
503 various species (Tsatsanis et al., 2007) makes regulatory mechanisms even more complex.

504 The humoral control of catecholamine-producing enzymes by ACTH and
505 glucocorticoids has long been known ((Axelrod, 1977; Hodel, 2001; Nankova & Sabban, 1999;
506 Wurtman, Pohorecky, & Baliga, 1972) for selected reviews). It differentially targets TH, DbH
507 and PNMT, the latter probably being the most responsive to HPA axis. From a functional point
508 of view, it is generally accepted that ACTH and glucocorticoids are required for maintaining
509 steady state levels and activity of TH, DbH and PNMT. Indeed, enzyme activity and/or adrenal
510 enzyme content are decreased in hypophysectomized animals, both for TH (Kvetnansky,
511 Gewirtz, Weise, & Kopin, 1970; Mueller, Thoenen, & Axelrod, 1970a; Wurtman & Axelrod,
512 1966), DbH (Ciaranello & Black, 1971; Ciaranello, Wooten, & Axelrod, 1975; Weinshilboum
513 & Axelrod, 1970) and PMNT (Wurtman & Axelrod, 1965, 1966). Treatment of
514 hypophysectomized animals with ACTH or the synthetic glucocorticoid dexamethasone or
515 corticosterone can restore, at least in part, enzyme activity (Ciaranello, Wooten, & Axelrod,
516 1976; Jiang, Uht, & Bohn, 1989; Wurtman & Axelrod, 1965, 1966), although there are notable
517 differences between enzymes. In this context, the most remarkable difference is the lack of
518 effect of glucocorticoids on TH activity (Mueller, Thoenen, & Axelrod, 1970a; Wurtman &
519 Axelrod, 1966). Mechanistically, glucocorticoids activate consensus sequence for a
520 glucocorticoid-response element (GRE) in the promotor region of *Th* and *Pnmt* genes (Hagerty,
521 Morgan, Elango, & Strong, 2001; Ross et al., 1990). Note that ACTH and glucocorticoids have
522 no significant effect in control animals, regardless of the enzyme (Ciaranello, Wooten, &
523 Axelrod, 1976; Mueller, Thoenen, & Axelrod, 1970a). The effects of ACTH on catecholamine-
524 synthesizing enzymes mainly involve a paracrine regulation from the cortex (*zona fasciculata*

525 in which most ACTH receptors (melanocortin 2 receptors (MC2R) are localized) to the
526 medulla. However, a direct effect of ACTH on chromaffin cells cannot be excluded (Michener,
527 Peach, & Creutz, 1985).

528 ACTH and glucocorticoids also act at transcriptional level. Hypophysectomy markedly
529 reduces *Pnmt* mRNA levels (Jiang, Uht, & Bohn, 1989; Stachowiak, Rigual, Lee, Viveros, &
530 Hong, 1988), which can be restored by ACTH or dexamethasone treatment (Evinger, Towle,
531 Park, Lee, & Joh, 1992). Conversely, hypophysectomy does not change *Th* mRNA levels
532 (Stachowiak, Rigual, Lee, Viveros, & Hong, 1988).

533 In response to stressful situations, both the sympathoadrenal and HPA systems are
534 markedly activated. In this context, ACTH and glucocorticoids, which are stress-associated
535 factors, strongly contribute to remodel the catecholamines synthesizing enzymes in the
536 'stressed' medullary tissue and further E synthesis and release (Kvetnansky et al., 1995;
537 Kvetnansky & Sabban, 1998; Wong, 2006; Wurtman, 2002). Because E is the main stress
538 hormone secreted by the adrenal medulla, we propose to focus here on PNMT, the enzyme that
539 converts NE into E. As summarized in tables 1 and 2, PNMT expression is increased in acutely
540 or chronically stressed animals (with the exception of the hypoxic stress). The increase is
541 observed at the transcriptional and protein levels, and also on the enzyme activity (Wong &
542 Tank, 2007). The rise in *Pnmt* mRNA levels in stressed animals depends on intact HPA axis
543 (Viskupic et al., 1994). Stress evokes an ACTH surge followed by an increase in endogenous
544 glucocorticoids production by the adrenal cortex. Glucocorticoids then bind to GRE sequences
545 in the *Pnmt* gene to promote its induction. Other transcription factors, such as Egr-1, Sp1, or
546 AP-2, can regulate the *Pnmt* gene through their binding to consensus sequences in the *Pnmt*
547 promoter. These factors are stress-induced (Tai et al., 2007) and are therefore part of the
548 mechanisms underlying increased PNMT expression and E secretion in stressed animals.
549 Interestingly discussed in (Ciaranello & Black, 1971), the slow turnover of PNMT suggests that

550 the mechanisms described here represent rather a long-term regulation of PNMT, and are
551 probably not involved in short-term adrenal medullary responses to acute stress. The increase
552 in PNMT would probably find its biologic role in chronic stress situations. As such, PNMT
553 regulation is undoubtedly complex and relies on mechanisms that can act distinctly on the
554 transcription activation of *Pnmt* gene and expression of active PNMT enzyme.

555

556 **3.3 Stress-induced remodeling of chromaffin cell excitability**

557 Chromaffin cell excitability is a key parameter of the adrenal stimulus-secretion coupling
558 (Carbone, Borges, Eiden, Garcia, & Hernandez-Cruz, 2019). It results from the interplay
559 between cellular/intercellular mechanisms, including the afferent cholinergic and peptidergic
560 innervation (Guerineau, 2020), the gap junctional communication between chromaffin cells
561 (Colomer, Martin, Desarmenien, & Guerineau, 2012; Guerineau, 2018; Hodson, Legros,
562 Desarmenien, & Guerineau, 2015) and the repertoire of ion channels expressed at the
563 chromaffin cell plasma membrane. In this regard, both voltage-gated and leak/background
564 channels contribute to pattern the spiking activity by regulating the resting membrane potential,
565 shaping the action potential (AP) waveform, and setting the frequency of AP trains (Lingle,
566 Martinez-Espinosa, Guarina, & Carbone, 2018). Depending on the species, chromaffin cells
567 exhibit spontaneous and/or depolarization-evoked APs. While a large fraction of rat and mouse
568 chromaffin cells fires spontaneously (Brandt, Hagiwara, Kidokoro, & Miyazaki, 1976; Busik,
569 Nakamura, Abe, Shibuya, & Kanno, 1996; Cena, Brocklehurst, Pollard, & Rojas, 1991;
570 Colomer, Lafont, & Guerineau, 2008; Desarmenien et al., 2013; Guarina, Vandael, Carabelli,
571 & Carbone, 2017; Martin, Mathieu, Chevillard, & Guerineau, 2001; Nassar-Gentina, Pollard,
572 & Rojas, 1988; Perez-Alvarez, Hernandez-Vivanco, Caba-Gonzalez, & Albillos, 2011; Sanz-
573 Lazaro et al., 2019; Vandael, Zuccotti, Striessnig, & Carbone, 2012), only 10% of chromaffin
574 cells in humans display spontaneous APs (Hernandez-Vivanco et al., 2017). However, it should

575 be noted that many 'experimental' parameters (cultured cell/organotypic culture/acute slice/*in*
576 *vivo* in anaesthetized animals, electrophysiological techniques used to monitor electrical
577 activity (extracellular/intracellular/patch-clamp (conventional/perforated/loose) recordings),
578 animal species, age and sex, physiological/pathological conditions ...) can impact the detection
579 of spontaneous spiking.

580 Intriguingly, chromaffin cells can fire in a bursting mode and/or a regular/tonic mode
581 (Fig. 2B). While bursts are well described in the mouse, both in *ex vivo* and *in vivo* preparations
582 (Desarmenien et al., 2013; Marcantoni et al., 2010; Milman et al., 2021; Vandael, Marcantoni,
583 & Carbone, 2015; Vandael et al., 2015), rat chromaffin cells appear to spike preferentially with
584 a regular pattern (Colomer, Lafont, & Guerineau, 2008). Interestingly, chromaffin cell AP
585 frequency increases in stressed animals (Colomer, Lafont, & Guerineau, 2008). This is
586 associated with changes in ion channels, both in their expression at the membrane and their
587 biophysical properties. Although all channels can be stress-related targets, let me focus on two
588 ion channel families, namely the voltage-gated Ca^{2+} and K^{+} channels, which play a crucial role
589 in chromaffin cell excitability (Fig. 3).

590

591 [Insert Figure 3 here]

592 **Fig. 3: Increased chromaffin cell excitability and ion channel remodeling in the stressed**
593 **adrenal medulla.** In general, the voltage-gated ion channels that are involved in the generation
594 and propagation of action potentials are up-regulated in response to stress. This is the case for
595 Na_v channels, Ca_v channels and K^{+} channels. Regarding Ca_v channels, stress induces the
596 expression of low voltage-dependent channels (Ca_v3 family), $\text{Ca}_v3.2$ channels in particular. The
597 coupling between $\text{Ca}_v1.3$ and SK/BK channels is also enhanced in response to stress.
598 Collectively, these changes in ion channel expression underlie the sustained electrical
599 discharges observed in chromaffin cells of stressed animals.

600

601

602 Although either absent or weakly expressed in adult chromaffin cells, T-type Ca^{2+}
603 channels are a primary target of stress response (Guerineau, Desarmenien, Carabelli, &
604 Carbone, 2012). Indeed, elevated neuronal stimulation of the adrenomedullary tissue or adrenal
605 stimulation with exogenous PACAP leads to the recruitment of a T-type Ca^{2+} channel-mediated
606 Ca^{2+} influx, which in turn promotes catecholamine exocytosis (Hill, Chan, Kuri, & Smith,
607 2011). Similarly, in neonatal rat chromaffin cells, intermittent hypoxia upregulates *Cacnalg*
608 and *Cacnalh* mRNA levels and increases T-type Ca^{2+} currents (Souvannakitti et al., 2010). T-
609 type Ca^{2+} channels can also be recruited following chronic hypoxia (Carabelli et al., 2007) or
610 long-term beta-adrenergic stimulation (Novara et al., 2004).

611 L-type Ca^{2+} channels and large conductance K^+ (BK) channels both contribute to the
612 spontaneous rhythmicity of chromaffin cells (Marcantoni et al., 2007; Marcantoni, Carabelli,
613 Comunanza, Hoddah, & Carbone, 2008; Marcantoni et al., 2010; Vandael et al., 2010) and are
614 therefore expected candidates in the listing of stress-modulated ion channels. Upon prolonged
615 or sustained splanchnic nerve discharges (as observed during stress episodes), L-type Ca^{2+}
616 channels functionally couple to small conductance K^+ (SK) channels. This slows down firing
617 and prevents chromaffin cell overexcitation and excessive accumulation of circulating
618 catecholamines (Vandael, Zuccotti, Striessnig, & Carbone, 2012). Regarding BK channels, the
619 stress hormone ACTH controls the alternative splicing of *Slo*, the gene encoding them. Indeed,
620 hypophysectomy drastically decreases the levels of *Slo* transcripts containing the optional
621 STress axis-regulated EXon (STREX). This is prevented by ACTH injections (Xie & McCobb,
622 1998). When incorporated in *Xenopus* oocytes, STREX is associated with enhanced firing. As
623 such, by acting on BK channel protein composition, STREX contributes to shape the intrinsic
624 excitability of chromaffin cells. This is relevant during stress response in particular, as STREX-

625 containing *Slo* transcripts have been found less abundant in adrenal glands of stressed rats
626 compared with unstressed animals (McCobb, Hara, Lai, Mahmoud, & Flugge, 2003) and in
627 loser *versus* winner mice subjected to a social-instability stress (Chatterjee et al., 2009). More
628 generally, the findings that i) hypophysectomy changes the biophysical properties of BK
629 channels in chromaffin cells (Lovell & McCobb, 2001) and ii) glucocorticoids modulate the
630 gating of BK channels (Lovell, King, & McCobb, 2004) set the HPA axis as a major player in
631 regulating BK channels and intrinsic electrical properties of adrenal chromaffin cells.

632 From a functional point of view, the spiking pattern of chromaffin cells crucially
633 governs the adrenal stimulus-secretion coupling, in the sense that the bursting mode is more
634 efficient to trigger catecholamine secretion in rodent chromaffin cells Duan, Yu, Zhang, &
635 Zhou, 2003; Vandael et al., 2015). As mentioned above, depending on species, the burst firing
636 mode is more or less present among the chromaffin cell population at rest and any manipulation
637 (physiological, pharmacological, genetic...) that allows to switch to a burst-firing pattern is
638 associated with an increased release of catecholamines (Lingle, Martinez-Espinosa, Guarina, &
639 Carbone, 2018; Vandael, Marcantoni, & Carbone, 2015; Vandael et al., 2015). Although stress
640 response is associated with enhanced catecholamine secretion, one critical aspect to be clarified
641 is whether a 'stressed' chromaffin cell is more prone to fire bursting rather than tonic APs. It is
642 however likely that the answer will be not unique but will depend on animal species, age and
643 sex and stress paradigm...

644

645 **3.4 Stress-induced remodeling of secretory granule composition**

646 Chromaffin cell secretory granules are dense core structures. They are fascinating organelles in
647 the sense that they contain a complex mixture of various components including structural
648 proteins, monoamines (mainly catecholamines (dopamine, NE and E) and to a lesser extent
649 serotonin), granins (chromogranins, secretogranins) and derived peptides, peptides

650 (neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), galanin, bombesin, neurotensin,
651 natriuretic peptides, opioid peptides...), the relative processing enzymes (such as DbH for
652 catecholamine biosynthesis), as well as protease inhibitors ((Crivellato, Nico, & Ribatti, 2008)
653 for review). In addition, granules store high level of ATP and calcium, and ascorbic acid. This
654 terrific abundance and diversity of molecules contained in a single granule support distinct
655 quantitative and qualitative composition of granules, either within a same chromaffin cell and
656 between chromaffin cells in a same individual. This provides basis for multiple regulatory
657 mechanisms and predicts almost unlimited combinations of differential release and co-release,
658 at least in theory. The more striking feature of chromaffin secretory granules is that they content
659 all compounds required for an individual to cope with stress (Crivellato, Nico, & Ribatti, 2008).
660 They contain molecules acting on the cardiovascular system, mainly catecholamines (E and
661 NE), the first hormones to be released in the blood circulation in response to stress and
662 increasing heart rhythm and arterial pressure, as well as other vasoactive molecules. Opioid
663 peptides and morphine-derived molecules promote analgesia and antimicrobial peptides (some
664 of them being chromogranin-derived fragments) shield against infections (Goumon et al., 2006;
665 Metz-Boutigue, Goumon, Lugardon, Strub, & Aunis, 1998; Scavello et al., 2022). The immune
666 system is also sustained by secretion of molecules with anti-inflammatory properties. To make
667 the situation even more intricate, the chromaffin cell function itself can be modulated by
668 peptides acting through an autocrine/paracrine activity.

669 Chromaffin granule content is released by exocytosis, in response to a variety of stimuli,
670 the splanchnic nerve stimulation being the main one occurring *in vivo*. Contributing to the
671 striking plasticity of the adrenal medullary tissue, the transmitter release (or co-release) from
672 chromaffin granules is a finely regulated, activity-dependent process (Fulop, Radabaugh, &
673 Smith, 2005). The first level of regulation comes from the sympathetic nerve activation. As
674 already mentioned, the splanchnic nerve responds to stress episodes by increasing its spiking

675 discharge. Depending on the firing frequency, acetylcholine first and then neuropeptides such
676 as PACAP or VIP are sequentially released in the synaptic cleft. Depending on the nature of
677 the secretagogues released by the nerve terminals, the evoked electrical response in chromaffin
678 cells can display various patterns. This brings an additional level of regulation as the release of
679 molecules contained in the secretory granules is coded by specific firing patterns. In this
680 context, the most striking example is illustrated by the sequential release of catecholamines,
681 followed, upon prolonged stimulation (as found during stress), by the release of peptides. This
682 differential release is achieved through an activity-dependent dilation of the granule fusion pore
683 (Fulop, Radabaugh, & Smith, 2005). Intriguingly, the profiles of secreted catecholamines (E,
684 NE and dopamine) in cultured bovine chromaffin cells appear to be similar irrespective of the
685 secretagogues (nicotine, PACAP, histamine, bradykinin...), with E secreted at the highest level,
686 a finding consistent with its key role in the stress response (Podvin, Bunday, Toneff, Ziegler,
687 & Hook, 2015). Regarding neuropeptides, their release quantitatively differs between
688 secretagogues, but the highest amount of peptide released is found for the same secretagogue.
689 In this context, carbachol appears the most potent secretagogue for releasing both enkephalin,
690 NPY, VIP and galanin. Individual peptide analysis shows that, enkephalin is secreted at a higher
691 level than NPY, VIP and galanin (Podvin, Bunday, Toneff, Ziegler, & Hook, 2015).

692 Another family of molecules that is found in abundance in chromaffin granules is the
693 granins. The purpose of this chapter is not to provide an extensive review of the literature, but
694 let us focus on chromogranin A (CgA). First, CgA critically regulates secretory granule
695 biogenesis (Courel et al., 2006; Kim, Zhang, Sun, Wu, & Loh, 2005; Koshimizu, Kim, Cawley,
696 & Loh, 2010; Montero-Hadjadje et al., 2009; Pasqua et al., 2016). It also contributes to
697 catecholamine storage and release (Montesinos et al., 2008; Pasqua et al., 2016). Second, CgA
698 and its derived fragment catestatin are of particular interest in the context of the regulation of
699 catecholamines secretion in response to stress. In the 1980s, a CgA-derived peptide (later

700 identified as catestatin) has been reported to exert a negative feedback control on chromaffin
701 cell secretory activity (Simon, Bader, & Aunis, 1988). Catestatin acts as a non-competitive
702 nAChR antagonist and reduces catecholamine release. This 'brake' effect is particularly relevant
703 during the stress response, to avoid excessive and deleterious levels of circulating
704 catecholamines.

705 The remodeling of chromaffin cell granules may involve both granule composition
706 and/or granule number. As already mentioned, the chromaffin granule is highly versatile in its
707 composition, which can vary, both qualitatively and quantitatively, in response to so many
708 physiological/pathological stimuli (including stress), so that it is illusory to draw up a summary
709 scheme at the present time. Not only the amine (catecholamine) transmitter content is modified
710 in stressed animals (as expected for E), but also the peptide content. Here are some examples
711 of the phenotypic remodeling occurring in secretory granules of 'stressed' chromaffin cells, and
712 as mentioned above, this may concern each constituent of the granules. In animal models of
713 arterial hypertension, chromaffin cell granules contain more catecholamines than normotensive
714 animals (Takiyuddin et al., 1993). On the peptide front, insulin-induced hypoglycemia
715 elevates levels of mRNAs encoding NPY and enkephalin peptides (Fischer-Colbrie, Iacangelo,
716 & Eiden, 1988). This increase is prevented in adrenal glands denervated by bilateral transection
717 of the splanchnic nerve, indicating that neuropeptide biosynthesis (at least NPY and enkephalin)
718 is regulated by a neurogenic mechanism. Along the same line, prolonged stimulation of
719 nAChRs upregulates the mRNA levels encoding proenkephalin A (Wan, Marley, & Livett,
720 1991). *Npy* mRNA and/or NPY peptide expression, synthesis and secretion are increased in
721 stressed rodents (Han et al., 2005; Hiremagalur et al., 1994; Wang & Whim, 2013). Intermittent
722 hypoxia also increases NPY peptide expression level in the rat adrenal medulla (Raghuraman,
723 Kalari, Dhingra, Prabhakar, & Kumar, 2011). By promoting a prolonged and intense
724 vasoconstriction (Westfall et al., 1990), NPY could therefore contribute to stress-induced

725 elevation of arterial blood pressure (Han et al., 2005). In this context, NPY may be considered
726 as a 'stress molecule' (Zukowska-Grojec, 1995). Contrasting with the neural regulation of
727 peptides, CgA biosynthesis is regulated by a hormonal pathway involving the HPA axis, as
728 CgA levels in chromaffin granules are reduced in hypophysectomized rats (Fischer-Colbrie,
729 Iacangelo, & Eiden, 1988). Intriguingly, CgB, another member of the chromogranin family, is
730 not regulated by the HPA axis, pointing thus a marked difference in regulation of CgA and CgB
731 biosynthesis.

732 Thus, by continuously adjust the composition and nature of the released compounds to
733 homeostasis changes, chromaffin cell secretory granules are major elements in the stimulus-
734 secretion coupling plasticity.

735

736 **3.5 Stress-induced remodeling of synaptic neurotransmission**

737 Adrenal chromaffin cells are innervated by the splanchnic nerve terminals. In response to stress
738 episodes, changes in synaptic transmission occur both at presynaptic and postsynaptic sites.
739 While at the pre-synapse, the main changes involve the nature of the transmitters released in
740 the synaptic cleft, at the post-synapse, the main stress-modified targets are the nicotinic
741 acetylcholine receptors (nAChRs) (Fig. 4).

742

743 [Insert Figure 4 here]

744 **Fig. 4: Remodeling of synaptic transmission in the stressed adrenal medulla.** (A) Changes
745 observed between control and stressed animals occur both at pre- and postsynaptic sites.
746 Presynaptically, the main stress-induced change relies on the nature of the neurotransmitter
747 released onto chromaffin cells. While ACh is the principal adrenomedullary neurotransmitter
748 in control animals, PACAP takes over in stressed animals. Postsynaptically, stress promotes a
749 change in nAChR subtypes expressed in chromaffin cells, with a switch from $\alpha 3$ - to $\alpha 9$ -

750 containing nAChRs. (Ba) Increased frequency and amplitude of spontaneous EPSCs in a
751 stressed rat (5 day-cold exposure). *Adapted from Colomer, C., Lafont, C., & Guérineau, N.C.*
752 *(2008). Stress-induced intercellular communication remodeling in the rat adrenal medulla.*
753 *Annals of the New York Academy of Sciences, 1148:106-111.*
754 <https://nyaspubs.onlinelibrary.wiley.com/doi/abs/10.1196/annals.1410.040>. (Bb) Changes in
755 the nature of nAChRs expressed in a 'stressed' chromaffin cell versus a 'control' chromaffin cell.
756 The pharmacological agent hexamethonium and the toxin α RgIA were used to antagonize α 3-
757 and α 9-containing nAChRs, respectively. *Adapted from Colomer, C., Olivos-Oré, L.A.,*
758 *Vincent, A., McIntosh, J.M., Artalejo, A.R., & Guérineau, N.C. (2010). Functional*
759 *characterization of alpha9-containing cholinergic nicotinic receptors in the rat adrenal*
760 *medulla: implication in stress-induced functional plasticity. Journal of Neuroscience, 30:6732-*
761 *6742. https://www.jneurosci.org/content/30/19/6732.*

762

763

764 *3.5.1 Presynaptic remodeling: switch from cholinergic to peptidergic neurotransmission*

765 *3.5.1.1 Prevailing contribution of PACAP*

766 Under resting ('unstressed') conditions, the sympathetic nervous system discharges at a
767 moderate firing rate (less than 1 Hz) (Fulop, Radabaugh, & Smith, 2005; Holman, Coleman,
768 Tonta, & Parkington, 1994; Wakade, 1981; Wolf, Zarkua, Chan, Sridhar, & Smith, 2016) and
769 the neurotransmission between the splanchnic nerve and chromaffin cells uses ACh as
770 neurotransmitter. In response to a stressful situation, the sympathetic tone drastically increases
771 and the splanchnic nerve can fire at frequencies above 10 Hz (Fulop, Radabaugh, & Smith,
772 2005; Holman, Coleman, Tonta, & Parkington, 1994; Wakade, 1981; Wolf, Zarkua, Chan,
773 Sridhar, & Smith, 2016), thus enhancing synaptic neurotransmission onto chromaffin cells (Fig.
774 4Ba). In cold exposed rats for example, the percentage of chromaffin cells exhibiting

775 spontaneous excitatory postsynaptic currents (EPSCs) is higher than in unstressed animals and
776 synaptic events occur more frequently (Colomer, Lafont, & Guerineau, 2008). To ensure the
777 sustained and prolonged secretion of catecholamines needed by the organism to cope with
778 stress, a switch in the nature of the neurotransmitter released by the splanchnic nerve endings
779 takes place. The 'resting' primary cholinergic neurotransmission gives way to a peptidergic
780 transmission. In this context, the neurotransmitter PACAP, proposed as the 'stress response'
781 neurotransmitter of the sympathoadrenal axis (Hamelink et al., 2002) and subsequently
782 identified as a master regulator of the stress response (Eiden, Emery, Zhang, & Smith, 2018;
783 Mustafa, 2013) plays a major role (Fig. 4A, right panel). Unlike the cholinergic transmission,
784 the high-frequency firing of splanchnic nerve in response to stress benefits to the preferential
785 release of neuropeptides. PACAP fulfills many criteria of an adrenomedullary neurotransmitter,
786 as i) PACAP-immunoreactive fibers innervate the medullary tissue (Moller & Sundler, 1996;
787 Tornoe et al., 2000), ii) PACAP colocalizes with the vesicular ACh transporter in presynaptic
788 nerve endings (Hamelink et al., 2002) and iii) PACAP receptors are present at the chromaffin
789 cell plasma membrane (Shivers, Gorcs, Gottschall, & Arimura, 1991). Not only PACAP can
790 act as neurotransmitter at the splanchnic nerve-chromaffin cell synapse, but its contribution
791 prevails during stress episode. Endogenous PACAP is released by presynaptic terminals when
792 the sympathoadrenal system is highly activated, as observed during severe hypotension
793 (Lamouche & Yamaguchi, 2003). PACAP specifically triggers catecholamine release under
794 elevated splanchnic nerve firing activity (Kuri, Chan, & Smith, 2009). Unlike ACh and nicotine
795 whose responses display rapid desensitization (Boksa & Livett, 1984), PACAP-evoked
796 secretion of catecholamines can last for several hours (Babinski, Bodart, Roy, De Lean, & Ong,
797 1996; Taupenot, Mahata, Mahata, & O'Connor, 1999), a duration consistent with prolonged or
798 repetitive stress. In addition, many molecules contained in chromaffin cell granule
799 (neuropeptides, cytokines, growth factors) are up-regulated by PACAP ((Ait-Ali, Samal,

800 Mustafa, & Eiden, 2010) for review). PACAP also enhances gap junction-mediated electrical
801 coupling between chromaffin cells, favouring the spread of electrical activation (Hill, Lee,
802 Samasilp, & Smith, 2012) and therefore the propagation of instructive signals for secretion
803 (Desarmenien et al., 2013; Martin, Mathieu, Chevillard, & Guerineau, 2001). Interestingly,
804 PACAP mostly stimulates the secretion of E (Guo & Wakade, 1994). Because PACAP, as
805 mentioned in the chapter 3.2.2, is a potent regulator of TH and PNMT, both at transcriptional
806 and post transcriptional level (Bobrovskaya, Gelain, Gilligan, Dickson, & Dunkley, 2007;
807 Corbitt, Hagerty, Fernandez, Morgan, & Strong, 2002; Haycock, 1996; Wong, Anderson, &
808 Tai, 2002), it is obvious that stimulus-secretion coupling that take place during stress is altered
809 in PACAP-deficient animals. As such, the sustained secretion of catecholamines triggered by
810 hypoglycemia or by direct high-frequency stimulation of the splanchnic nerve is impaired in
811 PACAP^{-/-} mice (Hamelink et al., 2002; Stroth et al., 2013). At the transcriptional level, *Th* and
812 *Pnmt* mRNA abundance in response to a restraint stress or hypoglycemia is lower in PACAP^{-/-}
813 compared to wild type animals (Stroth & Eiden, 2010; Stroth et al., 2013). The increase in TH
814 activity which follows the activation of the sympathetic nervous system is also impaired in
815 PACAP^{-/-} animals (Hamelink et al., 2002).

816

817 3.5.1.2 Other neuropeptides

818 Many others neuropeptides are present in the nerve fibers innervating the adrenomedullary
819 tissue ((Guerineau, 2020) for review), and may be involved in the presynaptic remodeling of
820 the neurotransmission. This is in particular the case for the vasoactive intestinal peptide (VIP),
821 a peptide belonging to the same neuropeptide superfamily as PACAP, substance P and
822 neuropeptide Y. Like PACAP, VIP satisfies essential criteria to ensure the role of
823 neurotransmitter in the adrenomedullary tissue. VIP is present in nerve terminals (Hokfelt,
824 Lundberg, Schultzberg, & Fahrenkrug, 1981; Holzwarth, 1984; Linnoila, Diaugustine,

825 Hervonen, & Miller, 1980). It is released upon splanchnic nerve stimulation and elicits
826 catecholamine secretion (Wakade, Blank, Malhotra, Pourcho, & Wakade., 1991). In addition,
827 VIP is a potent regulator of TH and PNMT, both at transcriptional and post transcriptional level
828 (Haycock, 1996). In bovine chromaffin cells, VIP increases phosphorylation and activation of
829 TH through a cyclic AMP-dependent pathway, stimulating catecholamine biosynthesis
830 (Waymire et al., 1991). Similarly, in PC12 cells, VIP stimulates a time- and concentration-
831 dependent increase in *Th* mRNA levels, followed by an increased TH protein expression
832 (Wessels-Reiker, Haycock, Howlett, & Strong, 1991). Substance P (SP) is present in the nerve
833 fibers innervating the adrenal medulla, and originating from the intermediolateral cell column
834 of the spinal cord and the dorsal root ganglia (Holets & Elde, 1982; Linnoila, Diaugustine,
835 Hervonen, & Miller, 1980; Zhou, Oldfield, & Livett, 1991). SP is released upon splanchnic
836 nerve stimulation and affects catecholamine secretion, functioning thus as a neuromodulator
837 (Livett & Zhou, 1991). In bovine chromaffin cells, SP selectively inhibits the nicotinic response
838 of isolated cells in a non-competitive manner and protects the nicotinic response against
839 agonist-induced desensitization. This effect is particularly relevant during stress (Khalil, Livett,
840 & Marley, 1986). Like VIP and SP, Neuropeptide Y (NPY) is present in nerve fibers
841 innervating the adrenomedullary tissue (Kuramoto, Kondo, & Fujita, 1986; Maubert, Tramu,
842 Croix, Beauvillain, & Dupouy, 1990; Varndell, Polak, Allen, Terenghi, & Bloom, 1984). NPY
843 may therefore function as a neurotransmitter/neuromodulator during stress response, although
844 this hypothesis remains to be demonstrated.

845

846 *3.5.2 Postsynaptic remodeling*

847 *3.5.2.1 Nicotinic acetylcholine receptors*

848 Chromaffin cells express neuronal nicotinic acetylcholine receptors (nAChRs) (Criado, 2018).
849 nAChRs are ligand-gated cation channels and are homo or heteropentameric assemblies.

850 Heteropentamers are built of a combination of α and β subunits, and the dominant nAChR in
851 rat and bovine chromaffin cells express $\alpha 3\beta 4$ subunits (Campos-Caro et al., 1997; Colomer et
852 al., 2010; Criado, Alamo, & Navarro, 1992; Di Angelantonio, Matteoni, Fabbretti, & Nistri,
853 2003). A recent study performed in the rat shows that the adrenal medullary tissue contains two
854 populations of chromaffin cells that express either $\alpha 3\beta 4$ nAChRs alone or $\alpha 3\beta 4$ together with
855 the $\alpha 3\beta 2\beta 4$ subtype (Hone et al., 2020). In addition to $\alpha 3\beta 4$ -containing nAChRs,
856 homopentameric $\alpha 7$ - and $\alpha 9/\alpha 10$ -built nAChRs are also expressed in bovine (Garcia-Guzman
857 et al., 1995) and rat chromaffin cells (Colomer et al., 2010; Martin, Mathieu, & Guerineau,
858 2003). nAChRs located in chromaffin cell plasma membrane are possible targets for stress-
859 induced postsynaptic remodeling (Guerineau, Desarmenien, Carabelli, & Carbone, 2012). At
860 rest in non-stressed animals (rat), ACh released by splanchnic nerve endings onto chromaffin
861 cells elicits post-synaptic excitatory events which are mediated by activation of $\alpha 3\beta 4$ nAChRs
862 and, to a much smaller extent, $\alpha 7$ - and/or $\alpha 9$ -containing nAChRs (Barbara & Takeda, 1996;
863 Colomer et al., 2010; Martin, Mathieu, & Guerineau, 2003) (Fig. 4Bb, left panel). In response
864 to cold stress exposure, both *chrna9* mRNA and $\alpha 9$ -containing nAChR protein levels are
865 upregulated and these receptors dominantly contribute to acetylcholine-induced current in
866 stressed rats (Colomer et al., 2010) (Fig. 4Bb, right panel). Under the same experimental
867 conditions, the expression of $\alpha 3$ and $\alpha 7$ nAChRs does not change. Contrasting with this, *chrna7*
868 transcript levels are decreased in response to long-term hypoxia (Ducsay et al., 2007). Making
869 the situation more complex, $\alpha 7$ nAChRs, at least at the transcriptional level, are positively
870 regulated by chronic stress and glucocorticoids (Carrasco-Serrano & Criado, 2004). More
871 generally, these few examples illustrate that the regulation of nAChR expression is stressor-
872 specific. This is of great importance because each nAChR channel (itself resulting from the
873 combination of various subunits) display specific biophysical properties (Ca^{2+} permeability,
874 gating, desensitization...) and may allow chromaffin cells to appropriately respond to a variety

875 of stressors. Because an increase in cytosolic Ca^{2+} is an initial step in exocytosis (Baker &
876 Knight, 1978), upregulation of homomeric nAChRs ($\alpha 7$ - and $\alpha 9$ -built channels) in 'stressed'
877 chromaffin cells is likely physiologically relevant since these two nAChRs exhibit a higher
878 Ca^{2+} permeability than $\alpha 3$ nAChRs (Fucile, Sucapane, & Eusebi, 2006; Katz et al., 2000;
879 Seguela, Wadiche, Dineley-Miller, Dani, & Patrick, 1993; Zhang, Vijayaraghavan, & Berg,
880 1994). ACh-mediated $\alpha 7/\alpha 9$ nAChR activation would trigger instructive signaling cascades
881 contributing to enhance catecholamine and/or neuropeptide release from 'stressed' chromaffin
882 cells. Although this hypothesis is attractive, it likely reflects only part of the regulatory
883 mechanisms that occurs in stressed animals. The truth is obviously more complex, as nAChRs
884 could interact with other cellular components such as ion channels. For example, the well-
885 described cross-talk between $\alpha 9/\alpha 10$ nAChRs and SK channels in the mouse cochlea (Nie et
886 al., 2004) might also occurs in adrenal chromaffin cells. The high Ca^{2+} permeability of $\alpha 9/\alpha 10$
887 channels combined with the involvement of SK channels in shaping chromaffin cell firing
888 frequency would negatively regulate cell excitability by shortening the depolarizing action of
889 synaptically released ACh. This would thereby prevent the formation of a depolarizing plateau
890 in case of high frequency synaptic activity at the splanchnic nerve-chromaffin cell junction.

891

892 *3.5.2.2 Muscarinic acetylcholine receptors*

893 The muscarinic signaling pathway in the adrenal medullary tissue occurs both at pre-and post-
894 synaptic sites. In this regard, it is noteworthy that, in contrast to the extensive literature on
895 postsynaptic mAChRs, very few studies have focused on the contribution of presynaptic
896 mAChRs to adrenal stimulus-secretion coupling. On the elegant preparation of acute adrenal
897 slice, which maintains the tissular architecture of the adrenal gland, Barbara and colleagues
898 reported the inhibitory role of presynaptic mAChRs (M3-M4 receptors) on ACh release at rat
899 chromaffin cell synapses leading to uncouple catecholamine secretion from synaptic activity

900 (Barbara, Lemos, & Takeda, 1998). The attractive hypothesis, but still in search of validation,
901 of the physiological relevance of this inhibitory function is that activation of presynaptic
902 mAChRs may serve as a feedback mechanism when large quantities of ACh are released
903 Barbara, Lemos, & Takeda, 1998). On the postsynaptic side, adrenal chromaffin cells express
904 nicotinic and muscarinic receptors (mAChRs), and both contribute to the secretion of
905 catecholamines. However, far fewer studies have focused on deciphering the role of mAChRs
906 during neuronal transmission, compared with nAChRs (likely because nAChRs, more than
907 mAChRs, mediate cholinergic synaptic transmission from the splanchnic nerve, at least in rat
908 (Wakade & Wakade, 1983)). One of the main differences between nAChR- and mAChR-
909 mediated catecholamine release is the response time. While nAChR-mediated secretory
910 response displays almost no latency, catecholamine release in response to mAChR activation
911 is elicited within few seconds (Chowdhury, Guo, Wakade, Przywara, & Wakade, 1994). The
912 contribution of mAChRs to the adrenal stimulus-secretion coupling varies appreciably among
913 mammals ((Inoue, Matsuoka, Harada, & Kao, 2018; Olivos & Artalejo, 2008) for reviews).
914 Nevertheless, convergent findings deserve to be highlighted. First, muscarinic agonists induce
915 increase in chromaffin cell excitability in the rat (Akaike, 1992; Akaike, Mine, Sasa, & Takaori,
916 1990a, 1990b; Barbara, Lemos, & Takeda, 1998; Neely & Lingle, 1992), cat (Ladona, Aunis,
917 Gandia, & Garcia, 1987; Uceda et al., 1992), guinea-pig (Holman, Tonta, Coleman, &
918 Parkington, 1998; Inoue, Harada, Matsuoka, Nakamura, & Warashina, 2012) and mouse
919 (Nassar-Gentina, Pollard, & Rojas, 1988). This is mediated by activation of M1 (Harada,
920 Matsuoka, Miyata, Matsui, & Inoue, 2015) and or M3/M4 receptors (Barbara, Lemos, &
921 Takeda, 1998). In rat and guinea pig chromaffin cells, muscarinic stimulation, through
922 activation of M1 receptors, induces membrane depolarization and triggers APs. This is due to
923 clathrin-dependent endocytosis of TWIK-related acid-sensitive K⁺ (TASK)1 channels, through
924 the PLC-PKC-Src pathway (Matsuoka & Inoue, 2017). In addition, in guinea pig, muscarinic

925 excitation is due not only to TASK1 channel inhibition, but also to activation of non-selective
926 cation channels (Inoue, Harada, Matsuoka, Nakamura, & Warashina, 2012; Inoue & Kuriyama,
927 1991). Second, in response to a muscarinic stimulation, a release of E over NE primarily occurs,
928 in the cat (Ballesta, Borges, Garcia, & Hidalgo, 1989; Douglas & Poisner, 1965) and rat (Chen
929 & Dixon, 1990). Third, the density of mAChRs present at chromaffin cell membrane is higher
930 in NE-containing cells, in calf (Michelena, Moro, Castillo, & Garcia, 1991) and in rat (Zaika,
931 Pochynyuk, Kostyuk, Yavorskaya, & Lukyanetz, 2004). This could be of particular interest
932 especially in determining the ratio of E/NE secreted from the adrenal medulla during stress,
933 considering that i) the selective activation of one chromaffin cell population over the other is
934 stressor-specific and ii) E- and NE-secreting cells are distinctly innervated (more ACh-positive
935 fibers contacting NE cells (Lewis & Shute, 1969)).

936 Muscarinic signaling also impacts the transcriptional regulation of catecholamine
937 biosynthesis. In rat, the mAChR agonist bethanechol increases *Th* gene transcription rate, both
938 in innervated and denervated adrenal glands indicating that the effect is independent on
939 transsynaptic influences (Piech-Dumas, Sterling, & Tank, 1999). In bovine chromaffin cells,
940 muscarinic agents elevate transcription of the *Pnmt* gene (Evinger, Ernsberger, Regunathan,
941 Joh, & Reis, 1994).

942

943 As such, by targeting both pre- and post-synaptic processes, remodeling of the
944 neurotransmission at the splanchnic nerve-chromaffin cell synapse covers almost all the targets
945 of the stimulation-secretion coupling, and therefore appears as a decisive mechanism during
946 stress response. In this chapter dedicated to the remodeling of splanchnic nerve-chromaffin cell
947 synapse, I have deliberately used the term "remodeling" and not "plasticity". Could we talk
948 about plasticity as observed at synapses in the central nervous system? Does the splanchnic
949 nerve-chromaffin cell synapse undergo short- and/or long-term potentiation? To my

950 knowledge, the only report of a plasticity mechanism at this synapse comes from Whim's
951 laboratory, which shows that fasting induces in chromaffin cells a decrease in the paired-pulse
952 ratio of evoked synaptic currents, thus leading to a long-lasting increase in the strength of the
953 preganglionic-chromaffin cell synapse (Gupta, Ma, Wang, & Whim, 2017; M. Wang, Wang, &
954 Whim, 2016). Obviously, this research field remains open to future investigation.

955

956 **3.6 Stress-induced remodeling of gap junctional communication**

957 In addition to the neural command arising from the splanchnic nerve, chromaffin cells are
958 coupled through gap junctions. This local intercellular communication pathway has long been
959 suspected to play a role in adrenal stimulus-secretion coupling of various species (Cena,
960 Nicolas, Sanchez-Garcia, Kirpekar, & Garcia, 1983; Ishikawa & Kanno, 1978; Moser, 1998),
961 but it was only in the early 2000s that their functional role was actually unveiled ((Martin,
962 Mathieu, Chevillard, & Guerineau, 2001) for the inaugural report and (Colomer, Desarmenien,
963 & Guerineau, 2009; Colomer, Martin, Desarmenien, & Guerineau, 2012; Guerineau, 2018;
964 Guerineau, Campos, Le Tissier, Hodson, & Mollard, 2022; Hodson, Legros, Desarmenien, &
965 Guerineau, 2015) for reviews). By promoting propagation of electrical (and metabolic) signals
966 between chromaffin cells, the gap junction-mediated route acts as a complement to synaptic
967 transmission to drive catecholamine exocytosis (Desarmenien et al., 2013; Martin, Mathieu,
968 Chevillard, & Guerineau, 2001). In addition, it displays a dynamic and continuous remodeling,
969 making it a key element of stimulus-secretion coupling (Guerineau & Desarmenien, 2010;
970 Guerineau, Desarmenien, Carabelli, & Carbone, 2012). In conditions associated with an
971 impairment of synaptic transmission (*i.e.* denervated gland or pharmacological blockade of
972 postsynaptic nAChRs for examples), gap junctional coupling undergoes a drastic upregulation
973 (Martin, Mathieu, & Guerineau, 2003). By compensating for the loss in chemical

974 neurotransmission, gap junction signaling could sustain catecholamine release by maintaining
975 communication within the chromaffin cell network.

976 Rodent chromaffin cells express connexin 43 (Cx43), a connexin found in a wide variety
977 of cell types including endocrine cells, and the neuronal connexin 36 (Cx36) (Desarmenien et
978 al., 2013; Hodson, Legros, Desarmenien, & Guérineau, 2015; Martin, Mathieu, Chevillard, &
979 Guérineau, 2001; Meda et al., 1993; Moser, 1998). In the human adrenal medullary tissue, the
980 predominant connexin expressed is Cx50, with a *de novo* expression of Cx26, Cx32 and Cx43
981 in pheochromocytomas (Willenberg et al., 2006). When connexin channels are open, they
982 provide electrical communication between coupled cells, allowing electrical signals (such as
983 APs) to be transmitted into neighboring cells, either as small depolarizations (weak coupling)
984 or as whole APs (robust coupling) (Fig. 5).

985

986 [Insert Figure 5 here]

987 **Fig. 5:** Remodeling of gap junction-mediated electrical coupling between chromaffin cells in
988 the stressed adrenal medulla. In response to stress (5 day-cold exposure, male rat), expression
989 of both Cx36 and Cx43, the two main connexins expressed in rat chromaffin cells, is up-
990 regulated. As functional consequences, i) the number of chromaffin cells coupled by gap
991 junctions increases and ii) the strength of the electrical coupling is also enhanced, as evidenced
992 by the appearance of a robust coupling in stressed animals. *Adapted from Colomer, C., Olivos-
993 Oré, L.A., Coutry, N., Mathieu, M.-N., Arthaud, S., Fontanaud, P., Iankova, I., Macari, F.,
994 Thouënnon, E., Yon, L., Anouar, Y., & Guérineau, N.C. (2008). Functional remodeling of gap
995 junction-mediated electrical communication between adrenal chromaffin cells in stressed rats.
996 Journal of Neuroscience, 28:6616-6626. <https://www.jneurosci.org/content/28/26/6616>.*

997

998

999 The occurrence of gap junctional coupling between chromaffin cells depends on various factors,
1000 including age, species, animal sex, physiological state (*i.e.* stressed/unstressed) and splanchnic
1001 innervation competence (reviewed in (Colomer, Desarmenien, & Guerineau, 2009; Hodson,
1002 Legros, Desarmenien, & Guerineau, 2015)). In adult female rats, the percentage of chromaffin
1003 cells exhibiting a gap junctional communication is about 40%, whereas it is only 20% in age-
1004 matched males (Colomer et al., 2008; Martin, Mathieu, Chevillard, & Guerineau, 2001). One
1005 possible explanation for this discrepancy could be that males and females differentially respond
1006 to stress, with female expressing a greater increase in plasma catecholamine (Livezey, Miller,
1007 & Vogel, 1985; Weinstock, Razin, Schorer-Apelbaum, Men, & McCarty, 1998). The extended
1008 gap junctional communication in female would be in anticipation of a greater secretory
1009 response.

1010 In stressed animals (cold exposed rats and mice), gap junctional communication in the
1011 adrenal medulla is enhanced (Colomer, Lafont, & Guerineau, 2008; Desarmenien et al., 2013).
1012 Not only the number of coupled chromaffin cells is increased but also the coupling strength, as
1013 evidenced by the appearance of robust coupling (Colomer et al., 2008). This is particularly
1014 relevant in terms of catecholamine secretion, whose demand is greatly increased in stressful
1015 situations. In parallel, expression of Cx36 and Cx43 is increased, both at the transcriptional and
1016 protein levels. Interestingly and fully consistent with the role of PACAP as stress
1017 neurotransmitter at the adrenomedullary synapse, is the finding that the electrical coupling and
1018 the spread of excitability between chromaffin cells is increased by PACAP (Hill, Lee, Samasilp,
1019 & Smith, 2012).

1020 Along the same line, neonate (postnatal day 0-1) chromaffin cells are extensively
1021 coupled by gap junctions (Martin, Alonso, & Guerineau, 2005; Martin, Mathieu, & Guerineau,
1022 2003). Indeed, although pre-and post-synaptic elements are already present prenatally
1023 (Daikoku, Kinutani, & Sako, 1977), the neurogenic control of catecholamine secretion is not

1024 fully competent at birth but becomes mature during the first postnatal week (Slotkin, 1986).
1025 This critical period is marked by a persistent anatomical and functional remodeling of the
1026 adrenomedullary tissue, that is crucial to the acquisition of the neurogenic control. The switch
1027 from non-neurogenic (gap junction-mediated) to neurogenic control of catecholamine secretion
1028 is accompanied by a decrease in the number of gap junction-coupled chromaffin cells and an
1029 increase in synaptogenesis (Martin, Alonso, & Guerineau, 2005). As such, a prominent and
1030 continuous interplay between synaptic activity and gap junctional coupling is necessary for
1031 normal function of the adrenal medulla.

1032

1033

1034 **4. Conclusion and futures directions**

1035 As illustrated throughout this chapter, the adrenal medulla is a fascinating but complex
1036 neuroendocrine tissue, whose cellular/multicellular responses are devoted to the maintenance
1037 of body homeostasis in response to harmful situations. Behind intricate regulatory mechanisms,
1038 there is however a simple reality: coping with stress and adapting the body to changes in its
1039 homeostasis.

1040 Further decoding of the intra/intercellular mechanisms governing the stimulus-secretion
1041 coupling will undoubtedly improve our knowledge not only of the functioning of the adrenal
1042 medulla itself, but also and above all of the role of its secretory products in maintaining body
1043 homeostasis during stress and in stress-related pathologies. By releasing catecholamines (and
1044 other biologically active products) into the systemic circulation, it is obvious that the adrenal
1045 medulla holds a central position, at the interface between several organs, both peripheral and
1046 central. To my opinion, this makes it even more fascinating. Its influence therefore goes far
1047 beyond its role as a neuroendocrine tissue secreting stress hormones. After having been used
1048 for a long time as a 'simple cellular model' for the study of the molecular mechanisms of

1049 exocytosis, it is high time that the adrenal medullary glands regain their credentials as key tissue
1050 in the inter-organ communication of the body. To achieve this, the first step requires to fully
1051 elucidate both the afferent and efferent neuronal and/or humoral circuits linking the adrenal
1052 gland to other organs. Using trans-neuronal transport of rabies virus in the rat and monkey, two
1053 studies have elegantly tackled this issue and successfully unveiled cortical brain regions that
1054 have access to the adrenal medulla (Dum, Levinthal, & Strick, 2016, 2019). Intriguingly, in
1055 monkeys, these cortical regions are involved in movement, cognition and affect. This
1056 fascinating finding shows how the adrenal medulla must be seen not as a single functioning
1057 tissue, but as a link in a more complex chain connecting organs. In a more provocative manner,
1058 it could be proposed that the adrenal medulla is part of the neural substrate that links cognition
1059 to internal organ function.

1060 This new view of adrenomedullary tissue as a link between peripheral and central organs
1061 is beginning to emerge in the field of gut-brain axis research. Growing evidence highlights the
1062 involvement of gut microbiota dysbioses in the pathophysiology of stress and stress-related
1063 disorders (Rea, Dinan, & Cryan, 2017). It is now well known the gut microbiota influences the
1064 HPA axis, as ascertained by an enhanced blood corticosterone level and a dysregulation of the
1065 expression of genes encoding CRH and glucocorticoid receptors (brain) and corticosterone
1066 (adrenal cortical tissue) in germ-free rodents exposed to acute psychological stressors
1067 (Vagnerova et al., 2019). In addition, recent data accredit the gut microbiota as a partner able
1068 to affect the adrenomedullary tissue function (Mir et al., 2020). The dialogue between the gut
1069 microbiota and the adrenal medulla is still in its infancy but is certainly worthy of future
1070 investigations.

1071 After having been used for a long time as a 'simple cellular model' for the study of the
1072 molecular mechanisms of exocytosis, it is high time that the adrenal medullary glands regain
1073 their credentials as key tissue in the inter-organ communication of the body. It is likely that new

1074 discoveries and innovative concepts targeting the adrenomedullary tissue will emerge in the
1075 coming years.

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1083 **References**

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Table 1: Effect of various acute stress on gene expression, protein level and enzymatic activity on catecholamine biosynthetic enzymes

ACUTE STRESS	species sex	TH			DbH			PNMT			References
		mRNA	protein	activity	mRNA	protein	activity	mRNA	protein	activity	
single IMMO (2-2.5 hours)	rat, Sprague-Dawley ♂, adult			↑						→	(Kvetnansky, Weise, & Kopin, 1970)
		↑		→	→						(McMahon et al., 1992)
		↑	→	→							(Nankova et al., 1994)
								↑			(Viskupic et al., 1994)
		↑	→	↑							(Kvetnansky, Rusnak, Dronjak, Krizanova, & Sabban, 2003)
		↑	→		→	↑		↑			(Kvetnansky et al., 2004)
		↑	→	→							(Xu, Chen, Sun, Sterling, & Tank, 2007)
								↑	↑		(Tai et al., 2007)
	mouse, C57B1/129SV ♂, adult							↑		→	(Kvetnansky et al., 2006)
	mouse, C57B1/129SV ♂+♀, adult	↑			↑			↑			(Kubovcakova et al., 2004)

hypotension	rat, Sprague-Dawley ♂, adult			↑									(Fluharty et al., 1985)
glucoprivation	rat, Sprague-Dawley ♂, adult			↑									(Fluharty et al., 1985)
		↑	↑	↑									(Kvetnansky et al., 2003)
		↑											(DeCristofaro & LaGamma, 1994)
		↑	→	↑									(Xu et al., 2007)
	rat, Sprague-Dawley, 4-d-old pups	↑											(DeCristofaro & LaGamma, 1994)
hypoglycemia (insulin bolus)	rat, Sprague-Dawley ♂, adult			↑									(Fluharty et al., 1985)
		↑	→	↑									(Kvetnansky et al., 2003)
		↑											(DeCristofaro & LaGamma, 1994)
	rat, Sprague-Dawley ♀, adult	→							→				(Adams, Legan, Ott, & Jackson, 2005)
	rat, Sprague-Dawley, 4-d-old pups	→											(DeCristofaro & LaGamma, 1994)
















	mouse C57BL/6N, ♂, adult												(Stroth et al., 2013)
nicotine injection	rat, Sprague- Dawley ♂, adult												(Jahng, Houpt, Joh, & Wessel, 1997)
													(Serova, Danailov, Chamas, & Sabban, 1999)
hypoxia (2 hours, nitrogen)	rat, Sprague- Dawley, 4-d- old pups												(DeCristofaro & LaGamma, 1994)
hypoxia (1-48 hours, 10% O ₂)	rat, Sprague- Dawley ♂, adult												(Millhorn, Czyzyk- Krzaska, Bayliss, & Lawson, 1993)
acute footshock (1 mA, 1 pulse/min for 10 min)	rat, Sprague- Dawley ♂, adult												(Ong et al., 2014)
acute footshock (1 mA, 1 pulse/min for 20-40 min)	rat, Sprague- Dawley ♂, adult												(Ong et al., 2014)
individual housing (24 hours)	rat, Wistar ♂, adult												(Armando et al., 2001)

Table 2: Effect of various chronic stress on gene expression, protein level and enzymatic activity on catecholamine biosynthetic enzymes

CHRONIC STRESS	species sex	TH			DbH			PNMT			References
		mRNA	protein	activity	mRNA	protein	activity	mRNA	protein	activity	
repeated IMMO (2.5 hours, 7-42 times)	rat, Sprague-Dawley ♂, adult			↑						↑	(Kvetnansky, Weise, & Kopin, 1970)
repeated IMMO (2 hours, daily, 2-7 days)	rat, Sprague-Dawley ♂, adult	↑		↑	↑						(McMahon et al., 1992)
		↑		↑							(Nankova et al., 1994)
								↑			(Viskupic et al., 1994)
		↑	↑		↑			↑			(Kvetnansky et al., 2004)
		↑	↑	↑							(Xu, Chen, Sun, Sterling, & Tank, 2007)
								↑	↑		(Tai et al., 2007)
	mouse, C57B1/129S ♂, adult							↑	↑		(Kvetnansky et al., 2006)
	mouse, C57B1/129S ♂+♀, adult	↑			↑			↑			(Kubovcakova et al., 2004)

cold exposure (4°C, 2-4 days)	rat, Wistar ♂, adult			↑								(Thoenen, 1970)
cold exposure (4-5°C, 3 days)	rat, Sprague-Dawley ♂, adult	↑	↑									(Tank, Lewis, Chikaraishi, & Weiner, 1985)
								↑	↑			(Weisberg et al., 1989)
		↑										(Stachowiak, Fluharty, Stricker, Zigmond, & Kaplan, 1986)
		↑	↑	↑								(Baruchin et al., 1990)
cold exposure (5°C, 4 days-3 weeks)	rat, Sprague-Dawley ♂, adult	↑	↑									(Fluharty, Snyder, Zigmond, & Stricker, 1985)
hypotension	rat, Sprague-Dawley ♂, adult			↑								(Fluharty et al., 1985)
hypoglycemia	rat, Sprague-Dawley ♂, adult			↑								(Fluharty et al., 1985)
glucoprivation	rat, Sprague-Dawley ♂, adult	↑	↑	↑								(Xu et al., 2007)
individual housing (8 months)	mouse (ND) ♂, adult			↓							↓	(Axelrod, Mueller, Henry, & Stephens, 1970)

individual housing (12 weeks)	rat, Wistar ♂, adult											(Gavrilovic, Spasojevic, Tanic, & Dronjak, 2008)
												(Gavrilovic, Spasojevic, & Dronjak, 2012)
												(Jovanovic, Stefanovic, Spasojevic, Puskas, & Dronjak, 2016)
increased social stimulation (communicating box system, 8 months)	mouse (ND) ♂, adult											(Axelrod et al., 1970)
unpredictable chronic mild stress (7 days)	mouse, C57/BL6 ♂, adult											(Santana et al., 2015)
unpredictable chronic mild stress (21 days)	mouse, C57/BL6 ♂, adult											(Santana et al., 2015)
unpredictable chronic mild stress (28 days)	rat, Wistar ♂, adult											(Spasojevic, Gavrilovic, & Dronjak, 2010)
												(Stefanovic, Spasojevic, Jovanovic, & Dronjak, 2019)
chronic footshock (2-5 mA, 1	rat, Sprague-Dawley ♂, adult											(Stone, Freedman, & Morgano, 1978)

pulse/20 sec for 1 hour, 9 days)													
repeated attacks by aggressor mice (10 min, daily, 2-14 days)	mouse, C57BR/cdJ ♂, adult			↑							↑		(Thoa, Tizabi, Kopin, & Maengwyn-Davies, 1976)
long-term prenatal hypoxia (high altitude (3,820 m, 60 mmHg)	ovine fetus (late gestation, 139-141 days)	↓	↓		↓	↓		↓	↓				(Ducsay et al., 2007)
prenatal hypoxia (10% O ₂ , from day 5th to day 20th of gestation)	rat, Sprague-Dawley, fetus, E19		↓						↓				(Mamet et al., 2002)
prenatal hypoxia (11-13% O ₂ , 2-3 days)	rat, Sprague-Dawley, fetus, E21	↑			↑								(Holgert, Pequignot, Lagercrantz, & Hokfelt, 1995)
	rat, Sprague-Dawley, newborn, P0.5	↓			↓								(Holgert et al., 1995)
prenatal hypoxia (10% O ₂ , from day 5th to day 20th of gestation)	rat, Sprague-Dawley, newborn, P0		↓							→			(Mamet et al., 2002)

	rat, Sprague-Dawley, pups, P7													(Mamet et al., 2002)
	rat, Sprague-Dawley, pups, P21													(Mamet et al., 2002)

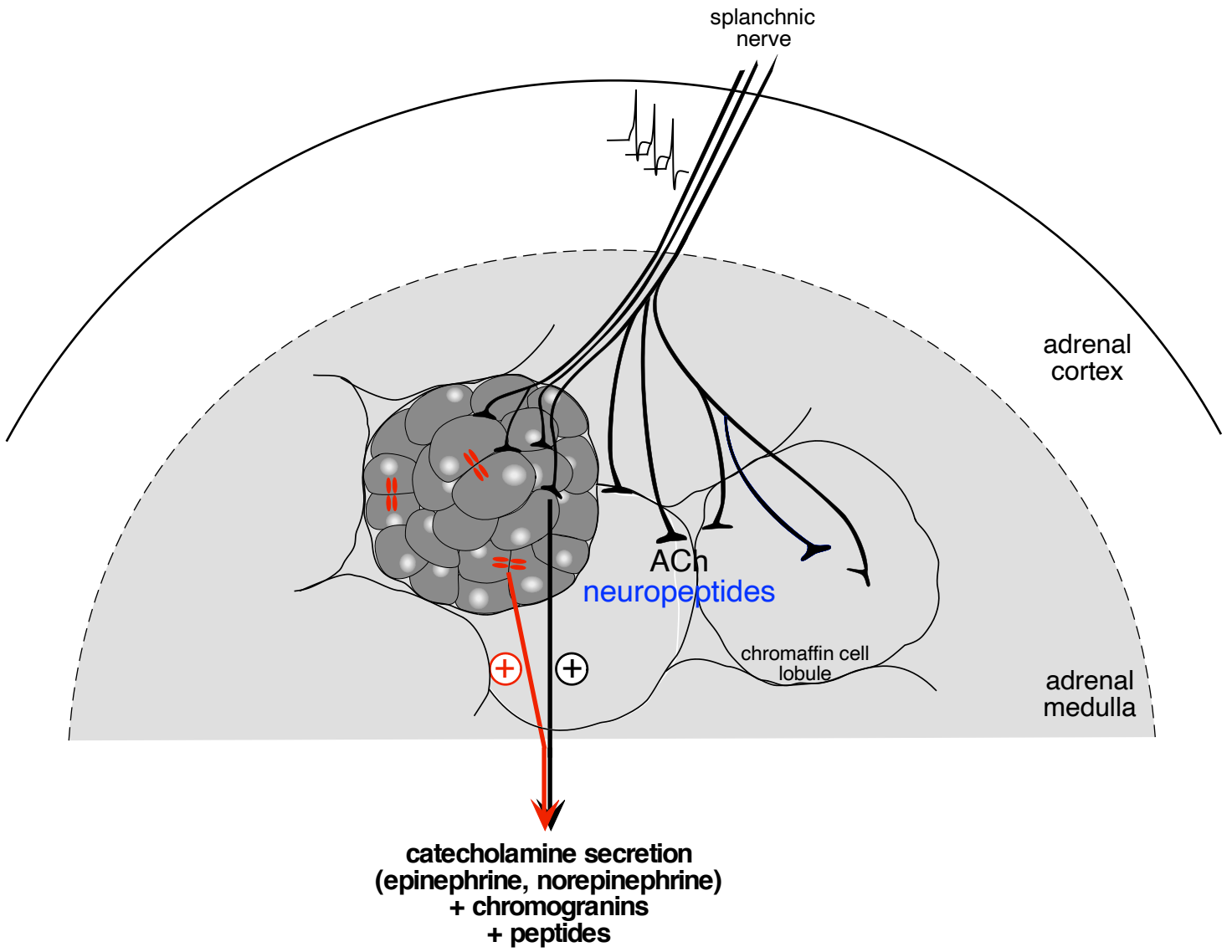


FIGURE 1

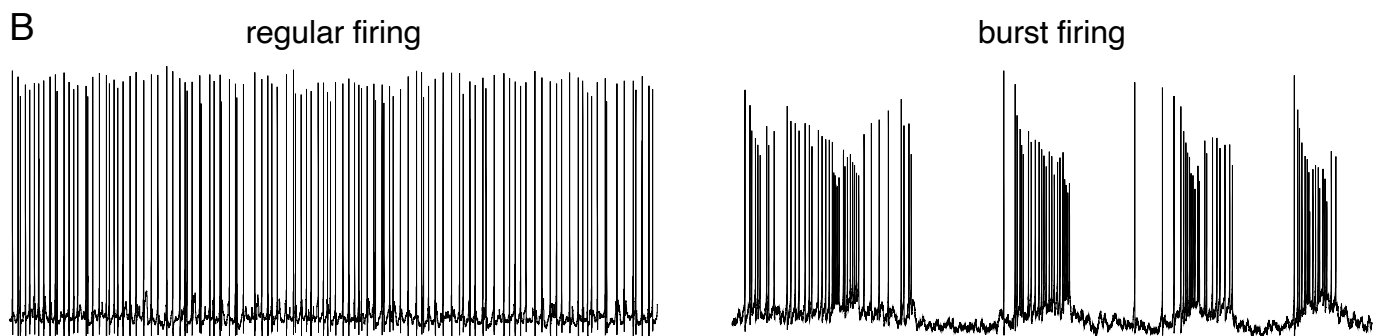
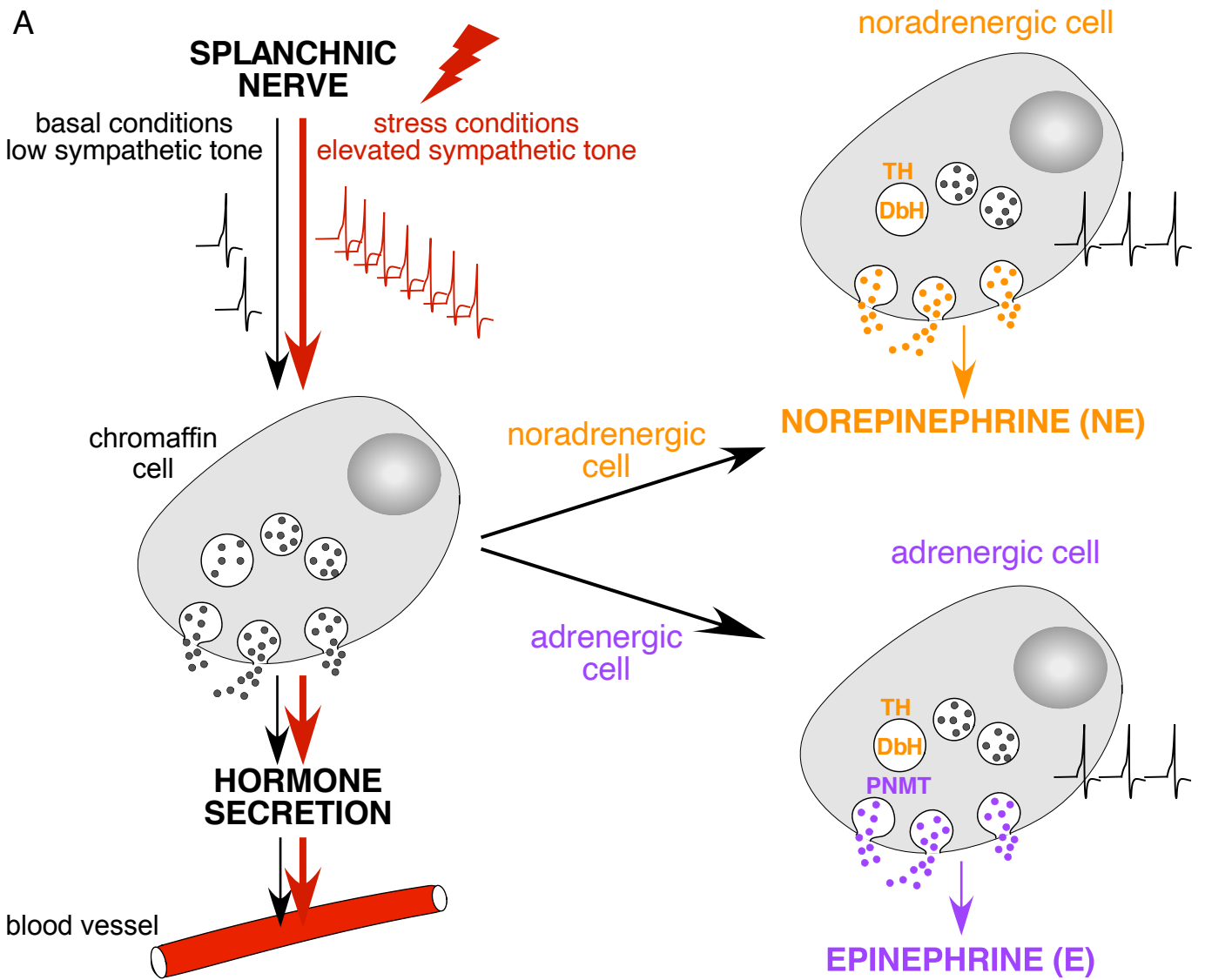


FIGURE 2

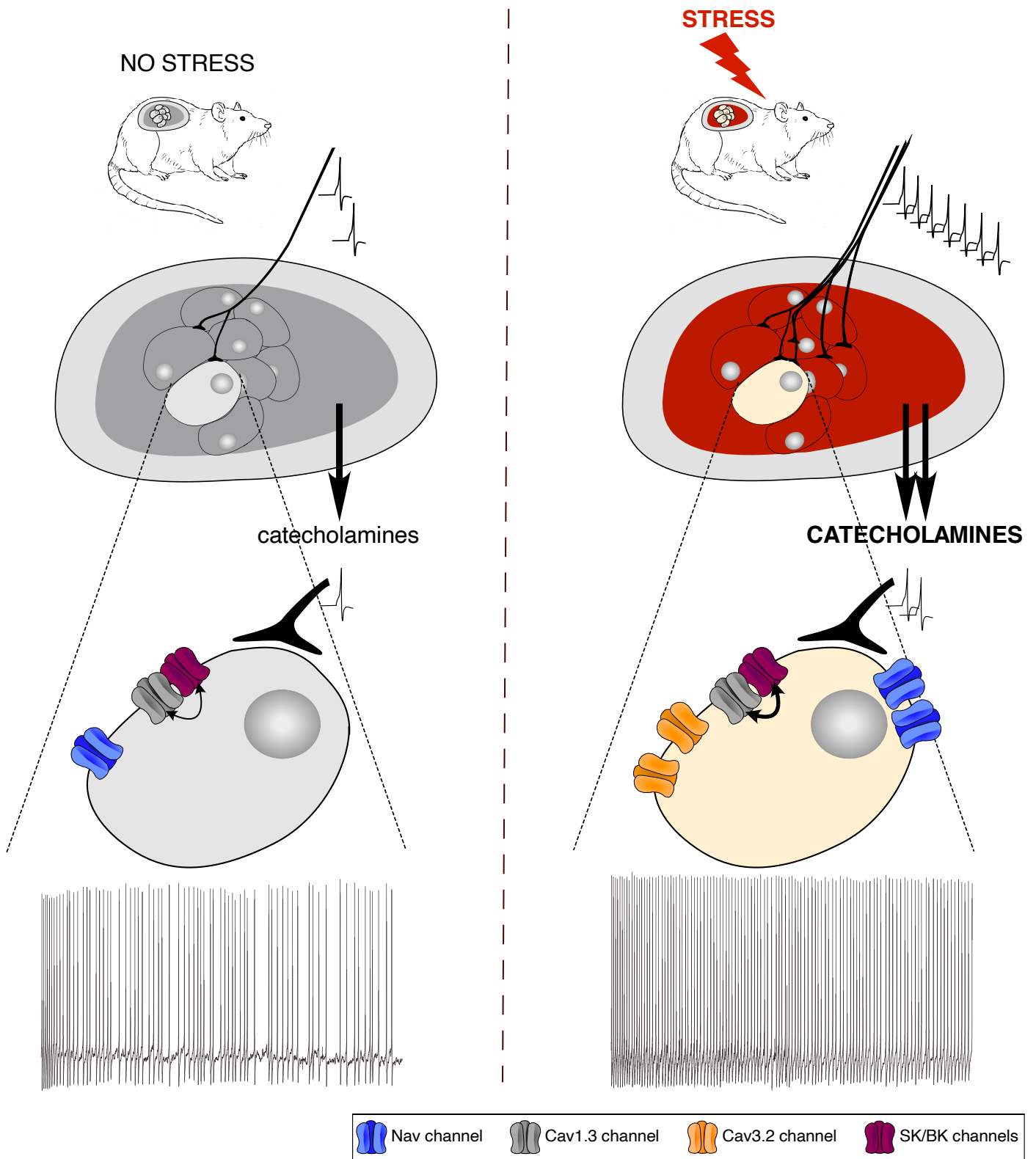


FIGURE 3

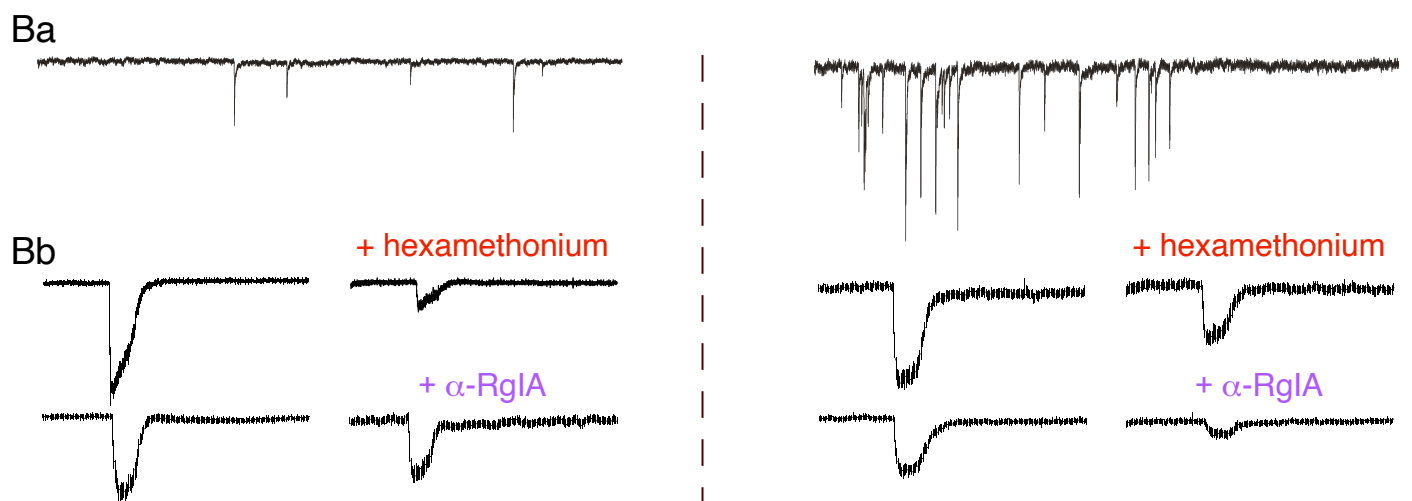
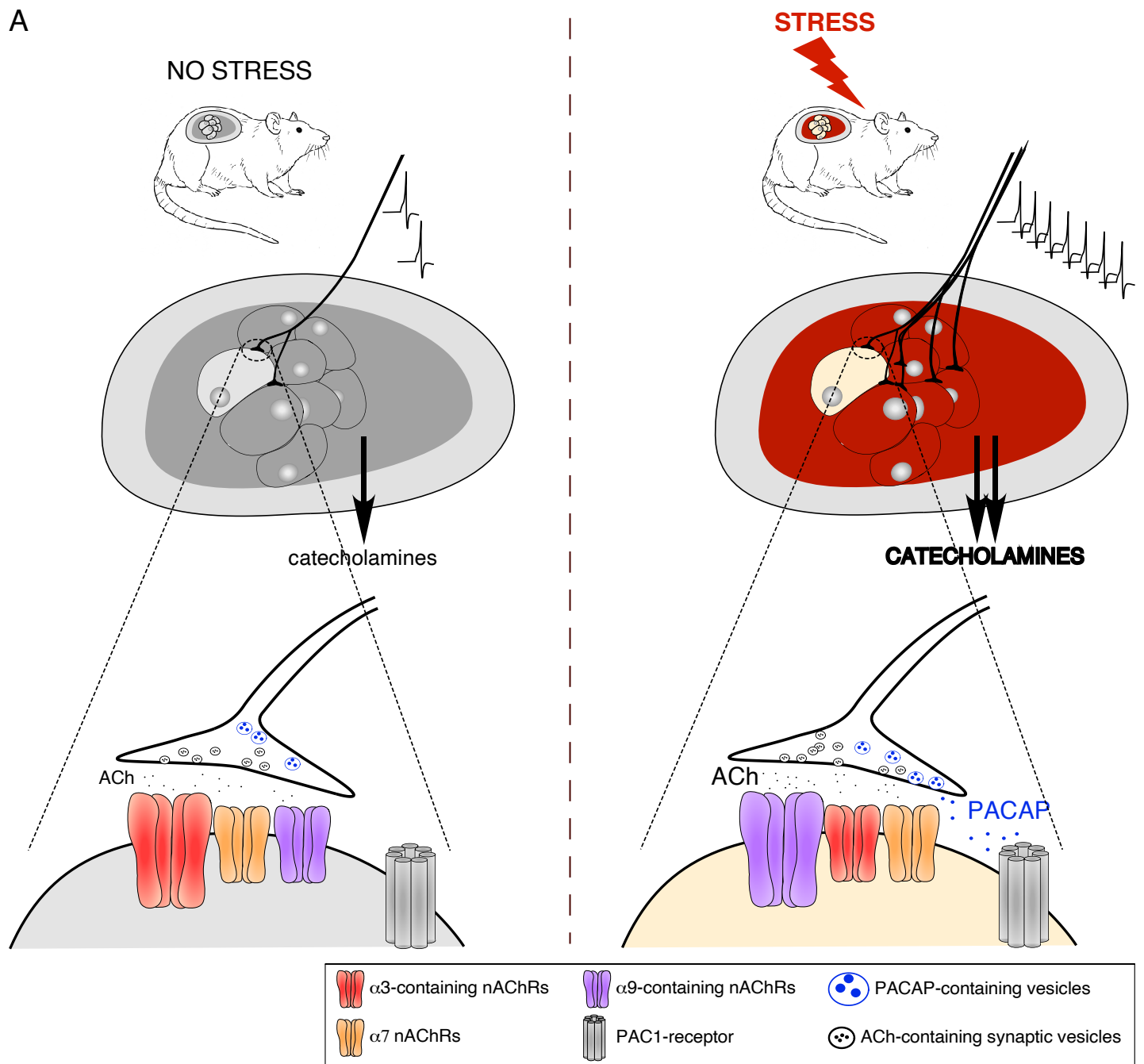


FIGURE 4

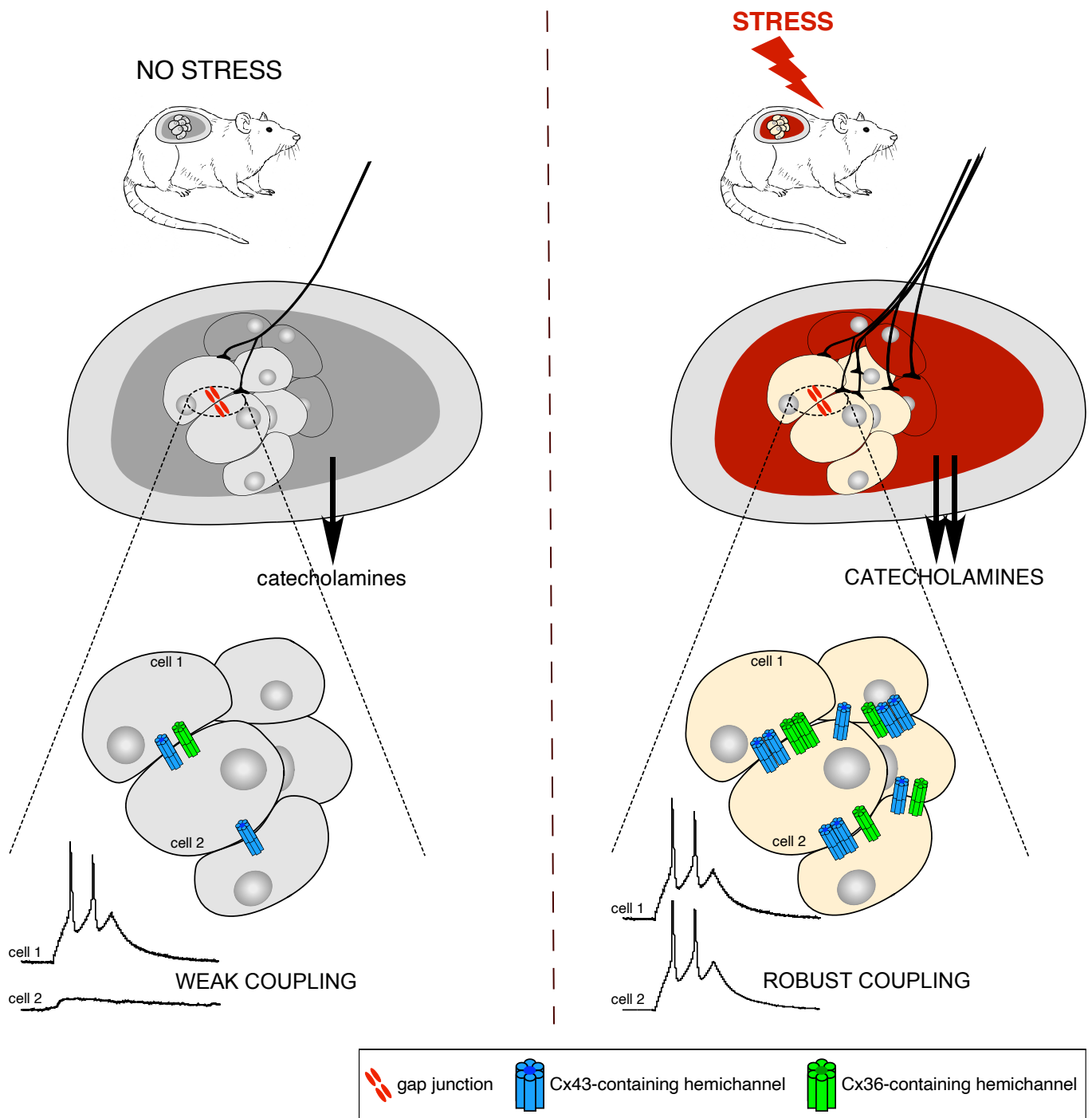


FIGURE 5