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► **To cite this version:**

Claude Colomer, Chrystel Lafont, Nathalie C. Guérineau. Stress-induced Intercellular Communication Remodeling in the Rat Adrenal Medulla. *Annals of the New York Academy of Sciences*, 2008, 1148 (1), pp.106-111. 10.1196/annals.1410.040 . hal-02484222

HAL Id: hal-02484222

<https://hal.science/hal-02484222v1>

Submitted on 19 Feb 2020

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Abbreviated running title: Colomer *et al.*: stress and cell-cell communication in adrenal medulla

Stress-induced intercellular communication remodeling in the rat adrenal medulla

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Keywords: intercellular communication, synaptic currents, gap junctional coupling, chromaffin cell excitability, cold exposure, acute rat adrenal slices, dye transfer

This work was supported by grants from Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Réseau National des Génopôles, and Région Languedoc-Roussillon.

Abstract

To understand the mechanisms by which a prolonged exposure to stress enhances catecholamine secretion, we examined the effects of a 5-day cold exposure on cell-cell communication pathways in the rat adrenal medulla. Upon stress, the neurosecretory tissue undergoes dramatic morpho-functional changes resulting in increased chromaffin cell excitability, up-regulation of both chemical transmission at the splanchnic nerve terminal-chromaffin cell synapses and spreading of gap junction-permeant Lucifer yellow between cells. All these changes converge to improve the stimulus-secretion coupling efficiency within the adrenal medulla and subsequently to adapt catecholamine release to a sustained organism demand.

Introduction

Stress triggers important adaptative responses that enable an organism to cope with a changing environment. An increase in catecholamine secretion (epinephrine and norepinephrine) by adrenal chromaffin cells is a key initial event in response to stressors. Prolonged or repetitive stress is commonly associated with a sustained catecholamine concentration in the blood circulation. Investigating the regulatory mechanisms involved in the secretion of catecholamines is therefore a crucial step to better understand adaptative responses to stress. *In vivo*, the secretion of catecholamines by chromaffin cells is chiefly controlled by the synaptic release of acetylcholine from splanchnic nerve terminals¹. In addition, as previously reported in adrenal acute slices², gap junction-mediated intercellular communication between chromaffin cells represent an efficient complement to synaptic transmission to amplify catecholamine release. The aim of this study is to characterize morphological and functional changes that occur in the adrenal medullary gland of stressed rats, using a 5-day cold exposure as a stress paradigm³. Our results show that stress remodels both chemical synaptic transmission and cell-cell communication likely mediated by gap junctions.

Material and Methods

Stress paradigm. Wistar male rats (6 week-old) were kept at an ambient temperature of 4°C for 5 days. The room was maintained on a 12-hour light/dark cycle. Food and water were provided *ad libitum*. The reversibility of stress-induced changes was examined 5 days after stress cessation.

Slices preparation. Acute rat adrenal slices were prepared as reported previously². Slices (200 µm-thick) were cut using a vibratome and transferred to a storage chamber containing Ringer's saline (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1,25 NaH₂PO₄, 26 NaHCO₃, 12 glucose, maintained at 37°C and buffered to pH 7.4. The saline was continuously bubbled with carbogen (95% O₂ / 5% CO₂).

Electrophysiological recordings. All experiments were performed in the whole-cell configuration of the patch-clamp technique⁴. Patch pipettes were filled with (in mM): 140 K-gluconate, 2 MgCl₂, 1.1 EGTA, 5 HEPES, pH 7.2. Recordings were acquired with an EPC-9 patch-clamp amplifier. Spontaneous post-synaptic excitatory currents EPSCs were recorded as described⁵.

Immunostaining. To process for immunolabeling, the adrenal glands were fixed by immersion in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (overnight at 4°C). They were then cut into 50- μ m-thick sections. The 200 kDa neurofilaments were labeled using a polyclonal antibody (NF-200, Sigma, 1:2000 dilution, 48h incubation, 4°C). Stained sections were imaged with a Biorad MRC-1024 confocal laser scanning microscope.

Scrape loading dye-transfer. This technique allows to introduce the gap junction-permeant fluorescent dye, Lucifer yellow CH (LY) into cells and to monitor its propagation into communication-competent cells within minutes after loading⁶. After ablation, adrenal glands were hemi-sectioned with a razor blade, incubated at room temperature for 3 min in 0.5% LY-containing saline and then fixed in 4% PFA in 0.1 M phosphate buffer (2h, room temperature). To visualize LY staining, each hemi-gland was then sliced (100 μ m thickness). The dye spreading was quantified by calculating the surface rendering of the fluorescence⁷ (Imaris® 4.5.2, Bitplane, Switzerland).

Statistics. Numerical data are expressed as the mean \pm sem. The Student t test was used to compare means. Percentages were compared using the Chi2 test. Differences of $p < 0.001$ were considered significant.

Results

Increased chromaffin cell excitability in stressed rats

The measurement of passive electrical properties of individual chromaffin cells indicates that both the membrane capacitance, the membrane input resistance and the resting potential significantly differed between stressed and unstressed rats ($p < 0.001$, table 1). Five days after cessation of the stress situation, these parameters recovered their control values. Regarding action potential firing, 82.6% of cells exhibited spontaneous action potentials at rest in stressed rats ($n = 799$) while only 27.5% of cells were spontaneously active in controls ($n = 590$, $p < 0.001$). In addition, stress increased the mean spontaneous discharge frequency (4.42 ± 0.16 Hz, $n = 209$ versus 2.85 ± 0.11 Hz, $n = 142$ in stress and control conditions, respectively, Figure 1A-B), thus reflecting an increase in cell excitability.

Stress-induced changes in synaptic transmission at the splanchnic nerve-chromaffin cell junction

Because the catecholamine release by chromaffin cells is chiefly regulated trans-synaptically via splanchnic nerve firing, we next investigated whether chromaffin cell innervation was affected upon

cold exposure. The innervation density was visualized by the immunofluorescent detection of the 200 kDa neurofilament, a neuron-specific intermediate filament protein⁸. As illustrated in figure 2A, stress increased the density of neuronal fibers synapsing onto chromaffin cells. To address the physiological relevance, spontaneous EPSCs were recorded in chromaffin cells voltage-clamped at -80 mV (Figure 2B). In cold exposed rats, not only the percentage of recorded cells exhibiting spontaneous EPSCs was higher compared with controls (22% *versus* 6%, n = 225), but also synaptic events occurred more frequently (0.12 ± 0.01 Hz, n = 50 *versus* 0.04 ± 0.01 Hz, n = 13 for stress and control, respectively).

Up-regulated intercellular communication in stressed rats

As previously reported², the gap junction-delineated route efficiently complements synaptic transmission to amplify catecholamine secretion from chromaffin cells. We therefore performed additional experiments to determine whether cell-cell coupling was modified upon stress. Lucifer yellow, a gap junction-permeant dye able to image coupled cells was introduced into cells by using the scrape loading technique⁶. Figure 3 illustrates representative examples of confocal stack image of LY diffusion in control and stressed rats. As shown by the 3D analysis of the surface rendering of fluorescent voxels, the spreading of LY was more extended in cold exposed rats as compared with unstressed rats. This strongly suggests that gap junction-mediated intercellular communication is up-regulated in stressed rats.

Discussion

The present study unveils morpho-functional changes that occur in the adrenal medulla after prolonged exposure to cold. All converge to an increased catecholamine secretion as commonly observed upon stressful conditions. Firstly, collective results from electrophysiological data including i) a more depolarized resting potential, ii) an increased number of cells exhibiting spontaneous action potentials and iii) an increased mean spontaneous discharge frequency argue in favor of stress-induced increased chromaffin cell excitability. In addition, the denser innervation of the adrenal medulla found in stressed rats and the subsequent increase in EPSC frequency in chromaffin cells also contribute to improve the stimulus-secretion coupling efficiency.

Although adrenomedullary secretory responses to cold are small compared to sympathetic neuronal responses, exposure to cold is known to increase the synthesis and the release of adrenomedullary catecholamines^{3,9}. Regarding catecholamine biosynthesis, our results describing an increased innervation of the medulla are consistent with the hypothesis of the involvement of neurotransmitters released from the splanchnic nerve in the induction of tyrosine hydroxylase in the adrenal medulla of stressed rats¹⁰. With regard to catecholamine secretion, stress-induced increase in action potential firing likely contributes to elevate cytosolic calcium concentration and ensuing catecholamine

release¹¹. Secondly, cell-cell communication that has been previously reported to amplify catecholamine secretion² is also up-regulated in stressed rats. This is supported by our scrape-loading experiments showing an extended spreading of LY in the adrenal medulla of cold exposed rats. As an independent confirmation, chromaffin cells from stressed rats display electrophysiological features that are consistent with an enhancement of electrical coupling, *i.e.* a significant increase in membrane capacitance and a significant decrease in membrane resistance. It is noteworthy that comparative morpho-functional modifications also occur in the adrenal medulla of rats exposed to a restraint stress (data not shown), suggesting that the reshapes reported here might commonly occur in response to various stressors. We propose that synaptic transmission and gap junctional coupling by acting in synergy on chromaffin cells likely contribute to improve the stimulus-secretion coupling efficiency in the adrenal gland and subsequent increased catecholamine secretion commonly observed in response to stressors.

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Figure legend

Figure 1: Increased frequency of spontaneous action potential firing in chromaffin cells of stressed rats

A. Representative example of spontaneous firing pattern recorded in a control (left chart) and in a stressed chromaffin cell (right chart). Cells were current-clamped at their resting membrane potential (-52 and -63 mV, respectively). B. Pooled data showing a significant increase in action potential mean frequency in stressed rats (*, $p < 0.001$). The number of recorded cells is indicated in parentheses.

Figure 2: Stress-induced morphological and associated functional changes of the adrenal medulla innervation

A. Immunofluorescent detection of 200 kDa neurofilaments (NF-200, polyclonal antibody, 1:2000 dilution ; secondary antibody conjugated to Alexa fluor 488, 1:2000 dilution) showing an increased density of neuronal fibers innervating the adrenal medulla in stressed rat (white arrows). B. Representative recordings of spontaneous EPSCs. Pooled data show a significant increase in synaptic current frequency in stressed rats (cells voltage-clamped at -80 mV, synaptic currents recorded for at least 10 min, *, $p < 0.001$).

Figure 3: Stress-induced increase in cell-cell communication in the adrenal medulla

The cell-cell communication extent was assessed by using the scrape-loading technique to label coupled cells. Left panel: confocal image stack of LY spreading (6 slices/stack, C: cortex, M: medulla). Right panel: 3D analysis by using surface rendering of fluorescent voxels.

	Cm (pF)	Rm (MΩ)	resting potential (mV)
control (n = 590)	10.3 ± 0.1	1597 ± 15	-64.4 ± 0.3
cold exposure (n = 799)	10.7 ± 0.1*	1430 ± 13*	-62.2 ± 0.3*
recovery (n = 142)	10.5 ± 0.1	1595 ± 9	-64.4 ± 0.2

TABLE 1: Comparison of passive electrophysiological properties of adrenal chromaffin cells between control and stressed rats.

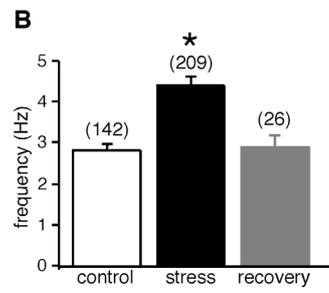
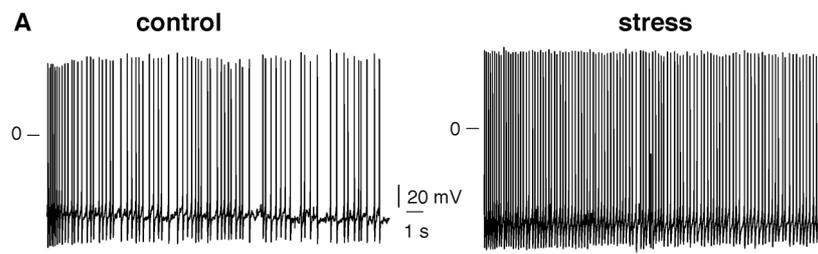


FIGURE 1

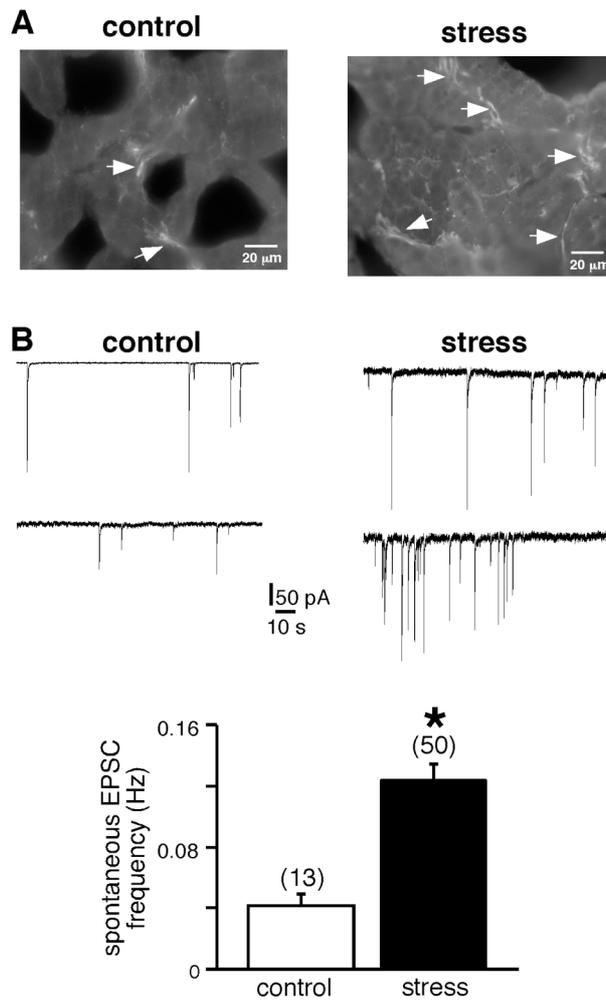


FIGURE 2

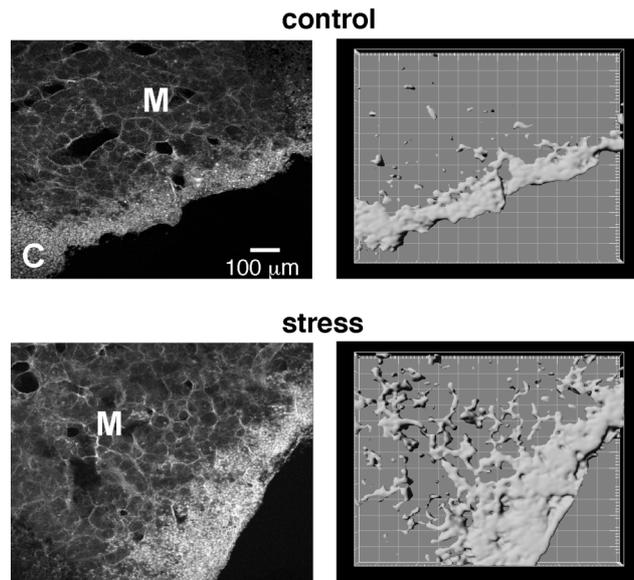


FIGURE 3