Rostral cuneiform nucleus and the defense reaction: Direct and indirect midbrain-medullary serotonin mechanisms in baroreflex inhibition

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Running title: Cuneiform nucleus and serotonin transmission in the defense reaction

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We declare no conflict of interests.

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The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Abstract

Background and Purpose: The activation of the defense reaction inhibits the baroreflex response through the B3 and nucleus tractus solitarius (NTS) regions. Our aim was to determine whether and how baroreflex inhibition induced by the disinhibition of the rostral cuneiform nucleus, part of the defense pathway, involves serotonin cells in B3 and 5-HT_3 receptors in the NTS.

Experimental Approach: We performed immunohistochemistry and anatomical experiments to determine whether raphe serotonin cells expressing Fos were directly targeted by the rostral cuneiform nucleus. The effect of blocking raphe serotonin transmission and NTS 5-HT_3 receptors, on cuneiform-induced inhibition of the baroreflex cardiac response, were also analyzed.

Key Results: Bicuculline microinjected into the rostral cuneiform nucleus induced an increase of double labeled Fos-5-HT IR cells in both the LPGi and Raphe Magnus. The anterograde tracer *Phaseolus vulgaris leucoaggutinin* into the rostral cuneiform nucleus revealed a dense projection to the LPGi but not Raphe Magnus. Cuneiform-induced baroreflex inhibition was prevented by B3 injection of 8-OH-DPAT, a specific agonist for 5-HT_{1A} receptors. Cuneiform disinhibition also failed to inhibit the baroreflex bradycardia after microinjection of a 5-HT_3 receptor antagonist (granisetron) into the NTS or in 5-HT_3 receptor knock-out mice.

Conclusion and Implications: In conclusion, the rostral cuneiform nucleus participates in the defense inhibition of the baroreflex bradycardia via direct activation of the LPGi and a relay to the Raphe Magnus, to activate NTS 5-HT_3 receptors and inhibit second-order baroreflex neurons. These data bring new insights in primary and secondary mechanisms involved in vital baroreflex prevention during stress.

Keywords: cuneiform, stress, caudal raphe, autonomic, serotonin, baroreflex

Bullets

What is already known

- The baroreflex bradycardia is inhibited during the defense reaction to acute fear, a key step for maximal oxygenation in skeletal muscles

What this study adds

- The rostral cuneiform disinhibition induces direct and indirect activation of serotonergic cells in the intermediate B3 region only.
- The release of serotonin targets 5-HT_3 receptors in the NTS

Clinical significance

- Multiple protective medullary pathways are involved in the vital stress-induced inhibition of the baroreflex response

Introduction

Arterial baroreflex represents a powerful negative feedback control mechanism to maintain blood pressure homeostasis. Activation of major arterial baroreceptors (of aortic and carotid origin) following an increase in blood pressure causes an immediate parasympathetic cardiovagal excitation. The rapid (<1 s) decrease in heart rate will secondarily provoke a decrease in blood pressure after a few seconds (baroreflex loop) (Vaschillo et al., 2006). Under specific circumstances such as during the defense reaction, blood flow is specifically redistributed among organs to increase blood supply in the skeletal muscles. This is the consequence of vessel constriction but also increase in cardiac output, both producing an increase in blood pressure (Nosaka et al., 1996). This would trigger a baroreflex bradycardia; however, this reflex response would counteract the vital cardiac output increase, necessary for muscles oxygenation and body reactions facing stress. Several studies found that the cardiovagal effect on the heart (bradycardia) is suppressed during the defense reaction (Nosaka et al., 1996; Sevoz-Couche et al., 2003; Comet et al., 2004, 2005).

The nucleus tractus solitarius (NTS) plays a major role in the reduction of the baroreflex cardiovagal response during the defense reaction. Specific blockade of local 5-HT_3 receptors, the only ligand-gated ion channel among serotonin receptors (Hoyer, 1990), prevents the reflex cardiac inhibition induced by activation of a key region of the defense reaction, the dorsolateral periaqueductal gray (dorsolateral PAG) (Comet et al., 2004, 2005). The possible source of serotonin released into the NTS to activate 5-HT_3 receptors following dorsolateral PAG activation was the intermediate rostro-ventromedial medulla region (intermediate B3 region), including the lateral paragigantocellularis nucleus (LPGi) and the Raphe Magnus, because serotoninergic cells in the LPGi and the Raphe Magnus (but not other raphe nuclei) expressed the Fos protein after dorsolateral PAG activation (Bernard et al., 2008).

The rostral cuneiform nucleus may also be part of the defense reaction as its disinhibition through GABAergic stimulation (using bicuculline microinjection) induces an inhibitory effect on baroreflex response (Netzer et al., 2011). This nucleus sends direct projections to the dorsolateral PAG (Netzer et al., 2011). Altogether, these data suggested that, as for the dorsolateral PAG, B3 activation could be at the origin of the baroreflex inhibition obtained during cuneiform disinhibition. To answer this question, we performed double immunolabeling in the B3 region (LPGi and Raphe

Magnus), as well as in other raphe nuclei (Raphe Dorsalis, Raphe Pallidus, and Raphe Obscurus). We analyzed the expression of Fos protein within serotonin immunoreactive (Fos-5-HT IR) cells in these nuclei after bicuculline injection into the rostral cuneiform nucleus. Despite the fact that activation of serotonergic cells was seen in the entire B3 intermediate region following the dorsolateral PAG stimulation (Bernard et al., 2008), there is a direct link from the latter to the LPGi but not the Raphe Magnus (Babic and Ciriello, 2004). This raised the possibility that activation of passing fibers, like those from the cuneiform nucleus, and reaching the Raphe Magnus, may have been at the origin of dorsolateral PAG-induced serotonin activation in the Raphe Magnus. To answer this question, we injected an anterograde tracer (*Phaseolus vulgaris leucoaggutinin)* into the rostral cuneiform nucleus to determine whether descending projections towards the intermediate B3 region (including the Raphe Magnus) could be found.

To definitely establish whether raphe serotonergic cells seen to be activated by rostral cuneiform disinhibition in the first set of experiments, as well as NTS 5-HT_3 receptors, are involved in defense-induced baroreflex inhibition, we performed pharmacological experiments. We analyzed the baroreflex response reduction obtained normally during rostral cuneiform disinhibition, before and after i/ local raphe inactivation using injections of muscimol, a GABAergic receptor agonist, ii/ raphe serotonergic cell inhibition using microinjection of 8-OH-DPAT, and iii/ blockade of NTS 5-HT_3 receptors by granisetron in rats, or wild-type versus 5-HT_3 receptor knock-out mice.

Methods

Experiments were performed using randomized 52 male Sprague-Dawley rats (330-350 g) and 12 male mice (25g) homozygous Htr3a KO (n=6) and WT littermates (n=6) born from heterozygous mutants on a C57BL/6J genetic background (>10 generations) and genotyped as described by Zeitz et al (2002). Animals were kept under controlled environmental conditions (ambient temperature: 21 ± 1 °C, 60% relative humidity, unrestricted access to food and water, alternate 12 h light/ 12 h dark cycles) for a least one week after receipt from the breeding center (CER Janvier, Le Genest-St Isle, France). Procedures involving animals and their care were all conducted following institutional guidelines, which comply with national and international law policies (Council directive 87-848, 19 October 1987, Ministère de

l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale; permission n° 75-855 to C.S.-C.).

Sustained chemical disinhibition of the rostral cuneiform nucleus to induce Fos expression in raphe nuclei

Rostral cuneiform nucleus long-term disinhibition

Twelve rats were anesthetized with chloral hydrate (400 mg/kg i.p.) for immunohistological experiments. The size of the samples was based on previous experiments (Bernard et al., 2008). The depth of anesthesia was assessed regularly by pinching a hind paw and monitoring the stability of the arterial blood pressure and heart rate recordings. Rectal temperature was maintained at 37°C with a thermostatically controlled heating blanket. The femoral vein was catheterized for the administration of additional anesthesia or drugs. Mean blood pressure (MBP) was monitored via a femoral arterial catheter connected to a pressure transducer and DC amplifier (Gould, Courtaboeuf, France). The electrocardiogram (ECG) was recorded using stainless steel pins placed subcutaneously into fore- and hind-paws. Signals were amplified and filtered (Universal Amplifier, Gould). Heart rate (HR) was computed from the ECG (R wave pulses) and displayed as mean frequency per minute (bin size=1 s).

Briefly, in Experimental rats (n=6), two unilateral (left) microinjections (with 10 min interval) at rostral cuneiform sites (-8.2 to -8.5 mm from bregma, Netzer et al., 2011) filled with bicuculline methiodide (50 pmol/50 nl) were necessary to maintain cuneiform activation for 20 min, as confirmed by a sustained increase in MBP and HR (Netzer et al., 2011). Defense reaction activation was identified by the observation of somatic responses such as mydriasis, vibrissae and body movements, and tail erection. Sham animals (n=6) received vehicle treatment (saline). No somatic responses were observed.

Visualization of Fos and/or serotonin immunoreactive (Fos-IR and/or 5-HT-IR) neurons

Two hours after the end of the 2nd microinjection of bicuculline or saline into the rostral cuneiform, anesthetized rats were perfused intracardially. The brain was removed and cryoprotected as described above. Coronal frozen sections (50 µm thick) of the whole brainstem were collected in four containers filled with phosphate buffer saline, allowing their parallel processing as serial groups of floating sections.

Processing is described in Supplemental Methods. Neurons were observed under brightfield illumination in 50 µm-thick coronal sections (200 µm apart) in regions containing serotonergic neurons (the lateral paragigantocellular reticular [LPGi] nucleus, the raphe magnus [RMg], the raphe pallidus [RPa], the raphe obscurus [ROb], and the raphe dorsalis [RDr] nuclei). The location of brainstem nuclei containing 5-HT neurons was based on observation of adjacent Nissl stained sections.

Brain tissue sections were analyzed after careful individual examination of each neuron at high magnification (x 40). The outline of the section and the main structures were drawn at low magnification (x 4 and x10), the computer providing continuous synchronization between plotting and section location during moving or magnification changes. For each animal, a total of four brain sections for each region of interest was analyzed under brightfield illumination. Neurons were plotted using Mercator software (Explora Nova, La Rochelle, France). The computer was connected to 1) a CCD color video camera that captured images and supplied red, green, and blue output, and 2) an XY stage microscope stage controller which sent the micrometric location of the section boundaries to the computer.

Anterograde tracing of rostral cuneiform nucleus efferents to the B3 region

Tracer injection

Ten rats were anesthetized with chloral hydrate (400 mg/kg i.p.) for anatomical experiments. The size of the sample was based on previous experiments (Netzer et al., 2011). The depth of anesthesia was assessed regularly by pinching a hind paw and monitoring the stability of the arterial blood pressure and heart rate recordings. Rectal temperature was maintained at 37°C with a thermostatically controlled heating blanket. Application of *Phaseolus vulgaris* leucoagglutinin lectin (PHA-L) was made by passing direct current (2-6µA, electrode tip positive) through a micropipette directed to the left rostral cuneiform nucleus (approximately -8.2 to -8.5 mm from bregma) for 20 seconds per 30-second period, for 20 minutes (Netzer et al., 2011). Following a postoperative survival time of 15 days, animals were deeply anesthetized with pentobarbital (200 mg/kg, i.p.). They were perfused transcardially and the brain was removed and cryoprotected in a 20% sucrose solution overnight. Frozen sections (50 µm thick) of the whole brainstem were collected in four containers filled

with PBS, allowing their parallel processing as 3 serial groups of free-floating sections.

Immunohistochemical processing for anterograde labeling

Processing is described in detail in Supplemental Methods.

Labeled fibers in the regions identified in the first set of experiments (identification of double-labeled Fos-5-HT-IR cells) were observed under brightfield illumination in coronal sections. Terminals were differentiated from fibers of passage using classical morphological criteria (ramification, varicosities, thickness, and pattern).

Pharmacological experiments

Acute rostral cuneiform disinhibition in rats and mice

Animals were anesthetized with urethane (1.5 g/kg, i.p.) to ensure similar experimental conditions as those performed previously (Bernard et al., 2008; Gau et al., 2009; Netzer et al., 2011). The femoral vein was catheterized, MBP and HR were recorded as mentioned above. Anesthetized animals were placed in a stereotaxic frame with the head fixed in the flat skull position. The size of the samples was based on previous experiments (Bernard et al., 2008; Netzer et a., 2011; Zafar et al., 2018). Briefly, rostral cuneiform disinhibition was caused by left microinjection of bicuculline in rats (50 pmol/50 nl, n=30, n=6 in each group [1/ B3 muscimol, 2/ B3 8-OH-DPAT, 3/ B3 saline, 4/ NTS granisetron and 5/ NTS saline]) and mice (25 pmol/25 nl, n=6 wild-type and n=6 5-HT_3 knock-out animals).

Evaluation of the baroreflex sensitivity in Control and Experimental conditions in rats and mice

In the Control condition, baroreceptors were unloaded using the vasodilator agent sodium nitroprusside (SNP, 100 µgkg−1, I.V.) followed by baroreceptor stimulation using the vasoconstrictor agent phenylephrine (PE, 10 µgkg−1, i.v.) (Sevoz-Couche et al., 2013). Administration of nitroprusside followed by phenylephrine allowed the generation of baroreceptor function curves by fitting the data to the sigmoid logistic function: Y = Bottom+(Top−Bottom) /(1+exp((V50 −X)/Slope), using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA), where Top-Bottom was the maximal baroreflex range. Rectilinear baroreflex slopes were calculated from the baroreceptor curves.

In the same animal (rat or mice), after 15 min, left rostral cuneiform disinhibition was performed. When blood pressure increase was stable (2-4 min after bicuculline), SNP+PE was administered again (Experimental condition).

Microinjections into the B3 region and NTS in rats

A micropipette connected to a Hamilton microsyringe was lowered into the target areas for the first 100 nl injection and refilled with the same volume for the second injection; the time interval between the two microinjections was less than 1 minute.

Bilateral microinjections of bodipy TMR-X conjugate muscimol (Ref M-23400, Invitrogen, Pontoise, France) (500 pmol/100 nl), a specific $GABA_A$ receptor antagonist (Johnston et al., 1968) were made in the intermediate B3 region. The injection sites were precisely located owing to the orange light emission (572 nm) of the fluorophore linked to muscimol. 5-HT cells were visualized in coronal sections thanks to the green fluorescent emission (519 nm) of Alexa Fluor goat 488 anti-rabbit (Invitrogen) used after incubation of sections with rabbit anti-5-HT antibody (Gau et al., 2009). Bilateral microinjections of 8-OH-DPAT HBr (1 nmol/ 100 nl), a specific 5-HT_{1A} autoreceptor agonist (Hamon *et al*., 1988), were made into the intermediate B3 region (Ootsuka et al., 2006). Bilateral microinjections of granisetron (250 pmol/100 nl), a specific 5-HT_3 receptor antagonist (Carmichael et al., 1989), were made into the commissural NTS (Sevoz-Couche et al., 2013). Bilateral microinjections of saline for control were made into the intermediate B3 region and the NTS.

Baroreflex Control and Experimental responses (15 min apart) were both elicited in the same rat before and 15 min after microinjections of saline or either bodipy muscimol and 8-OH-DPAT into the B3 region, and saline or granisetron into the NTS.

Histological localization of microinjection and stimulation sites

Microinjection sites were identified by the location of the micropipette track and pontamine sky blue deposit in 70 µm thick sections of brain tissue previously fixed in 10% formalin solution and cryoprotected in 20% sucrose solution for 5 days.

Statistical analyses

Student's unpaired *t*-test (GraphPad Prism 6) was used to compare Fos expression in serotonergic cells in control (saline) or experimental (rostral cuneiform disinhibtion) rats.

Two-way repeated measures ANOVA (GraphPad Prism 6, San Diego, CA, USA) was used to compare the baroreflex range responses and slopes in Control vs

Experimental conditions, before vs after local microinjections in B3 and NTS in rats or wild-type vs 5-HT_3 receptor knock-out mice.

Bonferroni correction was applied to all ANOVAs, and differences were considered significant at $P < 0.05$.

Results

Functional interaction between the rostral cuneiform nucleus and serotonergic raphe nuclei in rats

Fos immunohistochemical labeling after vehicle (Sham) or bicuculline (Experimental) into the rostral cuneiform nucleus

B3 nuclei

An increase in Fos-5-HT IR cell number in the Experimental condition compared to Sham was confined to the mid-rostrocaudal extent of the B3 serotonergic group, from -10.2 to -11.8 mm from bregma (Paxinos and Watson, 2005). Considering absolute values, i.e., mean number of neurons per rat, the total number of 5-HT-IR cells was similar in Sham vs Experimental animals in ipsilateral (130±10 vs 120±8, P=0.78) or contralateral (125±7 vs 122±6, P=0.85) LPGi, as well as in the Raphe Magnus (280±5 vs 250±10, P=0.45). Among total LPGi 5-HT-IR cells, unilateral rostral cuneiform disinhibition (Experimental conditions) induced an increase in Fos expression by more than twenty-fold in each side compared to Sham (Fig 1A). A representative example in ipsilateral LPGi is given Fig 1B. The number of double-labeled cells in Raphe Magnus was also significantly higher in Experimental than in Sham conditions, though the increase (ten-fold) was slightly less than in total LPGi (Fig 1A).

Non-serotonergic cells expressing Fos slightly increased in Experimental compared to Sham conditions in total LPGi, as well as in Raphe Magnus (Fig 2A).

Raphe Dorsalis

Raphe Dorsalis extends from -7.0 to -8.5 mm from bregma. In this region, the total number of 5-HT-IR cells was similar in Sham vs Experimental animals in Raphe Dorsalis (2550±110 vs 2420±130, Fig 2A). We observed a slight but not significant (P=0.18) increase of 5-HT-IR cells expressing Fos in Experimental compared to Sham conditions (Fig 1A). In contrast, bicuculline into the rostral cuneiform nucleus (Experimental) induced a large increase in Fos-IR cells in the non-serotonergic population compared to Sham (Fig 2A), especially in lateral wings at mid-

rostrocaudal levels (-7.5 to 8.0 mm from bregma). A representative example in the Experimental condition is given in Fig 2B.

Raphe Pallidus and Raphe Obscurus

Analyses were performed between -10.0 and -13.0 mm from bregma. The total number of 5-HT-IR cells was similar in Sham vs Experimental animals in Raphe Pallidus (27±2 vs 22±3, P=0.69) or Raphe Obscurus (153±8 vs 138±6, P=0.25). 5-HT-IR cells expressing Fos in these two regions were not affected by rostral cuneiform disinhibition compared to Sham (Fig 1A). No increase in Fos expression was seen in the non-serotonergic population in the Raphe Pallidus (P=0.2) or Raphe Obscurus (P=0.40) after bicuculline into the rostral cuneiformis nucleus compared to Sham (Fig 2A).

Anatomical link between the rostral cuneiform nucleus and B3

The anterograde tracer was unilaterally applied in the rostral cuneiform nucleus (Fig 3A). Labeled fine fibers and ramifications with numerous varicosities were highly concentrated in the middle B3 (Fig 3B). The maximum density of labeled fibers was found in the intermediate B3 region, between -10.4 to -11.6 mm from bregma (Paxinos and Watson, 2005). The majority of the labeled fibers were found bilaterally in the LPGi, and only scarce fibers were observed in the Raphe Magnus (Fig 3C).

Inhibitory effects of rostral cuneiform disinhibition on the baroreflex cardiac response in rats and mice

Involvement of the intermediate serotonin B3 region: Effects of muscimol and 8-OH-DPAT on baroreflex cardiac response induced by rostral cuneiform disinhibition in rats

According to results depicted in Fig 1B, microinjections of muscimol and 8-OH-DPAT were performed at intermediate B3 coordinates (Fig 4A). The baroreflex response was performed in the same rat alone (Control condition) or during (Experimental condition) rostral cuneiform disinhibition, before and after intra-B3 treatment. Interaction between conditions and intra-B3 muscimol or 8-OH-DPAT but not saline was seen for maximal cardiac range (Table 1) and rectilinear slopes derived from baroreflex sigmoid curves (Table 2).

An example of injections of muscimol conjugated to bodipy into B3 targeting the LPGi or the Raphe Magnus, is given Fig 4B-1 and Fig 4B-2, respectively. We pooled our

results as no difference between LPGi and Raphe Magnus microinjections were observed. A representative example of Control and Experimental baroreflex responses obtained before and after intra-B3 muscimol injection is given Fig 5A-1. The maximal baroreflex cardiac range obtained from sigmoid curves (Fig 5A-2) was significantly reduced in Experimental conditions before (75±1 bpm, P<0.0001) but not after (145±3 bpm, P=0.53) intra-B3 treatment, compared with Control conditions (165±3 bpm and 155±6 bpm, respectively, P=0.7). In the same manner, Experimental rectilinear slopes derived from the sigmoid curves were lower before (2.1±0.1 bpm/mmHg, P<0.0001) but not after (5.1±0.2 bpm/mmHg, P=0.0003) intra-B3 muscimol, than Control slopes (6.4±0.1 and 6.2±0.1 bpm/mmHg, respectively, P=0.07).

The maximal baroreflex cardiac range obtained from sigmoid curves (Fig 5B) was significantly reduced in Experimental conditions before (59±4 bpm, P=0.0008) but not after (130±7 bpm, P=0.87) intra-B3 8-OH-DPAT compared with Control conditions (160±3 bpm and 147±4 bpm, respectively, P=0.65). In the same manner, Experimental rectilinear slopes derived from sigmoid curves were lower before (1.10±0.05 bpm/mmHg, P<0.0001) but not after (5.53±0.09 bpm/mmHg, P=0.10) intra-B3 8-OH-DPAT, than Control slopes (5.91±0.07 and 6.21±0.19 bpm/mmHg, respectively, P=0.08).

Experimental cardiac baroreflex ranges from the baroreflex sigmoid curves before (151±4 bpm, P=0.89) and after (vs 154±5 bpm, P=0.54) intra-B3 saline were not different from Controls (156±3 and 163±3 bpm, respectively, P=0.8). Similarly, Control and Experimental rectilinear slopes before (5.6±0.1 vs 5.6±1.1 bpm/mmHg, P=0.95) and after (5.7±0.1 vs 5.5±0.1 bpm/mmHg, P=0.65) saline were not different.

Involvement of 5-HT$_3$ receptors

Effects of granisetron on baroreflex cardiac response inhibition induced by rostral cuneiform disinhibition in rats

The baroreflex response was performed in the same rat before (Control condition) and during (Experimental condition) intra-cuneiform bicuculline, before and after intra-NTS injection.

Interaction between conditions and intra-NTS granisetron but not saline was seen for maximal baroreflex range (Table 1) and rectilinear slopes (Table 2). The maximal cardiac range obtained from the baroreflex sigmoid curves (Fig 5C) in Experimental conditions was significantly reduced before (38±3 bpm, P<0.0001) but not after (130±5 bpm, P=0.35) intra-NTS granisetron, compared with Control conditions (160±5 bpm and 141±4 bpm, respectively, P=0.10). In the same manner, the Experimental rectilinear slopes derived from the sigmoid curves were lower before (1.4±0.2 bpm/mmHg, P<0.0001) but not after (5.4±0.1 bpm/mmHg, P=0.1) granisetron, than Control slopes (5.9±0.1 and 6.0±0.1 bpm/mmHg, respectively, P=0.85).

Experimental cardiac baroreflex ranges from the baroreflex sigmoid curves before (156±1 bpm, P=0.71) and after (153±4 bpm, P=0.82) intra-NTS saline were not different from Control conditions (163±3 and 157±4 bpm, P=0.8). Similarly, Control and Experimental rectilinear slopes were not different before (5.7±0.1 vs 5.7±0.1 bpm/mmHg, P=0.95) and after (5.8±0.1 vs 5.9±0.1 bpm/mmHg, P=0.55) this treatment.

Involvement of 5-HT_3 receptors in mice: Effects of rostral cuneiform disinhibition on baroreflex cardiac response in wild-type and 5-HT_3 knock-out mice

Injections of bicuculline into the rostral cuneiform were performed in wild-type or 5-HT_3 knock-out mice (Fig 6A). An example of injection at 4.6 mm from bregma is given Fig 6B. The baroreflex response was performed in the same animal before (Control condition) and during (Experimental condition), either in wild-type or in 5-HT_3 knock-out mice. A representative example is given Fig 7A.

Interaction between condition and mice genotype was seen for maximal baroreflex range and rectilinear slopes (Tables 1 and 2). The maximal cardiac range obtained from the baroreflex sigmoid curves in Experimental Conditions were significantly reduced in wild-type (125±5 bpm, P=0.03) but not in knock-out (243±7 bpm, P=0.93) mice (Fig 7B), compared with Control conditions (231±7 bpm and 243±5 bpm P=0.85). In the same manner, Experimental rectilinear slopes derived from the sigmoid curves were lower in wild-type (3.3±0.3 bpm/mmHg, P=0.0005) but not in knock-out (7.9±0.1 bpm/mmHg, P=0.95) mice, than Control slopes (8.3±0.3 and 8.0±0.1 bpm/mmHg, respectively, P=0.75).

Discussion

This study confirms and extends previous observations on the baroreflex inhibition during rostral cuneiform disinhibition. This negative control involves both LPGi and the Raphe Magnus in the intermediate B3, but direct links were found to reach only LPGi. Serotonin cells in B3 are at the origin of the release of serotonin into the NTS to activate 5-HT_3 receptors and block second-order baroreceptor neurons.

Fos expression in raphe nuclei

The defense reaction is associated with an inhibition of the baroreflex response (Nosaka, 1996). We showed in different studies that this effect involved the activation of NTS 5-HT_3 receptors (Sevoz-Couche et al., 2003; Comet et al., 2004, 2005; Bernard et al., 2008). The NTS receives serotonergic projections from various raphe nuclei, including the Raphe Pallidus and the Raphe Obscurus (Palkovits et al., 1986), as well as the Raphe Dorsalis (Schaffar et al., 1988), and in B3 the Raphe Magnus (Thor et al., 1987; Schaffar et al., 1988) and the LPGi (Babic and Ciriello, 2004; Schaffar et al., 1988). Therefore, these raphe nuclei are susceptible to take part in NTS 5-HT_3 receptor activation during the defense reaction. We showed that activation of the dorsolateral PAG, a key area of the defense reaction, induced a baroreflex inhibition through intermediate B3 raphe nuclei only (Bernard et al., 2008). Upstream to the dorsolateral PAG, disinhibition of local cells at specific coordinates (between -8.0 and -8.4 mm from bregma) in the rostral cuneiform nucleus following bicuculline microinjections, induced tachypnea, tachycardia, and hypertension associated with the inhibition of the baroreflex response (Netzer et al., 2011). We first examined whether B3 but also other raphe nuclei were activated during left rostral cuneiform disinhibition. As found for dorsolateral PAG activation (Bernard et al., 2008), only raphe nuclei in the intermediate B3 exhibited bilateral double-labeled Fos-5-HT cells following left rostral cuneiform disinhibition, with a higher proportion found in the LPGi than in the Raphe Magnus. It is unlikely that this result in B3 was a consequence of indirect feedback evoked from the resulting increases in blood pressure and heart rate. Indeed, no increase of Fos expression was described in the B3 region in response to blood pressure changes (Dampney and Horiuchi, 2003). No change was observed in other portions of the serotonergic system, including the Raphe Dorsalis. To note, a single exposure to social defeat has been found to selectively activate serotonergic neurons in the mid-rostrocaudal and caudal dorsal

raphe nucleus (Gardner et al., 2005; Lkhagvasuren et al., 2014). Similarly, chronic emotional stress (maternal separation or chronic mild stress, Pollano et al., 2018) increases the number of Fos-5-HT immunoreactive cells in the dorso- and ventro-lateral part of the Raphe Dorsalis. Therefore, the mechanisms involved in defense reaction (associated with panic, Johnson et al., 2008) and social emotional stress (closer from anxiety and/or depression [Blugeot et al., 2011; Sevoz-Couche et al., 2013]) seem to present discrepancies (at least concerning Raphe Dorsalis serotonergic cell activation).

Rostral cuneiform disinhibition also produced Fos expression in non-serotonergic cells in B3. The Raphe Magnus and LPGi contain non-serotonergic cells that may be ON and OFF cells responding to noxious tail heat (Gao and Mason, 2000). These specific cells have been proposed to play an important role in modulating the alertness evoked by any brief external stimulus, either noxious or innocuous. ON cells may facilitate alertness during waking and OFF cells suppress arousals during sleep (Foo and Mason, 2003). Therefore, activation of non-serotonergic cells in B3 induced by cuneiform disinhibition may participate in increased alertness during the defense reaction.

Interestingly, non-5-HT-Fos IR cells have also been found in the caudal part of the Raphe Dorsalis, especially in the mid-rostrocaudal lateral divisions of the nucleus. To note, active escape expression in the elevated T-maze recruited non-serotonergic neurons within the lateral wings of the Raphe Dorsalis (Spiacci et al., 2012) and systemic injection of sodium lactate in a group of pharmacologically-induced panic-prone rats was accompanied by the expression of a greater number of Fos-immunopositive non-serotonergic neurons preponderantly within the lateral mid-rostro-caudal Raphe Dorsalis (Johnson et al., 2008). Activation of Raphe Dorsalis non-serotonergic cells during cuneiform disinhibition or panic expression may be involved in descending analgesia because a double fluorescence immunohistochemistry study revealed that Raphe Dorsalis neurons expressing Fos after picrotoxin administration were non-serotonergic (Koyama et al., 2000).

Anterograde tracing from the rostral cuneiform nucleus to the intermediate B3

The rostral cuneiform nucleus targets the dorsolateral PAG (Netzer et al., 2011) and the dorsolateral PAG sends projections to the intermediate B3 region, at the level of the LPGi (Cameron et al., 1995; Babic and Ciriello, 2004). Previous reports using

different retrograde tracers including horseradish peroxidase (HRP) and colloidal gold-labeled wheat germ agglutinin conjugated to HRP showed that neurons labeled from the Raphe Magnus are distributed throughout the dorsal, lateral, ventrolateral PAG, but not the dorsolateral column (Abols and Basbaum, 1981; Babic and Ciriello, 2004; Yin et al., 2014). In the same manner, using anterograde tracing from the left rostral cuneiform nucleus, we show here that massive projections reach the LPGi, at the area (intermediate level of B3) where were identified Fos-5-HT-IR cells in the first set of experiments. In addition, as these projections were seen bilaterally, these data explain why disinhibition performed on one side can induce bilateral LPGi activation. However, only scarce labeling from the rostral cuneiform nucleus to the Raphe Magnus was observed in the present study, though projections exist from the pre-cuneiform nucleus to the caudal Raphe Magnus (Abols and Basbaum, 1981; Zemlan and Behbehani, 1988; Carlton et al., 1983). Therefore, the origin of Raphe Magnus 5-HT cell activation after cuneiform desinhibition (and dorsolateral PAG activation) remains unclear. One possibility could be that rostral cuneiform disinhibition partly activates the dorsal and/or lateral PAG along with dorsolateral PAG, even if very few Fos expression is observed here or in previous study (Netzer et al., 2011). Another possibility is that the LPGi or the gigantocellular nucleus pars alpha (shown to receive numerous projections from the dorsolateral PAG [Cameron et al., 1995] and rostral cuneiform nucleus [present study]), can downstream activate serotonergic cells in the Raphe Magnus following either rostral cuneiform disinhibition and dorsolateral PAG activation. In support of this idea, both regions are known to send projections to the Raphe Magnus (Zagon, 1993; Hermann et al., 1997).

Pharmacological experiments

The chemical blockade of the intermediate B3 region (including both the LPGi and Raphe Magnus) reduced the baroreflex inhibition induced by dorsolateral PAG activation (Bernard et al., 2008). In this context, it is relevant that muscimol-induced inactivation of the intermediate portion of the B3 region suppressed almost completely the inhibitory effect of rostral cuneiform disinhibition on vagal baroreflex response range and rectilinear slope. However, none of the data recalled above specifically demonstrated the involvement of serotonergic neurons in the B3 area. 8-OH-DPAT is a highly selective and centrally active potent ligand preferentially binding

to the somatodendritic 5-HT_{1A} autoreceptor (Middlemiss and Fozard, 1983). 8-OH-DPAT prevents the serotonergic-induced shivering during cooling when administered in the LPGi (Brown et al., 2008) and reduce thermoregulatory responses to peripheral thermal challenges when given into the Raphe Magnus (Berner et al., 1999). Here we observed similar prevention of the baroreflex bradycardia induced by rostral cuneiform disinhibition to that obtained after muscimol. Though a higher proportion of serotonin cells were activated in the LPGi than in the Raphe Magnus, there was a drastic increase in both regions. Thus we microinjected muscimol or 8-OH-DPAT in either LPGi or Raphe Magnus, and we pooled our data. Our data is clear-cut evidence of the key role played by both groups of serotonergic cells in the intermediate B3 in baroreflex inhibition during the defense reaction.

These cells are most presumably responsible for serotonin release into the NTS. We showed the baroreflex inhibition induced by the dorsolateral PAG involved the activation of NTS 5-HT_3 receptors (Sevoz-Couche et al., 2003; Comet et al., 2004, 2005; Bernard et al., 2008). Both microinjection of granisetron into the NTS or the use of mice lacking the 5-HT_3 receptor prevented dorsolateral PAG-induced inhibition of chemoreflex responses (Zafar et al., 2018). In the same manner, the cuneiform-induced baroreflex inhibition was prevented by the blockade of NTS 5-HT_3 receptors with granisetron and was not observed in 5-HT_3 knock-out mice. These results evidence the role of 5-HT_3 receptors in the cuneiform-induced baroreflex inhibition, most presumably at NTS location.

Conclusions

Our results are in favor of a key role of the rostral cuneiform nucleus in the defense reaction, with direct and indirect activation of intermediate B3 serotonergic cells to release serotonin into the NTS and stimulate 5-HT_3 receptors, to ultimately inhibit the baroreflex vagal bradycardia (Fig 8). These data bring new insights in the multiple mechanisms involved in baroreflex prevention during the defense reaction. Direct and indirect links to B3 appear to be a safety assurance for baroreflex inhibition, which is vital to allow cardiac output increase and blood flow redistribution when blood pressure increases during stress. A better knowledge of mechanisms involved in vagal disruption induced by acute stress is essential because it is a factor of risk for ventricular arrhythmia in chronic stress (Brouillard et al., 2020).

Acknowledgements

We thank Sorbonne University and INSERM for their support. The authors would also thank Julie Schneider for proofreading the manuscript.

References

Abols LA, Basbaum AI. 1981. Afferent Connections of the Rostral Medulla of the Cat: A Neural Substrate for Midbrain-Medullary Interactions in the Modulation of Pain. J Comp Neurol. 201:285-297.

Babic T, Ciriello J. 2004. Medullary and spinal cord projections from cardiovascular responsive sites in the rostral ventromedial medulla. Comparative Study. J Comp Neurol. 469 (3):391-412.

Bernard JF, Netzer F, Gau R, Hamon M, Laguzzi R, Sevoz-Couche C. 2008. Critical role of B3 serotonergic cells in baroreflex inhibition during the defense reaction triggere by dorsal periaqueductal gray stimulation. J Comp Neurol. 506:108–121.

Berner NJ, Grahn DA, Heller HC. 1999. 8-OH-DPAT-sensitive neurons in the nucleus raphe magnus modulate thermoregulatory output in rats. Brain Research. 831,1–2:155-164

Blugeot A, Rivat C, Bouvier E, Molet J, Mouchard A, Zeau B, Bernard, C, Benoliel J-J, Becker C. 2011. Vulnerability to depression: from brain neuroplasticity to identification of biomarkers. J. Neurosci. 31 :12889–12899.

Brouillard C, Carrive P, Sevoz couche. 2020. Social Defeat: vagal reduction and vulnerability to ventricular arrhythmias. Neurobiol Stress. Epub.

Brown JW, Sirlin EA, Benoit AM, Hoffman JM, Darnall RA. 2008. Activation of 5-HT1A receptors in medullary raphé disrupts sleep and decreases shivering during cooling in the conscious piglet. Am J Physiol Regul Integr Comp Physiol. 294(3):884-94.

Cameron AA, Khan IA, Westlund KN, Willis WD. 1995. The efferent projections of the periaqueductal gray in the rat: a Phaseolus vulgaris leucoagglutinin study. II. Descending projections. J Comp Neurol. 351:585–601.

Carlton SM, Leichnetz GR, Young EG, Mayer DJ. 1983. Supramedullary afferents of the nucleus raphe magnus in the rat: a study using the transcannula HRP gel and autoradiographic techniques. J Comp Neurol. 214(1):43-58.

Carmichael J, Cantwell BM, Edwards CM, Zussman BD, Thompson S, Rapeport WG, Harris AL. 1989. A pharmacokinetic study of granisetron (BRL 43694A), a selective 5-HT3 receptor antagonist: correlation with anti-emetic response. Cancer Chemother Pharmacol.24(1):45-9.

Comet MA, Sevoz-Couche C, Hanoun N, Hamon M, Laguzzi R. 2004. 5-HT-mediated inhibition of cardiac baroreceptor reflex response during defense reaction in the rat. Am J Physiol Heart Circ Physiol. 287:H1641–H1649.

Comet MA, Laguzzi R, Hamon M, Sevoz-Couche C. 2005. Functional interaction between nucleus tractus solitarius NK1 and 5-HT3 receptors in the inhibition of baroreflex in rats. Cardiovasc Res. 65:930–939.

Dampney RA, Horiuchi J. 2003. Functional organisation of central cardiovascular pathways: studies using c-fos gene expression. Prog Neurobiol. 71(5):359-84.

Foo H, Mason P. 2003. Brainstem modulation of pain during sleep and waking. Sleep Medicine Reviews. 7(2):145-154.

Gao K, Mason P. 2000. Serotonergic Raphe Magnus Cells That Respond to Noxious Tail Heat Are Not ON or OFF Cells. J Neurophysiol. 84:719–1725.

Gardner KL, Thrivikraman KV, Lightman SL, Plotsky PM, Lowry CA. 2005. Early life experience alters behavior during social defeat: focus on serotonergic systems. Neuroscience. 136(1):181-91.

Gau R, Sévoz-Couche C, Laguzzi R, Hamon M, Bernard JF. 2009. Inhibition of cardiac baroreflex by noxious thermal stimuli: a key role for lateral paragigantocellular serotonergic cells. Pain. 146(3):315-24.

Hamon M, Fattaccini CM, Adrien J, Gallissot MC, Martin P, Gozlan H. 1988. Alterations of central serotonin and dopamine turnover in rats treated with ipsapirone and other 5-hydroxytryptamine1A agonists with potential anxiolytic properties. J Pharmacol Exp Ther. 246(2):745-52.

Hermann DM, Luppi PH, Peyron C, Hinckel P, Jouvet M. 1997. Afferent projections to the rat nuclei raphe magnus, raphe pallidus and reticularis gigantocellularis pars alpha demonstrated by iontophoretic application of choleratoxin (subunit b). J Chem Neuroanat. 13:1–21.

Hoyer D. 1990. Serotonin 5-HT3, 5-HT4, and 5-HT-M receptors. Neuropsychopharmacology. 3(5-6):371-83.

Johnson PL, Truitt WA, Fitz SD, Lowry CA, Shekhar A. 2008. Neural pathways underlying lactate-induced panic. Neuropsychopharmacology. 33(9):2093-107.

Johnston GA, Curtis DR, De Groat WC, Duggan AW. 1968. Central actions of ibotenic acid and muscimol. Biochem Pharmacol. 17(12):2488-9.

Koyama N, Nishio T, Yokota T. 2000. Non-serotonergic midbrain neurons are involved in picrotoxin-induced analgesia. An immunohistochemical study in the rat. Neurosci Lett. 291(3):147-50.

Lkhagvasuren B, Oka T, Nakamura Y, Hayashi H, Sudo N, Nakamura K. 2014. Distribution of fos-immunoreactive cells in rat forebrain and midbrain following social defeat stress and diazepam treatment. Neuroscience. 272:34-57

Middlemiss DN, Fozard JR. 1983. 8-Hydroxy-2-(di-n-propylamino)-tetralin discriminates between subtypes of the 5-HT1 recognition site. Eur J Pharmacol. 90(1):151-3.

Netzer F, Bernard JF, Verberne AJ, Hamon M, Camus F, Benoliel JJ, Sevoz-Couche C. 2011. Brain circuits mediating baroreflex bradycardia inhibition in rats: an anatomical and functional link between the cuneiform nucleus and the periaqueductal grey. J Physiol. 589:2079–2091.

Nosaka S. 1996. Modifications of arterial baroreflexes: obligatory roles in cardiovascular regulation in stress and poststress recovery. Jpn J Physiol. 46(4):271-88.

Ootsuka Y, Blessing WW. 2006. Activation of 5-HT1A receptors in rostral medullary raphé inhibits cutaneous vasoconstriction elicited by cold exposure in rabbits. Brain Res. 1074:252-61.

Palkovits M, Mezey E, Eskay RL, Brownstein MJ. 1986. Innervation of the nucleus of the solitary tract and the dorsal vagal nucleus by thyrotropin-releasing hormone-containing raphe neurons. Brain Res. 373(1-2):246-51.

Paxinos P, Watson C. 2005. The rat brain in stereotaxic coordinates, 5th ed. London: Academic Press.

Pollano A, Trujillo V, Suárez MM. 2018. How does early maternal separation and chronic stress in adult rats affect the immunoreactivity of serotonergic neurons within the dorsal raphe nucleus? Stress. 21(1):59-68.

Schaffar N, Kessler JP, Bosler O, Jean A. 1988. Central serotonergic projections to the nucleus tractus solitarii: evidence from a double labeling study in the rat. Neuro cience. 26:951–958.

Sevoz-Couche C, Comet M-A, Hamon M, Laguzzi R. 2003. Role of nucleus tractus solitarius 5-HT3 receptors in the defense reaction-induced inhibition of the aortic baroreflex in rats. J Neurophysiol. 90(4):2521-30.

Sevoz-Couche C, Brouillard C, Camus F, Laude D, De Boer SF, Becker C, Benoliel J-J. 2013. Involvement of the dorsomedial hypothalamus and the nucleus tractus solitarii in chronic cardiovascular changes associated with anxiety in rats. J. Physiol. 591:1871–1887.

Spiacci A Jr, Coimbra NC, Zangrossi H Jr (2012) Differential involvement of dorsal raphe subnuclei in the regulation of anxiety- and panic-related defensive behaviors. Neuroscience. 227:350–360

Thor KB, Helke CJ. 1987. Serotonin- and substance P-containing projections to the nucleus tractus solitarii of the rat. J Comp Neurol. 265:275–293.

Vaschillo EG, Vaschillo B, Lehrer PM. 2006. Characteristics of Resonance in Heart Rate Variability Stimulated by Biofeedback. Applied Psychophysiol Biofeedback. 31(2):129-42.

Yin JB, Wu HH, Dong YL, Zhang T, Wang J, Zhang Y, Wei YY, Lu YC, Wu SX, Wang W, Li YQ. 2014. Neurochemical properties of BDNF-containing neurons projecting to rostral ventromedial medulla in the ventrolateral periaqueductal gray. Frontiers Neural Circ. 8:137-143.

Zafar T, Brouillard C, Lanfumey L, Sévoz-Couche C. 2018. A hypothalamo-midbrain-medullary pathway involved in the inhibition of the respiratory chemoreflex response induced by potassium cyanide in rodents. Neuropharmacol. 128:152-167.

Zagon A. 1993. Innervation of serotonergic medullary raphe neurons from cells of the rostral ventrolateral medulla in rats. Neuroscience. 55(3):849-67.

Zeitz KP, Guy N, Malmberg AB, Dirajlal S, Martin WJ, Sun L, Bonhaus DW, Stucky CL, Julius D, Basbaum AI. 2002. The 5-HT3 subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors. J Neurosci. 22:1010–1019.

Zemlan FP, Behbehani MM. 1988. Nucleus cuneiformis and pain modulation: anatomy and behavioral pharmacology. Brain Res. 453(1-2):89-102.

Table 1: Statistical analysis of the interaction between the Condition and intra-cerebral microinjections or mice genotyping, on the maximal baroreflex range using two-way repeated measures ANOVA

	Control vs Experimental Condition	**Before vs after Treatment in rats/ Or Wild-type vs KO mice**	**Interaction**
Intra-B3 Muscimol	**F (1,5) = 1920 P<0.0001**	**F (1,5) =254.01 P<0.0001**	**F (1,5) = 119.10 P<0.0001**
Intra-B3 8OH-DPAT	**F (1,5) = 72.82 P=0.0004**	**F (1,5) = 30.70 P=0.0026**	**F (1,5) = 37.81 P=0.0017**
Intra-B3 Saline	F (1,5) = 4.39 P=0.0902	F (1,5) = 1.71 P=0.2474	F (1,5) = 0.44 P=0.5343
Intra-NTS Granisetron NTS	**F (1,5) = 341.31 P<0.0001**	**F (1,5) = 48.88 P=0.0009**	**F (1,5) = 299.40 P<0.0001**
Intra-NTS Saline	F (1,5) = 5.56 P=0.0648	F (1,5) = 2.85 P=0.1518	F (1,5) = 0.07 P=0.7971
Mice	**F (1,5) = 60.36 P=0006**	**F (1,5) = 26.76 P=0.0035**	**F (1,5) = 10.50 P=0.0229**

Table 2: Statistical analysis of the interaction between the Condition and intra-cerebral microinjections or mice genotyping, on the baroreflex slope using two-way repeated measures ANOVA

	Experimental vs Control Condition	**Before vs after Treatment in rats/ Or Wild-type vs KO mice**	**Interaction**
Intra-B3 Muscimol	**F (1,5) = 653.3 P<0.0001**	**F (1,5) = 111.0 P=0.0001**	**F (1,5) = 95.6 P=0.0002**
Intra-B3 8OH-DPAT	**F (1,5) = 445.1 P<0.0001**	**F (1,5) = 660.3 P<0.0001**	**F (1,5) = 95.6 P<0.0001**
Intra-B3 Saline	F (1,5) = 3.9 P=0.10	F (1,5) = 0.02 P=0.88	F (1,5) = 1.51 P=0.27
Intra-NTS Granisetron NTS	**F (1,5) = 361.4 P<0.0001**	**F (1,5) = 119.7 P=0.0001**	**F (1,5) = 121.0 P=0.0001**
Intra-NTS Saline	F (1,5) = 0.25 P=0.63	F (1,5) = 0.09 P=0.77	F (1,5) = 0.59 P=0.47
Mice	**F (1,5) = 57.7 P=0.0006**	**F (1,5) = 287.0 P<0.0001**	**F (1,5) = 64.65 P=0.0005**

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Legends of figures

Figure 1. Increase in Fos expression in serotonin cells after rostral CnF stimulation

A. Bar graphs showing an increased number of serotonin cells (in four brain sections) expressing Fos protein immunoreactivity in ipsilateral (ipsi) as well as contralateral (contra) lateral paragigantocellular nucleus (LPGi), total LPGi, and Raphe Magnus (RMg), but not in Raphe Dorsalis (RDors), Raphe Pallidus (RPall) and Raphe Obscurus (RObsc), in Experimental (intra-cuneiform bicuculline, filled bars) compared to Sham (intra-cuneiform saline, open bars) conditions. Each bar is the mean ± SEM. *p< 0.05 and *** p<0.001.

B. Photomicrographs of the LPGi at approximately -11.2 mm from bregma in Sham and Experimental condition. The high magnification microphotograph is the region framed in the contralateral LPGi of the Experimental condition.

Arrows with d, s, and f indicate examples of Fos +5-HT double labeled (black core surrounded by brown staining), 5-HT single labeled (cytoplasm brown staining), and Fos single-labeled (nucleus black staining) neurons, respectively.

Figure 2. Increase in Fos expression in non-serotonin cells after rostral cuneiform stimulation

A. Bar graphs showing an increased number of non-serotonin cells expressing Fos protein immunoreactivity in total LPGi, RMg and RDors, but not in RPall and RObs, in Experimental compared to Sham conditions. Each bar is the mean ± SEM. *p< 0.05, **p<0.01 and *** p<0.001. Abbreviations as in Fig 1.

B. Photomicrographs of the Raphe Dorsalis in Sham and Experimental (Exp) condition. The high magnification microphotograph is the Raphe Dorsalis region framed in the Experimental condition.

Abbreviations: Aq: aqueduct; dPAG: dorsal column of the periaqueductal grey area; dlPAG: dorsolateral column of the periaqueductal grey area; lPAG: lateral column of the periaqueductal grey area; RDors: Raphe Dorsalis; vPAG: ventrolateral column of the periaqueductal grey area.

Figure 3. Projections from the rostral cuneiform nucleus to the B3 region

A. Photomicrograph displaying the site of Phaseolus leucoagglutinin (PHA-L) unilateral injection in the rostral cuneiform nucleus (CnF, arrow), at approximately 8.2 mm caudal to bregma.

B. Camera lucida drawings of anterograde labeling of descending axons and terminal boutons in B3 from injection of PHA-L in the rostral cuneiform nucleus seen in A. Series of transverse sections. Numbers indicate the distance (in mm) from bregma.
C. Photomicrographs corresponding to approximately -11.4 mm from bregma. PHA-L immunolabeled fibers and varicosities in the LPGi are shown in framed ×20 magnification photomicrograph.
Abbreviations: 7: facial motor nucleus; CIC: central nucleus of the inferior colliculus; GiA: gigantocellular reticular nucleus, alpha; ml: medial lemniscus; p7: perifacial zone; py: pyramidal tract ; sp5: spinal trigeminal tract; VTg: ventral tegmentum. Other abbreviations as in Fig 1 and 2.

Figure 4. Microinjection sites of muscimol into the B3 group
A. Coronal sections at the level of B3 group showing bilateral microinjections of bodipy muscimol (black circle), 8-OH-DPAT (grey circle) or saline (white circle). Abbreviations as in Fig 3.
B. Reconstructive photomicrograph showing the bilateral microinjection of bodipy muscimol (orange fluorescence) within the serotonergic neuron area (green cells) of LPGi (B-1) and RMg (B-2) shown by arrows in A at -11.2 and -11.4 mm from bregma, respectively.

Figure 5. Inhibition of the baroreflex curves after disinhibition of the rostral cuneiform nucleus
A-1. Representative recordings illustrating that pharmacological disinhibition of the rostral cuneiform nucleus by local application of bicuculline (Bic CnF, Experimental) did not affect the baroreflex tachycardia induced by administration of the vasodilator nitroprusside (SNP) but inhibited the reflex bradycardia induced by administration of the vasoconstrictor phenylephrine (PE) compared to the Control condition, before but not after intra-B3 muscimol.
A-2. Sigmoid baroreflex curves corresponding to the animal shown in A-1 showing that the decrease in HR in response to MBP changes was reduced in Experimental compared to Control conditions, before but not after intra-B3 muscimol.
B and C. Sigmoid baroreflex curves showing that the decrease in HR in response to MBP changes was reduced in Experimental compared to Control conditions, before but not after intra-B3 8-OH-DPAT (B) or intra-NTS granisetron (C).

Figure 6. Microinjection sites of bicuculline into the rostral cuneiform nucleus in mice

A. Coronal sections at the level of the rostral CnF showing unilateral microinjections of bicuculline in wild-type (grey circle) or 5-HT_3 knock-out (black circle) mice.

B. Photomicrograph showing pontamine blue deposit (arrow) below the tip of the micropipette used to inject bicuculline into the CnF at -4.6 mm from bregma, as indicated by the arrow in A. Abbreviations as in Fig 3.

Figure 7. Inhibition of the baroreflex bradycardia after disinhibition of the rostral cuneiform nucleus

A. Representative recordings illustrating that pharmacological disinhibition of the rostral cuneiform nucleus by local application of bicuculline (Bic CnF, Experimental) did not affect the baroreflex tachycardia induced by administration of the vasodilator nitroprusside (SNP) but inhibited the reflex bradycardia induced by administration of the vasoconstrictor phenylephrine (PE) compared to the Control condition, in wild-type but not in 5-HT_3 knock-out mice.

B. Sigmoid baroreflex curves corresponding to the animal shown in A showing that the decrease in HR in response to MBP changes was reduced in Experimental compared to Control conditions, in wild-type but not in 5-HT_3 knock-out mice.

Figure 8. Schematic representation of putative medullary pathways producing baroreflex inhibition during the defense reaction.

Baroreceptor glutamatergic vagal afferents are known to excite barosensitive NTS cells (A) through the activation of excitatory amino acid receptors (grey square) to induce vagal baroreceptor reflex bradycardia after activation of preganglionic (PG) cells in the nucleus ambiguous (NAmb). Previous and present findings show that, during the defense-like reaction triggered by rostral cuneiform nucleus (rCnF) disinhibition, activation of i/ direct and indirect (through the dorsolateral PAG, dlPAG) projections to the LPGi and ii/ indirect projections to the RMg, both located in the intermediate B3 (iB3) region, produces the release of 5-HT into the NTS. Serotonin downstream stimulates 5-HT_3 receptors (small grey circle) localized presynaptically on gastrointestinal (GI) vagal glutamatergic afferents in the NTS. The resulting facilitation of glutamate release ends with the excitation of NTS GABAergic interneurons, which, in turn, inhibit baroreflex bradycardia through the stimulation of

$GABA_A$ receptors (small black circle) on barosensitive A cells. + excitatory effect; - inhibitory effect.