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ORIGINAL PAPER

Somatosensory regulation of serotonin release in the central nucleus of the amygdala is mediated via corticotropin releasing factor and gamma-aminobutyric acid in the dorsal raphe nucleus

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Abstract Noxious cutaneous stimulation increases, whereas innocuous cutaneous stimulation decreases serotonin (5-HT) release in the central nucleus of the amygdala (CeA) in anesthetized rats. In the present study, we investigated the contribution of corticotropin releasing factor (CRF) receptors and gamma-aminobutyric acid (GABA) receptors in the dorsal raphe nucleus (DRN) to those responses. Release of 5-HT in the CeA was monitored by microdialysis before and after 10-min stimulation by pinching or stroking. Increased 5-HT release in the CeA in response to pinching was abolished by CRF_2 receptor antagonism in the DRN. Decreased 5-HT release in the CeA in response to stroking was abolished by either CRF_1 receptor antagonism or $GABA_A$ receptor antagonism in the DRN. These results suggest that opposite responses of 5-HT release in the CeA to noxious versus innocuous stimulation of the skin are due to separate contributions of CRF_2, CRF_1 and $GABA_A$ receptors in the DRN.

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Introduction

Somatosensory stimulation can produce emotional responses in addition to eliciting sensations. For example, noxious stimulation often evokes fear and anxiety [1, 2], whereas innocuous tactile stimulation can be pleasurable or even anxiolytic [3–6]. Recently, we showed that serotonin (5-HT) release in the central nucleus of the amygdala (CeA), an area important for emotional responsivity [7, 8], changes in response to somatosensory stimulation in anesthetized animals [9]. Specifically, 5-HT release in the CeA was found to increase in response to noxious mechanical stimulation (i.e., pinching) of the skin, but decrease in response to innocuous mechanical stimulation (i.e., stroking) of the skin. Together with studies suggesting that 5-HT in the CeA is involved in the triggering of anxiety and fear [10, 11], these findings suggest a serotonergic neural mechanism by which emotion can be affected by somatosensory stimulation.

Brain corticotropin releasing factor (CRF) has also been associated with anxiety and fear [12–14]. Furthermore, immobilization stress-induced 5-HT release in the CeA can be blocked by intracerebroventricular (icv) injection of a non-selective CRF antagonist [15]. These findings led us to question whether changes in 5-HT release in the CeA in response to pinching and stroking, demonstrated in our previous study [9], might also be modulated via CRF receptors.

CRF in the dorsal raphe nucleus (DRN), the origin of the serotonergic neurons that project to the CeA, appears to be critical for evoking fear- and anxiety-related behaviors

[16, 17]. In fact, intra-DRN CRF administration has been shown to increase 5-HT release in the CeA, and those 5-HT levels correlate with time spent freezing, a common behavioral index of fear in rodents [11].

There are two CRF receptor subtypes, CRF_1 and CRF_2. CRF_1 receptors have a broad distribution throughout the brain, whereas CRF_2 receptor expression is restricted to subcortical areas, such as the lateral septum, hypothalamus, amygdala, and raphe nucleus [18, 19]. The DRN is one of the few regions in which both CRF_1 and CRF_2 receptors are found [20, 21]. Freezing behavior in response to uncontrollable stress can be attenuated by either stimulation of CRF_1 receptors or blockade of CRF_2 receptors in the DRN [22, 23]. Hence, functions of CRF_1 and CRF_2 receptors in the DRN appear to oppose each other.

In the present study, we tested the hypothesis that opposite effects of pinching and stroking on 5-HT release in the CeA would involve CRF acting on different CRF receptor subtypes in the DRN. For this purpose, non-selective and selective CRF receptor antagonists were administered into the DRN. Furthermore, because serotonergic neurons in the DRN are inhibited by $GABA_A$ receptor activation within the DRN [24], we investigated whether $GABA_A$ receptors in the DRN are involved in pinching-induced and stroking-induced 5-HT responses in the CeA.

Materials and methods

All experiments were conducted in accordance with the Japanese Physiological Society's Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the animal ethics committee of the International University of Health and Welfare.

Animals

The experiments were performed on 27 male Wistar rats (280–340 g). The animals were kept in a temperature-controlled room (23 ± 1 °C) that was lit between 08:00 and 20:00 h (Showa, Tokyo). Commercial rodent chow (Labo-MR stock, Nosan, Kanagawa) and tap water were provided ad libitum.

Cannula implantation

One or two days prior to the experiment, the rats were anesthetized with sodium pentobarbital (50 mg kg^{-1}, intraperitoneal injection) and implanted with a guide cannula (diameter 0.5 mm; AG-12, Eicom, Kyoto) for a microdialysis probe aimed at the CeA as described in detail previously [9]. The placement coordinates for the guide cannula were as follows: 2.3 mm posterior to bregma, 4.0 mm lateral of the midline, and 6.4 mm below the dura.

In the same operation, a guide cannula (AG-8, Eicom) for an injection needle aimed at the DRN or the lateral cerebroventricle was implanted. The DRN cannula was implanted 7.6 mm posterior to and 2.8 mm lateral to the bregma and lowered at a 26° angle from vertical to a depth of 4.4 mm below the dura. A ventricular cannula was implanted 0.8 mm posterior to and 1.5 mm lateral to the bregma and lowered to 1.7 mm below the dura. All guide cannulae were secured to the skull with a screw and dental cement. After surgery, each animal was transferred to an individual cage.

Anesthesia during the experiment

The experiments were performed under urethane anesthesia (1.1 g kg^{-1}, intraperitoneal injection). The trachea was intubated for spontaneous breathing. Core temperature was maintained at 37.5 ± 0.1 °C with a heating pad and an infrared lamp (ATB-1100, Nihon-Kohden, Tokyo). Throughout the experiment, depth of anesthesia was assessed routinely by checking the respiration rate (counting breaths min^{-1}) and observing corneal and flexion reflexes.

Microdialysis probe implantation and dialysate sampling

Microdialysis probe placement and dialysate sampling were performed according to the methods described by Tokunaga et al. [9]. Briefly, on the morning of the experiment day, a concentric microdialysis probe with a 1-mm membrane (220-μm outer diameter, 50-kDa molecular-weight cut-off; A-I-12-01, Eicom) was inserted into the left CeA via a previously implanted guide cannula. The probe was perfused with modified Ringer's solution (147 mM Na^+, 4 mM K^+, and 1.15 mM Ca^{2+}) at a rate of 1 μl min^{-1}, and the dialysate was collected from the outlet tube for 10 min. Pooled dialysate samples were injected manually into a high-performance liquid chromatograph every 10 min for analysis. The in vitro recovery rate for 5-HT recorded with individual microdialysis probes ranged from 7.5 to 12.5 %. To avoid inter-probe differences in recovery rate, the 5-HT concentration in the dialysate was calculated based on a 10.0 % recovery rate.

Measurement of 5-HT

The 5-HT was measured by a high-performance liquid chromatograph equipped with an electrochemical detector (HTEC-500, Eicom), as described previously [9]. The coefficient of variation of this method for a standard

solution of 0.06 fmol μl^{-1} concentration was 0.95 % ($n = 8$).

Pharmacology

Injection into the lateral cerebroventricle

Ten microliters of α-helical CRF(9–41) (50 μg, Tocris Bioscience, Bristol, UK) diluted with a mixture of 50 % modified Ringer's solution and 50 % distilled water, or vehicle (50 % modified Ringer's solution and 50 % distilled water) in control experiments, was injected through an injection cannula (AMI-10, Eicom) into the lateral cerebroventricle via a surgically implanted guide cannula. The injections were propelled by an electric microinjector (IMS-10, Narishige, Tokyo) at a rate of 5 μl min^{-1}. In each animal, vehicle was injected first (control experiment) before α-helical CRF(9–41) was injected in the same animal.

Injection into the DRN

The non-selective CRF antagonist α-helical CRF(9–41) (0.5 μg in 0.1 μl) or vehicle was injected through an injection cannula (AMI-10, Eicom) into the DRN at a rate of 0.04 μl mim^{-1} by an electric microinjector (same as above). Furthermore, 0.5 μl of antalarmin hydrochloride (0.25 μg, Sigma-Aldrich, St Louis, MO, USA), a CRF_1 receptor antagonist, or 0.5 μl of antisauvagine-30 (ASV-30) (1 μg, Phoenix Pharmaceuticals, Burlingame, CA, USA), a CRF_2 receptor antagonist, was similarly injected into the DRN. Antalarmin was diluted in a mixture of 95 % modified Ringer's solution and 5 % DMSO. ASV-30 was diluted in a mixture of 50 % modified Ringer's solution and 50 % distilled water. Also, 0.1 μl of bicuculline methiodide (0.5 μg, Tocris Bioscience, Bristol, UK), a $GABA_A$ receptor antagonist, was injected into the DRN with the same method. Bicuculline was diluted in modified Ringer's solution. In each animal, vehicle solution was injected first (control experiment) and then an antagonist was injected in the same animal.

Cutaneous stimulation

Noxious mechanical stimulation (pinching) and innocuous mechanical stimulation (stroking) were applied as described in our previous study [9]. Briefly, pinching was applied with a surgical clamp at a force of 3–5 kg to the back (between the inferior angle of the scapula and the iliac crest) for 10 min. Stroking was applied manually to the back for 10 min with a pressure of 80–100 mm H_2O and at the frequency 65–75 strokes min^{-1} (1.08–1.25 Hz). Each stimulus type was applied once or twice per rat after administration with vehicle and antagonist; data from identical procedures were pooled to produce an averaged response data for each animal. After monitoring basal 5-HT levels in the CeA for 60 min, the two cutaneous stimulations were applied in random order.

Probe placement verification

After completion of the experiment, each rat was anesthetized deeply with sodium pentobarbital. Its brain was then removed after transcardial perfusion of formalin as described previously [9]. Placement of the probe was confirmed to be in the CeA for all of the rats used in this study.

Statistical analysis

Data are expressed as mean ± SD. Changes over time within a group were analyzed by a repeated measures one-way ANOVA followed by Dunnett's multiple range test for post hoc comparisons. The pre-stimulus basal values of 5-HT concentration in the CeA were compared to antagonist and respective vehicle treatment data sets by Student's *t* test. Probability values of less than 5 % were considered significant.

Results

icv injection of α-helical CRF(9–41)

Basal release of 5-HT

The basal concentration of 5-HT in the CeA dialysates of six animals was 0.89 ± 0.34 fmol 10 μl^{-1} (i.e., release of 0.89 ± 0.34 fmol 10 min^{-1}). The 5-HT levels in subsequent sequential dialysate samples decreased to 10–15 % below basal levels following icv injection of α-helical CRF(9–41), a non-selective CRF receptor antagonist (see Table 1), but remained stable (0.81 ± 0.18 fmol 10 μl^{-1}) for 60 min in the same six animals following prior icv administration of vehicle (Table 1).

Responses to pinching

When pinching was applied to the back after icv injection of vehicle, 5-HT concentrations in CeA dialysate samples increased significantly during the stimulation period (119 ± 10 % of basal value). The concentration returned to basal levels by the subsequent 10-min sampling period (10–20 min after onset of stimulation) (Fig. 1a, open

Table 1 Effects of CRF and GABA receptor antagonists (or vehicle) on basal 5-HT release in the CeA

Treatment	Time (min)							
	−20 to −10	−10 to 0	0–10	10–20	20–30	30–40	40–50	50–60
α-helical CRF (icv)	99 ± 2	100	85 ± 10*	84 ± 5 **	84 ± 6 **	84 ± 7 **	85 ± 7**	83 ± 6**
Vehicle	101 ± 6	100	99 ± 5	99 ± 4	98 ± 6	100 ± 8	100 ± 7	98 ± 9
α-helical CRF (intra-DRN)	99 ± 3	100	100 ± 3	101 ± 7	93 ± 10	98 ± 5	101 ± 10	99 ± 4
Vehicle	99 ± 2	100	100 ± 1	98 ± 3	99 ± 3	96 ± 4	97 ± 2	95 ± 6
Antalarmin (intra-DRN)	100 ± 3	100	101 ± 3	100 ± 6	100 ± 8	105 ± 10	95 ± 8	99 ± 4
Vehicle	101 ± 6	100	101 ± 7	99 ± 3	100 ± 4	101 ± 2	98 ± 4	101 ± 5
ASV-30 (intra-DRN)	100 ± 7	100	101 ± 9	101 ± 4	104 ± 1	101 ± 4	107 ± 5	105 ± 6
Vehicle	103 ± 10	100	98 ± 3	99 ± 3	100 ± 8	97 ± 3	97 ± 5	99 ± 7
Bicuculline (intra-DRN)	101 ± 2	100	109 ± 12	127 ± 11**	122 ± 10**	128 ± 18**	115 ± 16	118 ± 11
Vehicle	101 ± 4	100	101 ± 3	100 ± 5	102 ± 4	98 ± 6	103 ± 14	100 ± 4

0 indicates the onset of administration. Data are expressed as a percentage (mean ± SD) of the pre-injection control value (the value obtained for baseline period of −10 to 0 min)

* $p < 0.05$, ** $p < 0.01$, vs. pre-injection control values

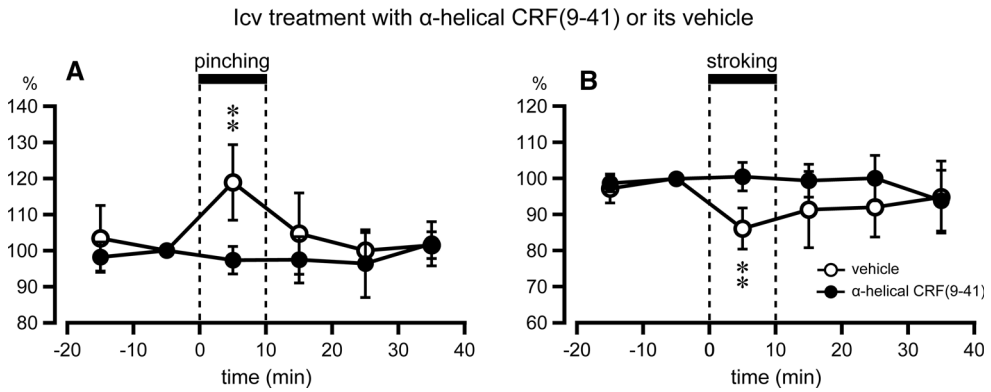

Fig. 1 Effects of icv administration of α-helical CRF(9–41), or vehicle, on 5-HT release responses in the CeA to pinching (**a**) and stroking (**b**) of the back. Ordinates: response magnitude is expressed as a percentage (mean ± SD) of the prestimulus control value. Abscissa: 0 indicates onset of stimulation. *Horizontal bar* indicates the 10-min stimulus period. $^{**}p < 0.01$ vs. prestimulus control values. $n = 6$

circles). After icv injection with α-helical CRF(9–41), 5-HT concentrations in the CeA dialysate showed no changes in response to pinching (Fig. 1a, closed circles).

Responses to stroking

When stroking was applied to the back after icv injection of vehicle, 5-HT concentrations in CeA dialysate samples decreased significantly (86 ± 6 % of basal value) during the stimulation period (Fig. 1b, open circles). On the other hand, 5-HT concentrations showed no changes in response to stroking after subsequent icv injection with α-helical CRF(9–41) (Fig. 1b, closed circles).

Effects of intra-DRN injection of α-helical CRF(9–41)

Basal release of 5-HT

The basal 5-HT concentration in the CeA dialysate in five animals was 0.97 ± 0.16 fmol 10 μl^{-1}, and subsequent dialysate sequential samplings showed no significant changes after intra-DRN injection of α-helical CRF(9–41) (see Table 1). The 5-HT concentrations in the CeA dialysate samples from the same five animals (0.89 ± 0.12 fmol 10 μl^{-1}) were stable for 60 min after prior intra-DRN administration of vehicle (Table 1).

Responses to pinching

When pinching was applied to the back after intra-DRN vehicle injection, 5-HT concentrations in CeA dialysate samples increased significantly (121 ± 8 % of basal value) during the stimulation period (Fig. 2a, open circles). On the other hand, intra-DRN injection with α-helical CRF(9–41) abolished pinching-induced increases in amygdalar 5-HT release (Fig. 2a, closed circles).

Responses to stroking

When stroking was applied to the back after intra-DRN treatment with vehicle, 5-HT concentrations in CeA dialysate samples decreased significantly (86 ± 8 % of basal value) during the stimulation period (Fig. 2b, open circles). On the other hand, intra-DRN injection of α-helical CRF(9–41) abolished stroking-induced decreases in amygdalar 5-HT release (Fig. 2b, closed circles).

Effects of intra-DRN injection of antalarmin

Basal release of 5-HT

Basal 5-HT concentration in CeA dialysate samples from five animals was 0.80 ± 0.20 fmol 10 μl^{-1}, and remained stable after intra-DRN injection of antalarmin, a selective CRF_1 receptor antagonist (see Table 1). The 5-HT concentrations in CeA dialysate samples from the same five animals were also stable (0.88 ± 0.15 fmol 10 μl^{-1}) for 60 min after intra-DRN administration of vehicle (Table 1).

Responses to pinching

As shown in Fig. 3a (open circles), the aforementioned stimulatory effect of pinching on 5-HT release in the CeA remained after intra-DRN vehicle injection (120 ± 11 % of basal value during the stimulation period). Concentrations of 5-HT in CeA dialysate samples returned to basal levels during the subsequent 10-min sampling period (10–20 min after the onset of stimulation). After intra-DRN injection of antalarmin, the pinching-induced 5-HT increases not only persisted during the stimulation period (118 ± 5 % of basal value), but remained evident in the subsequent 10-min sampling period (10–20 min after the onset of stimulation) (Fig. 3a, closed circles).

Responses to stroking

The aforementioned depressing effect of cutaneous stroking on 5-HT release in the CeA also remained (83 ± 7 % of basal value) during the stimulation period after intra-DRN injection of vehicle (Fig. 3b, open circles). In the presence of intra-DRN antalarmin, this 5-HT reduction effect in the CeA in response to stroking was abolished (Fig. 3b, closed circles).

Effects of intra-DRN injection of ASV-30

Basal release of 5-HT

The mean basal 5-HT concentration of CeA dialysate samples from five animals was 0.72 ± 0.07 fmol 10 μl^{-1}, and sequential samplings of the dialysate showed no significant changes in 5-HT output after intra-DRN injection of antisauvagine-30 (ASV-30), a selective CRF_2 receptor antagonist (see Table 1). The mean 5-HT concentration of CeA dialysate samples from the same five animals (0.88 ± 0.12 fmol 10 μl^{-1}) was stable for 60 min after intra-DRN injection of vehicle (Table 1).

Responses to pinching

The aforementioned pinching-induced increase in 5-HT release in the CeA was replicated (119 ± 8 % of basal value during the stimulation period) after intra-DRN vehicle injection (Fig. 4a, open circles). However, this pinching-induced 5-HT response was abolished after intra-DRN ASV-30 injection (Fig. 4a, closed circles).

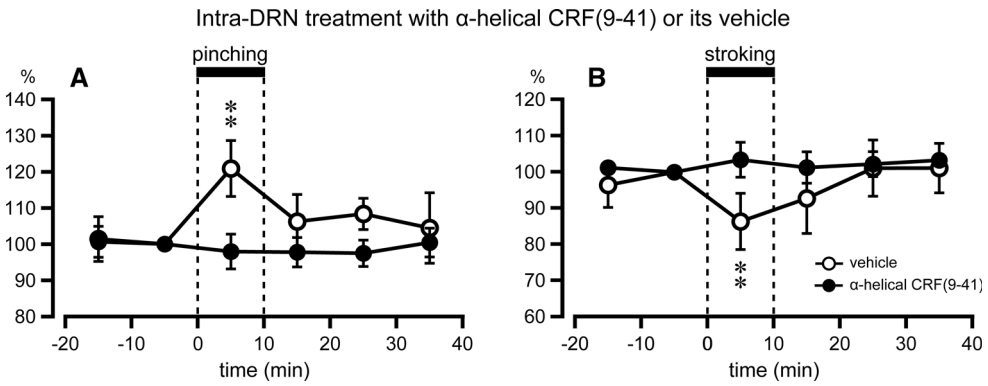

Fig. 2 Effects of intra-DRN α-helical CRF(9–41), or vehicle, administration on 5-HT release responses in the CeA to pinching (**a**) and stroking (**b**) of the back. $n = 5$. *Graphs* are set up as in Fig. 1

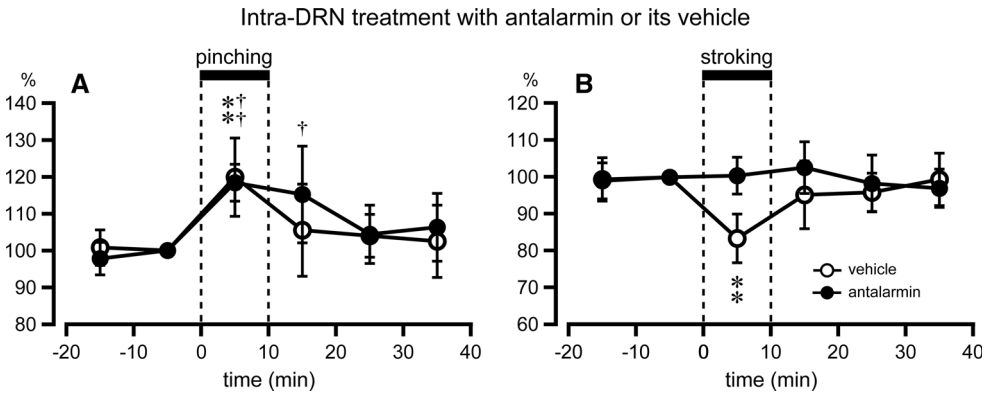

Fig. 3 Effects of intra-DRN antalarmin, or vehicle, administration on 5-HT release responses in the CeA to pinching (**a**) and stroking (**b**) of the back. $^{\dagger\dagger}p < 0.01$ (antalarmin), $^{\dagger}p < 0.05$ (antalarmin), $^{**}p < 0.01$ (vehicle) vs. prestimulus control values. $n = 5$. *Graphs* are set up as in Fig. 1

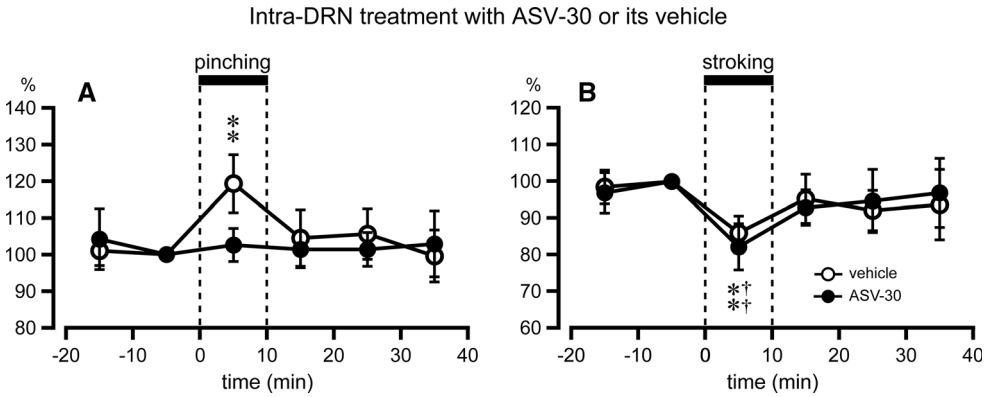

Fig. 4 Effects of intra-DRN ASV-30, or vehicle, administration on 5-HT release responses in the CeA to pinching (**a**) and stroking (**b**) of the back. $^{\dagger\dagger}p < 0.01$ (ASV-30), $^{**}p < 0.01$ (vehicle) vs. prestimulus control values. $n = 5$. *Graphs* are set up as in Fig. 1

Responses to stroking

The stroking-induced decrease in 5-HT release in the CeA was maintained following intra-DRN vehicle injection (86 ± 5 % of basal value; Fig. 4b, open circles) or intra-DRN ASV-30 injection (82 ± 6 % of basal value; Fig. 4b, closed circles).

Effects of intra-DRN injection of bicuculline

Basal release of 5-HT

The mean basal 5-HT concentration in CeA dialysate samples from six animals was 1.04 ± 0.31 fmol 10 μl^{-1} and then increased significantly after intra-DRN injection of the selective $GABA_A$ receptor antagonist bicuculline (10–40-min sampling periods; Table 1). CeA-dialysate 5-HT concentrations moved gradually toward pre-injection levels thereafter (40–60-min sampling periods). The mean 5-HT concentration of CeA dialysate samples from the same six animals (1.13 ± 0.35 fmol 10 μl^{-1}) was stable for 60 min after intra-DRN administration of vehicle (Table 1).

Responses to pinching

As shown in Fig. 5a, pinching-induced increases in CeA 5-HT levels were retained after intra-DRN injection of vehicle (115 ± 8 % of basal value; open circles) or bicuculline (118 ± 5 % of basal value; closed circles).

Responses to stroking

As shown in Fig. 5b, stroking-induced decreases in CeA 5-HT levels were retained after intra-DRN injection of vehicle (86 ± 5 % of basal value; open circles), but abolished after intra-DRN injection of bicuculline (closed circles).

Discussion

The present study showed for the first time that noxious mechanical stimulation (i.e., pinching) increased 5-HT release in the CeA in a manner that was dependent upon CRF_2 receptor activation in the DRN and that innocuous mechanical stimulation (i.e., stroking) decreased 5-HT

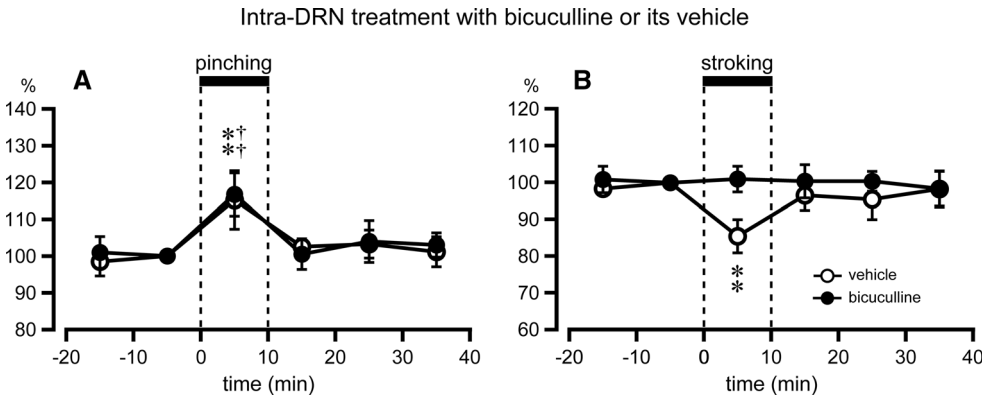

Fig. 5 Effects of intra-DRN bicuculline administration on 5-HT release responses in the CeA to pinching (**a**) and stroking (**b**) of the back. †† $p < 0.01$ (bicuculline), $^{**}p < 0.01$ (vehicle) vs. prestimulus control values. $n = 6$. *Graphs* are set up as in Fig. 1

release in the CeA in a manner that was dependent upon CRF_1 receptor activation in the DRN. These results indicate that the opposite 5-HT release responses to pinching and stroking in the CeA can be attributed to the involvement of different CRF receptors within the DRN.

Following icv injection of the non-selective CRF receptor antagonist α-helical CRF(9–41), basal release of 5-HT in the CeA remained suppressed for more than an hour. On the other hand, local administration of α-helical CRF(9–41) into the DRN did not alter basal release of 5-HT in the CeA, suggesting that basal 5-HT release in the CeA is regulated by CRF receptors outside of the DRN. Determining the site of the CRF receptors responsible for this tonic regulation will require further exploration of brain regions that express CRF receptors, including the paraventricular nucleus of the hypothalamus, bed nucleus of the stria terminalis, and CeA [25].

Similar to our results with α-helical CRF(9–41), basal release of 5-HT in the CeA was not affected by selective CRF_1 or CRF_2 receptor antagonism in the DRN, suggesting that neither CRF_1 nor CRF_2 receptors in the DRN were tonically activated in the present experimental conditions. These findings fit with Scholl et al.'s prior study showing no changes in 5-HT release in the CeA in response to intra-DRN injection with the selective CRF_2 receptor antagonist ASV-30 in conscious rats [26].

Our present findings of blocked CeA 5-HT responses to both pinching and stroking after icv α-helical CRF(9–41) injection indicate that both responses are mediated via CRF receptors in the brain. These results are consistent with Mo and colleagues' prior demonstration that increases in 5-HT release in response to immobilization stress disappeared after icv infusion of a non-selective CRF receptor antagonist in conscious rats [15]. However, in those prior experiments with conscious animals, it was unclear whether the neurophysiological responses were triggered by physical stimulation only or if they involved psychological factors. Regarding this point, the present study performed in anesthetized animals rules out psychological factors. That is, here, we showed that 5-HT release in the CeA is altered via CRF receptors in the brain in response to physical stimulation.

Furthermore, our findings showing that local injection of α-helical CRF(9–41) into the DRN also blocked the effects of pinching and of stroking on 5-HT release in the CeA demonstrated that these serotonergic responses were mediated directly via CRF receptors in the DRN. Although it has been reported previously that CRF injection into the DRN increases 5-HT levels in the CeA [10, 15], the contribution of CRF receptor activation in the DRN to the actual physiological responses had not yet been shown. The present study is the first demonstration that CRF receptor activation within the DRN mediates 5-HT release responses to somatosensory stimulation. Because basal release of 5-HT in the CeA was not affected by α-helical CRF(9–41) injection, we can deduce that CRF release in the DRN is elicited in response to somatosensory stimulation.

Kirby et al. found that intra-DRN injection of a low dose of CRF (3 ng) decreased, whereas injection of a ten-fold larger dose of CRF (30 ng) increased serotonergic neuronal activity in the DRN [27]. Similarly, Lukkes et al. [28] found that intra-DRN administration of a 100-ng CRF dose decreased extracellular 5-HT release in the nucleus accumbens, whereas a higher 500-ng CRF dose increased the release. Meanwhile, Forster et al. observed increases in 5-HT levels in the CeA following intra-DRN injection of a 500-ng dose of CRF [11]. Given that the dose in Forster et al.'s study [11] was the same as the higher dose in Lukkes et al.'s study [28], it seems reasonable to consider it another high dose in a wider biphasic dose-response phenomenon. We did not measure CRF levels in the DRN in the present study but, based on the pattern of findings summarized above, postulate that pinching may cause larger increases in CRF release within the DRN than stroking does.

DRN is one of the few regions in the brain that contains both CRF_1 and CRF_2 receptors [19]. Because CRF_1 receptors have a high binding affinity [29], injection of

relatively small amounts of CRF into the DRN would be expected to bind CRF_1 receptors selectively, or at least preferentially. And it has been shown that stimulation of CRF_1 receptors in the DRN causes a decrease in 5-HT neuronal activity in the DRN [27, 30]. On the other hand, injection of amounts of CRF into the DRN that are sufficient to activate CRF_2 receptors, which have a lower affinity to CRF than do CRF_1 receptors, increases 5-HT neuronal activity [27, 31]. The present findings of increased 5-HT release responses in the CeA after pinching requiring CRF_2 receptor availability in the DRN, and of decreased 5-HT release responses in the CeA after stroking requiring CRF_1 receptor availability in the DRN, suggest that CRF release in the DRN is increased more by pinching than by stroking. That is, the findings suggest that, in response to pinching, there is a relatively large amount of CRF released into the DRN, sufficient to stimulate CRF_2 receptors and thereby increasing 5-HT release in the CeA. Conversely, the evidence suggests that stroking induces a smaller (relative to pinching) release of CRF in the DRN, which can stimulate high-affinity CRF_1 receptors, thereby decreasing 5-HT release in the CeA.

The effects of stroking on 5-HT release in the CeA could be blocked by pretreatment with the CRF_1 receptor antagonist antalarmin or the $GABA_A$ receptor antagonist bicuculline. CRF_1 receptors are located on terminals of non-serotonergic fibers in the DRN and the non-serotonergic fibers in the DRN have been described as GABAergic [32, 33]. Furthermore, $GABA_A$ receptors are expressed by serotonergic neurons in the DRN [33, 34], the activities of which are inhibited by $GABA_A$ receptor agonism [24, 33]. Taken together, this convergence of evidence has led us to suppose that stroking may stimulate CRF_1 receptors on GABAergic terminals, stimulating the release of GABA, which inhibits the serotonergic neurons that project to the CeA via $GABA_A$ receptors, ultimately reducing 5-HT release in the CeA (see Fig. 6b).

On the other hand, CRF_2 receptors are expressed mostly in the cell bodies of serotonergic neurons within the caudal DRN, an area rich with serotonergic fibers to the amygdala [21, 35]. Because pinching-induced increases in 5-HT release in the CeA disappeared after treatment with ASV-30, a CRF_2 receptor antagonist, we suppose that pinching-induced release of CRF may activate CRF_2 receptors on serotonergic neurons, which project to the CeA, leading to increases in 5-HT release within the CeA (see Fig. 6a).

We stimulated the back area in both the pinching and stroking experiments. There is a possibility that the involvement of CRF receptors differs depending on the stimulus sites although our previous study [9] demonstrated that the responses of 5-HT release to pinching and stroking were similar across the different skin areas (forelimb and hindlimb).

Finally, we found that intra-DRN administration of the $GABA_A$ receptor antagonist bicuculline increased basal 5-HT release in the CeA. These results indicate that serotonergic projection neurons innervating the CeA are tonically inhibited by GABAergic neurons via $GABA_A$ receptors. On the other hand, blockade of CRF_1 receptors did not alter basal 5-HT release in the CeA, demonstrating that CRF-containing neurons, which stimulate GABA release via CRF_1 receptor activation, are not spontaneously active.

Limitations

One limitation of the present study is that we did not assess the influence of CRF receptor manipulations on 5-HT release in the CeA and emotional behavior (such as freezing, which correlates with 5-HT release changes in the CeA) in response to somatic stimulation in conscious animals. Another limitation is that we have not determined how CRF release in the DRN is affected by pinching and stroking.

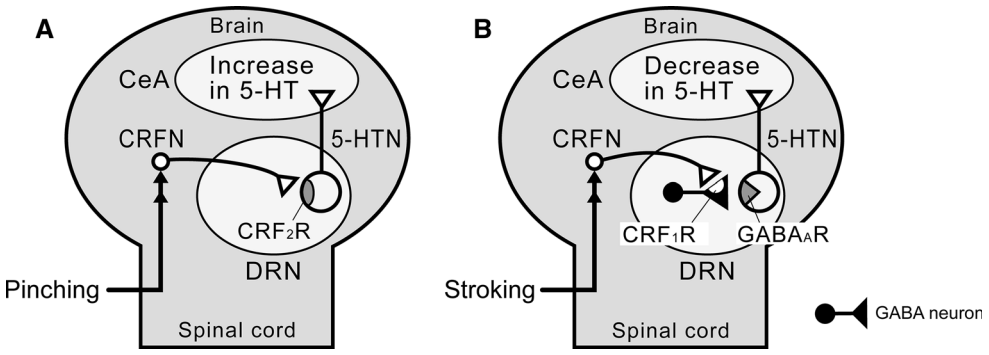

Fig. 6 Diagram summarizing the present results. **a** Pinching of the skin increases 5-HT release in the CeA via CRF_2 receptors in the DRN. **b** Stroking of the skin decreases 5-HT release in the CeA via CRF_1 receptors and $GABA_A$ receptors in the DRN. *CeA* the central nucleus of the amygdala, *DRN* the dorsal raphe nucleus, *CRFN* CRF-containing neuron, *5-HTN* serotonergic neuron, *CRF_1R* CRF_1 receptor, *CRF_2R* CRF_2 receptor, *$GABA_AR$* $GABA_A$ receptor

Conclusion

The present study demonstrated that opposite 5-HT release changes in the CeA in response to pinching and stroking can be attributed to independent stimulation of CRF_2 and CRF_1 receptors, respectively, in the DRN. Given that CRF_2 receptors in the DRN and 5-HT in the CeA have both been implicated in the occurrence of anxiety-related behavior [10, 11], increases in 5-HT release in the CeA stimulated by CRF_2 receptor activation in the DRN may be an important mediator of anxiety-related behavior. By contrast, stimulation of CRF_1 receptors in the DRN decreases freezing behavior in response to uncontrollable stress [23], suggesting that stimulation of CRF_1 receptors in the DRN may be anxiolytic. The observation that increased release of 5-HT in the CeA in response to pinching can be masked by simultaneous pinching and stroking [9] further suggests that stress responses elicited via CRF_2 receptors in the DRN may be suppressible by stimulation of CRF_1 receptors in the DRN.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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