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Postsynaptic 5-HT_{1A} receptors mediate 5-hydroxytryptamine release in the amygdala through a feedback to the caudal linear raphe

Fokko J. Bosker, André Klompmakers, Herman G.M. Westenberg *

Department of Psychiatry, Rudolf Magnus Institute for Neurosciences, P.O. Box 85500, 3508 GA Utrecht, The Netherlands

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Abstract

Using brain microdialysis, it was demonstrated that the release of 5-hydroxytryptamine (5-HT) in the central nucleus of the amygdala is under inhibitory control of somatodendritic and postsynaptic 5-HT_{1A} receptors. Systemic administration of flesinoxan, a selective 5-HT_{1A} receptor agonist, significantly reduced the extracellular levels of 5-HT in the central nucleus of the amygdala. This effect could be completely antagonized by the 5-HT_{1A} receptor antagonist *N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)-*N*-(2-pyridyl)cyclohexane carboxamine trihydrochloride (WAY 100635). Local administration of these compounds by reversed microdialysis into the raphe nuclei revealed that extracellular 5-HT levels in the central nucleus of the amygdala can be regulated through 5-HT_{1A} receptors in the caudal linear raphe nucleus, but not in the dorsal and median raphe nuclei. Interestingly, administration of flesinoxan into the central nucleus of the amygdala also decreased dialysate 5-HT levels both locally and in the caudal linear raphe nucleus. The former effect could be blocked by pretreatment with WAY 100635 when applied into the central nucleus of the amygdala, but not when applied into the caudal linear raphe nucleus. These data provide circumstantial evidence for the existence of a 5-HT_{1A} receptor mediated feedback loop from the central nucleus of the amygdala to the caudal linear raphe nucleus. © 1997 Elsevier Science B.V.

Keywords: Flesinoxan; WAY 100635; 5-HT (5-hydroxytryptamine, serotonin); 5-HT_{1A} receptor; Feedback regulation; Microdialysis; (Rat)

1. Introduction

Central serotonergic pathways are associated with an array of neuropsychiatric conditions such as depression (Schildkraut, 1965), schizophrenia (Iqbal and Van Praag, 1995), anxiety (Kahn et al., 1988) and panic disorder (Westenberg and den Boer, 1994). This broad range of functions and dysfunctions parallels the striking diversity and complexity, particularly in terms of receptor heterogeneity, of the serotonergic system in the brain.

The 5-hydroxytryptamine (5-HT) innervation of the forebrain areas is derived principally from the dorsal raphe nucleus and the median raphe nucleus, but the caudal linear raphe nucleus, the most rostral 5-HT cell groups in the brain stem, and the B9 cell group in the nucleus pontis centralis just dorsal to the medial lemnicus, contribute also to the 5-HT inputs in forebrain regions (Jacobs and Azmitia, 1992). Neurons arising from the caudal linear raphe nucleus bare some similarity to those described for the dorsal raphe nucleus, whereas the efferents from the median raphe nucleus closely resemble those from the B9 group (Imai et al., 1986). The ascending 5-HT projections arising from the rostral group (dorsal raphe nucleus and caudal linear raphe nucleus) and the caudal group (median raphe nucleus and B9) appear to innervate forebrain structures differentially. The striatum and amygdala predominantly receive serotonergic input from the rostral group, whereas the dorsal hippocampus is mainly innervated by the caudal group, viz., the median raphe nucleus (Azmitia and Segal, 1978). In contrast, the amygdala is almost exclusively innervated by the dorsal raphe nucleus. Most other forebrain regions receive input from both groups (Jacobs and Azmitia, 1992). There is mounting evidence that 5-HT neuronal activity is tightly controlled through feedback mechanisms. At the terminal level, stimulation of 5-HT_{1B} and its homologue 5-HT_{1D} autoreceptors results in a reduction of the amount of 5-HT released from nerve endings (Limberger et al., 1991), while at the somatodendritic level 5-HT_{1A} receptors, which are found with high density in raphe nuclei on 5-HT neurons (Sotelo et al., 1990), modulate the spontaneous firing rate of 5-HT neu-

* Corresponding author. Tel.: (31-30) 250-7472; Fax: (31-30) 250-5443; e-mail: h.g.m.westenberg@psych.azu.nl

rons (Sprouse and Aghajanian, 1987) and consequently the amount of 5-HT released from nerve endings (Bosker et al., 1994). In addition, 5-HT_{1A} autoreceptors also mediate the release of 5-HT in the somatodendritic region, as has been measured by in vivo microdialysis (Bosker et al., 1996). More recently, 5-HT_{1D} receptors have also been identified in the dorsal raphe nucleus, where they inhibit extracellular 5-HT in this cell body region (Starkey and Skingle, 1994; Davidson and Stamford, 1995). A direct coupling of this receptor subtype to 5-HT cell firing has yet to be established, but the decrease in 5-HT levels resulting from stimulation of this receptor, may indirectly affect the firing rate through the 5-HT_{1A} receptors.

Postsynaptic 5-HT_{1A} receptors are found with high density in the hippocampus, but high concentrations are also observed in the septum, hypothalamus, some cortical layers and the amygdala, particularly in the central nucleus of the amygdala (Pazos and Palacios, 1985; Ohuoha et al., 1993).

There is substantial evidence to suggest that 5-HT_{1A} receptors in the septo-hippocampal system and the amygdaloid complex are important targets for anxiolytic drugs (Traber and Glaser, 1987; Westenberg and den Boer, 1994; De Vry, 1995). However, there is no agreement on the pre- or postsynaptic location of the 5-HT_{1A} receptors mediating these anxiolytic effects. Most evidence points to the involvement of the somatodendritic 5-HT_{1A} receptors (Hogg et al., 1994; De Vry, 1995), but some studies also suggest postsynaptic 5-HT_{1A} receptors to play a role (Fernández-Guasti et al., 1992; Schreiber and De Vry, 1993a,b; File and Gonzales, 1996). An important confounding factor is the existence of reciprocal circuits. Indeed, many innervated areas project back to the raphe nuclei and these nuclei are probably interconnected (Jacobs and Azmitia, 1992). Blier and De Montigny (1987) have hypothesized that stimulation of the (hippocampal) postsynaptic 5-HT_{1A} receptors may suppress the dorsal raphe nucleus 5-HT neuronal firing, but the existence of such a feedback regulation is still controversial. Indirect evidence for the existence of 5-HT_{1A}-mediated feedback projections to the raphe nuclei has been presented for the frontal cortex and the striatum (Ceci et al., 1994; Romero et al., 1994), but others have questioned the role of postsynaptic 5-HT_{1A} receptors in the regulation of 5-HT neuronal activity (Jolas et al., 1995) and have pointed out that diffusion of the 5-HT_{1A} receptor agonists from, e.g., the striatum to the dorsal raphe nucleus may explain the observed effects.

The present study investigated the possible relevance of postsynaptic 5-HT_{1A} receptors in the control of 5-HT neuronal activity in the central nucleus of the amygdala. First, the effects of flesinoxan and WAY 100635, a selective 5-HT_{1A} receptor agonist and antagonist, respectively, on extracellular 5-HT in the central nucleus of the amygdala were examined by microdialysis following systemic administration of the compounds. In a subsequent series of experiments the contribution of the somatodendritic 5-HT_{1A} autoreceptors on 5-HT release in the central nucleus of the amygdala was studied using a dual-probe technique (Bosker et al., 1994, 1996). Flesinoxan was applied locally into the raphe regions by reversed dialysis and the effects on extracellular 5-HT were assessed in both the central nucleus of the amygdala and three raphe nuclei, viz., the dorsal raphe nucleus, median raphe nucleus and caudal linear raphe nucleus. Based on the results of these studies, experiments on feedback regulation were performed with probes in the caudal linear raphe nucleus and central nucleus of the amygdala. For this purpose we studied the effects of 5-HT_{1A} receptor stimulation in the central nucleus of the amygdala on 5-HT neuronal activity by measuring extracellular 5-HT levels in the caudal linear raphe nucleus and central nucleus of the amygdala. WAY 100635 was applied locally into the central nucleus of the amygdala or caudal linear raphe nucleus to verify the involvement of pre- and postsynaptic 5-HT_{1A} receptors.

2. Materials and methods

2.1. Animals

Male Wistar rats (GDL, Utrecht) weighing 250–300 g were housed three per cage under standard conditions (22–24°C, 12/12 h light/dark cycle, food and water ad libitum) for at least three days prior to the microdialysis experiments. After implantation the rats were housed separately. All animal experiments were according to the Governmental guidelines for care and use of laboratory animals and were approved by the Committee for Animal Research of the Medical Faculty of the Utrecht University.

2.2. Surgery

Rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.). A concentric microdialysis probe as described previously (Bosker et al., 1994) was stereotaxically implanted in the brain and aimed at central nucleus of the amygdala using the following coordinates: incisorbar at −3.5 mm, A: −2.6 mm, L: 4.2 mm, V: 9.1 mm, from bregma and skull surface (Paxinos and Watson, 1982). In the dual probe experiments an additional probe was implanted in the raphe area. Coordinates for the three raphe nuclei were as follows. Dorsal and median raphe nucleus: (lateral angle of 10°, incisorbar at −3.5 mm) A: +1.2 mm, L: 1.4 mm, V: 7.0 mm (dorsal raphe nucleus) or V: 9.0 mm (median raphe nucleus) taken from interaural line and skull surface (Paxinos and Watson, 1982). Caudal linear raphe nucleus: (lateral angle of 10°, incisorbar at −7.0 mm) A: +1.2 mm, L: 1.4 mm, V: 9.0 mm. The latter incisorbar setting corresponds to an anterior–posterior coordinate of +2.2 mm for the probe tip under standard conditions (incisorbar: −3.5 mm). AP coordinates were corrected for individual distances between in-

teraural line and bregma. Exposed tip lengths of the probes (ID 220 μm, OD: 310 μm) were 1 mm (central nucleus of the amygdala and caudal linear raphe nucleus), 1.5 mm (dorsal raphe nucleus) and 2 mm (median raphe nucleus). The probes were secured in place with dental cement.

2.3. Microdialysis experiments

To minimize the number of animals, microdialysis experiments were performed on both the first and second day after surgery. To avoid order (or carry over) effects, if any, treatments were randomly allocated for each experiment to the first or second day test using a balanced design. Thus, all animal received two different treatments in a randomized fashion. The order term was entered as a 'between' subject factor into the statistical analyses. Because the experiments with local infusion through the probes in the caudal linear raphe nucleus and central nucleus of the amygdala were designed as a joined experiment, the control group for data based on this experiment (Figs. 2–4) is identical.

The probes were perfused with Ringer (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$, pH 6–7), using a Harvard micro infusion pump (Harvard, USA) at a constant flow rate of 1.5 μl/min. Microdialysis in the central nucleus of the amygdala was performed in the presence of a 5-HT reuptake inhibitor (fluvoxamine 10 μM) in the perfusion fluid to allow measurement of decreases in 5-HT reliably. Microdialysis in the dorsal raphe nucleus, median raphe nucleus or caudal linear raphe nucleus was performed without reuptake inhibitor. Two hours after the beginning of an experiment, 15 min samples were collected into vials containing 7.5 μl 0.1 M acetic acid. Samples were mixed and immediately frozen at −80°C. Flesinoxan and WAY 100635 were administered systemically (single probe experiments) and locally (dual probe experiments). The vehicle for systemic administration of flesinoxan and WAY 100635 was saline. In the single probe experiments flesinoxan and WAY 100635 were injected subcutaneously (1 ml/kg) into the neck region. In the dual probe experiments, flesinoxan and WAY 100635 were dissolved in the perfusate and infused into the brain region of interest for 30 and 60 min, respectively. In the figures the point of injection or the period of local infusion is corrected for the lagtime of the microdialysis system. All experiments were performed in conscious and freely moving animals.

2.4. Analytical procedure

Analysis of 5-HT was performed by high-performance liquid chromatography (HPLC) with electrochemical detection. Briefly, 20 μl samples were injected into a high performance liquid chromatograph (LKB, Woerden, The Netherlands) equipped with a 10 cm reversed-phase column (Hypersil RP-18, 3 μm, 2.0 mm, Shandon) and an electrochemical detector (ANTEC Leyden, Leiden, The Netherlands) at a potential setting of 600 mV vs. Ag/AgCl reference electrode. A column oven (LKB, Woerden) set at 40°C was used for both column and electrochemical detector. The mobile phase consisted of 5 g/l $(NH_4)_2SO_4$, 50 mg/l heptane sulphonic acid sodium salt, 500 mg/l EDTA, 4% methanol, 30 μl/l triethylamine, adjusted to pH 4.65 with acetic acid. The flow rate was 0.4 ml/min. The detection limit for 5-HT was 0.5 fmol/20 μl sample (signal/noise ratio 2).

2.5. Histology

Following the termination of each experiment, animals were anaesthetized with chloral hydrate, the probes were flushed successively with 5% solutions of $FeCl_3$ and $K_4Fe(CN)_6$ in water for 15 min (Kendrick, 1990). The iron deposited in the tissue surrounding the probe tip is stained green–blue. The diffusion area was typically not larger than 1 mm from the membrane. The animals were killed by decapitation and the brains removed, fixed in a 5% formaldehyde solution, frozen and cut in 150 μm slices. The position of the probe was verified microscopically by the track of the probe through the brain and the green–blue staining of the tissue surrounding the probe tip. Data from few animals with improper probe placement were excluded. In this respect, a distance > 150 μm from the nucleus aimed at was used as exclusion criterion. Due to the occasional obstruction of a microdialysis probe or analytical failure the overall success rates were lower and amounted to approximately 70%.

2.6. Statistics

The data are presented as percentage of basal values calculated as individual means of the first four consecutive samples. Statistical analysis of the microdialysis data was performed on raw data using analysis of variance (ANOVA) for repeated measures with time as 'within' and treatment and order of treatment (first or second day) as 'between' subject factors (SPSS, Chicago, IL, USA). When appropriate, the degrees of freedom were adjusted using the Huynh-Feldt epsilon factor to correct for lack of compound symmetry. When appropriate, data were broken down on treatment and tested by ANOVA with repeated measures for pairwise comparison of the time profiles. The area under the concentration–time curve (AUC) for the post-treatment period was calculated and expressed as changes from baseline. AUC values were analyzed using Kruskal–Wallis analysis of variance followed by Mann–Whitney *U* tests where appropriate. The Student's *t*-test was used to compare the baseline 5-HT values on the first and second test day. Probabilities of 0.05 were considered to be statistically significant.

2.7. Chemicals

Reagents were from Merck (Darmstadt, Germany) except for heptane sulphonic acid sodium salt (Kodak,

Rochester, NY, USA) and methanol (Riedel-de Haën, Seelze, Germany). 5-HT was from Sigma (St. Louis, MO, USA). Fluvoxamine maleate and flesinoxan hydrochloride were generously donated by Solvay-Duphar (Weesp, The Netherlands). WAY 100635 was obtained from Solvay Duphar. Artificial kidney (AN 69 filtral 16) was a gift from Hospal (Uden, The Netherlands).

3. Results

3.1. Baseline 5-HT levels in the central nucleus of the amygdala, the dorsal raphe, median raphe and caudal linear raphe nucleus

The average basal 5-HT levels in the dialysates of the central nucleus of the amygdala on the first and second test day were 20.3 ± 1.0 fmol/15 min ($n = 75$) and 20.5 ± 1.3 fmol/15 min ($n = 69$), respectively. The values for the dorsal raphe, median raphe and caudal linear raphe nuclei were 17.2 ± 4.2 fmol/15 min ($n = 14$), 15.1 ± 1.3 fmol/15 min ($n = 12$) and 6.3 ± 0.6 fmol/15 min ($n = 34$), respectively, for the first test day and 14.9 ± 3.7 fmol/15 min ($n = 9$), 13.4 ± 1.1 fmol/15 min ($n = 9$) and 7.3 ± 0.7 fmol/15 min ($n = 29$), respectively, for the second test day. There was no statistically significant difference between the baseline values on both days in either area and the values on both days were highly correlated, indicating that the previous experiment had not affected the basal 5-HT release. The results in the central nucleus of the amygdala were obtained in the presence of 10 μM fluvoxamine, a 5-HT reuptake inhibitor. Dialysis in the raphe nuclei was performed without a 5-HT uptake inhibitor in the perfusion fluid.

3.2. Effect of systemic administration of flesinoxan on 5-HT levels in the central nucleus of the amygdala: Antagonism by WAY 100635

Systemic administration of 0.3 mg/kg of flesinoxan decreased dialysate 5-HT levels in the central nucleus of the amygdala to $51.6 \pm 3.0\%$ of the baseline value (Fig. 1). The 5-HT_{1A} receptor antagonist WAY 100635 (0.05 mg/kg) completely blocked this effect. The dosages of the agonist and antagonist were based on results reported previously for the hippocampus (Bosker et al., 1996). Multivariate analysis of variance revealed a highly significant time by treatment interaction ($F(48) = 3.6$, $P < 0.001$). No order effect was observed. Contrast analyses showed that flesinoxan was significantly different from saline, WAY 100635 and the combination of flesinoxan and WAY 100635. Taking the area under the concentration time curve (AUC) as outcome variable, the flesinoxan induced decrease in extracellular 5-HT in the central nucleus of the amygdala was completely antagonized by WAY 100635 ($P < 0.001$). WAY 100635 (0.05 mg/kg s.c.) alone did not affect dialysate 5-HT in the central nucleus of the amygdala, indicating that it had no intrinsic activity at the dosage used in this experiment.

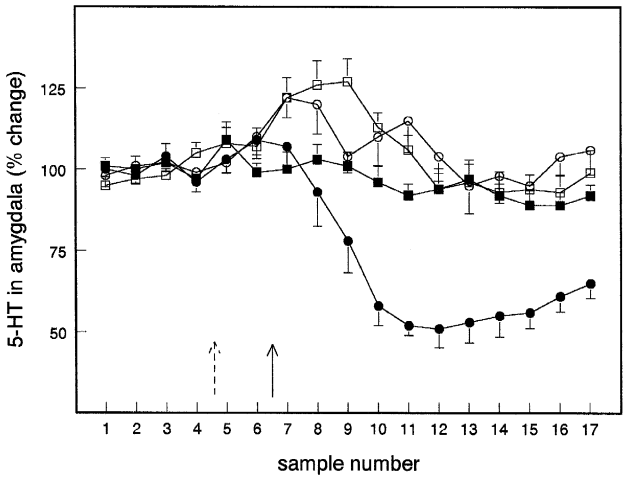

Fig. 1. Effect of systemic administration of flesinoxan and WAY 100635 on dialysate 5-HT levels in the central nucleus of the amygdala. Inhibition by WAY 100635 (0.05 mg/kg s.c.) on the flesinoxan (0.3 mg/kg s.c.) induced decrease of extracellular 5-HT in the central nucleus of the amygdala. Measurements in the central nucleus of the amygdala were performed in the presence of 10 μM fluvoxamine. WAY 100635 was given at the first arrow and flesinoxan 30 min later at the second arrow. The points of administration are corrected for the lagtime in the microdialysis system. Key: ■, saline ($n = 7$); ●, flesinoxan ($n = 8$); □, WAY 100635 ($n = 8$); ○, flesinoxan and WAY 100635 ($n = 9$).

Table 1
Effect of local perfusion of 1 μM flesinoxan for 30 min through the dialysis probes into dorsal raphe nucleus, median raphe nucleus and caudal linear raphe nucleus on 5-HT dialysates from the central nucleus of the amygdala and raphe region into which the drug was administered

Drug applied to	Effect in					
	dorsal raphe nucleus	median raphe nucleus	caudal linear raphe nucleus	*P* value	central nucleus of the amygdala	*P* value
Dorsal raphe nucleus	69.0 ± 7.0	—	—	< 0.05	104.9 ± 2.5	n.s.
Median raphe nucleus	—	65.9 ± 3.9	—	< 0.05	95.5 ± 4.4	n.s.
Caudal linear raphe nucleus	—	—	82.0 ± 4.9	< 0.05	81.8 ± 3.6	< 0.01

AUCs were evaluated statistically by one way ANOVA. Values (mean ± S.E.M.) are expressed as percentage change in area under the 5-HT concentration time curve (AUC) from baseline. Dialysis in the central nucleus of the amygdala was performed in the presence of 10 μM fluvoxamine.
P value: statistically different from the corresponding Ringer data (Kruskal–Wallis analysis of variance followed by Mann–Whitney).
n.s., not significant; —, not determined.

3.3. Local infusion of flesinoxan into the dorsal raphe nucleus and median raphe nucleus

Perfusion of 1 μM of flesinoxan for 30 min through the microdialysis probe into the dorsal raphe nucleus region resulted in a decrease in 5-HT levels to $57.5 \pm 6.1\%$ ($F(13) = 2.1$, $P < 0.05$). No order effects were observed. Simultaneous measurements in the central nucleus of the amygdala failed to detect any significant changes in extracellular 5-HT in this region. Similar results were obtained following local administration of flesinoxan (1 μM for 30 min) into the median raphe nucleus region. 5-HT levels in the median raphe nucleus decreased significantly to $54.4 \pm 4.4\%$ of baseline value ($F(13) = 7.7$, $P < 0.001$), but no effect was observed on dialysate 5-HT from the central nucleus of the amygdala. There was no order effect. These data were confirmed by the analyses of the corresponding AUC values (Table 1).

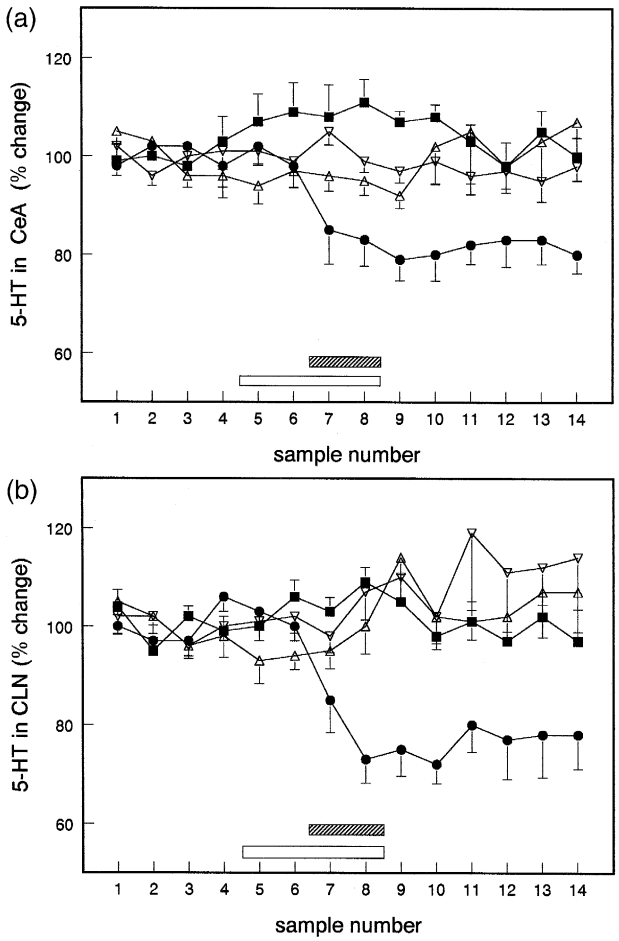

Fig. 2. Effect of administration of flesinoxan and WAY 100635 into caudal linear raphe nucleus on extracellular 5-HT in the central nucleus of the amygdala and caudal linear raphe nucleus. Perfusion with 1 μM of flesinoxan for 30 min through the microdialysis probe into the caudal linear raphe nucleus reduced the extracellular levels of 5-HT in the caudal linear raphe nucleus (upper panel) and central nucleus of the amygdala (lower panel). Coperfusion with WAY 100635 (1 μM for 60 min) antagonized this effect in both regions. Measurements in the central nucleus of the amygdala were performed in the presence of 10 μM fluvoxamine. Horizontal bars indicate the perfusion period of WAY 100635 (open bar) and flesinoxan (dashed bar). Key upper panel: ■, Ringer ($n = 7$); ●, flesinoxan ($n = 8$); ▽, WAY 100635 ($n = 10$); △, flesinoxan and WAY 100635 ($n = 6$). Key lower panel: ■, Ringer ($n = 8$); ●, flesinoxan ($n = 7$); ▽, WAY 100635 ($n = 10$); △, flesinoxan and WAY 100635 ($n = 6$).

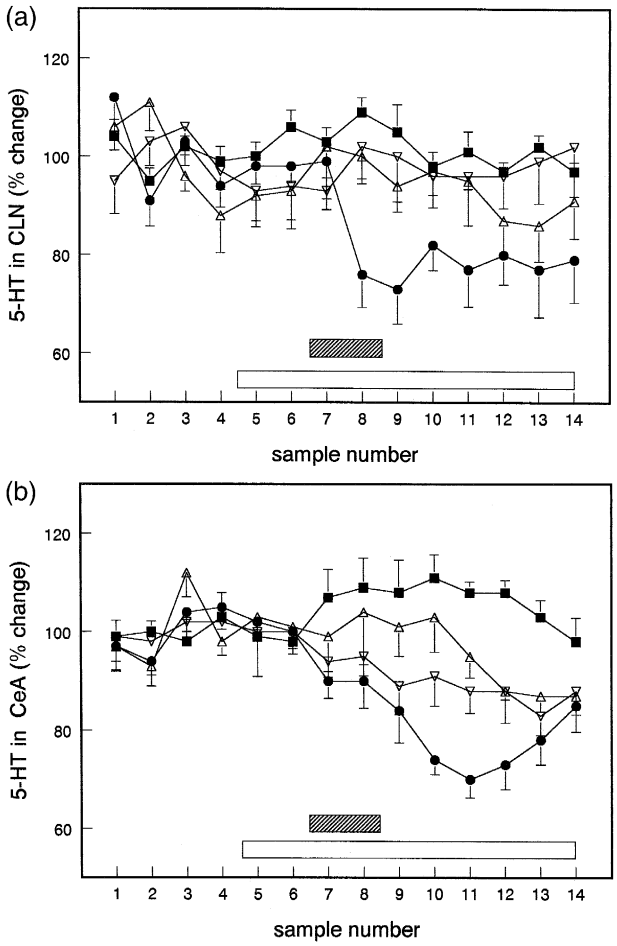

Fig. 3. Administration of flesinoxan and WAY 100635 into the central nucleus of the amygdala. Local administration of flesinoxan (1 μM for 30 min) through the dialysis probe into the central nucleus of the amygdala significantly decreased the extracellular 5-HT concentrations in the caudal linear raphe nucleus (upper panel) and central nucleus of the amygdala (lower panel). Co-administration of WAY 100635 (1 μM through the probe into the central nucleus of the amygdala) completely blocked the effects of flesinoxan in both regions. WAY 100635 alone had no effect on extracellular levels of 5-HT in the caudal linear raphe nucleus, but decreased the amount of 5-HT released into the central nucleus of the amygdala. Measurements in the central nucleus of the amygdala were performed in the presence of 10 μM fluvoxamine. Horizontal bars represent the perfusion period of WAY 100635 (open bar) and of flesinoxan (dashed bar). The Ringer groups depicted in these figures are identical to those depicted in Fig. 2. Key upper panel: ■, Ringer ($n = 7$); ●, flesinoxan ($n = 6$); ▽, WAY 100635 ($n = 5$); △, flesinoxan and WAY 100635 ($n = 6$). Key lower panel: ■, Ringer ($n = 8$); ●, flesinoxan ($n = 10$); ▽, WAY 100635 ($n = 5$); △, flesinoxan and WAY 100635 ($n = 6$).

3.4. Local infusion of flesinoxan and WAY 100635 into the caudal linear raphe nucleus

Administration of 1 μM of flesinoxan for 30 min through the microdialysis probe into the caudal linear raphe nucleus decreased dialysate 5-HT levels to 71.7 ± 3.9% and 78.7 ± 4.2% of the baseline values in the caudal linear raphe nucleus and central nucleus of the amygdala, respectively (Fig. 2). Coadministration of 1 μM of WAY 100635 into the caudal linear raphe nucleus for 60 min blocked the effects of flesinoxan on extracellular 5-HT levels in both brain regions. Administration of WAY 100635 was started 30 min before the perfusion of flesinoxan. Multivariate analysis of variance revealed a statistically significant time by treatment effect for the caudal linear raphe nucleus ($F(39) = 5.5,\ P < 0.001$) and central nucleus of the amygdala ($F(39) = 3.4,\ P < 0.001$). No order effect was observed. Contrast analyses revealed that the 5-HT concentration–time profile following flesinoxan administration was significantly different from those after Ringer, WAY 100635 alone, or the combination of flesinoxan and WAY 100635, in both regions. Administration of WAY 100635 alone into the caudal linear raphe nucleus did not affect dialysate 5-HT content in either region.

3.5. Local infusion of flesinoxan and WAY 100635 into the central nucleus of the amygdala

Administration of 1 μM of flesinoxan for 30 min through the microdialysis probe into the central nucleus of the amygdala also decreased 5-HT levels in the dialysates from the central nucleus of the amygdala and caudal linear raphe nucleus to 70.2 ± 3.7% and 72.9 ± 7.0% of the baseline values, respectively (Fig. 3). It is of interest that the effects on extracellular 5-HT in the caudal linear raphe nucleus and central nucleus of the amygdala is delayed as compared to the experiment where the compounds were applied into the caudal linear raphe nucleus. Coadministration of 1 μM of WAY 100635 into the central nucleus of the amygdala antagonized the effects of flesinoxan on extracellular 5-HT levels in both brain regions. Perfusion with WAY 100635 started 30 min before the administration of flexinoxan and lasted until the end of the experiment. Multivariate analysis of variance revealed a statistically significant time by treatment effect for the central nucleus of the amygdala ($F(39) = 2.3,\ P < 0.001$) and caudal linear raphe nucleus ($F(39) = 2.7,\ P < 0.001$). There were no order effects. Contrasts among the four treatment conditions showed that the 5-HT concentration–time profiles in rats treated with flesinoxan were different from those treated with Ringer or the combination of flesinoxan and WAY 100635, in both regions. Administration of WAY 100635 alone did not affect 5-HT dialysate content from the caudal linear raphe nucleus, but decreased extracellular 5-HT from the central nucleus of the amygdala significantly. Analyses of the AUC values confirmed these findings (Table 2). The decrease in extracellular 5-HT in the caudal linear raphe nucleus following application of flesinoxan into the central nucleus of the amygdala suggests a neuronal feedback loop from the central nucleus of the amygdala to the caudal linear raphe nucleus. The delayed and brief effects on 5-HT levels in the central nucleus of the amygdala are also in keeping with the idea that these effects are obtained indirectly through an effect on 5-HT cell firing.

3.6. Local infusion of flesinoxan into the central nucleus of the amygdala with simultaneous administration of WAY 100635 into the caudal linear raphe nucleus

To exclude the possibility that diffusion of flesinoxan from the central nucleus of the amygdala to the raphe region may account for the effects described above, WAY 100635 was applied into the caudal linear raphe nucleus (1 μM for 60 min through the probe) while flesinoxan (1 μM for 30 min) was administered into the central nucleus of the amygdala. Perfusion with WAY 100635 started 30 min before the administration of flesinoxan (see Fig. 4). Measurement of 5-HT dialysates from the central nucleus of the amygdala revealed that flesinoxan administration into the central nucleus of the amygdala could not be antagonized by a blockade of the somatodendritic 5-HT_{1A} autoreceptors in the caudal linear raphe nucleus by WAY 100635 at a concentration capable to block the same dose of flesinoxan applied directly into the caudal linear raphe

Table 2
Effect of local perfusion of 1 μM of flesinoxan, 1 μM WAY 100635 or the combination of both through the dialysis probe into the central nucleus of the amygdala

Drug	Central nucleus of the amygdala	*P* value	Caudal linear raphe nucleus	*P* value
Flesinoxan	79.5 ± 3.6	< 0.01	79.2 ± 6.1	< 0.05
WAY 100635	89.5 ± 4.1	< 0.05	98.2 ± 4.7	n.s.
Flesinoxan + WAY 100635	95.9 ± 4.6	n.s.	92.2 ± 5.1	n.s.
Ringer	106.9 ± 3.5		102.9 ± 1.9	

Measurements in the central nucleus of the amygdala were carried out in the presence of 10 μM fluvoxamine. Values are expressed as percentage change in area under the 5-HT concentration time curve (AUC) from baseline.
P value: statistically different from Ringer (Kruskal–Wallis analysis of variance followed by Mann–Whitney).
n.s., not significant.

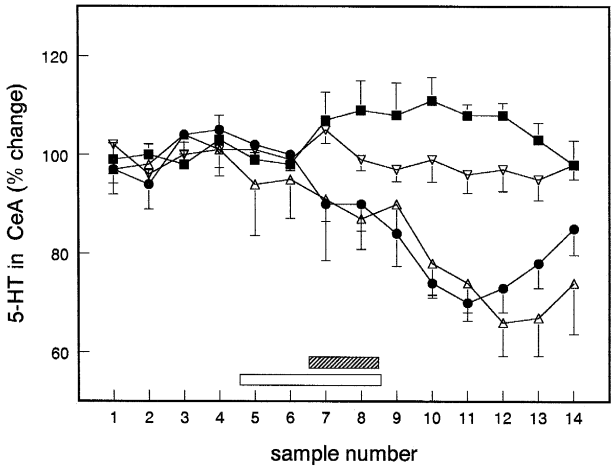

Fig. 4. Concomitant administration of flesinoxan into the central nucleus of the amygdala and WAY 100635 into the caudal linear raphe nucleus by reversed microdialysis. The decrease in extracellular 5-HT in the central nucleus of the amygdala induced by local application of flesinoxan (1 μM) could not be attenuated by simultaneous administration of WAY 100635 (1 μM) into the caudal linear raphe nucleus. Administration of WAY 100635 into the caudal linear raphe nucleus and simultaneous perfusion with Ringer in the central nucleus of the amygdala had no effect on 5-HT output in the central nucleus of the amygdala. Measurements in the central nucleus of the amygdala were performed in the presence of 10 μM fluvoxamine. Horizontal bars represent perfusion period of WAY 100635 (open bar) and flesinoxan (dashed bar). The Ringer group depicted in this figure is identical to that depicted in Fig. 2 for the central nucleus of the amygdala. Key: ■, Ringer ($n = 8$); ●, flesinoxan ($n = 10$); ▽ WAY 100635 ($n = 10$); △, flesinoxan and WAY 100635 ($n = 5$).

nucleus. Concomitant administration of flesinoxan through the probe in the central nucleus of the amygdala and WAY 100635 through the probe in the caudal linear raphe nucleus resulted in a decrease in dialysate 5-HT content from the central nucleus of the amygdala to 66.2 ± 6.8% of the baseline value. Multivariate analysis of variance revealed a statistically significant time by treatment effect ($F(26) = 2.4$, $P < 0.001$) among the four treatment conditions. Contrast analyses revealed that the 5-HT concentration–time profiles of rats treated with flesinoxan alone or the combination of flesinoxan and WAY 100635 differed from rats receiving Ringer in both regions and rats receiving WAY 100635 into the caudal linear raphe nucleus and Ringer into the central nucleus of the amygdala. The concentration–time profiles of flesinoxan alone and the combination of WAY 100635 and flesinoxan were not different. WAY 100635 alone was not different from Ringer. To be absolutely sure that no diffusion of flesinoxan had taken place, the latter experiment was repeated with WAY 100635 (1 μM) perfused into the caudal linear raphe nucleus until the end of the experiment (data not shown). Perfusion of WAY 100635 into the caudal linear raphe nucleus until the end of the experiment and concomitant perfusion of flesinoxan for 30 min into the central nucleus of the amygdala resulted in a decrease in extracellular 5-HT levels in the central nucleus of the amygdala to 60.3 ± 5.2% of the baseline value. This decrease was statistically significant from Ringer ($F(11) = 9.2$, $P < 0.001$).

4. Discussion

The present results reveal that stimulation of the 5-HT_{1A} receptors in the central nucleus of the amygdala decrease the extracellular 5-HT levels in both the central nucleus of the amygdala and the caudal linear raphe nucleus. Blockade of the 5-HT_{1A} receptors in the central nucleus of the amygdala abolished the effects in both regions, whereas blockade of the somatodendritic 5-HT_{1A} autoreceptors in the caudal linear raphe nucleus had no effect on extracellular 5-HT levels in the central nucleus of the amygdala, indicating that these effects were not mediated through somatodendritic 5-HT_{1A} autoreceptors in the caudal linear raphe nucleus and could not be accounted for by diffusion of flesinoxan from the central nucleus of the amygdala to the caudal linear raphe nucleus. These findings, therefore, provide circumstantial evidence for the existence of a 5-HT_{1A} receptor mediated feedback projection from the central nucleus of the amygdala to the caudal linear raphe nucleus. The data also show that somatodendritic 5-HT_{1A} autoreceptors in the caudal linear raphe nucleus, but not those in the dorsal raphe nucleus and median raphe nucleus, mediate 5-HT release in the central nucleus of the amygdala, suggesting that 5-HT neurons arising from the caudal linear raphe nucleus control the amount of 5-HT released in the central nucleus of the amygdala for the most part.

4.1. Serotonergic interaction between the central nucleus of the amygdala and raphe nuclei

Previous studies have shown that systemic administration of 5-HT_{1A} receptor agonists dose-dependently reduce the extracellular 5-HT levels in the hippocampus and amygdala (Bosker et al., 1994, 1996, 1997). This effect has been attributed to stimulation of the inhibitory somatodendritic 5-HT_{1A} receptors in the raphe nuclei. These autoreceptors have shown to exert tight control over 5-HT neurotransmission (Sprouse and Aghajanian, 1987). Stimulation of the somatodendritic 5-HT_{1A} autoreceptors slows cell firing, resulting in a decreased amount of 5-HT released in the terminal regions. The results of the present study show that similar mechanisms may be involved in the 5-HT afferent traffic to the central nucleus of the amygdala. Systemic administration of flesinoxan (0.3 mg/kg s.c.) significantly reduced extracellular 5-HT in the central nucleus of the amygdala and this effect could be completely antagonized by the potent and selective 5-HT_{1A} receptor antagonist WAY 100635 (0.05 mg/kg s.c.). The dosages of flesinoxan and WAY 100635 were based on

data reported previously for the amygdala (Bosker et al., 1997). To investigate whether the extent to which rostral (dorsal raphe nucleus) or caudal (median raphe nucleus) raphe nuclei are implicated in this effect, flesinoxan was also applied locally by reversed microdialysis (1 μM/30 min) into these two major superior raphe nuclei. Although it is generally assumed that 5-HT input into the amygdala originates for the most part from cells located in the dorsal raphe nucleus with some additional contribution from the median raphe nucleus (Mehler, 1980; Parent et al., 1981; Matsuzaki et al., 1993), we were unable to modulate 5-HT release in the central nucleus of the amygdala by stimulating the somatodendritic 5-HT_{1A} autoreceptors in these cell body regions. Previous studies with the same compounds and doses applied into the median raphe nucleus significantly reduced the levels of 5-HT in the dorsal hippocampus (Bosker et al., 1996). In view of the morphological and functional similarities between the caudal linear raphe nucleus and dorsal raphe nucleus — its caudal most part is contiguous with the dorsal raphe nucleus — it was decided to investigate the possible role of the caudal linear raphe nucleus in the regulation of 5-HT neuronal release in the central nucleus of the amygdala. The present data show that somatodendritic 5-HT_{1A} autoreceptors in the caudal linear raphe nucleus are capable to modulate the extracellular 5-HT levels in the central nucleus of the amygdala. This finding suggests that the 5-HT_{1A} autoreceptors in the caudal linear raphe nucleus, but not those in the dorsal raphe nucleus and median raphe nucleus, exert control over 5-HT neurotransmission to the central nucleus of the amygdala. This does not exclude the presence of such connections — we studied the functional interaction using a pharmacological probe for the somatodendritic 5-HT_{1A} autoreceptors — but the data do show that the decrease in 5-HT released in the central nucleus of the amygdala produced by systemic administration of flesinoxan cannot be accounted for by stimulation of somatodendritic 5-HT_{1A} autoreceptors in the dorsal raphe nucleus and median raphe nucleus. The caudal linear raphe nucleus is the most rostral member of the serotonergic raphe system. Most of the ascending serotonergic fibers pass through it (Azmitia and Segal, 1978) and electrical stimulation of cells in this nucleus has been shown to increase cortical 5-HT release (Aghajanian et al., 1967). Ashby et al. (1992) have shown that electrical stimulation of the caudal linear raphe nucleus reduces the spontaneous cell firing in the medial prefrontal cortex. The authors proposed this effect to be mediated via the amygdala or other sub-cortical areas.

Besides their effect on cell firing, somatodendritic 5-HT_{1A} autoreceptors also mediate 5-HT release in the cell body region in which they are found (Bosker et al., 1994, 1996). The present findings corroborate these findings. Local administration of flesinoxan decreased 5-HT levels in all three cell body regions tested. The capability of WAY 100635, a potent and selective 5-HT_{1A} receptor antagonist (Fletcher et al., 1996), to completely antagonize this effect in the caudal linear raphe nucleus, supports the role of the somatodendritic 5-HT_{1A} autoreceptors in the mechanism underlying this phenomenon. It is noteworthy, that the maximal effect on extracellular 5-HT from the central nucleus of the amygdala following flesinoxan administration through the probe in the caudal linear raphe nucleus was smaller than the maximal effect achieved following systemic administration of the compound. There are several explanations for this difference in effect size between local and systemic administration. Firstly, the concentration of flesinoxan in the perfusion fluid may have been too low. The flesinoxan concentration in the present experiments was based on a previous study with probes implanted in the median raphe and dorsal hippocampus (Bosker et al., 1996). In the latter study, 1 μM of flesinoxan for 30 min perfused through the probe into the median raphe produced a maximal effect in the hippocampus, the effect size for this region being identical to the effect obtained following systemic administration of flesinoxan (0.3 mg/kg s.c.). Secondly, the diffusion around the probe tip may have been insufficient to stimulate all 5-HT_{1A} receptors in the caudal linear raphe nucleus projecting to the central nucleus of the amygdala. Preliminary data with two probes implanted at various distances in the cortex have shown that the diffusion of flesinoxan around the probe tip is 1 mm or less. Thirdly, 5-HT_{1A} receptors in other brain regions, e.g., the central nucleus of the amygdala itself, may have contributed to the effect of flesinoxan in the central nucleus of the amygdala following systemic administration. The latter explanation would suggest the existence of a neuronal feedback loop to the raphe region.

4.2. Neuronal feedback loop between central nucleus of the amygdala and the caudal linear raphe nucleus

The existence of a neuronal feedback loop between projection areas and raphe nuclei has already been hypothesized by Blier and De Montigny (1987), but studies addressing this issue have yielded controversial data. Romero et al. (1994) found that systemic administration of 8-hydroxy-2-(di-*n*-propylamino)tetraline (8-OH-DPAT) reduced 5-HT levels in the striatum after inactivation of 5-HT_{1A} autoreceptors in the dorsal raphe nucleus. Although 5-HT input in the striatum is thought to be derived predominantly from the dorsal raphe nucleus (Azmitia and Segal, 1978; Kreiss and Lucki, 1994), it cannot be excluded that 5-HT_{1A} autoreceptors in other raphe nuclei have contributed to this effect (Hillegaart et al., 1990). Ceci et al. (1994) reported a diminished effect of 8-OH-DPAT on dorsal raphe nucleus cell firing after acute fronto-cortical deafferentation. They suggested that the inhibitory effect of 8-OH-DPAT is partly derived by effects in the frontal cortex. Jolas et al. (1995) studying the role of the postsynaptic 5-HT_{1A} receptors in the anxiolytic effects of 5-HT_{1A} agonists, reported intrahippocampal administration of 8-OH-DPAT (5 μg) to induce complete

inhibition of the neuronal discharge in the dorsal raphe nucleus. However, injection of 8-OH-DPAT into the striatum, where 5-HT_{1A} receptors are hardly detectable, or in the lateral ventricle, also yielded a dose-dependent reduction in dorsal raphe nucleus firing. Moreover, lesions of the postsynaptic 5-HT_{1A} receptors did not alter the inhibitory effect of intrahippocampal application of 8-OH-DPAT on dorsal raphe nucleus 5-HT neuronal activity. The authors suggest therefore that diffusion of 8-OH-DPAT from the site of injection to the dorsal raphe nucleus might explain the observed phenomena. A confounding factor here is the relatively high concentrations of the agonist (25 mM). In the present study, local perfusion of 1 μM of flesinoxan through the dialysis probe into the central nucleus of the amygdala significantly reduced 5-HT output locally and in the caudal linear raphe nucleus. Both effects were antagonized by perfusion of 1 μM of WAY 100635 into the central nucleus of the amygdala, but not by perfusion of the antagonist into the caudal linear raphe nucleus, suggesting that the effects were not mediated through somatodendritic 5-HT_{1A} receptors in the caudal linear raphe nucleus. If diffusion from the central nucleus of the amygdala to the caudal linear raphe nucleus had taken place, one would expect WAY 100635 applied into the caudal linear raphe nucleus to be capable to antagonize this effect. Moreover, based on the 8-OH-DPAT data published by Jolas et al. (1995) and the results of our preliminary diffusion experiments with flesinoxan (unpublished data), one would expect flesinoxan concentrations in the caudal linear raphe nucleus far below the concentration able to affect the 5-HT cell firing (Bosker et al., 1996). It is unlikely, therefore, that the observed effects could be accounted for by a diffusion process as suggested by Jolas et al. (1995). The present data support the notion that alterations in the 5-HT neuronal activity by stimulation of the postsynaptic 5-HT_{1A} receptor in the central nucleus of the amygdala are produced by a feedback pathway from the central nucleus of the amygdala to the caudal linear raphe nucleus. The possible existence of presynaptic 5-HT_{1A} autoreceptors in the central nucleus of the amygdala controlling 5-HT release might also explain the decrease in 5-HT efflux in the central nucleus of the amygdala, but cannot account for the decreased 5-HT efflux in the caudal linear raphe nucleus. Moreover, the existence of these receptors have, to the best of our knowledge not been reported. Similarly, a short feedback loop within the central nucleus of the amygdala regulating the 5-HT release in the central nucleus of the amygdala cannot explain the observed reduction in 5-HT cell firing as reflected by the decrease in 5-HT release in the caudal linear raphe nucleus. The existence of a negative feedback loop to the caudal linear raphe nucleus may also explain the smaller effect of flesinoxan on extracellular 5-HT in the central nucleus of the amygdala following local application into this raphe region as compared to systemic administration. The present finding is at variance with data reported previously by Sharp et al. (1989), who did not observe an effect on 5-HT release in the ventral hippocampus following local application of 10 μM of 8-OH-DPAT. Regional differences in feedback regulation may explain this discrepancy, but differences in specificity of the receptor agonists cannot be excluded either (Ahlenius et al., 1991). The existence of a feedback loop from the amygdala to the raphe might explain several discrepancies reported in behavioral studies following local administration of 5-HT_{1A} receptor agonists and urge to a reappraisal of the observed findings (Schreiber and De Vry, 1993a,b; Hodges et al., 1987).

Alterations in the firing rate through a neuronal feedback mechanism have also been suggested for the dopaminergic system (Bunney and Aghajanian, 1976; Iwatsubo and Clouet, 1977; Kondo and Iwatsubo, 1980), but there is as yet no general agreement on the existence and function of such a feed-back loop.

The present data add an additional level of complexity to the already intricate field of 5-HT_{1A} receptors. Moreover, it has strong implications for studies aiming at neuroanatomical mechanisms underlying the behavioral effects of 5-HT_{1A} receptor agonists and antagonists. Thus, local administration of a 5-HT_{1A} receptor agonist into the central nucleus of the amygdala cannot offer a fair appraisal of the relative contribution of the pre- or postsynaptic receptors in the behavioral responses of these compounds. The present study does not shed light on the nature of the feedback loop. Clearly, more work is needed to unravel the precise nature of this feedback mechanism. It is also interesting to know whether similar mechanisms are operating in other brain areas innervated by the caudal linear raphe nucleus or other raphe nuclei. It is noteworthy in this respect, that preliminary findings of our group with flesinoxan (1 μM through the probe) in the dorsal hippocampus and prefrontal cortex have also revealed effects on 5-HT output, suggesting that 5-HT_{1A}-mediated feedback mechanisms may also be operational in other brain areas (unpublished data).

In summary, the present data suggest that 5-HT efflux in the central nucleus of the amygdala is under inhibitory control of somatodendritic 5-HT_{1A} receptors in the caudal linear raphe nucleus. Stimulation of the postsynaptic 5-HT_{1A} receptors in the central nucleus of the amygdala affects the extracellular levels of 5-HT in the central nucleus of the amygdala, probably by turning down 5-HT cell firing in the caudal linear raphe nucleus by an as yet unknown neuronal pathway.

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