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Kainate Microinjection into the Dorsal Raphe Nucleus Induces 5-HT Release in the Amygdala and Periaqueductal Gray

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VIANA, M. B., F. G. GRAEFF AND P.-A. LÖSCHMANN. *Kainate microinjection into the dorsal raphe nucleus induces 5-HT release in the amygdala and periaqueductal gray.* PHARMACOL BIOCHEM BEHAV **58**(1) 167–172, 1997.—Earlier results obtained in one of our laboratories showed that microinjection into the dorsal raphe nucleus (DRN) of the excitatory amino acid kainic acid, the benzodiazepine (BZD) inverse agonist FG 7142, and the 5-HT_{1A} receptor agonist 8-OH-DPAT changed the behavior of rats in the elevated T-maze, an animal model of anxiety. The present study investigates biochemical correlates of these results in awake rats by measuring 5-HT release with in vivo microdialysis in two brain structures innervated by the DRN—the amygdala (Am) and the dorsal periaqueductal gray matter (DPAG)—that have been implicated in anxiety. Microinjection of kainic acid (60 pmol) into the DRN significantly increased 5-HT release in both the Am and the DPAG. In the DPAG, the increase was 14-fold higher with respect to the baseline and occurred only at the first sample, which was collected 30 min after the injection. In the Am, the increase was less pronounced (nearly fourfold) but persistent, lasting until the fourth sample, which was collected 120 min from the injection. FG 7142 (40 pmol) and 8-OH-DPAT (8 nmol) were ineffective. Because only intra-DRN kainate both increased inhibitory avoidance and decreased one-way escape in the elevated T-maze, the present behavioral results support the suggestion that 5-HT facilitates conditioned fear in the Am and inhibits unconditioned fear in the DPAG. © 1997 Elsevier Science Inc.

5-HT Microdialysis Amygdala Dorsal periaqueductal gray Dorsal raphe nucleus Kainic acid FG 7142 8-OH-DPAT

TO reconcile contradictory evidence concerning the role of 5-HT in anxiety, Deakin and Graeff (7) proposed that distinct 5-HT pathways and receptor subtypes modulate different classes of anxiety. The ascending 5-HT pathway, which arises in the dorsal raphe nucleus (DRN) and innervates the amygdala (Am) and the frontal cortex, would facilitate defensive behaviors that occur in response to potential or distal threat. Because these behavior strategies usually involve learning and memory, they represent conditioned fear and may be related to clinical anticipatory anxiety and generalized anxiety disorder. In turn, the DRN–periventricular pathway, which innervates the medial hypothalamus and the dorsal periaqueductal gray matter (DPAG), would inhibit inborn flight-or-fight reactions that arise in response to proximal danger. The latter may be related to panic disorder (11).

The results of a recent behavioral study (13) performed in one of our laboratories have shown that three drug treatments supposed to increase 5-HT release from DRN terminals facilitate inhibitory avoidance in a new animal model of anxiety, the elevated T-maze (26). Avoidance of the open arms of the maze is supposed to represent conditioned fear. On the other hand, one-way escape from the open to the enclosed arms, thought to represent unconditioned fear, was impaired by two of these drug treatments. Conversely, drug treatments supposed to inhibit 5-HT release from DRN terminals impaired inhibitory avoidance in the elevated T-maze, although one-

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way escape was not affected (14). According to Deakin and Graeff (7), the changes in inhibitory avoidance and one-way escape observed in the elevated T-maze would be mediated by 5-HT released from DRN nerve terminals in the Am and DPAG, respectively. To test this hypothesis, we presently measured 5-HT release in the Am and DPAG of awake rats by in vivo microdialysis using the same drug treatments as in the above-mentioned behavioral studies, namely, microinjection into the DRN of 40 pmol of FG 7142, 60 pmol of kainic acid, and 8 nmol of 8-OH-DPAT.

The β-carboline derivative FG 7142 has been shown to act as a benzodiazepine partial inverse agonist and to induce anxiety in laboratory animals as well as in human beings (6,8). Intraraphe administration of FG 7142 is supposed to stimulate 5-HT neurons by counteracting tonic GABAergic inhibition (15,25). Although high doses (nanomolar range) of the excitatory amino acid kainic acid cause cell body lesion without affecting neuronal axons (5), subtoxic doses (picomolar range) of the same compound have been shown to stimulate somadendrites of central nervous system neurons (10,24). Therefore, intra-DRN injection of kainate is expected to directly stimulate 5-HT neurons. Finally, 8-OH-DPAT is a selective 5-HT_{1A} receptor agonist that behaves as a full agonist at both postsynaptic and somatodendritic autoreceptors (22). Intraraphe administration of 8-OH-DPAT has been shown to decrease the firing rate of 5-HT neurons (2).

METHOD

Animals and Housing

Twenty-eight male Wistar rats (Interfauna, Tuebingen, Germany) weighing 250–300 g were used. Rats were housed four or five per cage and maintained in a temperature-controlled room (23 ± 1°C) with lights on from 0700 to 1900 h. Food pellets (Altromin Spezialdiäten GmbH, Tuebingen, Germany) and water were available ad lib. After surgery, the animals were housed individually until the day of the experiment.

Surgery

Animals were anesthetized with sodium pentobarbital (45 mg/kg intraperitoneally) and placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA) with the incisors bar set at −2.5 mm. Burr holes were drilled in the skull above the DRN, the DPAG, and the Am according to coordinates from the atlas of Paxinos and Watson (21). For intracerebral injection, a stainless steel guide cannula (10 mm long) was inserted into the DRN at a 28° angle with the sagittal plane, 3.2 mm lateral to lambda, 2.5 mm below the skull surface. Guide cannulae provided with a dummy probe (CMA 12, Carnegie Medicine, Stockholm, Sweden) were positioned in the DPAG (22° angle, 1.9 mm lateral to lambda, 5.0 mm below the skull surface) and Am (2.12 mm posterior and 4.0 mm lateral to bregma, 8.5 mm below the skull surface). Following surgery, the rats were housed individually for 3–6 days prior to the experiment.

Dialysis Procedure

Microdialysis experiments were performed in freely moving animals in which the dummy probes were replaced by dialysis probes (outer diameter 0.5 mm, type CMA 12, Carnegie Medicine) exceeding the tip of the guide cannulae by 2 mm. The probes were perfused with artificial cerebrospinal fluid (composition, mM: NaCl 147, KCl 5.3, $CaCl_2$ 1.9, $MgCl_2$ 1.1, ascorbic acid 0.02; pH 7.4) using a syringe pump (Braun, Melsungen, Germany). Recovery rates of individual probes were determined in vitro by placing the probes in standard solutions containing 0.1 pmol/μl of each of the compounds analyzed. The flow rate was 0.77 μl/min, and samples were collected at 30-min intervals into vials located 5 cm distant from the outlet of the dialysis probes. Two samples were used to determine basal amine concentrations. After that, animals were randomly divided into four treatment groups and injected into the DRN with control (saline or 10% cremophor, BASF, Ludwigshafen, Germany) solution, FG 7142 (40 pmol), kainic acid (60 pmol), or 8-OH-DPAT (8 nmol). During this procedure, animals were gently held by the experimenter and a needle was introduced into the guide cannula until its tip was 2 mm below the cannula end. A volume of 0.2 μl was injected over a period of 2 min, using a 10-μl microsyringe (Hamilton, Reno, NV, USA). The displacement of an air bubble inside the polyethylene catheter connecting the syringe needle to the intracerebral needle was used to monitor the microinjections. The intracerebral needle was removed 1 min after each injection was finished. Following injection, four more dialysate samples were collected from each structure. Samples were stored on ice and protected from light until high-performance liquid chromatography (HPLC) analysis. At the end, animals were sacrificed and their brains were removed and stored at −80°C. Frozen 40-μm sections were cut with a cryostat (CM 3000, Leica, Nusslah, Germany) for determination of cannula and probe location.

HPLC System

Analysis of biogenic amines and metabolites was performed using HPLC with amperometric detection (electrochemical detector: M20 Gynkotek, Munich, Germany; electrode: Bioanalytical Systems, West Lafayette, IN, USA; pump: Gynkotek; M 480-microinjector: Rheodyne, Cotati, CA, USA) for simultaneous determination of dopamine (DA), DOPAC (3,4-dihydroxyphenylacetic acid), HVA (homovanillic acid), 3-MT (3-methoxytyramine), 5-HT, and 5-HIAA (5-hydroxyindolacetic acid). The oxidation potential was set at 685 mV, and readings were taken at 0.02 nA full scale. Separation of amines was performed on a C18 reversed-phase column (100 × 1 mm, 3-μm particles, Step-Stick, Bioanalytical Systems) in 5-μl samples. The mobile phase consisted of 100 mM sodium phosphate buffer, pH 3.1, containing 1 mM octansulfonic acid and 6% acetonitrile in deionized water (Millipore, Saint Quentin, France), and the flow was set at 70 μl/min. Quantification was performed on a 486 computer system using integration software (Turbochrome 4, PE Nelson, Norwalk, CT, USA) and employing a three-point external standard calibration curve (5, 50, and 500 fmol in 5 μl). Raw amounts were corrected for probe recovery and are expressed as percentage of the efflux under baseline conditions.

Drugs and Solutions

Kainic acid (Sigma, St. Louis, MO, USA) and 8-OH-DPAT [8-hydroxy-2-(di-*n*-propylamino)tetralin; Schering, Berlin, Germany] were dissolved in saline (0.9% NaCl). FG 7142 (*n*-methyl-β-carboline-3-carboxamine; Schering) was suspended in saline containing 10% (w/v) cremophor. Control animals were injected with either saline or 10% cremophor solution.

Statistical Analysis

Only data from animals with probes located into the DPAG and Am as well as the cannula in the DRN were used. Data were expressed as a percentage of change from baseline values, calculated as the mean of the last two fractions before

injection. For the four fractions collected following the injection, a multiple analysis of variance (MANOVA) with repeated measures was performed, with "treatment" as the between-subjects factor and "time" as the within-subjects factor. Whenever a significant treatment × time interaction was found, a post hoc test of contrasts with α-adjustment was used to compare treatment groups at different time points. Otherwise, the cumulative drug effect was analyzed. For this, a sum of the four fractions measured after injection was obtained for each rat. The differences between these summed values were then analyzed by one-factor analysis of variance (ANOVA), followed by the multiple-comparison test of Tukey. Statistical significance was set at $p < 0.05$.

RESULTS

As shown in Fig. 1, the injection sites were localized inside the DRN and the dialysis probes were placed both in the Am and in the DPAG.

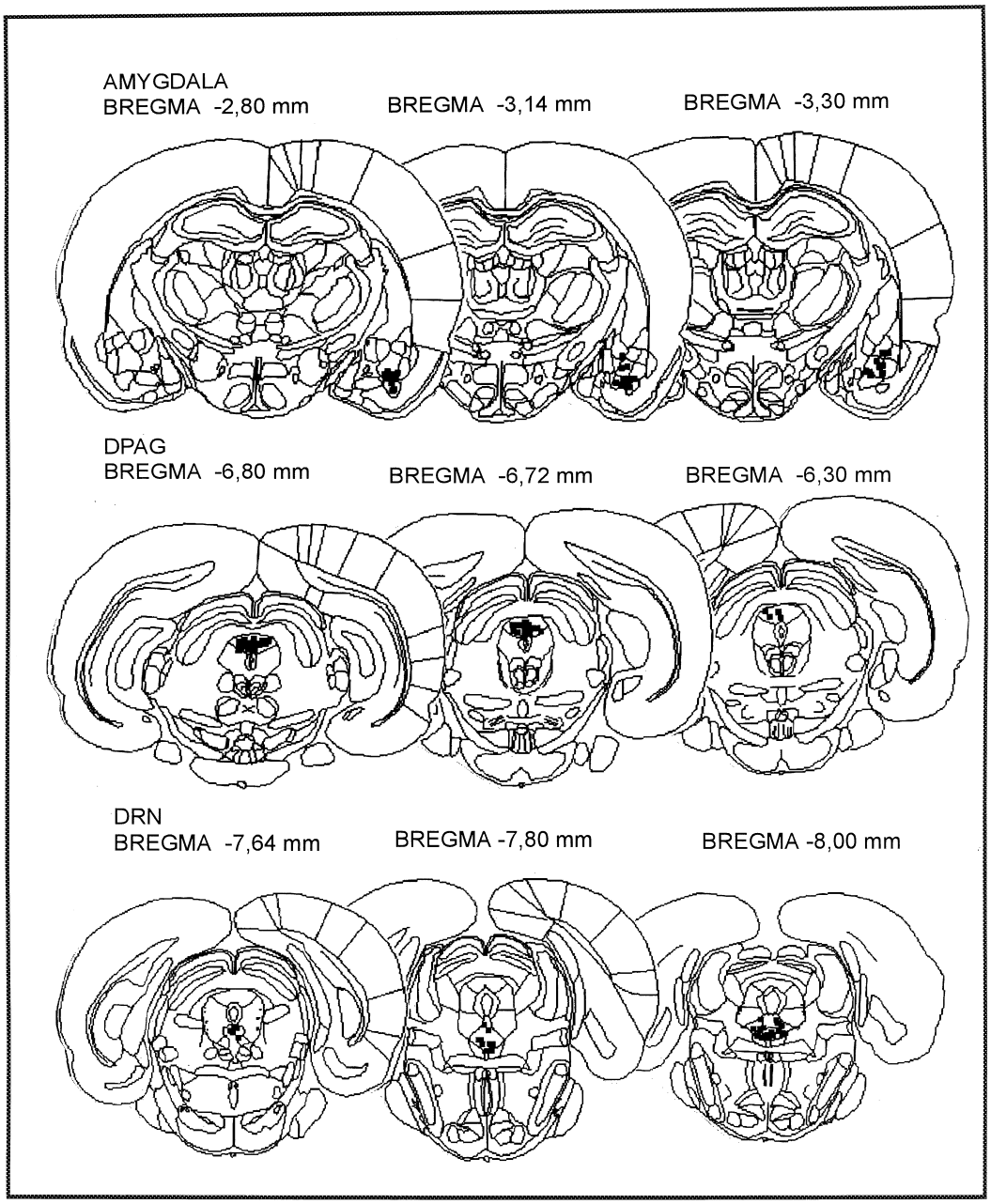

FIG. 1. Localization of injection sites in the dorsal raphe nucleus (DRN) and probe placements (tip) in the amygdala and dorsal periaqueductal gray matter (DPAG). Diagrams taken from a computer-based atlas of the rat brain are presented (3). Figures represent coordinates from Paxinos and Watson's (21) rat brain atlas, posterior to bregma.

The analytical system used in this study allowed reproducible determination of 5-HT in dialysates without addition of reuptake inhibitor. Under control conditions, the mean basal 5-HT efflux was 79 ± 12 fmol/20 μl in DPAG samples and 110 ± 22 fmol/20 μl in the Am. The concentrations of 5-HIAA were 100-fold greater than those of 5-HT. Because these concentrations were above the upper detection limit of the system, which was optimized for sensitive 5-HT determination, these data were not analyzed.

Concentrations of DA and its main metabolites DOPAC, HVA, and 3-MT were very low in all samples. Dopamine was only occasionally detected, and none of the metabolites changed over time or in correlation with the drug treatments (data not shown).

Figure 2 shows the effect of the different treatments on 5-HT release in dialysates collected from the DPAG after the injection. MANOVA showed a significant effect of treatment [$F(3, 26) = 12.98, p < 0.001$] and time [$F(3, 78) = 24.37, p < 0.001$], as well as a significant treatment × time interaction [$F(9, 78) = 14.82, p < 0.001$]. Post hoc analysis performed by the test of contrasts with α-adjustment showed a significant difference between the control and kainate groups in the first sample ($p < 0.01$). Nevertheless, no significant difference was detected between the control group and the groups treated with either FG 7142 or 8-OH-DPAT ($p > 0.05$).

Figure 3 shows the same drug effects in dialysates from the Am. MANOVA showed a significant effect of treatment [$F(3, 25) = 5.12, p < 0.01$], but neither an effect of time nor a significant treatment × time interaction. Post hoc comparisons with the Tukey test (cumulative data) showed a significant difference between the control and kainate groups ($p < 0.05$), but no significant difference between the control group and the groups treated with either FG 7142 or 8-OH-DPAT ($p > 0.05$).

DISCUSSION

Although the role of 5-HT in anxiety has been extensively investigated (12), few studies have addressed the changes in extracellular levels of 5-HT in brain regions controlling defensive behavior (16,19). The present work is a first attempt to measure the effect of direct pharmacological manipulation of 5-HT neurons in two structures belonging to the brain defense system, the Am and the DPAG (9).

The main finding in this study is that microinjection of kainate into the DRN significantly increased the release of 5-HT in both the Am and the DPAG. The release of 5-HT in the Am was long lasting as compared with that in the DPAG. Although it is difficult to interpret this temporal difference, it may be speculated that terminal autoregulation of 5-HT release (4) might be more effective in the DPAG than in the Am. Another possibility is a regional difference in effectiveness of the 5-HT reuptake mechanism (1).

Similar microdialysis studies performed with electrical stimulation of median and dorsal raphe nuclei showed small and variable increases in extracellular 5-HT in the rat forebrain. Only after addition of a reuptake inhibitor to the perfusion medium were consistent increases of 5-HT release detected (20,23). In this regard, the absence of reuptake inhibition in the present study may explain the lack of effect of FG 7142 on 5-HT release. Whereas kainate powerfully stimulates neuronal cells directly (10), intra-DRN FG 7142 seemingly attenuates tonic GABAergic inhibition of 5-HT neurons, as suggested by its anxiogenic effect in rats exposed to animal models of anxiety (15,25). The latter mechanism may not provide enough neuronal activation to produce measurable increases in extracellular 5-HT without a reuptake inhibitor in the perfusion medium. Furthermore, electrophysiological results indicate that tonic GABAergic inhibition occurs during slow wave sleep but is absent during quiet waking, which was the behavioral state of the rats in the present study (18).

The present results have additionally shown that intra-DRN 8-OH-DPAT failed to decrease 5-HT extracellular concentration. In contrast, a previous study performed in anesthetized rats (17)—also without a reuptake inhibitor in the perfusion medium—showed that intra-DRN 8-OH-DPAT significantly reduced 5-HT release. Because both the dose of 8-OH-DPAT (9.8 nmol) and the baseline 5-HT concentration (nearly 10 fmol/5μl) were similar to those of the present study, the cause of the discrepancy is not clear. Possible explanations are the different procedures used, awake vs. anesthetized animals, and the brain structures studied, because Kreiss and Lucki's (17) measurements were performed in the striatum.

The present work was aimed at correlating neurochemical data with previously reported behavioral changes in the ele-

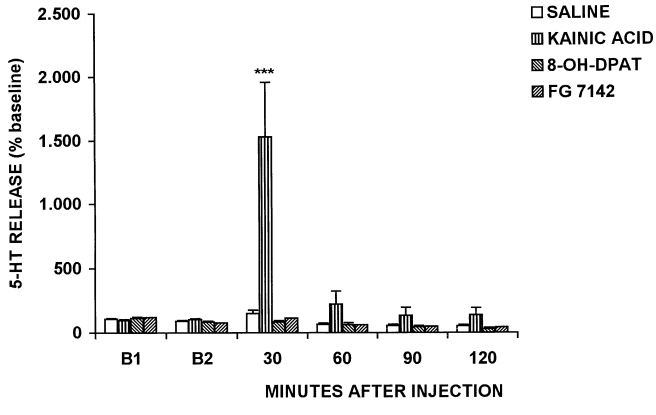

FIG. 2. Effects of intraraphe drug injections on extracellular 5-HT concentration in the DPAG as percentage of baseline (mean of fractions B1 and B2, collected at −60 and −30 min from the injection, respectively). Bars represent mean ± SEM. $n = 7$ (saline), 6 (kainate), 9 (8-OH-DPAT), and 8 (FG 7142). ***$p < 0.001$.

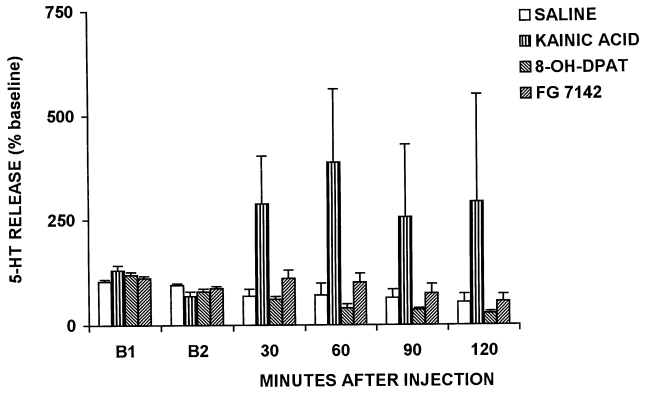

FIG. 3. Effects of intraraphe drug injections on extracellular 5-HT concentration in the Am. For further specifications, see legend of Fig. 2.

TABLE 1
QUALITATIVE BEHAVIORAL EFFECTS IN THE ELEVATED T-MAZE OF DRUGS MICROINJECTED INTO THE DRN

Intra-DRN Injection	Inhibitory Avoidance	Escape
Kainate	↑	↓
FG 7142	↑	0
8-OH-DPAT	↓	0

↑, Facilitation; ↓, inhibition; 0, no change. Original data were reported by Graeff and colleagues (12,13).

vated T-maze (13,14). Table 1 summarizes the behavioral effects of the drug treatments used in the present study. It may be seen that kainate—the only drug that caused measurable increases of 5-HT release in the Am and DPAG—both enhanced inhibitory avoidance and impaired one-way escape in the elevated T-maze. Such correlation between neurochemical and behavioral events agrees with Deakin and Graeff's (7) suggestion that release of 5-HT in the Am enhances conditioned fear (inhibitory avoidance), whereas release of 5-HT in the DPAG decreases unconditioned fear (escape). Nevertheless, it remains to be shown whether there is a causal relationship between 5-HT action in each brain structure and the respective behavioral effect.

No change in 5-HT release was detected in the Am following either FG 7142 or 8-OH-DPAT administration, despite the fact that inhibitory avoidance was facilitated by the former and impaired by the latter drug (Table 1). If FG 7142 and 8-OH-DPAT did not really alter 5-HT function, there is a lack of behavioral–neurochemical correlation that does not support the dual 5-HT–fear hypothesis under scrutiny (7). However, the low sensitivity of the present method (discussed above) might have prevented detection of drug-induced changes in 5-HT concentration. Further experiments are necessary to establish whether the addition of a reuptake inhibitor to the perfusion medium and/or the use of higher doses of FG 7142 and 8-OH-DPAT will result in detectable changes in 5-HT concentration in the Am and/or the DPAG.

In summary, the present results have shown that microinjection of a behaviorally active dose of kainic acid into the DRN increased 5-HT release in both the Am and the DPAG, as predicted by the dual 5-HT–fear hypothesis (7). Despite the negative results with intra-DRN FG 7142 and 8-OH-DPAT, further investigation along this line of inquiry is warranted.

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