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Serotonin Neuronal Release From Dorsal Hippocampus Following Electrical Stimulation of the Dorsal and Median Raphé Nuclei in Conscious Rats

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ABSTRACT: We have studied 5-hydroxytryptamine (5-HT) release in the hippocampal formation following electrical stimulation of the dorsal and median raphé nuclei in the behaving rat. The primary finding in this study is a decrease in neuronal release of serotonin in the dorsal hippocampal formation following electrical stimulation of either the dorsal or median raphé nucleus in conscious rats. At no time did electrical stimulation of either raphé nucleus result in behavioral, including vigilance state, changes. The amount of 5-HT released was found to be frequency dependent with higher frequencies (20 Hz) producing larger decreases in release of 5-HT. However, the pattern of release differs between the two raphé nuclei. Extracellular levels of 5-HT decrease during stimulation of the dorsal raphé, whereas levels decrease only following cessation of stimulation of the median raphé nucleus. This may relate to the patterns of innervation of the dorsal hippocampal formation by these two midbrain raphé nuclei and also may reflect an inhibition of median raphé cell firing during stimulation of the dorsal raphé. Electrical stimulation of the dorsal raphé in anesthetized animals resulted in an enhanced release of 5-HT. The suppression of 5-HT release in the dorsal hippocampal formation in behaving animals was long-lasting (over 2 h), suggesting that the control mechanisms that regulate 5-HT release operate over a long time-course. This difference in release between non-anesthetized and anesthetized animals may relate to anesthesia blocking long- and/or short-loop serotonin recurrent axonal collaterals negatively feeding back onto 5-HT$_{1A}$ and 5-HT$_{1D}$ somatodendritic autoreceptors on raphé neurons. Further, the anesthetized animal has diminished monoaminergic "gating" influences on the hippocampal formation, whereas the behaving animal is more complex with behavioral (vigilance) states associated with different patterns of gating of information flow through the hippocampal formation. *Hippocampus 1998;8:262–273.*

KEY WORDS: microdialysis; serotonin release; 5-HT autoreceptors; hippocampal formation; raphé and behavior

INTRODUCTION

Various neurochemical approaches have been used to monitor the release of 5-hydroxytryptamine (5-HT) from neurons in the forebrain. In particular, microdialysis has proven to be a valuable approach to the neurochemical study of the functioning of intact serotonergic neurons in the rat brain in vivo (Sharp et al., 1989). In the present studies, we have examined the relationship between release of 5-HT in the hippocampal formation and electrical stimulation of the midbrain raphé in conscious animals. Raphé neurons were stimulated using electrodes implanted in the dorsal and median raphé nuclei and 5-HT release was measured in the dorsal and ventral hippocampal formation using in vivo microdialysis.

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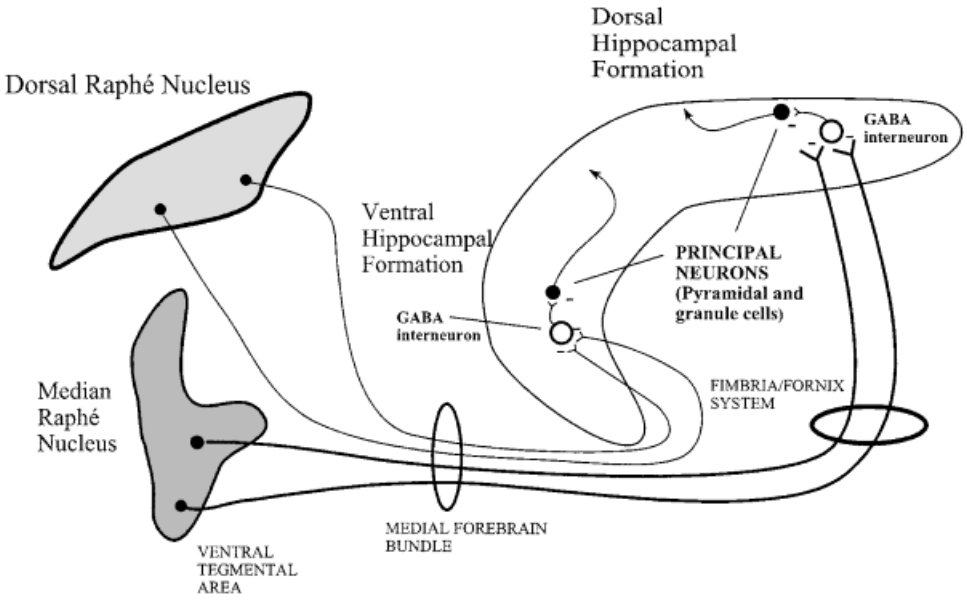


FIGURE 1. Simplified schema of raphé-hippocampal system. The dorsal raphé nucleus predominately innervates the ventral hippocampal formation with lesser input into the dorsal hippocampal formation. The median raphé nucleus projects principally to the dorsal hippocampal formation with lesser input to the ventral hippocampal formation. Raphé inputs are able to differentially modulate different aspects of hippocampal formation information processing by innervating specialized subsets of inhibitory interneurons in the hippocampal formation. The raphé system influences hippocampal activity through regulation of the activity of these interneurons.

Based largely on neuroanatomical evidence, the 5-HT innervation of the hippocampal formation, and the forebrain in general, comprises a dual system (Fig. 1). One group of projections with fine axons and small fusiform varicosities arises from the dorsal raphé nucleus while the other, having course axons and bead-like terminal varicosities, arises from the median raphé nucleus (Molliver et al., 1987; Mamounas and Molliver, 1988; Mamounas et al., 1991). The median raphé projection innervates largely the dorsal hippocampal formation, whereas the dorsal raphé nucleus projects heaviest to the ventral hippocampal formation (Azmitia and Segal, 1978). McQuade and Sharp (1995), using anesthetized animals, showed that electrical stimulation of the dorsal raphé has little effect on 5-HT release from the dorsal hippocampal formation but a marked effect on release from the ventromedial hippocampal formation. Kreiss and Lucki (1994) reported that the median raphé, but not the dorsal raphé, regulates release of 5-HT in the dorsal hippocampal formation. Thus, release of 5-HT in the dorsal and ventral hippocampal formation is, to a considerable extent, dorsal raphé and median raphé pathway selective.

It is now clear that there are several regulatory mechanisms controlling 5-HT neurotransmission (Fig. 2). The release of 5-HT from nerve terminals is under control of inhibitory serotonin autoreceptors (Briley and Moret, 1993). Further, there are more than one subtype of 5-HT autoreceptor controlling the release of serotonin (Fig. 3). The three principal regulatory feedback mechanisms controlling serotonin release are: 1) the firing rate of serotonergic neurons in the dorsal and median raphé nuclei is under control of somatodendritic 5-HT_{1A} and 5-HT_{1D} autoreceptors, 2) the terminal release of 5-HT is under control of presynaptic 5-HT autoreceptors (5-HT_{1B} subtype in rodents and 5-HT_{1D} subtype in other species), and 3) the synthesis of 5-HT. In any consideration of the functional organization of the raphé serotonergic-hippocampal system each of these feedback mechanisms must be considered.

Some details of the control mechanisms of 5-HT neurotransmission are necessary to interpret results of electrical stimulation of the raphé nuclei in relation to 5-HT release. The firing rate of raphé neurons is under the control of 5-HT_{1A} receptors located on soma and dendrites in the raphé neurons (Verge et al., 1985; Weissman-Nanopoulas et al., 1985; Piñeyro, 1995). Stimulation of these receptors by systemic administration of 5-HT_{1A} agonists reduces firing of these neurons (Chaput et al., 1986; Sprouse and Aghajanian, 1987). Increased levels of extracellular serotonin resulting from uptake inhibition may activate 5-HT_{1A} autoreceptors in the raphé nuclei leading to feedback inhibition of release in the terminal regions through decreased firing of raphé serotonergic neurons. Various studies have shown that stimulation of these receptors by systemic administration of 5-HT_{1A} agonists attenuates the firing rate of these neurons, thus reducing 5-HT release in the hippocampal formation (Kreiss and Lucki, 1994; Starkey and Skingle, 1994). Recent studies have also suggested a role for the 5-HT_{1D} receptor in the control of raphé cell firing (Piñeyro et al., 1996; Sprouse et al., 1997). In addition, there are release controlling 5-HT feedback mechanisms in terminal regions of 5-HT neurons. It is now clear that release of 5-HT from nerve terminals is under control of inhibitory 5-HT_{1B} autoreceptors (Moret, 1985; Moret and Briley, 1988). Stimulation of terminal 5-HT_{1B} autoreceptors activates feedback inhibition that then reduces release. Finally, serotonergic feedback mechanisms are

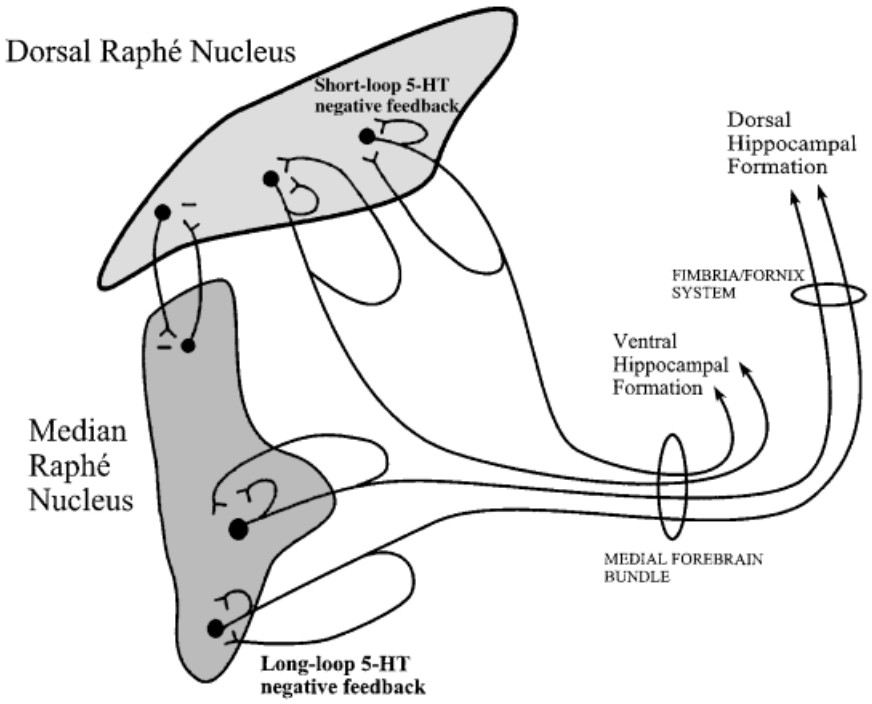


FIGURE 2. Many experiments have confirmed the distinctive nature of the several clusters of serotonergic neurons in the midbrain raphé (nuclei raphé dorsalis and medianus). Dorsal and median raphé neurons are clearly distinguishable on the basis of their forebrain projections, their behavioral roles and their pharmacological responses. Dense reciprocal connections have been demonstrated between the dorsal and median raphé nuclei. These reciprocal connections are inhibitory and are likely to be 5-HT or possibly GABAergic projection neurons. This figure also illustrates the long and short axon collaterals from dorsal and median raphé nuclei feeding back to serotonergic neurons and inhibiting raphé neuron firing via somatodendritic 5-HT_{1A} and 5-HT_{1D} receptors. Wang and Aghajanian (1978) demonstrated antidromic effects on raphé serotonergic neurons providing evidence that these effects are mediated via axon collaterals. The axon collateral inhibitory system may have a physiological role in maintaining the slow, regular spontaneous firing of serotonergic neurons.

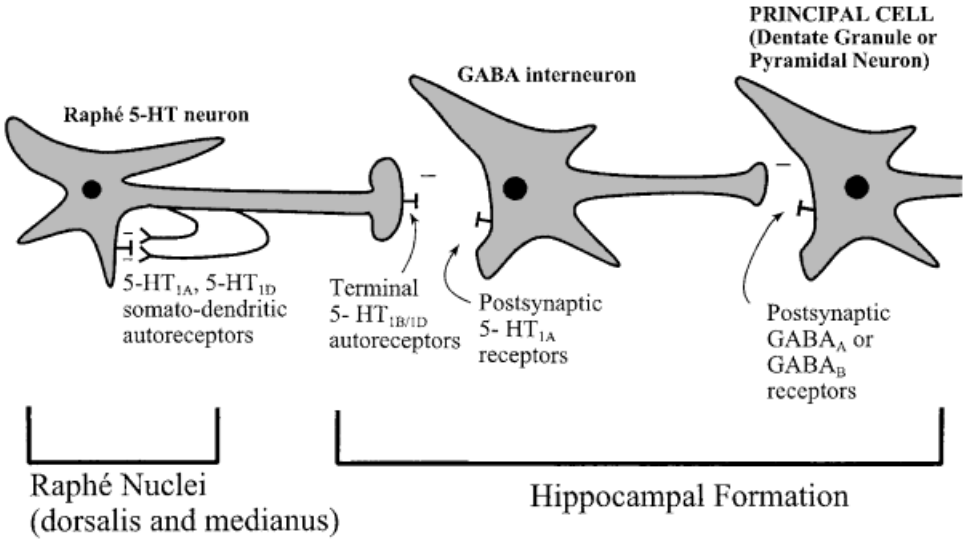


FIGURE 3. Schema of raphé-hippocampal serotonergic neuron indicating somatodendritic serotonergic autoreceptors (5-HT_{1A} and 5-HT_{1D}) in the raphé nuclei (firing-rate controlling auto-receptors) and axon terminals (5-$HT_{1B/1D}$) in the hippocampal formation (release-modulating autoreceptors). Intra-raphé and long-loop negative feedback axon collaterals are shown onto 5-HT_{1A} and 5-HT_{1D} somato-dendritic autoreceptors. Both the dorsal raphé and median raphé projections terminate on selectively distinct populations of GABA interneurons in the hippocampal formation and these, in turn, terminate on principal neurons (dentate granule cells or pyramidal neurons of the hippocampus proper) forming the basis for raphé activation resulting in disinhibition of principal neurons in the hippocampal formation.

important in controlling 5-HT synthesis. For example, the activity of the rate-limiting enzyme of serotonin synthesis, tryptophan hydroxylase, is under feedback control (Briley and Moret, 1993). Systemic administration of 5-HT_{1A} receptor agonists reduces the activity of tryptophan hydroxylase (Hamon et al., 1988), the control of tryptophan hydroxylase activity likely being mediated through the stimulation of both 5-HT_{1A} and 5-$HT_{1B/D}$ receptors.

As opposed to most earlier studies using the intact, anesthetized animal or brain slices, we have used the behaving, intact rat. Having established conditions under which 5-HT output was detectable and stable prior to stimulation, experiments were carried out to determine whether 5-HT release in the hippocampal formation was similar in the behaving animal to that previously reported in anesthetized rats. Furthermore, we studied the frequency dependence of 5-HT release following electrical stimulation of the dorsal and median raphé nuclei in these conscious animals. Sharp et al. (1989) presented the first findings using the microdialysis technique that the output of 5-HT in the hippocampal formation increased proportionately with raphé stimulation at frequencies ranging from 2 to 20 Hz for 20 min. Their data are clear evidence that raphé-hippocampal serotonergic neurons are able to maintain levels of transmission well above normal firing rates of the slow-firing raphé neurons. Their data on the decrease of 5-HT in hippocampal dialysates induced by injection of the 5-HT_{1A} agonist (8-OH-DPAT) into the dorsal raphé nucleus supports the electrophysiological data that 8-OH-DPAT reduces 5-HT neurotransmission by a direct action on 5-HT neurons in the raphé nuclei (Sprouse and Aghajanian, 1987, 1988). Thus, 5-HT release in the hippocampal formation reflects raphé cell firing.

The issue of studies using anesthetized vs. those using behaving, awake animals is important since they result in diametrically opposite results in terms of release of 5-HT following raphé stimulation (this study and Sharp et al., 1990). Winson (1980), in an important study on the influence of the raphé system on neuronal transmission through the trisynaptic circuit of the dentate gyrus, showed clearly that transmission through the hippocampal formation is under vigilance state control. Hence, studies of the behaving (unanesthetized) animal are critical to understanding the functional organization of raphé-hippocampal system. The brainstem inputs, especially from the raphé 5-HT system, form an essential part of this vigilance-state dependent "gating" system. In the anesthetized animal, gating processes are relatively inactive in relation to vigilance (sleep-waking) states.

MATERIALS AND METHODS

Surgery

Male Sprague-Dawley rats (Charles River-derived) bred in the animal colony at UNE, raised to weaning with natural dam and littermates, and ranging from 275 to 375 g, were used in these experiments. Prior to surgery, animals were housed with same-sex littermates in plexiglass cages in animal rooms with constant temperature (22–24°C) and humidity. Lights were on from 0600 to 1800 daily. Experiments were begun between 0800 and 0900. Animals had free access to food and water throughout the experiments.

Animals were implanted with bipolar, concentric electrodes (280 μm diameter tip, Plastics One, Roanoke, VA) into the raphé nuclei under pentobarbital (35 mg/kg, ip) and chloral hydrate (160 mg/kg, ip) anesthesia. The coordinates for the DRN were A 1.2: L 0.0: V −3.5 with reference to intraural zero, and for the MRN A 1.2: L 0.0: V −1.5 with reference to intraural zero (Paxinos and Watson, 1982). Electrodes were implanted at an angle of 20 degrees from the vertical to avoid blocking the cerebral aqueduct and to prevent damage to the midline blood vessels. Guide cannulae (CMA10, CMA/Microdialysis AB, Acton, MA) were implanted at the same time aimed at the dorsal hippocampal formation (AP −3.8, L ± 1.5, DV −3.5) (Paxinos and Watson, 1982). Once the microdialysis probe was inserted into the guide the probe was positioned into the dorso-medial hippocampal formation. Animals received intradermal infiltration of bupivacaine, a long-acting local anesthetic, and epinephrine at the site of the incision to decrease bleeding during surgery and pain following surgery. After surgery, animals were housed individually. Animals which lost electrodes were euthanized with an overdose of pentobarbital.

Electrical Stimulation and Microdialysis

After allowing 3 days for recovery, animals were placed into a deep plexiglass bowl (CMA 120 Awake Animal System, CMA/Microdialysis AB, Acton, MA) with clean animal bedding at between 0800 and 0900. The animal was then connected by an electrode lead to an AM Systems Model 2100 Isolated Pulse Stimulator (AM Systems, Seattle, WA). The electrode lead was connected through a two-lead commutator allowing the animal to move freely about the plastic cage (Plastics One, Roanoke, VA). Brief behavioral observations of vigilance state were made every 20 min throughout the experiment. Based on movement, posture and eye opening or closure, we stimulated the raphé nuclei only during the still, alert vigilance state.

The electrical stimulus consisted of constant current biphasic pulse pairs with each stimulus being 0.10 ms in duration with a stimulus current of 200 μA. Stimulation was done at frequencies of 5, 10 and 20 Hz. These stimulus parameters were carefully chosen to reflect a number of criteria. Many previous experiments have used relatively high levels of stimulation, both in terms of frequency and current (Sheard and Aghajanian, 1968; Kostowski et al., 1969; Duda and Moore, 1985; Shannon et al., 1986; Sharp et al., 1989, 1990). Current levels in excess of 300 μA are supramaximal and are aversive in conscious animals. The stimulus frequency of 20 Hz is a moderately high level of stimulation for 5-HT neurons, but a stimulus frequency that can be followed by raphé neurons in terms of 5-HT release (Sharp et al., 1989). Furthermore, this was the frequency of stimulation used by Hirschhorn et al. (1975) and Mokler et al. (1994, 1997) in behavioral experiments which showed that this stimulation is

discriminated by rats as being similar to the hallucinogenic drugs LSD and DOI. The raphé typically fires at 0.5–2.0 Hz in anesthetized animals and up to 5 Hz in active waking states. The firing rate for 5-HT neurons is clearly vigilant-state-dependent, varying from 5 Hz in active waking to almost complete silence during REM sleep.

Microdialysis probes (3 mm, CMA 10, CMA/Microdialysis AB, Acton, MA) were perfused at a rate of 1.0 μl/min with artificial CSF (147 mM NaCl, 1.26 mM $CaCl_2$, 2.5 mM KCl, 1.18 mM $MgCl_2$ in sterile water). Artificial CSF (aCSF) contained 1 mM sertraline (Pfizer Pharmaceuticals, Groton, CT), a selective 5-HT uptake inhibitor, to stabilize levels of extracellular 5-HT and reduce variability in results. Preliminary experiments showed that perfusion of the terminal fields in the hippocampal formation with artCSF containing sertraline did not alter the dynamics of 5-HT release. Recovery of 5-HT by these probes has been verified at 30 to 40% using in vitro recovery at 37 degrees C. Levels of 5-HT in dialysate are unadjusted for recovery in order to avoid underestimates of extracellular levels of 5-HT. Due to variability from animal to animal in the basal levels of extracellular 5-HT, values are converted to percent of basal levels.

On the day of the experiment, the microdialysis probe was inserted with minimal stress and handling into the guide cannula while the animal was alert. At least six 20-min samples were collected for baseline determination. Baseline was considered stable if microdialysis samples did not differ by more than 20% over three consecutive samples. Electrical stimulation was begun after levels of 5-HT in dialysate had stabilized and samples were then collected for an additional 2 h following raphé stimulation.

Neurochemical analysis was done using liquid chromatography with electrochemical detection (ESA Coulochem II, ESA Inc., Chelmsford, MA) using a C18 3-μm column. The area under the curve (AUC) for samples was compared with a regression analysis of AUC for three authentic standards injected onto the column at the beginning of each day to determine the levels of 5-HT. 5-HT was verified by examining the voltammogram for standards against that determined using microdialysis samples.

TTX, Ca^{++}-Free CSF, and K^+-Stimulated Release

In order to determine if the measured release of 5-HT was neuronal in origin, experiments were carried out to examine the reliance of levels of 5-HT in dialysate on Na^+ channels and extracellular Ca^{++}. In the first experiment, after 5-HT levels had stabilized, the aCSF was switched with one containing 10 mM tetrodotoxin (TTX, Sigma Chemicals, St. Louis, MO). TTX blocks Na^+ channels, thus decreasing neuronal transmission, thereby providing evidence for neuronal release.

After stable levels of 5-HT were obtained, the aCSF was changed to one without Ca^{++} for the remainder of the experiment. The artCSF with no Ca^{++} will then deplete the extracellular Ca^{++} levels around the probe over time, thus inhibiting the neuronal release of 5-HT from the pre-synaptic terminal.

To determine if the release of 5-HT is still possible following electrical stimulation, especially at the higher frequency of 20 Hz, experiments were done showing K^+-stimulated release of 5-HT following electrical stimulation of the dorsal raphé. Artificial CSF was replaced with an aCSF containing 120 mM KCl and 29 mM NaCl (to control for the osmolarity of the solution). This aCSF was continued for 80 min with samples being collected every 20 min and behavioral observations made every 10 min. The artCSF was then switched back to the normal 2 mM KCl and 147 mM NaCl for an additional 40 min.

Basal Release of 5-HT

In order to determine the effects of electrical stimulation of the dorsal raphé nuclei on the release of 5-HT in the dorsal hippocampal formation, basal release was determined. Animals were implanted with electrodes into the dorsal raphé nucleus and microdialysis probes into the dorsal hippocampus. The release of 5-HT was measured over a period of 6 h after probe insertion.

Electrical Stimulation of the Dorsal or Median Raphé Nucleus in Conscious Animals

After 5-HT levels stabilized, tonic electrical stimulation was done at 5, 10 or 20 Hz for 20 min. Animals were observed at the beginning of stimulation and every 20 min following the initiation of stimulation. Each animal received only one 20-min period of stimulation. Behavioral signs of aversive or proconvulsant behavior at the onset of stimulation resulted in a cessation of stimulation. Electrical stimulation was stopped after 20 min and six additional samples were collected at 20-min intervals over a 2-hour period.

In anatomical control animals, the stimulating electrode was implanted 1 mm lateral to the lateral border of the median raphé nucleus to serve as a control for specificity of the median raphé 5-HT release in the dorsal hippocampal formation. Dialysis and stimulation were carried out as outlined above.

Electrical Stimulation of the Dorsal Raphé Nucleus in Anesthetized Animals

In order to assess effects of electrical stimulation of the raphé on 5-HT release and in line with the experiments of Sharp et al. (1990), we also carried out a series of studies in anesthetized animals. Rats were anesthetized with 360 mg/kg chloral hydrate and were mounted in the stereotaxic apparatus. An electrode was implanted into the dorsal raphé nucleus and a microdialysis probe into the dorsal hippocampus. The probe was perfused with artCSF for 2 to 3 h until basal levels were stable. Electrical stimulation at 20 Hz was delivered for 20 min using the same stimulus parameters used in unanesthetized animals. Microdialysis continued for an additional 2 h after cessation of stimulation.

Histology

Following microdialysis studies, animals were anesthetized with pentobarbital. After a surgical level of anesthesia was reached, the heart was exposed and the animal perfused by intracardiac cannula with 100 cc buffered saline followed by 100 cc of 10% buffered formalin. The brain was removed and stored in buffered formalin. Brains were blocked to include the raphé nuclei and

hippocampal formation and were sectioned on a cryostat at 30–40 μm to verify the location of the stimulating electrode and the microdialysis probe. Slices were mounted and stained with cresyl violet (Nissl stain).

Statistical Analysis

Statistics were performed using SigmaStat v.1.01 (Jandel Scientific, San Rafael, CA). One-way ANOVAs were used to evaluate the effects of the experimental variables on the release of 5-HT as expressed as a percent of control. The transformation was done to normalize the data for variablity from animal to animal in the basal levels of 5-HT. A least significant differences (lsd) test was used for post-hoc comparisons. A value of $P < 0.05$ was considered significant.

RESULTS

Stable levels of 5-HT were routinely found in the dorsal hippocampal formation 2 to 3 h after insertion of a microdialysis probe. Basal levels of 5-HT in the dorsal hippocampus prior to experimental manipulation were between 1.5 and 3.5 fmol/20 μl of dialysate. As noted, the probe was inserted through a previously implanted guide cannula into a conscious, unrestrained animal. The animal was naive to the dialysis chamber at the beginning of the experimental day. During the initial hooking up of the electrode lead and insertion of the probe, animals showed exploratory activity. The animals showed no signs of stress or hyperarousal either before or after insertion of the probe. No behavioral changes were noted after insertion of the probe since this involved minimal handling. Soon (within 5 min) after insertion of the probe, animals were in a quiet, waking state. Histological verification showed probe tracts restricted to the dorsal hippocampal formation (Fig. 4A). Electrodes tips were localized in either the dorsal or median raphé nucleus (Fig. 4B and 4C, respectively).

We also showed that the release of 5-HT from the dorsal hippocampal formation was Na^+- and Ca^{++}-dependent. Addition of 10 μM tetrodotoxin (TTX) to the artCSF perfusing the probe resulted in an immediate, significant decrease in the release of 5-HT which persisted throughout the period of TTX perfusion ($F(8,26) = 12.3$, $P < 0.05$: Fig. 5). Levels of 5-HT in the dialysate were decreased to 17% of control levels after 1 h of TTX infusion. No gross behavioral changes were noted in these animals following TTX infusion.

Substitution of artCSF with Ca^{++}-free artCSF resulted in a decrease in extracellular 5-HT in the dorsal hippocampal formation to 33% percent of control levels (Fig. 6). This decrease was not as dramatic as the decrease seen with TTX infusion, but was significant over time ($F(8,24) = 5.46$, $P < 0.05$). The animals showed no changes in the still, alert state they were in prior to switching artCSF solutions.

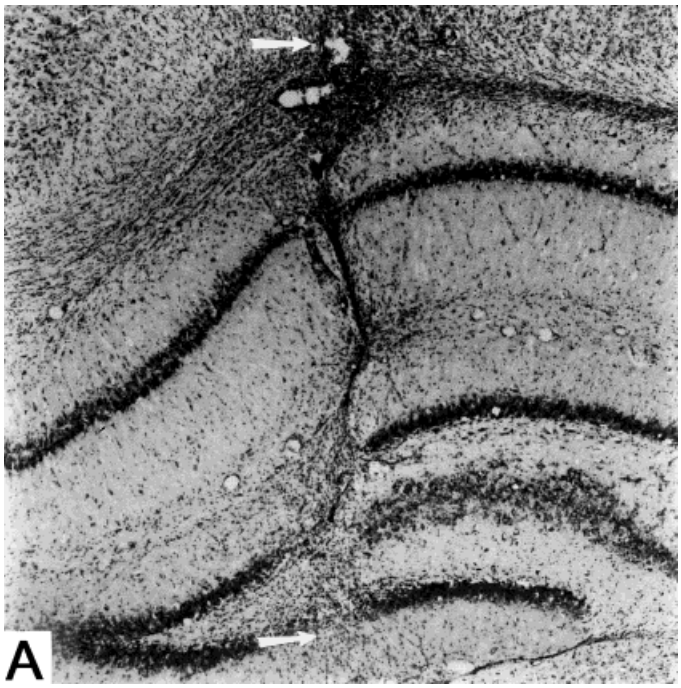
This marked decrease in release is in contrast to the pattern of 5-HT release in control animals. Over a period of 3 h of perfusion following stable basal release, there was a slow, non-significant decline in 5-HT release ($F(6,32) = 0.883$, n.s.: Fig. 7). Behaviorally, these animals showed a quiet, waking behavior throughout the experimental period. We found that some animals, toward the end of the control experimental period, appeared to showed brief periods of slow-wave sleep as indicated by eye closure and a curled-up position. No periods of hyperarousal were seen.

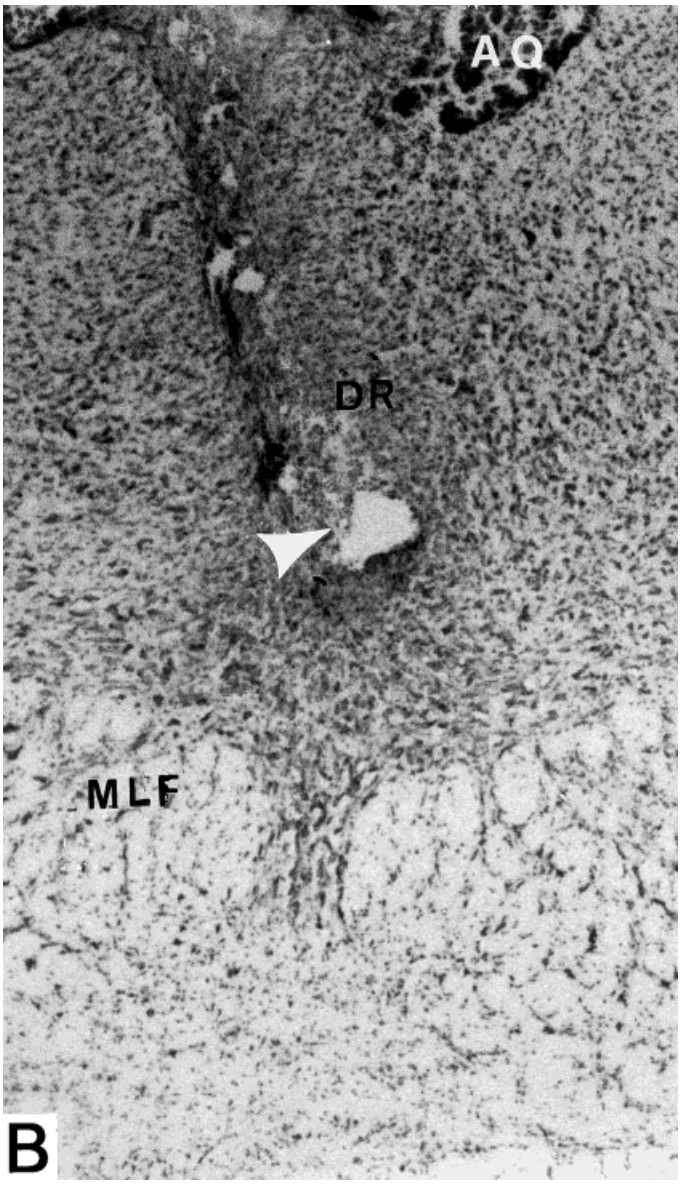
In a small number of animals, we increased the concentration of K^+ in the artCSF to 120 mM following studies showing decreases in release after electrical stimulation of the raphé. Over a period of 80 min, 5-HT in dialysate was increased fivefold ($F(7,15) = 8.01$, $P < 0.05$: Fig. 8). The levels of 5-HT declined throughout the period of KCl infusion and returned to control levels prior to the artCSF being switched back to 2 mM K^+. As in other experiments, these animals were in the still, alert state prior to the infusion of K^+. We observed significant increases in the level of arousal in these animals during high K^+ infusion. These behaviors included exploratory behavior, grooming and head shakes. This is of interest since relationships have been shown between raphé neuronal activity and 5-HT release, hippocampal theta and sniffing frequency during exploration. Furthermore, stimulation of postsynaptic 5-HT_2 receptors produces headshakes (Mokler et al., 1992; Darmani and Gerdes, 1995), suggesting that 5-HT release in the hippocampal formation may stimulate 5-HT_2 receptors resulting in headshakes.

Our studies have examined release of 5-HT from the dorsal hippocampal formation following electrical stimulation of the dorsal and median raphé nuclei in conscious rats. Tonic stimulation of the dorsal raphé at 20 Hz, 0.1 ms duration at 200 μA for 20 min produced an immediate decrease in 5-HT release during stimulation. This decrease in 5-HT release persisted for 2 h following cessation of stimulation (Fig. 9). This effect of raphé stimulation on 5-HT release was significant over the entire 2-h time period ($F(8,25) = 2.62$, $P < 0.05$). The behavioral state of the animals prior to stimulation was one of quiet waking. There was generally a brief alerting response and a short (5 s) period of exploration (moving about the cage) that accompanied both the onset and offset of stimulation. Otherwise, the animals remained in the quiet, waking vigilance state.

We also determined the effect of chloral hydrate anesthesia on release of 5-HT after stimulation of the raphé nucleus. In these studies, we used the same stimulus parameters used in behaving rats (above) and similar to those of Sharp et al. (1990). Release of 5-HT from the dorsal hippocampal formation increased to 252% of control values in a stimulus-bound manner (Fig. 10). This increase occurred over a 1-h period and then levels declined to non-significant levels.

Since the heaviest serotonin projections to the dorsal hippocampus are from the median raphé nucleus, we examined the release of 5-HT from the dorsal hippocampus following stimulation of the median raphé nucleus using the same stimulus parameters. Again, stimulation of the median raphé nucleus produced a significant decrease in the release of 5-HT from the hippocampal formation ($F(9,44) = 9.11$, $P < 0.05$; Fig. 11). This decrease occurred only





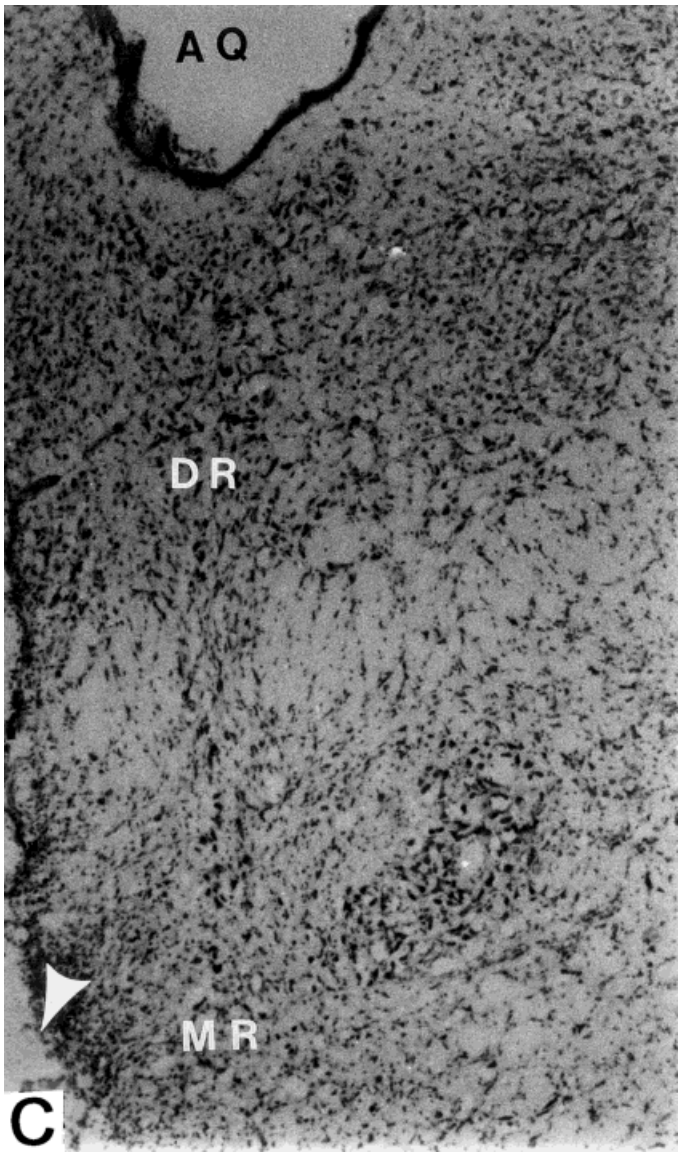


FIGURE 4. **A: Histological frontal section showing microdialysis sampling site in dorsal hippocampal formation. The active membrane length of the dialysis probe (3.0 mm) is designated by arrows. Note the closed, seam-like probe track limited to the dorsal hippocampal formation. The stimulating electrode in this instance is in the median raphé nucleus. Section is 50 μm thick. Nissl stain. Approximately 100×. B: Histological frontal section showing stimulating electrode with tip in dorsal raphé nucleus (arrowhead). Electrode is inserted at 20 degree angle to avoid blockage of aqueduct. Stimulation at this site in quiet-waking behavior was associated with decreased release of serotonin in the dorsal hippocampal formation. DR, dorsal raphé nucleus; Aq, aqueduct; MLF, medial longitudinal fasciculus. Section is 50 μm thick. Nissl stain. Approximately 100×. C: Histological frontal section showing stimulating electrode with tip in median raphé nucleus (arrowhead). Electrode is inserted at 20 degree angle to avoid blockage of aqueduct. Stimulation at this site in quiet-waking behavior was associated with decreased release of serotonin in the dorsal hippocampal formation. DR, dorsal raphé nucleus; MR, median raphé nucleus; Aq, aqueduct. Section is 50 μm thick. Nissl stain. Approximately 100×.**

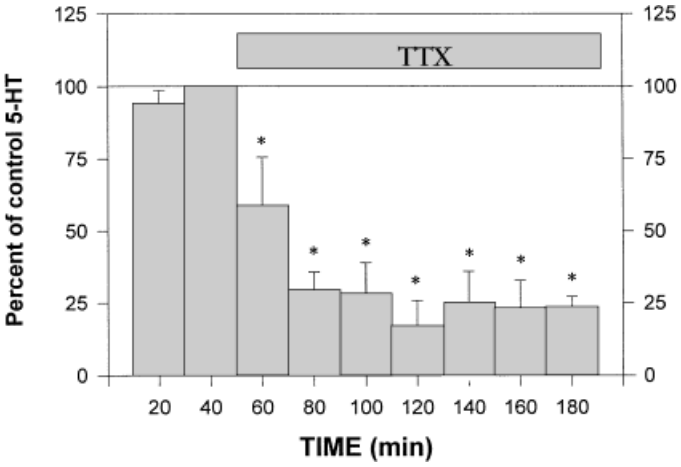


FIGURE 5. Release of 5-HT from the dorsal hippocampus of the rat before and after addition of tetrodotoxin (10 μM) to the artCSF. N = 3. *Significantly different from baseline (100%).

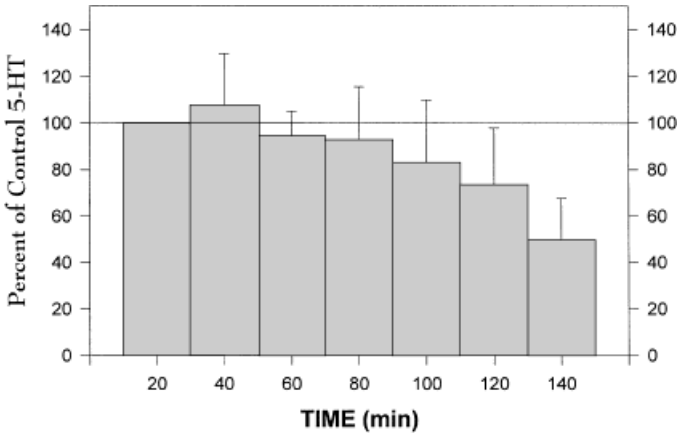


FIGURE 7. Basal release of 5-HT from the dorsal hippocampus of rats. Rats were in a quiet waking state throughout. N = 5. No significant changes were seen in release over the time course of the experiment.

after the cessation of stimulation and lasted for the duration of the experiment (another 2 h). Again, such stimulation of the median raphé did not alter behavior. In experiments where the electrode was placed 1 mm lateral to the lateral border of the median raphé nucleus, stimulation at 20 Hz did not alter 5-HT release in the hippocampal formation in a manner different from unstimulated controls.

Finally, in order to determine the frequency dependence of this stimulation effect, we also stimulated the median raphé at 5 and 10 Hz. Stimulation at 10 Hz produced a variable response in three animals (Fig. 12), but overall produced an effect intermediate between 5 and 20 Hz (Fig. 13). Stimulation at 5 Hz did not alter 5-HT release in the dorsal hippocampal formation ($F(6,28) = 1.51$, n.s., Fig. 12). Thus, there was a frequency dependent decline in the release of 5-HT from the dorsal hippocampus following electrical stimulation of the median raphé nucleus (Fig. 13).

DISCUSSION

The serotonergic raphé-hippocampal pathways have powerful effects on hippocampal EEG activity, synaptic plasticity and cognitive behavior. The functions of the two distinct serotonin forebrain projection systems (one from the nucleus raphé dorsalis and one from the nucleus raphé medianus) include a behavioral (vigilance) state-dependent control of hippocampal activity closely related to information flow (through-put) in the hippocampal trisynaptic circuit and information processing. These latter can only be studied in behaving animals in which the functional integrity of these serotonergic systems is not compromised by anesthesia. Since self-regulation of the activity of neurons relates directly to the amplitude of the neuron's chemical signal, it is essential to examine this signal by in vivo microdialysis in awake animals in which all afferent pathways are functionally intact.

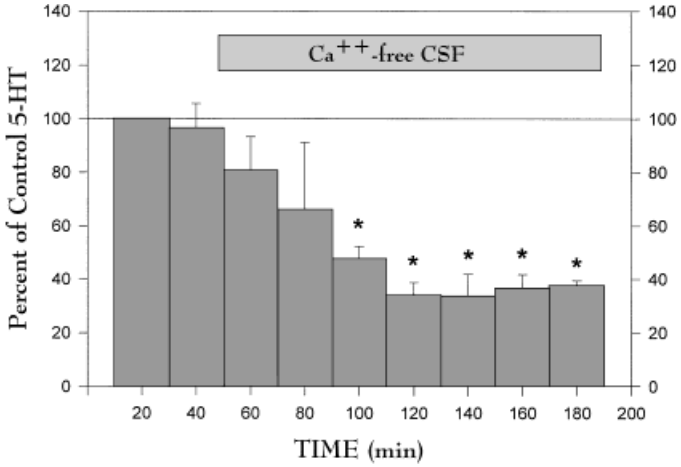


FIGURE 6. Effects of switching to a Ca^{++}-free artCSF on the release of 5-HT from the dorsal hippocampus. N = 3. *Significantly different from control (100%) levels, $P < 0.05$.

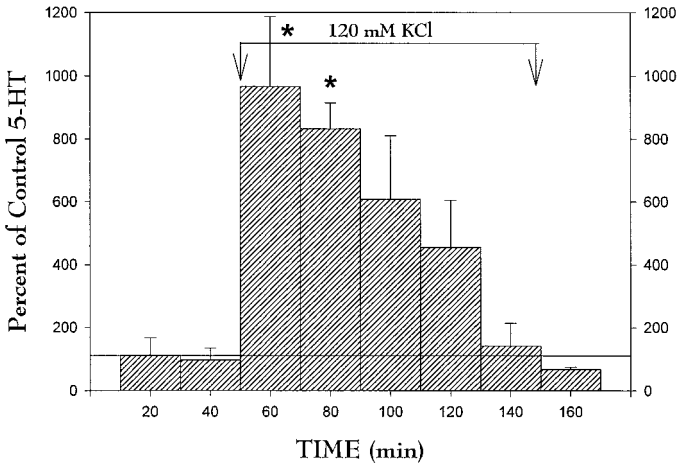


FIGURE 8. Effects of KCl on the release of 5-HT from the dorsal hippocampus. Artificial CSF was changed after stimulation. N = 2. *Significantly different from control, $P < 0.05$.

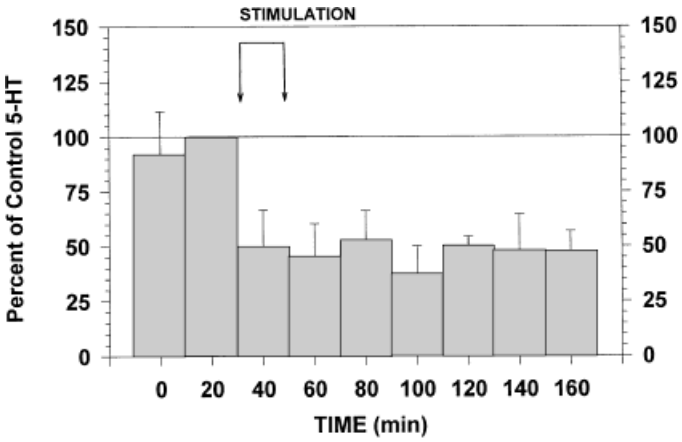


FIGURE 9. Electrical stimulation of the dorsal raphé at 20 Hz and the release of 5-HT from the dorsal hippocampus in unanesthetized rats. Bars represent mean level of 5-HT in dialysate during 20 min period. There was a significant effect of stimulation on 5-HT levels over time, although there were no significant differences between points. N = 5.

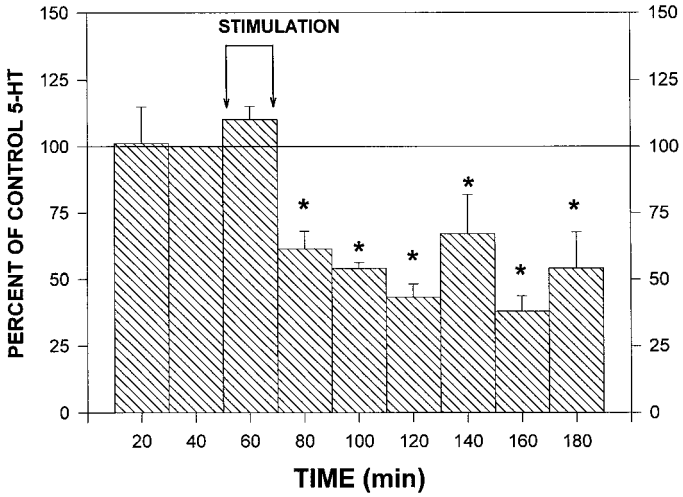


FIGURE 11. Release of 5-HT from the dorsal hippocampus of conscious rat following electrical (20 Hz) stimulation of the median raphé nucleus. Note the difference during stimulation with Figure 9, dorsal raphé stimulation. N = 5. *Significantly different from control levels.

The functional specialization between the two midbrain raphé nuclei in terms of pathway projections and termination on different types of GABAergic interneurons in the hippocampal formation is of considerable interest relative to the present findings (Freund et al., 1990; Halasy et al., 1992). While both the nucleus raphé dorsalis and nucleus raphé medianus utilize serotonin as their principal neurotransmitter, they differ in several important ways: 1) they give rise to separate, but partially overlapping, projections to the hippocampal formation and other areas of the forebrain. In particular, they each project dominantly to specific regions of the hippocampal formation, i.e., the ventral and dorsal hippocampal formation, respectively; 2) each nucleus has axons that are structurally distinct with two types of morphologically identified axons and axon terminals; 3) they differ in their pharmacological properties, particularly their susceptibility to neurotoxic amphetamines; and 4) each nucleus projects to distinct types of GABA interneurons in the hippocampal formation. All of these distinctions likely relate to important functional differences between the nucleus raphé dorsalis and nucleus raphé medianus.

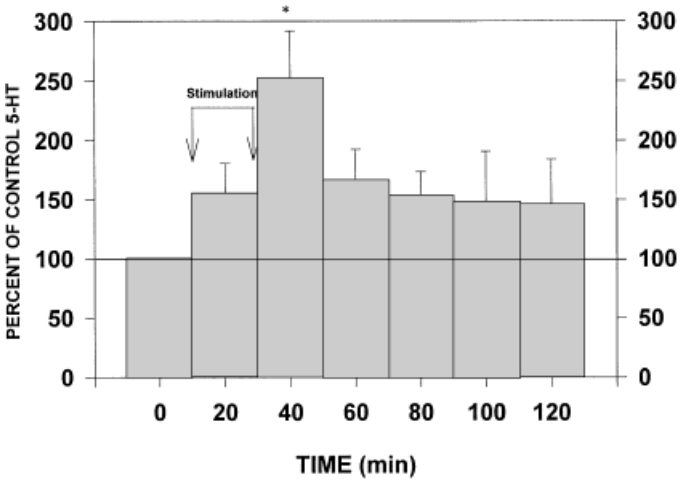


FIGURE 10. Effects of electrical stimulation of the dorsal raphé nucleus on the release of 5-HT from the dorsal hippocampus of anesthetized rats. Note difference with Figure 9 (unanesthetized animals). N = 4. *Significantly different from control levels, $P < 0.05$.

Our principal finding is that electrical stimulation of each raphé nucleus in conscious animals results in a distinct pattern of decreased release of 5-HT in the dorsal hippocampal formation. In studies similar to those of Sharp et al. (1990), we have replicated their findings in anesthetized animals, i.e. dorsal raphé stimulation resulted in a significant increase in 5-HT release in the hippocampal formation. Thus, it is clear from our results that 5-HT release in the hippocampal formation is consciousness-dependent. As is well known, the sleep-waking (vigilance) states are clearly related to raphé neuronal activity and, hence, 5-HT release in the hippocampal formation relates directly to raphé cell firing. In the anesthetized animal there are many factors involved in "release" of raphé neurons from feedback inhibition. Thus, anesthesia may block short and/or long-loop negative feedback regulation, thereby enhancing raphé cell firing and subsequently 5-HT release in terminal areas.

One essential factor in assessing the relative roles of the dorsal and median raphé nuclei in serotonin release in the hippocampal formation relates to the interrelations between these two nuclei. It is clear from a variety of studies that they are complexly interlinked by reciprocal connections (for review see Vertes and Kocsis, 1994). These investigators showed that the nucleus raphé dorsalis projects heavily to the nucleus raphé medianus. Two other studies using retrograde tracing techniques showed dense projections from the dorsal raphé to the median raphé (Marcinkiewicz et al., 1989; Behzadi et al., 1990). Kalen et al. (1988a) and Vertes and Martin (1988) demonstrated heavy projections from the

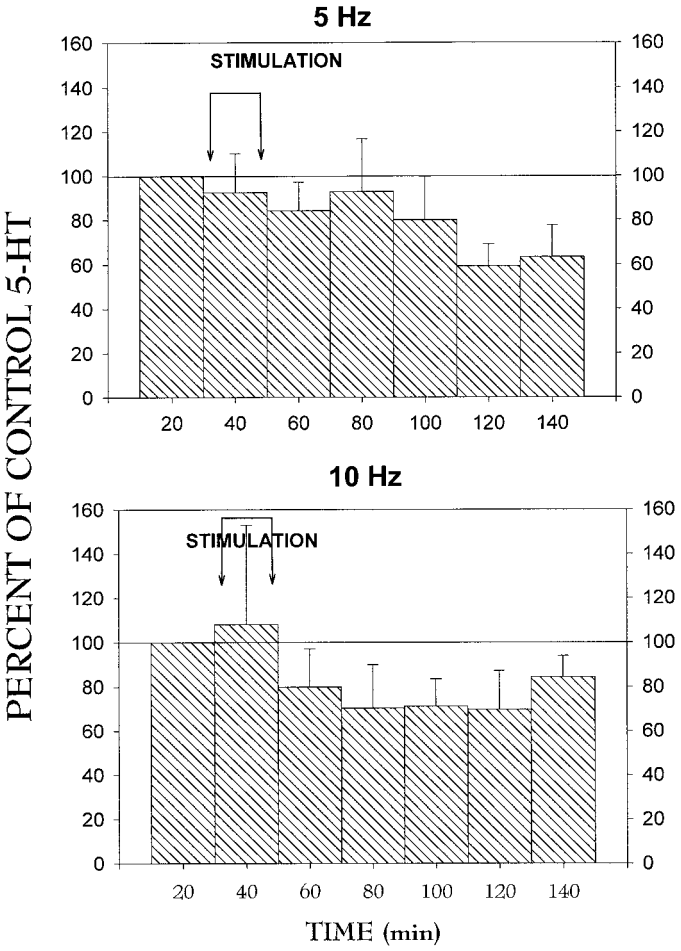


FIGURE 12. Effects of electrical stimulation of the median raphé at 5 and 10 Hz on release of 5-HT from the dorsal hippocampus. Neither 5 nor 10 Hz stimulation produced a significant effect of extracellular 5-HT release.

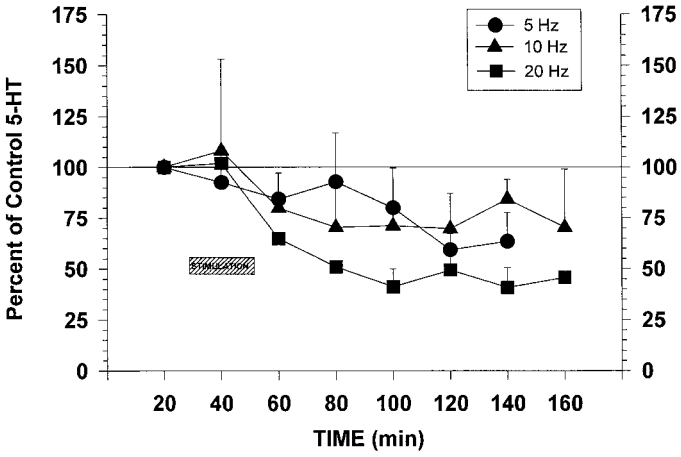


FIGURE 13. Electrical stimulation of the median raphé at 5, 10 and 20 Hz and the release of 5-HT from the dorsal hippocampus of the conscious rat. 5 Hz and 10 Hz stimulation did not alter 5-HT release from basal levels (Figure 7).

median raphé to the dorsal raphé, thus confirming reciprocal connections between these two midbrain raphé nuclei. The dorsal raphé and median raphé are clearly involved in separate, rather non-overlapping sets of functions, despite the strong reciprocities between these nuclei, thus pointing to strong functional interplay between them (Vertes, 1990). In terms of serotonin release in the hippocampal formation following electrical stimulation of each midbrain raphé nucleus, it seems likely that stimulation of one would directly affect the other, thus potentially complicating interpretation of the effects of stimulation of each nucleus.

The present study presents two distinct patterns of 5-HT release following stimulation of the dorsal raphé and the median raphé. In the case of dorsal raphé stimulation, 5-HT levels in the dorsal hippocampal formation decline during the period of stimulation and remain low for the remainder of the experiment (2 h). In contrast, stimulation of the median raphé nucleus does not produce a decline until after cessation of stimulation. At the cessation of stimulation 5-HT levels decline to 60% of control and remain low throughout the remainder of the experiment (Fig. 11). A possible explanation for this difference may be found by examining the relationships between these raphé nuclei and the selective innervation of the hippocampal formation by each of these nuclei. Reciprocal innervation between the two nuclei is, in large part, likely to be inhibitory, i.e., serotonergic and/or GABAergic, though this remains to be tested experimentally. Given this reciprocity, then dorsal raphé stimulation would result in inhibition of median raphé cell firing and, hence, produce an immediate decrease in extracellular 5-HT in the dorsal hippocampal formation. Electrical stimulation of the median raphé nucleus, however, would not produce an immediate 5-HT decline in the hippocampal formation since the electrical stimulation would continue to drive the neurons to release 5-HT, even while feedback mechanisms are operating to inhibit cell firing. This becomes apparent when stimulation ceases, in that we see an immediate decrease in hippocampal extracellular 5-HT (Fig. 11).

The time resolution of technique in this experiment is 20 min. This is a very long period to measure changes that may be occurring at the synapse over periods of milliseconds or seconds. Thus, the lack of change that is seen during median raphé stimulation may be the result of an initial increase in release followed by a prolonged suppression, ending in a net change that was not different from baseline. Further experiments using shorter time periods would be needed to test this hypothesis.

Another important consideration is the role of autoreceptors. As is well known, the regulation of raphé activity is under complex control by processes involving autoreceptors on raphé soma and dendrites and on terminals of raphé projections. Of interest in regard to the present study is the long duration of the suppression of 5-HT release following electrical stimulation of the dorsal raphé or median raphé nucleus. Unlike data in anesthetized animals, where electrical stimulation results in a stimulus-bound increase in release of 5-HT (the present study and Sharp et al., 1990), electrical stimulation of either raphé nucleus in conscious animals produces a long-term inhibition of release. The diminished 5-HT release is not due to exhaustion of 5-HT stores since KCl stimulation immediately results in a marked enhancement of

release (Fig. 8). Therefore, the decrease in serotonin release is likely due to long-term negative feedback depressing raphé cell firing. It is possible that activation of somato-dendritic 5-HT_{1A} and/or 5-HT_{1D} autoreceptors, which regulate raphé cell firing, may result in this long-term suppression of 5-HT release in the hippocampal formation. The present studies cannot rule out a role for 5-HT_{1B} receptor involvement on terminals, or inhibition of synthesis of 5-HT in this suppression of release in the hippocampal formation. Hallucinogenic drugs also may produce a long-term suppression of raphé cell firing (Aghajanian et al., 1972; Haigler and Aghajanian, 1973; Jacobs, 1978) and suppression of 5-HT release in the hippocampus and frontal cortex (Garratt et al., 1991; Johnston et al., 1997) by activation of long-loop feedback mechanisms from the forebrain to the raphé nuclei (Wang and Aghajanian, 1977). This may also explain the long course of action of hallucinogenic drugs in humans.

The current experiments have established that the release of 5-HT is stable in the hippocampal formation of conscious, behaving animals in a quiet-waking state, 2–3 h after insertion of a microdialysis probe through a previously implanted guide cannula. This release of 5-HT is dependent upon Na^+ channels and extracellular Ca^{++}, and is markedly enhanced by increases in extracellular K^+. Of interest is that we also have found that the insertion of the probe into a conscious, behaving animal without restraint or undue stress is essential for stable 5-HT levels to be achieved quickly. Furthermore, dialysis of the hippocampal formation for up to 8 h did not produce significant damage to the neuropil as indicated by histological examination. Previous studies have shown that at least 12 h post-implantation time is required for stable release of 5-HT to be attained. However, those studies involved implantation of the probe under surgical anesthesia without the use of a guide cannula (Kalen et al., 1988b). Thus, the effects of tissue damage, as well as anesthesia, are considerations in interpreting previous findings.

Our current experiments also point to the frequency dependence of effects of electrical stimulation on 5-HT neurons. Stimulation at frequencies close to the physiological frequency of raphé neurons (0.5 to 2 Hz) did not produce sufficient 5-HT release to alter basal levels, whereas higher frequencies resulted in decreased 5-HT release dependent on the frequency, i.e., 20 Hz resulted in a greater decrease in release than 10 Hz, whereas 5 Hz produced minimal changes in 5-HT release. This agrees with the work of Sharp et al. (1990), in that stimulation of the raphé at frequencies close to physiological frequencies does not evoke a detectable change in the release of 5-HT in terminal regions. Florin-Lechner et al. (1996) found similar results in the levels of norepinephrine in the prefrontal cortex after stimulation of the locus coeruleus.

In summary, using the intact, non-anesthetized preparation, we can better provide a physiological preparation in which vigilance state control of 5-HT release, via monoamine and cholinergic inputs from the brain stem, provides the most useful paradigm to assess normal function in the 5-HT-hippocampal system. Further studies examining the relations between the nucleus raphé dorsalis and medianus by electrical stimulation of each and microdialysis of the other may shed additional light on the complexities of the raphé-hippocampal system. Pharmacological studies are planned to elucidate the role of the several 5-HT autoreceptors in the regulation of 5-HT release in the raphé nuclei, hippocampal formation, and other forebrain areas. Additionally, studies are in progress to examine 5-HT release across the vigilance state, which have been shown by Winson (1980) to selectively gate information flow through the hippocampal trisynaptic circuit.

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