Decreased dorsal raphe nucleus neuronal activity in adult chloral hydrate anesthetized rats following neonatal clomipramine treatment: implications for endogenous depression

Gene G. Kinney, Gerald W. Vogel, Pingfu Feng

Abstract

Although the biological cause of endogenous depression is unknown, one commonly held hypothesis proposes that depression results, in part, from decreased central serotonin (5-HT) neurotransmission. Previous research found that clomipramine (CLI) treatment of neonatal rats produced, in adult rats, a variety of behavioral and physiological dysfunctions resembling those found in human endogenous depression. It was later reported that adult CLI-treated rats exhibited a decreased discharge of 5-HT neurons in the dorsal raphe nucleus (DRN) compared with control rats. This finding, however, was not replicated in subsequent studies that detected differences in DRN receptor function. Several factors were identified that may have contributed to the inability of the latter studies to detect CLI vs. control differences in DRN firing rates and interspike interval histograms (ISIH). Among these were the anesthetic used, the age at which the adult rats were tested, and the location of the recording electrode. The present study controlled these variables by using chloral hydrate anesthesia, testing ‘depressed’ rats at both 2 and 3 months of age, and verifying electrode location using standard histological techniques. We found that DRN unit firing in ‘depressed’ rats (0.417 ± 0.071 spikes/s) was less than half that of ‘non-depressed’ control rats (i.e. neonatal saline treatment 0.968 ± 0.12 spikes/s). Additionally, ISIH’s indicated that, in addition to the lower firing rate of 5-HT DRN neurons, adult CLI rats had an altered temporal discharge pattern of these neurons. Thus, the ISIH of 5-HT DRN neurons recorded from CLI rats was characterized by a flat distribution suggesting random temporal firing patterns. These results confirm previous findings of decreased DRN firing rates and flat ISIH’s in ‘depressed’ rats and extend previous findings to younger rats of a different strain. The results thereby lend support to the hypothesis of a role for decreased central 5-HT as a substrate for the behavioral deficiencies observed in endogenous depression and suggest that these deficiencies may also result, in part, from a random, rather than orderly, temporal pattern of discharge in these neurons.

Keywords: Depression; Serotonin; Dorsal raphe nucleus; Neonatal clomipramine; Electrophysiology; Rat

1. Introduction

Chronic clomipramine (CLI) treatment of neonatal rat pups for 14 days produces a myriad of behavioral deficiencies in adulthood which, collectively, approximate symptomatology indicative of clinical endogenous depression [27,31]. For example, CLI-treated animals exhibit decreased sexual activity [18,34], increased open-field locomotor activity [10], decreased intracranial self-stimulation [32] and decreased aggressive behavior [29]. Adult CLI rats also exhibit rapid-eye-movement (REM) sleep abnormalities that are similar to those seen in human endogenously depressed patients [33]. For example, increased REM sleep percentage of total sleep time, decreased REM latency, an increase in phasic events associated with REM sleep, and an abnormal temporal pattern of REM rebound following REM sleep deprivation [33]. Further, similar to the human disorder, CLI rats have a higher core body temperature during periods dominated by sleep (i.e. the light phase) [20]. Preliminary tests have demonstrated that compromised behaviors exhibited by CLI-treated adult rats can be reversed following semi-chronic antidepressant treatment or REM sleep deprivation by arousal [30,31]. This later finding is consistent with the finding in humans...
that REM sleep deprivation alleviates symptomatology associated with endogenous depression [28].

It has been widely hypothesized that depression results from a decrease of central serotonergic activity and, thus, many antidepressants have been designed with the goal of increasing central serotonin [5,6,16]. The midbrain raphe nuclei (viz. median and dorsal) provide the majority of ascending serotonergic input to forebrain structures [12,17,23,25]. It is known that manipulation of the serotonergic systems within these nuclei produce abnormalities similar to those seen in depression (e.g. sexual activity, locomotion, and core body temperature [11]). Thus, it might be expected that the activity of neurons in the dorsal raphe nucleus (DRN) would be abnormal if this area is critically involved in clinical depression. Attempts to measure neurophysiological abnormalities in animal models of depression have been limited. Our laboratory has previously demonstrated a reduction in the firing rate of neurons in the DRN in adult rats that had been neonatally treated with CLI [35]. However, more recent reports found no differences in baseline firing rates between CLI-treated animals and normal animals but did find differences in DRN receptor function as indicated by reduced responsiveness to intravenously administered citalopram [14,15]. Several methodological differences between these sets of studies may, in part, account for the discrepancy regarding differences in baseline firing rates between CLI neonatally treated animals and those treated with saline (SAL). For example, in the study of Yavari et al. [35], data were collected from rats anesthetized under pentobarbital anesthesia, whereas in the latter case, the more common method of chloral hydrate anesthesia was used. Further, in one study performed by Mauduit and colleagues, 2 month old rats were utilized [14], whereas previous behavioral [10,18,30–34] and electrophysiological [35] studies typically used animals 3 months of age or older.

In an effort to reassess possible differences in firing rates between adult rats neonatally treated with either CLI or SAL, and to make those results comparable with studies which have utilized the more widely used anesthetic for such studies (i.e. chloral hydrate), the present study examined the effect of neonatal CLI treatment on the firing characteristics of DRN neurons in chloral hydrate anesthetized rats. Further, the spontaneous firing rates of DRN neurons in rats at both 2 and 3 months of age were assessed to control for the possibility of age-related differences.

2. Materials and methods

120 Male Long–Evans rat pups were cross fostered and treated as described previously [10,18,30–34]. Briefly, the rat pups were randomly assigned to receive either clomipramine or saline injections (n = 60 per group). Corresponding to their group, rat pups were given s.c. injections from days P8 to P21 of either clomipramine (20 mg/kg s.c. twice daily; Sigma Chemical Company, St. Louis, MO) or an equivolume of saline twice daily. Of the 60 rats in each group, 6 CLI-treated and 6 SAL-treated animals were randomly chosen for testing at 2 months of age (tested between 2 months 6 days and 2 months 15 days of age) and an additional 5 CLI-treated and 6 SAL-treated animals were randomly chosen for testing at 3 months of age. For each testing period, animals were anesthetized with chloral hydrate (250 mg/kg) and cortical blood flow was monitored by an infrared oximeter to ensure adequate anesthesia. DRN neurons were recorded in rats anesthetized with chloral hydrate and tested at 2 months of age. Of the 60 rats in each group, 6 CLI-treated and 6 SAL-treated animals were randomly chosen for testing at 2 months of age (tested between 2 months 6 days and 2 months 15 days of age) and an additional 5 CLI-treated and 6 SAL-treated animals were randomly chosen for testing at 3 months of age. For each testing period, animals were anesthetized with chloral hydrate (250 mg/kg) and cortical blood flow was monitored by an infrared oximeter to ensure adequate anesthesia. DRN neurons were recorded in rats anesthetized with chloral hydrate and tested at 2 months of age.

Table 1 Mean DRN firing rates (spikes/s ± S.E.M.) in rats treated neonatally with either saline (SAL) or clomipramine (CLI) and tested at 2 or 3 months of age

<table>
<thead>
<tr>
<th>Neonatal treatment</th>
<th>Age at time of testing (months)</th>
<th>Pooled average</th>
<th>Total n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 months</td>
<td>3 months</td>
<td></td>
</tr>
<tr>
<td>SAL</td>
<td>1.0998 ± 0.25 (n = 8)</td>
<td>0.898 ± 0.131 (n = 15)</td>
<td>0.968 ± 0.12</td>
</tr>
<tr>
<td>CLI</td>
<td>0.3963 ± 0.103 (n = 10)</td>
<td>0.4301 ± 0.099 (n = 15)</td>
<td>0.417 ± 0.071</td>
</tr>
<tr>
<td>Total n</td>
<td></td>
<td></td>
<td>48</td>
</tr>
</tbody>
</table>

Fig. 1. Mean firing rates (spikes/s) for all serotonergic DRN neurons recorded in 2- and 3-month-old rats which had been neonatally treated with saline (SAL, open bars) or clomipramine (CLI, shaded bars). Note significantly lower firing rates in CLI-treated animals tested at both 2 and 3 months of age. Error bars represent standard error from the mean (S.E.M.). * * P < 0.006, * * * P < 0.005.
age (tested between 3 months 0 days and 3 months 10 days of age). Prior to testing, rats were group housed in an isolated colony room with a reversed light-dark cycle (lights on at 1330 h), and allowed access to food and water ad libitum. Animals were cared for in strict compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (Pub. 85–23, 1985).

Rats were anesthetized with 400 mg/kg chloral hydrate injected intraperitoneally (i.p.). After rats were completely anesthetized (i.e. lack of hind-limb withdraw reflex and eye-blink reflex), rats were placed in a stereotaxic frame and an incision was made to expose the skull. Using a steel burr, a small hole was drilled over the dorsal raphe nucleus to allow for the passage of the recording electrode (AP + 1.0 mm from lambda, L ± 0.0, H = 5.5 to 7.0 mm) [19]. Single-unit recordings were made from the DRN using two (i.e. one active and one reference) Teflon insulated fine-stainless steel microelectrodes (250 μm in diameter; tip diameter = < 1 μm; impedance range = 4 to 6 MΩ) (Frederick Haer and Company, Brunswick, ME) which had been joined using standard epoxy such that the tips of the electrodes were separated by ≈ 1 mm. The electrodes were lowered into the DRN using a Narishige (model WR-60) manually operated hydraulic microdrive. Typically 2–5 cells were recorded per animal in testing sessions lasting approximately 3–4 h. Spontaneous unit activity was recorded for 15–40 min for each isolated cell. Unit activity was amplified, filtered (300–10 kHz) (WPI model

![Figure 2](image)

**Fig. 2.** Interspike interval histogram (A,B), action potential (C,D), and spike train (E,F) of a representative DRN 5-HT neuron in a SAL (A,C,E) and CLI (B,D,F) rat. Note the higher peak in the SAL-treated rat (A) as compared to a lower peak and more widely distributed distribution in the CLI-treated rat (B) indicating a lower firing rate and less regular discharge pattern in this CLI rat. The lower firing rate is further depicted in the actual record samples of these cells (E and F for SAL and CLI rats, respectively). Note also the characteristic shape of the serotonergic action potential in both the SAL-treated and CLI-treated rat (C and D, respectively). These neurons have a biphasic spike with a notch on the rising phase, typical of serotonergic neurons.
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DAM70 and iso-DAM8 differential amplifiers), and recorded on standard VHS tape following processing with an A.R. Vetter Co. PCM recording adaptor (model 4000).

At the conclusion of each experiment, a low DC current was passed through the recording electrode (20 μA for 15–20 s) and the rats were perfused transcendally (300 ml normal saline followed by 300 ml 10% phosphate-buffered formalin saturated with potassium ferrocyanide) under deep anesthesia. The brains were removed and stored in a 1:1 solution of 40% formaldehdy and 95% alcohol for no longer than 5 days. The brains were subsequently sectioned at 60 μm using a vibratome (WPI Vibroslice VSL). Sections were mounted on gelatin-coated slides, stained with Cresyl violet, and microscopically inspected for electrode placement.

Only neurons which met the following criteria were included for analysis: (1) the units were encountered at the appropriate stereotaxic coordinates [19]; (2) the units showed a bi-phasic action potential, preferably with a notch on the rising phase; (3) the units showed a slow non-bursting firing pattern; (4) the action potentials were 1–3 ms in duration; (5) the firing rates were ≤ 2.5 spikes/s; (6) the recording provided a sufficiently adequate signal to noise ratio, such that the unit could be clearly identified and isolated; (7) the histology verified that the unit recorded (or the last unit in a given tract) was within the DRN, as defined anatomically [19]. These criteria were incorporated from previous studies [1–4,14,15,34], and provided conservative inclusion criteria for serotonergic DRN neurons in the present study. The inclusion/exclusion of a spike train for analysis was done blind to experimental treatment. Firing rates and interspike intervals (ISIH; bin width = 0.1 s) were determined from DRN unit activity which was analyzed off-line using an analog-to-digital converter (12 bit resolution) and commercially available software (1401 plus A–D board and Spike2 software; Cambridge Electronic Design, Cambridge, UK).

Prior to digital sampling, the unit’s spike train was processed with a dual-window discriminator (model SA-23T; SA Instrumentation, San Diego, CA) to isolate single spikes. Differences between groups were determined using one-tailed t-tests and analyses of variance, when appropriate. A difference was considered significant when P ≤ 0.05.

3. Results

The cellular activity of 76 neurons was recorded on tape for further off-line analysis. Of these 76 neurons, 48 were included for final analysis based on the exclusionary criteria described above (18 of 22 in 2-month-old rats; 30 of 54 in 3-month-old rats; see also Table 1). As shown in Fig. 1 and Table 1, the firing rate of DRN neurons in CLI-treated animals was significantly lower than in SAL-treated animals in both 2-month-old (P = 0.006) and 3-month-old (P = 0.005) rats. A two-way analysis of variance confirmed a significant difference between DRN firing rates in CLI and SAL-treated rats (F(1,44) = 16.868, P < 0.0003), while there was no significant effect of age or treatment × age interaction (P’s > 0.55 and 0.41, respectively).

Fig. 2 shows the interspike interval histogram (A,B), action potential (C,D), and spike train (E,F) of a representative DRN 5-HT neuron in a SAL (A,C,E) and CLI (B,D,F) rat. As seen in Fig. 2 and previously shown in Fig. 1 (group data), the spontaneous firing rate of DRN neurons

![Fig. 3. The average interspike interval histogram (ISIH) for all SAL (fitted bars) and CLI (open bars) neurons recorded. Note the pronounced peak in SAL rats between 0.4 and 0.7 s, whereas the average ISIH for all CLI rats is flat and widely distributed indicating a non-consistent pattern of discharge. The average ISIH was constructed from 5-min segments, bin width = 0.1 s, 150 bins. Note that one atypical CLI neuron was excluded during the construction of the average ISIH for this group (see text for details).](image)
in CLI-treated animals is significantly lower than in SAL-treated animals. The ISIH of the SAL-treated rat contained a single peak at 0.653 s (Fig. 2A), whereas the ISIH of the CLI-treated rat was flattened and widely distributed indicating a lower firing rate as well as a randomized firing pattern (i.e. an approximately equal probability of all interspike intervals – see also Fig. 3). The reduced firing rate in CLI-treated rats is further illustrated in the trace of neuronal activity depicted in Fig. 2E,F. Note that the firing rate of the DRN 5-HT neuron in the SAL-treated rat (E) is approximately 2 to 3 times greater than seen in the CLI-treated rat (F).

With regard to temporal discharge patterns, Fig. 3 represents the ISIH averaged across all SAL and CLI neurons which were included for analysis. It should be noted that one CLI cell included in all other analyses, was excluded during the construction of the average ISIH for the CLI group. This was done following an assessment of the ISIH for each neuron in which one CLI cell was found to have an atypical response pattern (i.e. more similar to the distribution typically observed in SAL rats). Thus, in order to obtain a more representative depiction of the CLI 5-HT DRN population, this cell was excluded. As seen, there is a single peak at approximately 0.5–0.7 s in SAL

Fig. 4. Photomicrograph isolating a recording electrode site (small lesion created by DC current at the conclusion of the recording session) in the dorsal raphe nucleus. Abbreviations: DRN, dorsal raphe nucleus; CG, central gray; 4n, trochlear nucleus; Aq, cerebral aqueduct; mlf, medial longitudinal fasciculus; xscp, decussation of the superior cerebellar peduncle. Scale bar located in the lower right – 300 μm
rats. Note, however, the difference in distribution between the two groups. Most notably, the decay in SAL rats is quite rapid, whereas this decay is much slower in CLI-treated rats, such that, CLI-treated rats have higher counts than SAL rats from 2.5 s until over 6 s. The more variable, flattened distribution in the CLI group represents a more randomized (i.e. less regular) firing pattern of DRN neurons in these rats, in addition to the lower firing rate of these neurons.

It appears unlikely that the findings reported herein are a result of a small sample of neurons or rats with extreme firing patterns. Thus, the mean and median firing rates are similar for all units (pooled across age) recorded from SAL (0.968 and 0.878 spikes/s, respectively) and CLI rats (0.416 and 0.329 spikes/s, respectively). Further, when the mean of all the mean firing rates for each rat was determined: (a) mean firing rates for SAL (0.991 spikes/s) and CLI (0.398 spikes/s) rats were similar to those seen when the mean rates were determined for all units recorded (see above); and (b) the difference between SAL and CLI firing rates remained highly significant ($P < 0.005$).

Fig. 4 shows a photomicrograph of a coronal section at the level of the pons, localizing a typical recording electrode site in the DRN.

4. Discussion

The primary finding of the current study is that the baseline firing rates of 5-HT neurons located within the DRN are significantly lower in CLI-treated animals than in SAL-treated animals. Given the fact that neonatal CLI treatment has been previously shown to produce a behavioral syndrome that approximates clinical endogenous depression [27,31], the present findings lend support to the hypothesis that decreased central serotonin contributes to depressive disorders [5,6,16]. Thus, in this animal model of depression, decreased DRN activity may lead to lower serotonergic levels in DRN projection areas.

These results confirm the previous findings of Yavari et al. [35]. The mean firing rate of SAL- (0.968 vs. 0.93 spikes/s) and CLI- (0.417 vs. 0.21 spikes/s) treated animals in the present study is comparable to those reported by Yavari et al. [35]. Thus, a one-tailed $t$-test demonstrated no significant difference between the findings in the present study and those found by Yavari et al. [35] ($P's > 0.05$). The present study also extends the findings of Yavari et al. [35], which utilized 5–7-month-old male Sprague–Dawley rats, to 2- and 3-month-old male Long–Evans rats.

In similar studies, Maudhuit et al. [14,15] found no significant differences in firing rates of 5-HT DRN neurons between CLI- and SAL-treated rats. Several possibilities have been suggested to explain these different outcomes. (1) The anesthetic used: Yavari et al. [35] used sodium pentobarbital, an atypical anesthetic for such studies, while Maudhuit et al. [14,15] used chloral hydrate, the typical anesthetic for such studies. Maudhuit et al. [14] cited this difference as a possible reason for their failure to confirm the Yarvari et al. [35] finding. However, the results of the present study using chloral hydrate are in contradiction to this explanation. (2) Rat age: the study of Yavari et al. [35] used 5–7-month-old rats while the initial study of Maudhuit et al. [14] used 2-month-old rats, suggesting that rat age might account for the different outcomes. However, in a subsequent study [15], the Maudhuit group failed to find a CLI-SAL difference in 5–7-month-old rats while the present study found differences in 2- and 3-month-old rats. Thus, rat age cannot account for the differences in the observed outcomes. (3) Clomipramine dose: in the present study CLI neonatal rats were injected with $20 \text{ mg/kg}$ clomipramine twice daily, whereas in the studies of Maudhuit and colleagues [14,15] a dose of $15 \text{ mg/kg}$ twice daily was used. However, in the study of Yavari et al. [35], where results were found similar to those reported here, the dose of $15 \text{ mg/kg}$ twice daily was used. Thus, dose cannot account for these differences. (4) Recording time: in the present study baseline firing rates were collected for between 15 and 40 min (typically 30–35 min), whereas in the studies of Maudhuit and colleagues [14,15] only 5–10 min of unit activity was sampled prior to pharmacological manipulations. It may be the case that the initial transient increase in discharge typically seen following the isolation of a neuron artificially altered the computation of firing rates, whereas this would not be the case during longer periods of collection. (5) Location of recording electrodes: in the present study, as well as in the study of Yavari et al. [35], the atlas of Paxinos and Watson [19] was used to establish an anterior-posterior (A.P.) coordinate of 1.0 mm anterior to lambda. Further, histological verification of recording placement was used in both of these studies. In the studies of Maudhuit et al. [14,15] no atlas was referenced, no histological procedures were reported and no histology was shown. Further, the A.P. coordinate used by Maudhuit et al. [14,15], as reported by the authors of those studies, was 2.0 mm anterior to lambda, which, according to Paxinos and Watson [19] is approximately 2.3 mm anterior to the interaural line [19]. This A.P. position is anterior to the DRN at the level of the caudal linear nucleus (CLI). This electrode placement would present even more of a methodological problem when using younger animals (e.g. 2-month-old) where A.P. positioning must be more precise than in older animals. Interestingly, the CLI is rich in serotonergic neurons which are anatomically distinct from DRN serotonergic neurons (see Ref. [12]). Although the depth of the recording electrodes reported by Maudhuit et al. [14,15] was 5.5 mm ventral to the dura, which is consistent with part of the DRN, the A.P. position reported by these authors is clearly anterior to the DRN. Thus, without any histological verification and in light of the coordinates reported by Maudhuit et al. [14,15], it is...
impossible to say with any certainty that the unit activity shown in the studies [14,15] was recorded from the DRN and not the CLI. By contrast, the present study used an exclusionary criterion, histological verification of a recording site and/or tract. Although these considerations suggest that the most likely explanation of the different outcomes in the two laboratories was different locations of the recording electrodes, we cannot be certain that additional unidentified methodological differences did not contribute to the different observed results. In light of the above considerations and the present results, we conclude that the mean firing rate of 5-HT neurons in the DRN is significantly lower in adult CLI rats than in adult SAL rats. This finding may have clinical implications, in that, neonatal CLI treatment has been previously shown to produce a behavioral and physiological syndrome that approximates human endogenous depression [27,31]. The DRN is a major serotonergic input to forebrain structures [12,17,23,25]. Thus, the present findings add further support to the hypothesis that 5-HT neurotransmission is decreased in depressive disorders. Nevertheless, other findings about DRN firing rates are inconsistent with the hypothesis that decreased 5-HT neurotransmission is a substrate of depression. Tricyclic and monoamine oxidase inhibitor (MAOI) antidepressants drugs initially produce a marked decrease in the firing rate of serotonergic 5-HT neurons [3,7,21,22]. Thus, the 5-HT hypothesis of depression would predict that treating depressed patients with tricyclic’s or MAOI’s should acutely exacerbate the symptoms of depression. However, to our knowledge, there are no clinical reports of such an effect. Thus, this issue remains unresolved.

In addition to differences in firing rates, DRN neurons in CLI and SAL rats displayed different temporal firing characteristics as depicted by the different shape of their ISIH’s (Fig. 3). This finding again replicates findings of Yavari et al. [35], and extends them to a different age and rat strain. In SAL rats the ISIH of each individual neuron (or of the population as depicted by the average ISIH shown in Fig. 3) approximated an exponential decay curve. The differential equation of such a curve indicates that at any given moment, the rate of decrease of firing rate is proportional to the firing rate at that moment. This suggests that the firing rate of this population of neurons is modulated by a negative feedback mechanism that temporally pacifies cell discharge. It is well known that 5-HT DRN neurons are subject to a variety of negative feedback systems (see Ref. [12]) as well as intrinsic ‘pacemaking’ properties [8,24]. In the CLI rats the ISIH of individual neurons, with one exception (see Section 3), approximated a low horizontal line. Thus, the lower firing rate in CLI rats was neither a result of a shift to the right of a SAL-shaped interval histogram, nor a result of a lower amplitude of the SAL-shaped ISIH. Instead, the temporal pattern of unit discharge was different in CLI and SAL rats. The horizontal shape of the interval histogram for CLI rats indicated that all firing intervals had approximately equal probability. This suggests a random temporal order of 5-HT DRN unit discharge in CLI rats. As has been suggested [26], one implication of this finding may be that the CLI rats lack the feedback mechanism that controls the orderly firing of 5-HT DRN neurons, and that in its place is a system that results in reduced firing in a random pattern.

Two different views about neuronal information transfer have been proposed. One view holds that the transfer code depends mainly on mean firing frequency, whereas the other holds that the code depends on the temporal pattern of firing (see e.g. [9,13]). The firing patterns of 5-HT DRN neurons in CLI rats show alterations in both mean rate and temporal distribution. Thus, it is possible that a part of the neuronal substrate of depression is a random, rather than orderly, temporal firing pattern of 5-HT DRN neurons which may or may not be independent of decreased firing rates [26].

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References


