The lateral septum as a regulator of hippocampal theta oscillations and defensive behavior in rats

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Abstract

Hippocampal theta oscillations are linked to various processes, including locomotion, learning and memory, and defense and affect. The lateral septum (LS) has been implicated in the generation of the hippocampal theta rhythm, but its precise role in this process is not well understood. Here, we investigated the effects of direct pharmacological inhibition or disinhibition of the dorsal lateral septum (dLS) on the frequency of hippocampal theta activity elicited by stimulation of the reticular formation in urethane-anestheiazed rats. We found that bilateral infusions of the GABA_A receptor agonist muscimol into the dLS significantly increased theta frequency. Strikingly, intra-dLS infusions of the GABA_A receptor antagonist GABAzine largely abolished reticularly-elicited theta activity. We also locally injected these same compounds into the medial septum (MS) to test for neuroanatomical specificity. In contrast to the effects seen in the dLS, intra-MS infusions of muscimol had no effect on theta frequency, whereas intra-MS infusions of GABAzine increased theta frequency. Given the hypothesized role of hippocampal theta in behavioral defense, we also examined the effects of intra-dLS application of muscimol in two models of anxiety, the elevated plus maze and the novelty-induced suppression of feeding paradigm; both tests revealed clear, anxiolytic-like effects following muscimol infusions. The fact that dLS-muscimol increased theta frequency while also reducing anxiety-like behaviours challenges the influential theta suppression model of anxiolysis, which predicts a slowing of theta with anxiolytic compounds. More importantly, the experiments reveal a novel role of the LS, especially its dorsal aspects, as an important gating mechanism for the expression of theta oscillations in the rodent hippocampus.

Keywords: anxiety, GABAzine, muscimol, rat, theta frequency
Hippocampal theta activity is a large amplitude, near-sinusoidal oscillatory pattern with a frequency range of 4-14 Hz that can be recorded throughout the hippocampal formation (Vanderwolf et al. 1975; McNaughton et al. 2007). Theta activity has been implicated in various functions, including locomotion (Vanderwolf 1969; Whishaw and Vanderwolf 1973), spatial navigation (Buzsáki 2005), sensorimotor integration (Bland and Oddie 2001), and learning and memory (Winson 1978; Berry and Seager 2001). Notably, hippocampal theta appears when rodents exhibit immobility during fear conditioning (Whishaw 1972; Sainsbury et al. 1987a; Seidenbecher et al. 2003) or in the presence of a predator (Sainsbury et al. 1987b), suggesting a link between theta and behaviors related to defense and affect (i.e., fear and anxiety).

Theta activity in the hippocampus can be elicited with high frequency electrical stimulation of the midbrain reticular formation (Green and Arduini 1954; Stumpf 1965). In both freely moving and anesthetized rats, theta frequency increases linearly with increasing stimulation intensities (McNaughton et al. 2007). Interestingly, all classes of clinically effective anxiolytics (barbiturates, benzodiazepines, 5-HT1A receptor agonists, selective serotonin reuptake inhibitors), as well as experimental compounds that have been shown to reduce defensive behaviors in animal models of anxiety (e.g., somatostatin, phenytoin, the bradycardic agent ZD7288), decrease the frequency of reticular-elicited theta (McNaughton et al. 2007; Engin et al. 2008; Yeung et al. 2012a, 2012b). Critically, antipsychotics or drugs with sedative, but not anxiolytic effects (e.g., haloperidol, chlorpromazine) do not show this effect (McNaughton et al. 2007). Thus, a decrease in the frequency of reticularly-elicited theta seems to be a reliable, neurophysiological correlate of anxiolytic compounds; this hypothesis is referred to as the theta suppression model of anxiolysis (McNaughton et al. 2007; Yeung et al. 2012b).
Recently, some observations have provided evidence inconsistent with the theta suppression model. Bilateral infusions of histamine into the rat lateral septum (LS) exerted consistent anxiolytic-like effects in two models of anxiety, the elevated plus maze (EPM) and the novelty induced suppression of feeding paradigm (NISF; Chee et al. 2014). Remarkably, in urethane-anesthetized rats, the same infusions increased the frequency of reticularly-elicited theta, an effect opposite to that predicted by the theta suppression model (Chee et al. 2014).

The cellular effects of histamine receptor activation on LS neurons have, to the best of our knowledge, yet to be determined. As such, whether the increase in theta frequency following intra-LS application of histamine resulted from excitation or inhibition of the LS is unknown. Curiously, although the LS has been suggested to be an important mediator of the theta rhythm (Vinogradova 1995), few (e.g., McLennon and Miller 1974; McLennon and Miller 1976; Pedemont et al., 1998) have investigated its precise role in modulating theta generation. Thus, here we examined the effects of direct, pharmacological inhibition or disinhibition of dorsal LS (dLS) activity by local application of muscimol (GABA<sub>A</sub> receptor agonist) or GABAzine (GABA<sub>A</sub> receptor antagonist), respectively, on hippocampal theta frequency in urethane-anesthetized rats. To verify the neuroanatomical specificity of the intra-dLS infusions, we also tested the effects of muscimol or GABAzine on reticularly-elicited theta when infused into the MS. Further, given the links between the LS and hippocampal theta with defense and affect, we examined the behavioral effects of intra-dLS infusions of muscimol in the EPM and the NISF.

**Materials and Methods**
Subjects

A total of 71 experimentally naïve male Long-Evans rats (Charles River, Québec) were used. Rats subjected to the electrophysiological procedures \((n = 49, 300-425 \text{ g body weight})\) were housed in groups of four in polycarbonate cages \((51 \times 40 \times 21 \text{ cm})\) on a reversed 12:12 dark/light cycle (lights off at 0700h). Rats used for the behavioral experiments \((n = 22; 275-325 \text{ g body weight at the time of surgery})\) were initially pair-housed in polycarbonate cages \((45.5 \times 24 \times 21 \text{ cm})\) on a regular 12:12 light/dark cycle (lights on at 0700h). Following cannulae implantation, rats were singly housed in accordance with standard operating procedures for surgically prepared animals at Queen’s University.

The colonies were maintained at a temperature of approximately 21°C. Food and water were available \textit{ad libitum}. All rats were allowed to acclimatize to the colony for at least 1 week before surgery. All procedures were in compliance with the regulations established by the Canadian Council on Animal Care and were approved by the Queen’s University Animal Care Committee.

Drugs

The drugs used were muscimol \((\text{GABA}_A\text{ receptor agonist}; \text{Beaumont et al. 1978})\), GABAzine \((\text{SR 95531 hydrobromide, GABA}_A\text{ receptor antagonist}; \text{Heaulme et al. 1986})\), and buspirone hydrochloride \((\text{all obtained from Tocris Bioscience, R&D Systems, Inc., Minneapolis, MN, USA})\). All drugs were dissolved in 0.9% saline. Muscimol and GABAzine were stored in aliquots at -20°C until use, with a fresh aliquot used on each test day. Buspirone was freshly prepared on the day of the experiment.
Experiment I: Electrophysiology procedures

Surgical preparation

Rats were deeply anesthetized with urethane (1.5 g/kg total; administered as 0.5 g/kg x 3 every 15 minutes, i.p., with supplements given as required). After the third injection of urethane, the local anesthetic Marcaine (2 mg/kg) was injected subcutaneously into the skin near the incision site. Following complete anesthesia induction, the rat was placed into a stereotaxic instrument. Body temperature was monitored throughout the experiment with a rectal thermometer and maintained at approximately 37°C using an electrical heating pad and fleece insulating blankets.

The scalp was incised along the midline, exposing the skull. Burr holes were drilled into the skull above the following areas: hippocampus (from bregma: - 4.5 mm AP, + 2.0 mm ML), reticular formation (from bregma: - 7.0 mm AP, + 1.6 mm ML; same as McNaughton and Coop 1991), and dLS (from bregma: - 0.1 mm AP, ± 0.4 mm ML). An additional hole was drilled over the cerebellum to allow the insertion of a ground connection (jeweller’s screw attached to a miniature connector). A bipolar recording electrode (two 125-μm diameter Teflon-insulated stainless steel wires with a vertical tip separation of about 1 mm) was lowered into the hippocampus (from dura: -3.0 to -3.5 mm DV), and a stimulation electrode (Series 100 concentric bipolar electrode; Rhodes Medical Instruments, David Kopf Instruments) was lowered into the reticular formation (from dura: -7.2 mm; McNaughton and Coop 1991). A microinjector (Hamilton 10 μL syringe) was inserted into the dLS of one hemisphere (from dura: -4.4 mm DV) to infuse either 0.9% saline (0.25 μL), muscimol (5 ng in 0.25 μL), or GABAzine (25 ng in 0.25 μL) at a rate of 1.0 μL/min. The microinjector was left in position for 1 minute.
after the infusion to minimize fluid reflux up the injector track. The same procedure was then
repeated on the opposite hemisphere, with the infusion order (left versus right dLS) counterbalanced between rats. A second group of rats was prepared in the same manner as described above, with the exception that the microinjector was inserted into the MS (from bregma: -0.1 mm AP, 00 mm ML; from dura: -6.5 mm DV). These rats received a single 0.50 μL infusion of either muscimol (10 ng) or GABA\(\text{amine}\) (50 ng) at a rate of 1.0 μL/min. A third group of rats had recording and stimulating electrodes implanted as described above, but did not receive any infusions into the dLS or MS. Instead, they were given a systemic injection of buspirone hydrochloride (10 mg/kg, i.p.).

Electrophysiology and data collection

The bipolar hippocampal recording electrode was connected to an amplifier (BioAmp, AD Instruments, Toronto, Ontario) and A/D converter (PowerLab 4/s system running Chart software v.5.4; AD Instruments). The raw signal was digitally filtered between 4 Hz and 10 Hz and stored for subsequent offline analysis using Chart software. Stimulation of the reticular formation consisted of 5 s trains of 0.1 ms pulses delivered at 100 Hz, supplied by a stimulation isolation unit providing a constant current output (ML 180 Stimulus Isolator; AD Instruments).

Prior to the delivery of drug infusions or injections, an input-output curve for hippocampal theta activity was established by stimulating the reticular formation at sequentially increasing intensities (0.01-0.20 mA in 0.01 mA increments; one stimulation episode for each intensity; inter-stimulus interval of about 6 s). Because the stimulation intensity required to elicit theta activity differs between subjects (McNaughton and Sedgwick 1978), the threshold intensity required to elicit theta was established for each, individual rat. The stimulation intensities used
for formal data collection were 1.5, 2.0, 2.5, and 3.0 times the threshold intensity. These four stimulation intensities were randomized to create three sets of counterbalanced trials that were then used to calculate the average evoked theta frequency at each stimulation intensity. The average theta frequencies at each stimulation intensity were established before any drugs were administered and again afterwards, with five minutes post-infusion for rats receiving intra-dLS or intra-MS infusions, and ten minutes post-injection for rats receiving buspirone. Following this, a second input-out curve was established, in which the reticular formation was stimulated at sequentially increasing intensities (same parameters as described above). At the end of the experiment, each rat was given an overdose of urethane (1-2 mL) and transcardially perfused with 120 mL of 0.9% saline, followed by 120 mL of 10% phosphate buffered formalin.

Histology

Brains were extracted and submerged in formalin for at least 48 h before they were sliced into 40 μm coronal sections using a freezing cryostat. Sections were dry mounted onto gelatin-coated glass slides. An observer who was unaware of the corresponding data determined the location of the microinjector tips and electrode tracks and transcribed them onto brain atlas sheets (Paxinos and Watson 1998). Data obtained from rats with inaccurate microinjector or electrode placements were excluded from the data analysis.

Data analysis and statistics

Hippocampal activity during the 5 s epoch of reticular formation stimulation by the four stimulation intensities (1.5, 2.0, 2.5, and 3.0 threshold intensity) was analyzed for peak frequency
in the theta band (4 to 10 Hz) using power spectral analysis (Fast Fourier Transform (FFT),
windowed using Cosine-Bell function; FFTs computed by Chart software).

To verify the linear relationship between stimulation intensity and theta frequency, the
initial, pre-drug input-output curve was separately modeled for each individual rat using a linear
regression with peak theta frequency as the dependent variable and stimulation intensity (starting
at threshold and ending at 0.20 mA in 0.01 mA increments) as the independent variable. Data
from rats that failed to show a significant linear relationship between stimulation intensity and
theta frequency were removed from the analysis.

Theta frequency data from rats receiving intra-dLS infusions of saline or muscimol were
initially analyzed with a three-way omnibus mixed ANOVA, with TIME (pre- versus post-
infusion) and INTENSITY (1.5 x threshold, 2.0 x threshold, 2.5 x threshold, and 3.0 x threshold)
as the two within-subjects variables and DRUG (saline versus muscimol) as the between-subjects
variable. Subsequent pair-wise comparisons were computed with the Least Significant
Difference (LSD) test. Theta frequency data from rats receiving intra-dLS infusions of
GABAzine were not included in the analysis since reticular stimulation no longer elicited theta
activity after the intra-dLS GABAzine infusion had been delivered.

Theta frequency data from rats receiving intra-MS infusions of muscimol or GABAzine
were first analyzed with a three-way omnibus mixed ANOVA, with TIME and INTENSITY as
the two within-subjects variables and DRUG (muscimol versus GABAzine) as the between-
subjects variable. Follow-up pair-wise comparisons were computed using the LSD test.

Theta frequency data from rats given buspirone were analyzed with a two-way repeated
measures ANOVA, with TIME and INTENSITY as the two within-subjects variables, followed
by LSD tests.
The threshold intensities required to elicit theta activity for rats receiving intra-dLS infusions of saline or muscimol were analyzed with a two-factor mixed analysis of variance (ANOVA), with TIME (pre- versus post-infusion) and DRUG (saline versus muscimol) as the within- and between-subjects variables, respectively. The same analysis was performed for rats receiving intra-MS infusions of muscimol or GABA锌ine. For rats injected with buspirone, the threshold intensities were analyzed with a one-way repeated measures ANOVA with TIME as the within-subjects variable.

For all analyses, the alpha value was set at $p < 0.05$.

**Experiment II: Behavioral procedures**

**Surgery**

Rats were anesthesized with isoflurane (4.5% for induction; 1–2% for maintenance) in oxygen and administered Tramadol (20 mg/kg, s.c.) preoperatively to reduce pain; in addition, the local anesthetic Marcaine (2 mg/kg, s.c.) was injected into the incision site. Following anesthesia induction, rats were placed into a stereotaxic instrument. Ten minutes later, the scalp was disinfected and incised along the midline to expose the skull. Burr holes were drilled into the skull to allow two 23-gauge, stainless-steel guide cannulae to be lowered 1.5 mm above the left and right dLS (from bregma: $+0.5$ mm AP, $\pm1.2$ mm ML; from dura: $−3.4$ mm at a 7° medial angle; coordinates from Paxinos and Watson 1998). The guide cannulae were secured to the skull using four small jeweller's screws and dental acrylic. To maintain cannulae patency, a stylet was inserted into each cannula post-surgery. Immediately following surgery, the rats received injections of the analgesic Metacam (2 mg/kg, s.c.) and lactated ringer solution (5-10 mL, s.c.)
and were placed under a heat lamp to maintain body temperature. After waking from anesthesia, the rats were moved to a recovery room separate from the home colony, in which the temperature was set to 25°C. For three days post-surgery, rats received daily injections of Tramadol (20 mg/kg, s.c.) and Metacam (2 mg/kg, s.c.). Rats were returned to the home colony four days post-surgery and were given at least five additional recovery days before commencement of the behavioural procedures (see below).

**Infusions**

All rats were initially habituated to the infusion procedures by bringing them to the infusion room over three consecutive days. Here, rats were gently restrained with a towel and the cannula stylets were briefly removed and replaced. On test days (see below), rats were restrained as before, and the cannula stylets were removed to allow two 30-gauge stainless steel internal injectors to be lowered to a depth of 1.5 mm below the tip of the guide cannulae. Each internal injector was connected via polyethylene tubing (PE50) to a constant-rate 10 μL Hamilton microsyringe mounted onto a dual micro-syringe infusion pump (KD Scientific, MA). Bilateral infusions of muscimol \( (n = 11; \text{volume of } 0.25 \mu \text{L per hemisphere; concentration of } 20 \text{ ng/ } \mu \text{L}) \) or 0.9% saline \( (n = 11; \text{same volume}) \) were carried out using an infusion rate of 1 μL/min. At the end of the infusion, the injectors were left in place for an additional 60 s to allow diffusion of fluid away from the cannula tips and to reduce the possibility of reflux. The movement of a bubble inside the polyethylene tubing was monitored to verify drug flow. Prior to replacing the cannulae stylets, the tips of the internal injectors and tops of each cannula guide were examined for fluid efflux. Data from rats that showed infusion problems (e.g., cannulae guide blockage or
post-infusion efflux of fluid) were excluded from the data analysis. Five minutes passed between
the completion of the infusion and the start of behavioral testing.

Given that GABA antagonists are known to produce seizure activity in freely-moving
animals (Treiman 2001), we did not test the behavioral effects of intra-dLS infusions of
GABAzine.

Behavioral testing

Following complete recovery from the surgery, rats were first tested in the EPM,
followed 6 days later by testing in the NISF. All testing was conducted between 1000h and
1400h.

Elevated plus maze (EPM): The EPM was a wooden, cross-shaped maze with two
opposing open arms (50 x 10 cm) and two opposing closed arms (50 x 10 x 50 cm). All arms had
open roofs, and the maze was elevated 50 cm above the floor. The testing room was lit with a red
light so that the center of the maze was at 3.5 lx. Five minutes after completion of the infusion
procedure, rats were individually placed into the center of the maze, facing a closed arm, for a 5
minute free exploration test. An experimenter sat quietly in one corner of the testing room and
recorded the number of entries made into open and closed arms. An entry was defined as all four
paws placed in the arm. Testing sessions in the EPM were also recorded with a digital camcorder
and later coded by an experimenter blind to the subject’s drug condition using Observer
VideoPro (Noldus, MA) for the following behaviours: (a) the total time spent in the open arms
and (b) the total time spent in the closed arms. After each test session, the maze was cleaned with
a 5% alcohol solution to minimize odour cues. The percentage of open arm entries (open/open +
closed) and the percentage open arm time (open/open + closed) were used as measures of anxiety
in the EPM (Pellow et al. 1985). The numbers of total arm entries and closed arm entries were
used as measures of general locomotor activity (Pellow et al. 1985; Rodgers and Johnson 1995).

Novelty-induced suppression of feeding (NISF): Testing occurred over seven consecutive
days. Individual rats were given a dish containing 10 g of graham cracker pieces in their home
cage for 10 min/day for six consecutive days. The first five days served as habituation days,
while the sixth day was the home cage test. On the seventh day (the novel cage test), the rats
were brought to a new testing room, placed into an opaque cage lined with fresh bedding, and
given a dish containing 10 g of cracker pieces. The latency to initiate food consumption was
recorded on all seven days. The rats received infusions (5 min before testing) only on days 6 and
7, the home and novel cage test days, respectively. Rats that failed to habituate to the testing
procedure (their response latencies were 600 s throughout habituation days 1-5) were not tested
on days 6 and 7. Difference scores were calculated by subtracting home cage latency scores from
novel cage latency scores. Anxiety reduction in the NISF is indicated by a reduced latency to
initiate consumption of the graham cracker in the novel cage (day 7) in the absence of changes in
the latency to initiate snack consumption in the home cage (day 6). In contrast, an increase in
appetitive motivation would be indicated by a reduction in the latencies to initiate snack
consumption in both the home and novel cage tests (days 6 and 7, respectively; Merali et al.
2003).

Histology

The histological procedures were identical to those described above for the
electrophysiological experiments.
Statistics

Data from the EPM were first analyzed using one-way analyses of variance (ANOVA). Data from the NISF was analyzed with a two-factor mixed ANOVA, with DAY as the within-subjects variable and DRUG as the between-subjects variable. Subsequent pair-wise comparisons were computed with the Least Significant Difference (LSD) test. In addition, the difference scores for each rat (obtained by subtracting the home cage latency from the novel cage latency) were analyzed using a one-way ANOVA. For all analyses, significance was set at $p < 0.05$.

Results

Experiment I: Electrophysiology

Intra-dLS infusions of saline, muscimol, or GABAzine

Figure 1A is a photomicrograph of a coronal brain section with microinjector tips bilaterally placed into the dLS. Twenty-eight rats had microinjector tips placed into the dLS of each hemisphere (Figure 1B), yielding the following group numbers: saline ($n = 10$), muscimol ($n = 10$), and GABAzine ($n = 8$).

In all rats, stimulation of the reticular formation led to the appearance of pronounced hippocampal theta activity (Fig. 2A), with peak frequencies ranging from 4 Hz to 5.5 Hz, as revealed by power spectra analysis (Fig. 2B). Once the stimulation ended, theta activity rapidly ceased ($< 0.5$ s). Outside the periods of stimulation, spontaneous theta activity was never
observed; spontaneous hippocampal activity prior to and following reticular stimulation
consisted of irregular, mixed frequency activity.

Under control conditions (i.e., pre-infusion for all rats, regardless of infusion group),
theta frequency increased linearly with stimulation intensity (1.5 to 3 x threshold; Fig. 3).
Further, the linear regression analysis of the pre-drug input-output curves showed that, for each
individual subject, there was a significant linear relationship between stimulation intensity and
theta frequency (i.e., the slope of the linear function was significantly different from zero; all $p$’s
< 0.005).

As shown in Figures 2 and 3, intra-dLS infusions of saline had no obvious effects on
theta frequency, while intra-dLS infusions of muscimol increased theta frequency at all
stimulation intensities. In contrast, intra-dLS infusions of GABAzine completely abolished theta
activity at all stimulation intensities tested (Fig. 2 and 4). Thus, only the theta frequency data
from saline and muscimol-infused rats were statistically analyzed (see below).

An initial omnibus three-factor mixed ANOVA of the theta frequency data from saline
and muscimol-infused rats detected significant main effects of TIME ($F_{1,54} = 6.78; p = 0.02$) and
INTENSITY ($F_{1.45, 28.59} = 105.89; p < 0.001$), as well as significant TIME * DRUG ($F_{1,54} =
13.43; p = 0.002$) and INTENSITY * DRUG ($F_{1.45, 28.59} = 4.46; p = 0.03$) interactions. All other
main effects or interactions (DRUG, TIME * INTENSITY, TIME * INTENSITY * DRUG)
were non-significant (all $p$’s > 0.4). Subsequent pair-wise comparisons found that, in rats infused
with saline, theta frequencies did not change pre- or post-infusion at any stimulation intensity (all
$p$’s > 0.4). In contrast, intra-dLS infusions of muscimol significantly increased theta frequencies
across all stimulation intensities (all $p$’s < 0.01).
The effects of intra-dLS infusions of saline or muscimol on the threshold stimulation intensity needed to elicit theta activity were also examined. Again, as GABAzine infusions resulted in an inability to elicit theta activity, its effect on threshold could not be analyzed. Mean (± standard error of the mean) threshold currents before and after saline-infusions were 0.087 (± 0.011 mA) and 0.081 (± 0.011 mA) respectively, while muscimol-infused rats required 0.087 (± 0.011 mA) and 0.094 (± 0.011 mA) pre- and post-infusion, respectively. The two-way mixed ANOVA found a significant interaction of TIME * DRUG ($F_{1, 18} = 4.99; p = 0.04$), while the main effects of TIME and DRUG were non-significant (both $p$’s > 0.7). Subsequent post hoc tests found that threshold currents did not differ before or after intra-dLS infusions of saline or muscimol (both $p$’s > 0.1), nor did threshold currents differ between saline or muscimol-infused rats before or after the infusions (both $p$’s > 0.4).

Intra-MS infusions of muscimol or GABAzine

Figure 5A is a photomicrograph of a coronal brain section with the mircoinjector tip placed into the MS. Sixteen rats had microinjector tips placed into the MS (Figure 5B), yielding the following group numbers: muscimol ($n = 8$) and GABAzine ($n = 8$).

In all rats, theta activity appeared when the reticular formation was stimulated (Fig. 6A), with peak frequencies ranging from 4 Hz to 6.5 Hz (Fig. 6B). Spontaneous theta activity was never observed outside the periods of stimulation in any rats before the infusion. However, post-GABAzine infusion, 5 out of 8 rats displayed spontaneous theta activity.

Under control conditions (i.e., pre-infusion for all rats, irrespective of infusion group), theta frequency increased linearly with stimulation intensity (1.5 to 3 x threshold; Fig. 7). That is, for each individual rat, the linear regression analysis of the pre-infusion input-output curve
detected a significant linear relationship between stimulation intensity and theta frequency (all slopes significantly different from zero; all p’s < 0.005).

In contrast to the effects observed after intra-dLS application of muscimol or GABA
d, intra-MS infusions of muscimol had no effect on theta frequency, while intra-MS infusions of GABA increased theta frequency (Fig. 7). More specifically, an omnibus three-factor mixed ANOVA found significant main effects of TIME (F\(_{1.42} = 6.25; p = 0.03\)), INTENSITY (F\(_{1.21, 24.27} = 117.55; p < 0.001\)), and DRUG (F\(_{1.14} = 6.74; p = 0.02\)). None of the interactions (TIME * DRUG, TIME * INTENSITY, INTENSITY * DRUG, and TIME * INTENSITY * DRUG) achieved significance (all p’s > 0.05). Subsequent pair-wise comparisons failed to detect differences in theta frequency pre- or post-muscimol infusion at any stimulation intensity (all p’s > 0.5; Fig. 7). In contrast, intra-MS infusions of GABA increased theta frequencies across all stimulation intensities (Fig. 7). However, the increase was significant only at 1.5 and 2.0 x threshold intensities (both p’s < 0.001); the p-values at 2.5 and 3.0 x threshold were 0.09 and 0.48 respectively.

The effects of intra-MS application of muscimol or GABA on the threshold stimulation required to elicit theta activity were also examined. The mean (± standard error of the mean) threshold currents pre- and post-muscimol infusions were 0.077 (± 0.008 mA) and 0.077 (± 0.013 mA), respectively. In GABA-infused rats, the mean threshold currents before and after the infusions were 0.102 (± 0.015 mA) and 0.030 (± 0.014 mA), respectively. A two-way mixed ANOVA detected a significant TIME * DRUG interaction (F\(_{1.10} = 11.22; p = 0.007\), as well as a significant main effect of TIME (F\(_{1.10} = 11.22; p = 0.007\)). The main effect of DRUG was not significant (F\(_{1.10} = 0.67; p = 0.43\)). Follow-up comparisons found that threshold currents
did not differ before or after intra-MS infusions of muscimol ($p = 1.0$), while mean threshold currents were significantly reduced following intra-MS infusions of GABAzine ($p = 0.001$).

**Buspirone**

As a positive control, an additional group of animals ($n = 5$) were administered the clinically used anxiolytic agent buspirone (10 mg/kg; i.p.). Under control conditions (i.e., pre-buspirone administration), theta frequency increased linearly with stimulation intensity (Fig. 3). That is, the regression analysis for each individual rat showed that the pre-drug input-output curve significantly fitted a linear model (all slopes significantly differed from zero; all $p$’s < 0.001).

As depicted in Fig. 3, buspirone decreased theta frequencies at all stimulation intensities. This observation was confirmed with statistical analyses: a two-way repeated measures ANOVA detected significant main effects of TIME ($F_{1, 12} = 58.50; p = 0.002$) and INTENSITY ($F_{3, 12} = 61.34; p < 0.001$), as well as a significant TIME * INTENSITY interaction ($F_{3, 12} = 10.47; p = 0.001$). Subsequent pair-wise comparisons found that buspirone significantly reduced theta frequencies at all stimulation intensities (all $p$’s < 0.01).

The mean threshold current needed to elicit hippocampal theta activity did not change after buspirone administration, with threshold currents of 0.122 (± 0.012 mA) and 0.132 (± 0.011 mA) before and after drug administration respectively ($F_{1, 4} = 2.00; p = 0.23$).

**Experiment II: Behaviour**

**Histology**
Figure 8A is a photomicrograph of a coronal brain section with bilateral cannulae correctly implanted into the dLS. Twenty-two rats had cannulae tips correctly placed in the dLS (Figure 8B), yielding the following group numbers: saline ($n = 11$) and muscimol ($n = 11$).

The elevated plus maze

Rats that received bilateral intra-dLS infusions of muscimol displayed significantly higher percentages of open arm entries ($F_{1,20} = 7.81; p = 0.01$) and percentages of open arm time ($F_{1,20} = 4.23; p = 0.05$) than saline-infused controls (Fig. 9A). The number of total arm entries did not differ between infusion groups (saline [mean ± standard error of the mean]: $14.36 ± 1.06$, muscimol: $13.55 ± 1.52; F_{1,20} = 0.19; p = 0.67$); however, saline-infused rats made more closed arm entries than muscimol-infused animals (saline: $13.73 ± 1.03$, muscimol: $9.82 ± 1.26; F_{1,20} = 5.80; p = 0.03$).

Novelty induced suppression of feeding

One rat failed to habituate to the test procedure (600 s latency on each of the 5 habituation days) and was not included in the data analysis. A second rat failed to thrive and was therefore euthanized. The resulting group numbers were as follows: saline ($n = 10$) and muscimol ($n = 10$).

Figure 9B shows the latencies to initiate snack consumption over habituation days 1-5, the home cage test day (day 6), and the novel cage test day (day 7) for rats receiving bilateral intra-dLS infusions of either saline or muscimol (days 6 and 7 only). A mixed-model ANOVA detected a significant main effect of DAY ($F_{6,108} = 6.50; p < 0.001$) and a significant DAY x DRUG interaction ($F_{6,108} = 4.40; p = 0.001$). Subsequent pair-wise comparisons failed to find a
difference in latency scores on habituations day 1-5 or on day 6, the home cage test day (all p’s > 0.1). However, muscimol-infused rats showed significantly lower latency scores in the novel cage test day (day 7) relative to saline-infused rats ($F_{1,18} = 12.93; p = 0.002$). This difference is reflected in the analysis of the difference scores (Fig. 9B inset; novel cage latency minus home cage latency for each rat), in which muscimol-infused rats showed significantly smaller difference scores than saline-infused rats ($F_{1,18} = 14.92; p = 0.001$).

**Discussion**

In the present set of experiments, we examined the effects of bilateral modulation of cellular activity in the dorsal LS on hippocampal theta activity elicited by electrical stimulation of the reticular formation in urethane-anesthetized rats. We found that infusions of the selective GABA$_A$ receptor agonist muscimol, expected to inhibit neuronal activity (Arikan et al. 2002; Edeline et al. 2002) significantly increased theta frequency across a wide range of reticular stimulation intensities (1.5 – 3.0 x threshold to elicit theta). Conversely, dLS application of the competitive GABA$_A$ receptor antagonist GABAzine, known to produce cellular disinhibition/excitation (Sokal et al. 2000), largely abolished reticularly-elicited theta, demonstrating a surprising and novel role for the LS in gating hippocampal theta oscillations. These effects were neuroanatomically specific to the dLS, as infusions of muscimol into the MS had no effect on theta frequency, while intra-MS infusions of GABAzine increased theta frequency, reduced the threshold stimulation intensity to elicit theta, and resulted in the appearance of spontaneous theta activity. We did not observe any consistent effects of muscimol or GABAzine, when infused in either the dLS or MS, on the amplitude of theta (when present).
Given the proposed role of hippocampal theta activity in affect and defensive behaviour (McNaughton et al. 2007), we also tested the behavioral effects of intra-dLS infusions of muscimol and found anxiolytic-like effects in two rodent models of anxiety, the EPM and the NISF paradigm. These behavioral data constitute the second demonstration that a pharmacological treatment that increases theta frequency can induce behavioral anxiolysis, an observation that is inconsistent with the influential theta suppression model of anxiolytic drug action (McNaughton et al. 2007; Yeung et al. 2012b).

The data presented here clearly suggest that activity of the LS, in particular its dorsal aspects, exerts a powerful influence on hippocampal theta oscillations. Pharmacological inhibition (by muscimol) or disinhibition (by GABAzine) of the dLS resulted in the facilitation or cessation of reticularly-evoked theta, respectively, indicative of an important, inhibitory role of the LS in theta expression. Interestingly, the dorsocaudal LS is closely interconnected with many of the components of the ascending theta generation system. Generally, important theta generators are thought to be located in the reticular formation, specifically the nucleus pontis oralis (Vertes et al. 2004). Ascending fibers originating in the pontis oralis reach the supramammillary nucleus (SUM) of the posterior hypothalamus, which acts as a major regulator of the frequency of theta activity (Kirk 1998; Vertes et al. 2004). The SUM then sends fibers to the cholinergic and GABAergic pacemaker cells (and also glutamatergic cells) of the MS, which provides a final stage to influence theta expression at the level of the hippocampus (Thinschmidt et al. 1995; Vertes and Kocsis 1997; Dragoi et al. 1999; Buzsáki 2002; Vertes et al. 2004; Bland 2008). Notably, Risold and Swanson (1997) characterized dense, GABAergic projections from the dorsocaudal LS to the SUM. The same authors also noted projections from the LS to the MS, which, given that the majority of LS projection neurons are known to be GABAergic, likely
contain GABA (Risold and Swanson 1997; Sheehan et al. 2004). The dorsocaudal LS also sends GABAergic fibres to the lateral hypothalamus (LH), an area that has been implicated in theta activity, since electrolytic lesions of the LH decrease theta frequency (de Ryck and Teitelbaum 1978; Jurkowlaniec et al. 1989), while electrical LH stimulation leads to a current-related increase in theta frequency (Whishaw et al. 1972). Finally, the LS itself receives substantial glutamatergic input from the hippocampus (Risold and Swanson 1997), suggesting the possibility of a feed-forward mechanism to regulate theta activity. Indeed, it has been hypothesized that the LS modulates theta generation by using feedback from the hippocampus to regulate the firing of neurons in the MS (McLennon and Miller 1974; McLennon and Miller 1976; Vinogradova 1995; Pedemont et al., 1998).

This anatomical and neurochemical evidence, together with the results obtained in the present experiments, is consistent with the hypothesis that the LS, via its GABAergic projections to structures in the ascending theta generation pathway, normally exerts a profound, inhibitory influence on hippocampal theta activity. Under conditions of reduced LS activity, this inhibitory influence weakens, thereby resulting in a facilitation of theta oscillations (e.g., increased frequency). In contrast, strong excitation of the LS appears to be sufficient to largely abolish theta elicited by stimulation of the reticular formation. However, future experiments are needed to critically test this model, for example by characterizing the effects of LS inhibition or disinhibition on cellular activity of various theta generators and modulators (i.e., the MS, SUM, and/or the LH).

One important consideration of intracerebral drug infusions involves the diffusion area and neuroanatomical specificity of the infused compound. That is, it is possible that the effects of muscimol and GABAzine, when locally injected into the dLS, in actuality reflect drug diffusion...
from the dLS to the MS, known to play a critical role in theta regulation (see above). To address this possibility, we included additional groups of animals that received infusions of muscimol or GABA\textsubscript{zine} directly into the MS. The effects seen with these MS infusions were in clear contrast to those observed following dLS infusions. First, intra-MS infusions of muscimol had no effect on theta frequency. This finding contrasts with previous reports demonstrating that, in both freely moving and urethane-anesthetized rats, intra-MS infusions of muscimol abolished theta activity (Allen and Crawford 1984; Bland et al. 1996; Brandon et al., 2011). However, the doses used in these papers (100 ng/0.5 μL, 570 ng/0.5 μL, and 125 ng/0.5 μL, respectively) were substantially (at least 10 x) greater than the dose we used (10 ng/0.5 μL). As such, it is likely that the dose used in the current study was insufficient to eliminate theta. Unlike muscimol, intra-MS application of GABA\textsubscript{zine} significantly increased theta frequency at the 1.5 – 2.0 x threshold stimulation intensities, rather than suppressing theta, as was seen with the dLS infusions. In fact, in 5 of the 8 rats that received intra-MS infusions of GABA\textsubscript{zine}, spontaneous theta activity appeared post-infusion. These latter effects confirm the role of the MS in theta generation (Buzsáki 2002): its disinhibition should result in increased theta activity. The fact that modulation of cellular activity in the MS showed no effect (for muscimol), or an effect opposite to that seen with dLS infusions (for GABA\textsubscript{zine}), provides strong support for the notion that drug diffusion from the dLS to MS theta-generators cannot account for the effects observed in the present experiments.

Given the proposed role of theta oscillations in defensive and anxiety-like behaviors, we also examined the effects of dLS application of muscimol in two rodent models of anxiety, the EPM and NISF test. For both tests, muscimol infusions produced clear, anxiolytic effects, as indicated by increased open arm exploration in the EPM and reduced hyponeophagia in the NISF
test. These data are consistent with previous work demonstrating that intra-LS infusions of muscimol produce anxiolytic-like effects in a number of anxiety models, including the EPM, shock probe burying test, predator odor test, and Vogel punished responding test (Drugan et al. 1986; Degroot et al. 2001; Endres and Fendt 2008; Trent and Menard 2010); to the best of our knowledge, the effects of dLS muscimol application in the NISF task have not been examined in previous work. The NISF offers several advantages over these aforementioned paradigms, for example, reduced sensitivity to changes in general locomotor activity. Further, the design of the NISF test allows for the dissociation of treatment-induced effects on appetitive motivation from reductions in anxiety levels, since motivational effects are thought to decrease feeding latency scores in both the home cage and the novel cage tests (Merali et al. 2003). The fact that we did not observe changes in feeding latency in the home cage suggests that dLS infusions of muscimol did not exert significant effects on feeding-related motivational states in our animals.

For the EPM, we found no evidence that the muscimol-induced increase in open arm activity was secondary to treatment-related increases in locomotor activity (e.g., hyperactivity), as the total number of arm entries did not differ between the infusion groups. In fact, intra-dLS infusions of muscimol significantly reduced the number of closed arm entries, suggesting that muscimol shifted rats’ natural preferences for enclosed to open spaces. Overall, the anxiolytic-like effects of intra-dLS infusions of muscimol seen here confirm previous work demonstrating anxiolytic-like effects following either pharmacological or lesion-induced inactivation of the LS (Menard and Treit 1996; Pesold and Treit 1996; Degroot et al. 2001; Trent and Menard 2010; but also see Sheehan et al. 2004 for a contradictory hypothesis of the role of the LS in anxiety).

It is of interest to note that an influential model of anxiolytic drug action suggests that pharmacological compounds with anxiolytic properties share a common, neurophysiological
effect of reducing the frequency of reticular-elicited hippocampal theta activity (McNaughton et al. 2007). Clinically effective anxiolytics (e.g., barbiturates, benzodiazepines, the 5-HT$_{1A}$ agonist buspirone, selective serotonin reuptake inhibitors), as well as experimental compounds that reduce defensive behaviors in animal models of anxiety, have all been shown to reduce hippocampal theta frequency (McNaughton and Sedgwick 1978; McNaughton et al. 1986; McNaughton and Coop, 1991; McNaughton et al. 2007; Engin et al. 2008; Munn and McNaughton 2008; Yeung et al. 2012a, 2012b). Importantly, this effect is present in several experimental preparations, such as freely-behaving and urethane-anesthetized rats (McNaughton et al. 2007). Thus, it appears surprising that the present experiments did not confirm this proposed link between behavioral anxiolysis and theta reduction, given that intra-dLS application of muscimol (which produced behavioral anxiolysis) actually increased theta frequency in the experiments reported here. However, the present results are in agreement with data demonstrating that application of histamine in the LS also increases the frequency of hippocampal theta activity (Chee et al. 2014). Importantly, and similar to the effects reported here, intra-LS infusions of histamine exerted strong, anxiolytic effects in the EPM and NISF test (Chee et al. 2014). Thus, the data summarized here confirm our previous work and emphasize that behavioral anxiolysis can be dissociated from a reduction in hippocampal theta frequency, at least under specific, experimental conditions.

A possible explanation for the apparent discrepancy between our data and the work showing anxiolytic-induced theta suppression lies in the different routes of drug administration. In the previous reports, drugs were administered such that they can directly affect the hippocampal formation (i.e., either systemically, intracerebroventricularly, or directly into the hippocampus; McNaughton et al. 1986; Engin et al. 2008; Yeung et al. 2012a). In our
preparations (Chee et al. 2014), drugs were administered into extra-hippocampal tissue (i.e., the LS), allowing them to exert only indirect, modulatory effect on hippocampal theta activity. Thus, it is possible that the theta suppression model of anxiolysis applies only to conditions where drugs are acting directly on the hippocampus. Nevertheless, the data reported here and previously (Chee et al. 2014) demonstrate a clear dissociation between behavioral anxiolysis and a reduction in theta frequency. Indeed, the observation that systemic administration of anxiogenic compounds (e.g., yohimbine, FG7142) fails to increase theta frequency (Yeung et al. 2013) further highlights the contention that hippocampal theta frequency neither serves as a direct index of, nor as a mechanism generating anxiety in rodents.

To confirm that the methodology used here (anesthesia type and dose, electrode placements, stimulation parameters, etc.) was appropriate for the detection of pharmacologically-induced slowing of theta frequency, we included an additional experimental group of rats treated with the clinically used anxiolytic agent buspirone, a partial 5-HT\textsubscript{1A} agonist that has been shown to reduce hippocampal theta frequency (Coop and McNaughton 1991; McNaughton and Coop 1991). Indeed, we found that systemic injections of buspirone reliably reduced theta frequency. We also statistically verified the adequacy of our stimulation protocol by showing that, for each individual subject, pre-drug administration (e.g., saline, muscimol, GABAzine, or buspirone), theta frequency increased linearly with stimulation intensity. Thus, the failure to observe a reduction in theta frequency following intra-dLS infusions of muscimol or GABAzine cannot be attributed to some aspect of the electrophysiological or experimental procedures used in the current study.

In summary, our data provide evidence for an important gating role of the LS, especially its dorsal aspects, in the generation of hippocampal theta activity. Pharmacological inactivation
(by muscimol) of the dLS increases the frequency of reticularly-elicited hippocampal theta in urethane-anesthetized rats, while pharmacological dLS disinhibition (by GABA)ine) largely abolishes the ability to elicit theta activity. We also found that dLS infusions of muscimol exerted clear, anxiolytic-like behavioral effects in the EPM and NISF test, observations that further challenge the theta suppression model of anxiolysis (McNaughton et al. 2007; Yeung et al. 2012b). Future research is needed to clarify the exact role and mechanisms that allow the LS to exert its influence over hippocampal oscillatory activity, as well as behaviors related to anxiety and defense.
This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grants awarded to J.L.M. (Grant Number: RGPIN 261762-08) and H.C.D. (Grant Number: RGPIN 203175-12).


Figure Captions

Figure 1: A: Photomicrograph of a coronal brain section showing microinjector tips (indicated by arrows) correctly placed into the dLS of each hemisphere. B: Histological results. Circles represent the locations of the microinjector tips for bilateral infusions of saline (white circles), muscimol (black circles), or GABAzine (grey circles) into the dLS. Numbers refer to the distance (in mm) from bregma. Atlas plates are adapted from Paxinos and Watson (1998).

Figure 2: A: Representative examples of unfiltered (black) and filtered (4-10 Hz; gray) hippocampal activity prior to and during electrical stimulation (black bar; first 2 s of a 5 s stimulation episode, 100 Hz, 2 x threshold intensity) of the reticular formation before and after infusions of saline (left), muscimol (middle; 10 ng), or GABAzine (right; 50 ng) into the dLS. B. Corresponding power spectra for the filtered activity depicted in (A).

Figure 3: Mean (± S.E.M.) peak hippocampal theta frequency elicited by reticular formation stimulation (at four intensities) in rats given intra-dLS infusions of saline (n = 10; top), muscimol (10 ng; n = 10; middle), or systemic injections of buspirone (10 mg/kg; n = 5; bottom). Circles represent the average evoked theta frequency for each rat at each stimulation intensity. * denotes a significant (p < 0.05) difference between pre- and post-drug values at that particular stimulation intensity.
Figure 4: Averaged power spectra for filtered (4-10 Hz) hippocampal activity during electrical stimulation (5 s; 100 Hz, 1.5 x threshold intensity) of the reticular formation before and after intra-dLS infusions of GABAzone (50 ng; n = 8).

Figure 5: A: Photomicrograph of a coronal brain section showing a microinjector tip (indicated by arrow) correctly placed into the MS. B: Histological results. Circles represent the locations of the microinjector tips for unilateral infusions of muscimol (black circles) or GABAzone (grey circles) into the MS. Numbers refer to the distance (in mm) from bregma. Atlas plates are adapted from Paxinos and Watson (1998).

Figure 6: A: Representative examples of unfiltered (black) and filtered (4-10 Hz; gray) hippocampal activity prior to and during electrical stimulation (black bar; first 2 s of a 5 s stimulation episode, 100 Hz, 2 x threshold intensity) of the reticular formation before and after infusions of muscimol (left; 10 ng) or GABAzone (right; 50 ng) into the MS. B. Corresponding power spectra for the filtered activity depicted in (A).

Figure 7: Mean (± S.E.M.) peak hippocampal theta frequency elicited by reticular formation stimulation (at four intensities) in rats given intra-MS infusions of GABAzone (50 ng; n = 8; top) or muscimol (10 ng; n = 8; bottom). Circles represent the average evoked theta frequency for each rat at each stimulation intensity. * denotes a significant (p < 0.05) difference between pre- and post-drug values at that particular stimulation intensity.
Figure 8: A: Photomicrograph of a coronal brain section showing cannulae tips (indicated by arrows) correctly implanted into the dLS of each hemisphere. B: Histological results. Circles represent the locations of the cannula tips for bilateral infusions of saline (white circles) or muscimol (black circles) into the dLS. Numbers refer to the distance (in mm) from bregma. Atlas plates are adapted from Paxinos and Watson (1998).

Figure 9: A: Mean (± S.E.M.) percentage of open arm entries and open arm time displayed by rats in the EPM following bilateral infusions of saline (n = 11) or 10 ng muscimol (n = 11) into the dLS. B: Mean (± S.E.M.) feeding latencies over habituation days 1-5, the home cage test day (day 6), and the novel cage test day (day 7) in the NISF paradigm. Rats receiving bilateral infusions of saline into the dLS (n = 10) exhibited longer latencies on the novel cage test day relative to rats infused with muscimol (10 ng, n = 10). Inset: Mean (± S.E.M.) difference scores (novel cage latency – home cage latency) in rats bilaterally infused with saline or 10 ng muscimol in the dLS. * denotes a significant (p < 0.05) difference.
### Pre-Infusion

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![Power Spectrum Diagram](attachment:image.png)

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GABAzone

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Muscimol

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<th>Frequency (Hz)</th>
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<th>Post-Infusion</th>
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Stimulation Intensity

1.50 x Threshold | 2.00 x Threshold | 2.50 x Threshold | 3.00 x Threshold