Multiple Hypothalamic Sites Control the Frequency of Hippocampal Theta Rhythm

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ABSTRACT: Stimulation of a neural pathway originating in the brainstem reticular formation, with synapses in the medial hypothalamus, activates the hippocampal theta rhythm. The frequency of reticular-elicited theta is determined in the medial supramammillary nucleus (mSuM) completely in anaesthetised rats, but only partially when the animal is awake. We tested other medial hypothalamic sites for their capacity to control theta frequency in awake rats. Blockade of sodium channels (1/1000 procaine, experiment 1) or increased inhibition by GABA (Chlordiazepoxide [CDP], experiment 2) was found to reduce or increase the frequency of reticular-elicited theta, depending on the precise site of injection, in the region of the dorsomedial hypothalamic nucleus (DMH) and the posterior hypothalamic nucleus (PH). A band of null sites for CDP was located in the region of the ventral border of PH and dorsal border of mSuM. Using 0.5 and 1/1000 CDP, and slow infusions (experiment 3), it was found that effective PH sites were also separate from mSuM in the rostrocaudal direction. In experiment 4, the DMH/PH region was mapped with unilateral and bilateral slow infusions of 0.5/1000 CDP. CDP significantly reduced frequency in medial (periventricular) and dorsal PH, but not DMH. Bilateral injections appeared to generally sum the usual effects of unilateral injection, producing effects of intermediate size. However, the absolute frequency change in any given site, or with any pair of sites, did not exceed 1 Hz, which is similar to what is seen with single injections in mSuM. Overall, it appears that at any one time, theta frequency may be determined by a complex interplay between distinct but interacting modulatory regions in the medial hypothalamus. Hippocampus 2003;13:319–332. © 2003 Wiley-Liss, Inc.

KEY WORDS: RSA; theta; reticular; hypothalamus; medial supramammillary nucleus; posterior hypothalamic nucleus; dorsomedial hypothalamic nucleus; benzodiazepine

INTRODUCTION

Hippocampal theta rhythm is a large amplitude, sinusoidal, 4–12-Hz waveform that is seen in the hippocampal formation of the rat and other mammals during voluntary movements and information seeking behaviours (Vanderwolf, 1969; Winson, 1974; Kramis et al., 1975). It is also seen when the animal is stationary but can be presumed to be processing important information (Sainsbury, 1998). Theta is thought to play an important role in memory processing by the hippocampus (O’Keefe and Nadel, 1978). Indeed, it has been proposed that the specific frequency at which theta occurs may select which inputs to the hippocampus are preferentially processed (Miller, 1991; Vinogradova, 1995), or that specific bands of theta frequency may select different modes of hippocampal function (Gray, 1982).

It was thought for some time that the medial septum/vertical limb of the diagonal band of Broca (MS/vDBB) encoded the frequency of theta. However, chemical injections into MS/vDBB change the amplitude of theta or block its occurrence entirely without changing frequency (Veasey et al., 1982; Allen and Crawford, 1984; Monmaur and Breton, 1991; Bland et al., 1996). A number of distinct polysynaptic systems ascend from the brainstem and appear to be involved in the control of theta (Vertes, 1982). Until recently, the extent to which each of these controls frequency as opposed to other aspects of theta, or at what point in the ascending systems frequency is encoded, has been unclear.

One substantial system originates in the pedunculopontine tegmental nucleus (PPT; Vertes, 1982). This nucleus is predominantly cholinergic and cholinergic blockade affects the gating of theta (i.e., determines whether it occurs or not) but does not affect the frequency of theta when it does occur (McNaughton and Sedgwick, 1978). Injections of procaine (a local anaesthetic) into PPT efferents also block elicitation of theta by PPT stimulation without affecting frequency (Swain and McNaughton, 1996). Therefore, the ascending cholinergic system from PPT does not appear to contribute to the control of theta frequency.

A second substantial system originates in the nucleus reticularis pontis oralis (RPO) (Vertes, 1982) and has a relay in the medial supramammillary nucleus (mSuM) that is involved in encoding the frequency of theta. mSuM receives a denser innervation from RPO than does the MS/vDBB (Vertes, 1986, 1988; Vertes and Martin, 1988) and mSuM cells send projections to the MS/vDBB via the median forebrain bundle (Vertes,...

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Initial proof that mSuM encodes theta frequency was obtained in rats anaesthetised with urethane. Kirk and McNaughton (1991) elicited theta by stimulating RPO and found a population of mSuM neurons that discharged synchronously in bursts that were in phase with hippocampal theta. The rhythmic activity of these cells was not disrupted by temporary blockade of the MSn/DBB with procaine, even though this intervention abolished theta in the hippocampus. Later it was shown that procaine injected into sites between RPO and mSuM reduced theta frequency but not amplitude, whereas procaine injected between mSuM and MSn/DBB reduced theta amplitude, but not frequency (Kirk and McNaughton, 1993). Procaine in mSuM reduced both theta frequency and amplitude.

Pharmacological inhibition of mSuM cells by the benzodiazepine chlordiazepoxide (CDP) was less effective than procaine, but still produced a substantial mean frequency decrease (McNaughton et al., 1995). Taken together, these findings indicate that, in the urethane-anaesthetised rat, mSuM is critical for maintaining normal theta frequency. The linear increase in frequency with increasing stimulation intensity and the effect of systemic and supramammillary injections of benzodiazepines on theta frequency can both be simply explained if tonic excitation of supramammillary neurons is modulated by recurrent GABAAergic inhibition, depending on the benzodiazepine-GABA-chloride ionophore complex.

However, in awake rats, the contribution of mSuM to theta frequency control is apparently less complete. When procaine and CDP are injected into mSuM during behavioural immobility the frequency of theta elicited by RPO stimulation is reduced much less than in the anaeasthetised preparation (McNaughton et al., 1995). Under anaesthesia, RPO-elicited theta can be almost totally blocked. In freely moving animals frequency reduction is only to about one-third of the theoretical maximum. Further, Thinschmidt et al. (1995) found that with theta elicited by spontaneous movement, frequency was unaffected by large, albeit subtotal, mSuM lesions. These findings demonstrate that, in awake rats, mSuM is not the only site controlling theta frequency, and at times mSuM may not be involved at all. This article describes a search for some of the additional nuclei that should be involved.

It has been known for some time that theta can be elicited by stimulation of the posterior hypothalamic nucleus (PH) (Green and Arduini, 1954; Bland and Vanderwolf, 1972) and of the dorsomedial hypothalamic nucleus (DMH) (Destrade, 1982). These two nuclei are directly adjacent to one another (Paxinos and Watson, 1998), and in experimental work the distinction between them is sometimes not made clear. The boundaries of PH are also not clear and recent data suggest that the anterior extent of PH overlies part of posterior DMH in a region that has previously been designated as the dorsal hypothalamic area (Abrahamson and Moore, 2001) (see Fig. 3). However, the intensity of stimulation in the region of both PH and DMH shows a positive relationship with theta frequency in urethane-treated rats (Oddie et al., 1994) and awake rats (Oddie et al., 1996). Oddie et al. (1994) also used PH procaine to abolish RPO-elicited theta in their urethane-treated rats and concluded that the integrity of PH was critical for the RPO theta system. However, they used a very large volume of procaine (≤4 μl), and it is possible that the drug could have diffused to mSuM. The present series of experiments was undertaken to confirm the separate roles of PH and DMH, as distinct from mSuM, in the frequency of theta elicited by RPO stimulation in awake but immobile rats.

Initially (experiment 1) we mapped effective sites in the DMH/PH region with modest (1-μl) injections of procaine. This volume of procaine solution has a limited radius of diffusion (~500 μm) (McNaughton et al., 1995), enabling the assessment of drug effects in tissue proximal to the site of injection. Procaine was injected via a permanently implanted multiple glass cannula system described previously (McNaughton et al., 1995). Each array comprised five to seven individual cannulae, spaced apart horizontally. This array allowed the region between DMH/PH and mSuM to be mapped in the anteroposterior direction within a single rat as well as by comparing across rats. To discover whether frequency control in DMH/PH, like in mSuM, was sensitive to benzodiazepines, 1 μl CDP was also injected into this region via multiple glass arrays (experiment 2). For the final two experiments rats were implanted with standard stainless steel guide cannulae to allow repeated injections. In experiment 3, the effectiveness of small (0.5-μl) and standard (1-μl) CDP infusions in DMH/PH was compared concurrently, in an attempt to confirm the anterior limits of effective supramammillary sites. In experiment 4, the effective region for CDP was remapped with 0.5 μl CDP to attempt finer grain localisation of the specific sites involved in frequency control. Cannulae were implanted bilaterally to see whether injecting two cannulae in any location previously found to be effective would produce a larger change in frequency than a single injection.

**MATERIALS AND METHODS**

**Animals**

Seventy-six male Sprague-Dawley rats were obtained from the University of Otago Department of Laboratory Animal Sciences. They were maintained in groups of four with food and water ad libitum for ≥4 days before surgery, in a temperature controlled room (21°C) with incident daylight supplemented by fluorescent lighting on a 12:12-light/dark cycle. After surgery, the rats were housed singly either in hanging wire cages (steel guide cannulae) or in translucent plastic dustbins (glass cannula array). Food and water were available ad libitum.

**Cannula Construction and Drug Administration**

**Multiple cannula arrays**

Glass capillary tubing (YS-140-40, Scientific Glass Engineering, UK) was cut into lengths of ~5 cm. The tubes were aligned flat in columns on sheets of nonstick plastic (Aclar Embedding Film, Ted Pella). To ensure extended coverage of the target region, arrays were spaced by inserting shorter pieces of tubing (“spacers”)...
between each pair of cannulae. Adhesive was applied to the central portion of the column of tubes, and the upper free ends of the cannulae were inserted into stainless steel collars. The upper part of the array was then cemented together, leaving ∼2 cm of the cannula column exposed. The columns were then cut in a straight line either at right angles, or at an angle that resulted in 500-μm vertical distance separating the most anterior and most posterior cannulae once implanted. The latter design was an attempt to obtain arrays with a cannula in both an effective DMH/PH site at one end and an effective mSuM site at the other with null sites in between. Each cannula was separated from its neighbours by ∼140 μm horizontally. This resulted in tracks that allowed identification of individual cannulae in histological sections. Before implantation, Silastic caps filled with distilled water were slipped over the stainless steel collars protecting each cannula. If this indicated that a specific cannula had become blocked it was not tested later. Before testing each cannula, the Silastic cap was removed and refilled with 1 μl of drug solution (experiment 1: procaine hydrochloride, 20% wt/vol, 7.3 M, in saline; experiment 2: chloralozepoxide hydrochloride, 30 mg/ml, 0.9 M, in saline). The volume was controlled by the length and diameter of the cap (McNaughton et al., 1995). After baseline readings were obtained, a “fast” injection was made by slipping the cap over the collar so that the collar filled the cap and the solution was forced down the cannula. The stimulation protocol was then resumed. For four of the rats in experiment 2, cannulae were connected to a 10-μl Hamilton syringe via a length of Silastic tubing (0.4 mm internal diameter) that was partially filled with CDP solution. Unlike the caps used for the other rats, connection with this small bore tubing would have produced an injection with a maximum volume of 1 μl, at least part of which would be the saline with which the tube was originally filled. Baseline readings were obtained after connection with the syringe. An air bubble separated the CDP from the distilled water in the syringe end of the tubing. Injections were made manually via the Hamilton syringe. The movement of the air bubble in the Silastic tubing was monitored to assess the success of an injection. Testing of the next cannula in any rat did not commence until baseline values had been recovered.

**Stainless steel cannulae**

Guide cannulae (25 G, 20-mm, 0.5-mm outside diameter) were implanted unilaterally (experiment 3) or bilaterally (experiment 4). Glass injection cannulae were silica capillary tubes (as for multiple arrays), with one end glued into a stainless steel (25-G) collar. When the glass cannula was inserted into the guide up to the collar it extended ∼0.5 mm into the brain beyond the tip of the guide. Silastic tubing connected the injection cannula to a 10-μl Hamilton syringe driven by an electrical microdrive. A “slow” infusion of CDP (40 mg/ml, 1.2 M, in saline) was made over 2.5 min, after which the injection cannula was removed. The movement of an air bubble in the Silastic tubing was used to monitor the success of the infusions. In experiment 3, the first infusion was made at a volume of 0.5 μl. On a subsequent day the procedure was repeated but using 1 μl of the drug solution. In experiment 4, after baseline theta frequency readings were obtained rats received either a systemic injection of CDP (5 mg/kg, in saline, 5 mg/ml, i.p.), or bilateral or unilateral intracranial injections. The left side cannula was injected first, followed closely by the right when both cannulae were viable. However, injections could often not be made down one of the cannulae due to blockage. In this case testing resumed after the viable cannula had been injected.

**Surgery**

On the day of surgery, the rats weighed 250–600 g; 20–30 min before surgery, they were injected i.p. with atropine sulfate (25 mg/kg) to reduce muosal secretion. They were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.) and stereotaxically implanted with bipolar recording and stimulating electrodes and either a unilateral multiple glass cannula array or stainless steel guide cannulae, depending on the particular experiment.

The skull was adjusted to be level between lambda and bregma, and all coordinates were calculated with reference to the skull surface and obtained from the stereotaxic atlas of Paxinos and Watson (1998). Recording electrodes consisted of two strands of Teflon-coated stainless steel wire (70-μm outside diameter), twisted together with the tips separated vertically by 2.0 mm, and implanted in the left subicular region of the dorsal hippocampus (bregma, A-P -6.0, M-L 2.0, D-V 5.0; as in McNaughton et al., 1995). A stimulating electrode was constructed in the same way but with a vertical tip separation of 0.5 mm. This was implanted in the region of the left nucleus reticularis pontis oralis (RPO) of the rostral pons (A-P −7.0, ML 1.6, DV 8.5). A ground electrode, consisting of a length of uninsulated silver wire (25-μm diameter), was wound around a stainless steel jeweller’s screw. Multiple cannula arrays were implanted parallel to the midline (0.9–2.0 mm, lateral) at various depths (8.8–9.2 mm, deep) and angles (0–11°), selected to achieve tip placements in the medial DMH/PH region without damage to the sagittal sinus. The most posterior cannula was used to measure the anteroposterior position. In different rats, it targeted the region of posterior DMH/PH as well as anterior mSuM (bregma ~4.5mm). The rats in experiment 3 had their stainless steel guide cannula individually aimed at different antero-posterior coordinates (Bregma, −3.6 to −4.2 mm), but the same mediolateral and dorsoventral coordinates (0.9 mm lateral, 0.85 mm deep) were used for all rats. The rats in experiment 4 were implanted bilaterally with stainless steel guide cannulae aimed at the region of posterior DMH/anterior PH (bregma AP −3.3, ML 1.0, DV 8.5). For all experiments electrodes were inserted via Amphenol gold pins into a McIntyre miniconnector that was secured to the skull with dental cement anchored by jewellers’ screws.

**Apparatus and Procedure**

During electrophysiological testing the rats were kept in a square black plastic bin (34 × 34 × 33 cm) with a sawdust floor in which they could move freely. Electrodes were connected via a cable to a dual field effect transistor, the signal preamplified (Grass P511K, 1–30-Hz bandpass filter) and extracellular field activity digitised at 100 Hz for analysis. Stimulation (100 Hz, pulse width 0.1 ms,
0.75-s train duration) was delivered by a Grass SD9 constant voltage stimulator controlled by a BBC Microcomputer. Testing began when exploratory behaviour had ceased, and rats were still awake but motionless. An intensity-frequency function for each rat was first obtained. The stimulation level for each rat was then set at the intensity (2–8 V), which produced the highest frequency of theta without any accompanying movement for that particular rat. The hippocampal electroencephalogram (EEG) was recorded for 0.75 s during each delivery of stimulation. A computer algorithm detected the first three peaks of each sampled waveform and from the interval between these calculated the frequency. This procedure was used as the initial theta response to stimulation shows the highest frequencies and is most responsive to changes in stimulation. Peak detections were checked by the experimenter and adjusted where necessary. Where theta was absent, a missing value was recorded.

Readings were obtained at ∼30-s intervals with electrical stimulation being applied only when the rat was stationary. If the rat showed signs of drowsiness it was woken up by gentle manual stimulation. Baseline frequency readings were obtained every 30 s and were continued until they had been stable for ≥6 min. Drugs were then delivered by the various methods. Testing resumed after completion of drug delivery and stimulation was applied once every 30 s for ≥10 min and for ≤40 min in cases in which there were signs of a drug effect.

Data Analysis

McNaughton et al. (1995) found effects on theta frequency of procaine and of CDP in the vicinity of mSuM that were clearest within the first 6 min of infusion. With small effects, return to baseline was complete within 6 min. Effects starting >6 min after injection are also likely to represent diffusion to structures some distance from the tip of the cannula. For these reasons, and to allow direct comparison of the sizes of our effects with those reported previously for mSuM, our assessment of the effects on procaine and CDP were almost always based on the average frequency of theta recorded in the 6 min before infusion compared with the 6 min after infusion. In experiments 3 and 4, a more detailed temporal analysis was used to dissect more proximal (early) and distal (late) effects.

Histology

Rats that had received intracranial injections were deeply anaesthetised (60 mg sodium pentobarbital), and perfused transcardially with saline, then with 10% formalin. The brains were removed and kept in 30% sucrose-formalin for 7 days. Frozen coronal sections (60 μm) were mounted and stained with thionin. The positions of recording and stimulating electrodes and cannula tips were reconstructed according to the atlas of Paxinos and Watson (1998). If a lesion had formed around the cannula tip, or if the tip was clearly located in a ventricle, the data for that cannula were excluded from analysis. Representative examples are shown in Figure 1.

RESULTS

Experiment 1: Injections of Procaine Anterior to mSuM.

Arrays of five to seven cannulae were implanted in 21 rats. Thirteen of these rats were suitable for both electrophysiological testing and drug infusion. Up to seven injections (one per cannula) of procaine were made in each rat to obtain a total of 60 injected cannula tips in the medial hypothalamic region. Figure 2 shows the location of the tips and the effects on theta frequency that procaine produced at each site. Tips were distributed between the anterior hypothalamic nucleus through to mSuM over a range of ∼2.3 mm. Because of the staggered arrangement of many of the multiple cannula arrays, the most posterior tips were generally the most ventral. Procaine was effective at reducing theta frequency at a number of sites throughout the anterior to posterior range. Effective sites were mainly concentrated around the margins of the DMH nucleus and the ventral PH nucleus, in the lateral hypothalamic nucleus (LH), and around the mammillary recess of the third ventricle. Sites at the margin of, and dorsal to DMH, were close to or within PH as defined by Abrahamson and Moore (2001) (Fig. 3A). If these nominal PH sites and sites in LH are excluded what remains is a set of sites with large and small frequency reductions that are located ventrally in a consistent medioateral and dorsoventral position (0.4–0.6 mm lateral to midline, 9.4–9.6 mm ventral to skull surface) that runs along an anteroposterior trajectory (Fig. 3B). The most posterior of these were located in the medial mammillary nucleus (MM), and more anterior placements were in ventral DMH and the ventromedial hypothalamic nucleus (VMH). This pattern of effects is consistent with procaine acting on a relatively discrete fibre pathway passing through these nuclei.

In most cases in which procaine produced a drop in frequency, this drop was of a magnitude of <1 Hz. This differs from the size of effects produced by 1 μl of procaine in mSuM, which are mainly within the range of 1.2–1.5 Hz (McNaughton et al., 1995). However, large effects (>1 Hz) were not absent in the present study, but were rare and generally occurred outside of the DMH and PH nuclei. Several small (<0.5-Hz) frequency increases were observed outside the DMH nucleus. This is an effect we have not seen previously with intracranial procaine infusions using this method.

One rat had mean frequency decreases of >0.5 Hz in both its most anterior and its most posterior cannula, with smaller or null effects in between. These two cannulae were injected with procaine again, one immediately after the other, 2 h after the first testing was completed. Histology showed that the cannulae were located in the posterior part of DMH and on the anterior border of mSuM, respectively. Figure 4 shows the difference in the time course of effects for the single injections compared with the effect when both cannulae were injected. Procaine inactivation of posterior DMH and mSuM together produced a greater initial reduction in frequency than either of the effective single injections. Over the first 6 min, however, this greater effect was less than the sum of the single injection effects. This preliminary result suggests that the effects on
theta frequency of inactivation of separate frequency controlling regions may tend to summate but do so incompletely.

**Experiment 2: Injections of CDP in the DMH and PH Regions**

Eighteen rats provided 54 viable glass cannulae. The cannula tips (Fig. 5) were distributed over a range of 1.5 mm from anterior DMH through to mSuM. CDP was mainly effective at reducing theta frequency when injected into DMH, PH, and mSuM, but seldom when injected outside of these nuclei. There were also CDP-sensitive sites both dorsal and lateral to PH near the region identified as the rostral extension of PH by Abrahamson and Moore (2001) (Fig. 3A). In PH itself, CDP was more effective in the medial as opposed to lateral parts of the nucleus. The magnitude of the CDP effects were mainly small (0.4–0.8 Hz), which is comparable with the size of effects CDP produces in the region of mSuM (McNaughton et al., 1995). However some large effects (>0.8 Hz) were obtained in the vicinity of the posterior part of DMH. There were a few sites where CDP increased theta frequency. These were all located in the general region of ventral PH, or just dorsal to mSuM (PH/mSuM border), areas where CDP was otherwise mainly ineffective at changing frequency.

**Experiment 3: Effects of CDP Immediately Anterior to mSuM**

Five rats were implanted with a single stainless steel cannula. The locations of the cannula tips and the time course of effects for the two injection volumes for each location are shown in Figure 6. For each rat and each volume of CDP the mean frequency change for theta recorded during consecutive 3-min intervals (≤21 min post-injection) was calculated by subtracting the mean frequency from 9 min of baseline. The two most posterior cannulae were located near the anterior horns of mSuM. Neither volume of the CDP solution produced any substantial change in frequency.
within 6 min of injection at this location. For both rats, a frequency increase with the small volume and a frequency decrease with the large volume occurred at 6–12 min post-injection. Given the time course, these effects are likely to be due to diffusion of the drug to other sites and not to any local sensitivity. It is conceivable that the smaller volume might have diffused into the region containing frequency-increasing sites (PH/mSuM border) that were identified in experiment 2. The larger injection might have diffused further to the more posterior parts of mSuM where CDP decreases theta frequency (McNaughton et al., 1995). The anterior horns of mSuM therefore are probably less sensitive to CDP than the nucleus as a whole, which suggests that CDP acted locally when it decreased theta frequency anterior to this point in experiment 2. The next cannula, in the anterior direction, was located just in front of mSuM, in ventral PH. Frequency decreases in excess of 0.5 Hz were produced by both 0.5 and 1 μl CDP within 3 min of injection. The 1-μl injection produced a somewhat larger effect than the 0.5 μl. The two remaining cannulae were slightly more anterior. One was located on the lateral border of ventral PH. The small injection produced what appeared to be a very small (0.2 Hz) frequency decrease, while a much more robust frequency increase was seen after the large injection. The second cannula was located below the first, and outside of both PH and DMH. Both the 0.5- and 1-μl injections produced immediate frequency increases.

In all the locations in which CDP was injected, there was either a quantitative or qualitative difference in the effect on theta frequency between the two injection volumes. The qualitative differ-

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**FIGURE 2.** Effects on reticularis pontis oralis (RPO)-elicited theta frequency of 1 μl procaine (20% wt/vol) in a region mapped from the anterior hypothalamic nucleus to medial supramammillary (mSuM), which includes the entire anterior to posterior extent of the dorsomedial hypothalamic (DMH) and posterior hypothalamic (PH) nuclei. Effects are classified as large frequency decreases (>1.0 Hz, black circles), small frequency decreases (0.5–1.0 Hz, gray circles), no effect (frequency change <0.4 Hz, unfilled circles), or frequency increase (>0.5 Hz, crossed circles).
ences are due to the functional differentiation of this general region of the posterior hypothalamus with regard to the pharmacological effect of CDP. Therefore, the further a solution diffuses from the site of injection the greater the likelihood it will interact with the receptors of more than one functional region. This is illustrated by the case of the cannula placed on the lateral border of PH. A more medial placement than this cannula produced only frequency decreases, while a more ventral placement produced only frequency increases. The lateral PH cannula produced a weak decrease with the small injection, suggesting that it was just outside the effective medial PH zone. With a large injection volume a robust frequency increase was observed. This is consistent with a significant volume of the drug diffusing to nearby frequency increasing sites outside of PH. In our previous experiments, we wanted to detect the presence of frequency controlling sites outside of mSuM, and a large injection volume was suitable for this endeavour. However, based on the findings of the present experiment, a small injection volume should be favoured for the mapping of specific effects to specific locations in future.

Experiment 4: Comparison of the Effects of CDP in DMH and PH

Thirty-three rats had at least one RPO stimulation session. Of these, five had i.p. injections, 13 were injected in both cannulae
The effects of CDP on the frequency of reticularis pontis oralis (RPO)-elicited theta were assessed using fast infusions of 0.5 µl CDP (30 mg/ml) in the dorsomedial hypothalamic/posterior hypothalamic (DMH/PH) region. Effects were classified as large frequency decreases (>0.8 Hz, black circles), small decreases (0.4–0.8 Hz, gray circles), no effect (frequency change <0.4 Hz, unfilled circles), or frequency increases (>0.4 Hz, crossed circles).
group included rats with a cannula in the DMH nucleus. Two rats with cannulae within 500 μm of mSuM comprised the mSuM group. An anatomical control group included all rats with cannulae outside of PH, DMH, and mSuM. Given the specific ordering chosen of levels of the factor of site of injection (PH, DMH, control, mSuM) the quadratic component estimates variation common to PH and mSuM and not shared with DMH and controls. There was a significant site by time interaction. Figure 8 shows that CDP in PH and mSuM produced a drop in frequency that was not seen with CDP in DMH or control sites:

\[
\text{Site (quad)} \times \text{time (quad): } F(1,12) = 13.64, \, P < 0.01
\]

The mean frequency decrease during the 6 min post-injection was ~0.4–0.6 Hz for PH and ≤0.8 Hz for mSuM. Both groups showed a return towards baseline levels after 6 min. DMH/CDP appeared to increase frequency slightly, but post-hoc analysis restricted to the DMH group found no effect of time:

\[
\text{Time (quad): } F(1,4) = 3.251, \, \text{ns}
\]

The bilateral cannula pairs were often located in different structures and sometimes in different histological planes. When both cannulae had the same location in their hemisphere, the effects were what would be expected from the results with single cannulae. For example, a pair in anterior DMH and a pair lateral to PH both produced small frequency increases. A pair in the most posterior part of DMH and a remote (anterior) pair were both null. One pair in dorsal PH produced a moderate frequency decrease. When the cannulae of a pair were located in regions where different effects

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**FIGURE 6.** Locations of cannula tips and time course of effects on the frequency of reticularis pontis oralis (RPO)-elicited theta of 0.5 and 1 μl chlordiazepoxide (CDP) (40 mg/ml) infused at different coordinates relative to anterior medial supramammillary nucleus (mSuM). Placements represented on the right side of each coronal plane correspond to the right data plot, and placements on the left to the left data plot.
FIGURE 7. Effects on reticularis pontis oralis (RPO)-elicited theta frequency of (A) unilateral, and (B) bilateral infusions of 0.5 μl chlordiazepoxide (CDP) (40 mg/ml) into the dorsomedial hypothalamic/posterior hypothalamic (DMH/PH) region. B: lines connect the injected cannulae for individual rats. Effects are large frequency decreases (>0.4 Hz, black circles), small decreases (0.2–0.4 Hz, gray circles), no effect (frequency change <0.2 Hz, unfilled circles), small frequency increases (0.2–0.4 Hz, crossed gray circles) and large increases (>0.4 Hz, crossed black circles).
that the systems controlling theta frequency in awake rats are not homogenous, and are distributed in the brain. In contrast, mSuM appears to be the sole controller for frequency in anaesthetised rats (Kirk and McNaughton, 1993).

The medial (periventricular) part of PH was the most consistently effective region for the frequency decreasing effects of CDP, and there were also decreases in the anterodorsal extension of PH described recently by Abrahamson and Moore (2001). These effects could not have resulted from diffusion of the drug to mSuM, given the small injection volumes used, the distances separating mSuM from the majority of PH placements, and the fact that the mSuM/PH border dorsally and rostrally were regions in which CDP was generally ineffective in reducing frequency. It appears then that PH, like mSuM, participates in determining theta frequency via a benzodiazepine-sensitive mechanism, possibly phasic recurrent inhibition in GABAergic interneurons. With both large (1-μl), fast injections and small (0.5-μl), slow injections, frequency decreases in PH were mainly within the range of 0.4–0.8 Hz, whereas both types of injection into mSuM tended to produce decreases in excess of 0.8 Hz, in these experiments and previously (McNaughton et al., 1995). Because of the similar effectiveness of the two volumes in PH it is unlikely that this disparity is due to the CDP solutions diffusing more completely throughout mSuM than the larger PH. Rather, as indicated by experiment 4, there appears to be a limiting factor (or “ceiling”) on frequency change determined in a single functional region. Medial SuM probably determines a slightly greater proportion of benzodiazepine-mediated frequency control during RPO-stimulation, than PH. However, given that even small frequency changes (≤0.6 Hz) can be behaviourally significant (Pan and McNaughton, 1997; Woodnorth and McNaughton, 2002), the effective PH region must represent an important modulator of hippocampal function.

It should be noted that the posterior part of PH was not mapped extensively in these experiments so it is unclear if this region is involved with frequency control. In a study by Bland et al. (1995), a population of cells was identified, under urethane anaesthetic, which increased their firing rate during theta and were distributed throughout the medial part of the entire PH nucleus. Because mSUM is the sole controller of frequency under urethane, it is possible that this anaesthetic eliminated rhythmicity in these PH cells. If so then it might be expected that, in awake animals, CDP could be effective at reducing frequency in the posterior part of PH as well. However, PH as defined by Paxinos and Watson (1998) is definitely not involved as a whole. Injections of CDP near the lateral border of PH did not affect frequency, and a CDP-insensitive part of ventral PH appears to separate the two sensitive zones (in PH and mSuM). The latter is probably the same region where Bland et al. (1995) found a population of cells, under urethane, that reduced their firing, or ceased firing altogether, during theta states (tonic theta-off cells). While the functional significance of these theta-off cells is as yet unknown, Bland et al. (1995) suggested they may be the target of septohippocampal theta modulatory projections, or of desynchronising input from the median raphe. Activation of mSuM/PH border cells in both scenarios would most likely result in a reduction of hippocampal theta activity. Increased GABAergic inhibition of such cells by CDP would be likely to

**DISCUSSION**

These results provide the first evidence that the frequency of RPO-elicited theta in awake rats is modulated in regions anterior to, and separate from, mSuM. Also, for the first time with RPO elicitation, mapping identified sites where frequency was increased by procaine and CDP. These sites were generally quite separated from those where drugs reduced frequency. However, they are few and scattered, and it is difficult to draw any general conclusions about them. These findings provide general support for the view
increase the frequency of theta. This could counteract decreases in frequency produced above and below this area and so possibly lead to a slight overestimation of the size of the CDP-insensitive cell area.

The presence of adjacent areas that can generate frequency increases and frequency decreases, and the fact that these different effects can be achieved at a single site by varying injection volume, makes it difficult to provide a simple integration of the results of the current experiments. What is really required is an assessment of a range of injection volumes at each of a much larger number of sites than was tested in the present study. This would allow separation of null effects due to a drug-insensitive location from null effects resulting from drug diffusion to two adjacent locations with opposite functional effects.

With procaine and large CDP injections, frequency decreases were obtained from cannulae in the DMH nucleus. Small CDP injections on the other hand were not effective in reducing frequency in DMH, and often produced frequency increases. The results of experiment 3 suggest that this discrepancy might be explained in terms of a relatively limited radius of diffusion of the small injections. CDP produced qualitatively and quantitatively different effects in the same cannulae depending on the volume injected. A larger volume, then, which would diffuse more widely, could interact with a greater proportion of a frequency controlling area, or with two systems that exert different forms of control over theta frequency (i.e., increases or decreases). Large injections made into posterior DMH in experiment 2 might have diffused to proximal PH sites, where CDP reduced frequency. However, several of the 1-μl CDP effects in posterior DMH were very robust (>0.8 Hz), while effects of this magnitude were mainly absent in PH. Also, some of the effective DMH injections may arguably have been too distant to diffuse as far as PH (McNaughton et al., 1995).

In summary then, the nature of the involvement of DMH in the control of RPO-elicited theta frequency remains unclear. DMH and PH could comprise different parts of a functional region that is heterogeneous with regard to benzodiazepine sensitivity of receptor density. Alternatively, DMH may comprise part of a separate system, of which at least part of the function is to suppress theta.

There was a partial inverse relationship between procaine and CDP in the specific sites where frequency was reduced. McNaughton et al (1995) also found discrepancies between the sites where procaine and CDP were most effective at reducing frequency in the region of mSuM. Such discrepancies may depend on differences in the morphology of the sites in question. Procaine acts on sodium channels and was mainly effective on the margins of nuclei where neuronal fibres could course in relatively compact bundles. Many sites affected by procaine, but not CDP, were probably in pathways, of the type mapped by Kirk and McNaughton (1993), involved in transmitting tonic intensity information from the brainstem to frequency controllers. GABA/benzodiazepine receptors are more densely concentrated on neuronal soma than neuronal processes (Velazquez et al., 1989), so any effects with CDP would be expected to be, and were, localised mainly within nuclei. Although sodium channels are also present on soma, they are less concentrated there than in tightly packed fibre bundles and would be expected to me relatively less important than chloride channels, especially if the critical fibres make contact with dendrites at the margins of the nuclei concerned. It is not surprising, therefore, that there should be some differential sensitivity of the drugs.

Frequency increases with procaine were rare and not clearly concentrated near any particular structure, whereas with CDP there appeared to be some regular pattern with these effects. Small increases (0.2–0.6 Hz) in the lateral hypothalamic region (and particularly within the LH nucleus) were obtained with both large and small CDP injections. These findings were in contrast to our results with procaine where injections into LH often decreased frequency. The lateral hypothalamic region can exert a significant influence over the hippocampal EEG. Electrical stimulation of the same lateral hypothalamic site can elicit different hippocampal EEG patterns with different voltages (Whishaw et al., 1972), evidence that synchronising and desynchronising systems are adjacent in this region, and can be activated in parallel. Similarly, the effects of LH lesions on theta depend on their precise position (Jurkowianiec et al., 1989). Frequency increases with CDP indicate that the drug was affecting cell bodies that are normally involved in suppressing theta activity, possibly by limiting the activity of nuclei that control frequency. The LH effects of procaine agreed with those seen in more medial locations (i.e., around PH) and so it is unclear whether the LH/procaine sites correspond to the input pattern of critical PH afferents or to a separate theta-controlling pathway. Presumably, the fibres that are involved are suppressed by general anaesthetic since extensive mapping of LH in urethanised rats found no frequency effects of procaine (Kirk and McNaughton, 1993).

Small CDP injections in DMH also produced small magnitude (0.2–0.6 Hz) frequency increases, although decreases were found in comparable locations with 1-μl injections. This discrepancy cannot easily be explained, although it is possible that pharmacokinetic factors linked to the speed of injection (i.e., 1 μl “fast,” 0.5 μl “slow”) partially determine the overall effect of DMH/CDP.

Overall, considerably fewer frequency increases than decreases were found with both drugs, and the magnitude of the largest frequency increase produced was always less than that of the largest frequency decrease produced. The fact that an i.p. injection of CDP (which would make CDP available throughout the brain), is invariably reported to produce a large reduction of RPO-elicited theta frequency (McNaughton et al., 1986; McNaughton and Coop, 1991; Zhu and McNaughton, 1991; Coop et al., 1992), may therefore have two explanations. First, CDP reduces frequency in a greater proportion of the brain than that in which it reduces frequency. Second, frequency-reducing sites are generally more sensitive to the same plasma concentrations of the drug than frequency-increasing sites. As far as mapping anterior to mSuM can tell, both possibilities appear likely.

That the contributions from separate sites, which exert the same form of control over frequency, are at least partially cumulative is supported by preliminary data in experiment 1. A posterior DMH and mSuM site were infused with procaine simultaneously, and the resulting frequency decrease was greater than when either cannula was injected alone. It is plausible that simultaneous benzodiazepine injections into effective sites for CDP (e.g., medial or
showed that in the urethanised rat mSuM/procaine reduced the intensity of its tonic input by CDP, ought to attenuate mSuM of its PH input by local injections of procaine, or reduction brainstem afferents) to increasing theta frequencies. Depriving of PH activation (driven by its anaesthetic-sensitive system) to increasing theta frequencies in awake rats as it does under urethane (Kirk and McNaughton, 1993). We speculate that the band of effective ventral procaine sites in experiment 1 (Fig. 4) may represent the passage of fibres contributing to the anaesthetic-sensitive system. Vertes (1981) identified three fibre systems that ascend through the midbrain and caudal diencephalon and are involved in theta production. RPO was the most effective site for brainstem elicitation of theta, and RPO-stimulation was used to elicit theta in this experiment. Therefore, the putative pathway could originate in RPO. Projections from RPO to PH exist (Vertes and Martin, 1988), but the trajectory of this input has not been described. Alternatively, such an input could have originated in a nucleus that is innervated by RPO. This idea may have some merit since DMH does not appear to receive RPO afferents, but does appear to participate in controlling RPO-elicited theta frequency.

Regardless of the relative contributions to the two types of theta, mSuM and PH must cooperate in controlling theta frequency in awake rats. They cannot act as independent oscillators or beats would be apparent in the hippocampal theta rhythm (McNaughton et al., 1995). This favours an explanation in terms of mass action. In this situation, frequency controllers would summate their inputs, which would correspond to their joint innervation from excitatory projections, and synchronise their outputs, most likely by recurrent GABAergic inhibition in GABAergic collaterals within and between the relevant nuclei. Interconnections between mSuM and PH (Vertes, 1992; Vertes et al., 1995) might facilitate this process.

PH could transmit a frequency-coded signal to the MS/vDBB via a known projection (Vertes et al., 1993). Alternatively, mSuM might receive some proportion of its tonic input from brainstem afferents via PH. In this scenario, mSuM could transduce increasing levels of PH activation (driven by its anaesthetic-sensitive brainstem afferents) to increasing theta frequencies. Depriving mSuM of its PH input by local injections of procaine, or reduction of the intensity of its tonic input by CDP, ought to attenuate RPO-elicited theta frequency according to this model. Kirk (1993) showed that in the urethanised rat mSuM/procaine reduced the frequency and amplitude of DMH/PH-elicited theta in the same way as RPO-elicited theta. However, there are reasons why an mSuM connection is unlikely to facilitate the effects of PH treatments on frequency per se, in the awake rat. First, theta frequency occurs at normal levels during locomotor activity (Thiemschmidt et al., 1995) and defensive behaviour (Pan, 2000) in rats with mSuM lesions, whereas rats with PH lesions produce lower frequencies of theta during locomotion than controls (Robinson and Whishaw, 1974). If mSuM were critical for the effects of PH treatments on theta frequency, then both treatments would be expected to reduce frequency equivalently. Also, this scenario would represent PH as a mere relay between RPO and mSuM, whereas it is known that PH comprises a critical part of the ascending brainstem system that generates theta (Oddie et al., 1994). With all available evidence taken into consideration, it appears that cells in PH can contribute to the control of theta frequency in awake rats independent of mSuM. It should be noted that phasic cells that ought to be present for phasic recurrent inhibition, have not been found in PH under urethane (Bland et al., 1995). However, as has been discussed, at least part of the excitatory brainstem afferent input to PH must be anaesthetic-sensitive. Therefore, the activity of theta-related PH cells may become phasic when the rat is awake, or else an additional population of phasic cells becomes involved in the rhythmic process. Future experiments ought to address this issue.

REFERENCES


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