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REDUCTION OF THE EXTRACELLULAR LEVEL OF GLUTAMATE IN THE MEDIAN RAPHE NUCLEUS ASSOCIATED WITH HIPPOCAMPAL THETA ACTIVITY IN THE ANAESTHETIZED RAT

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Abstract—The relationship between hippocampal activity and the extracellular level of excitatory amino acids in the median raphe nucleus has been studied in urethane anaesthetized rats, using the *in vivo* microdialysis technique. Dialysates were collected from the median raphe nucleus during two to eight sampling periods of equal length (20 min) and hippocampal electroencephalogram was continuously monitored. For each observation period, the average glutamate level in the median raphe nucleus was determined and the percentage of theta and non-theta segments in the hippocampal recordings was calculated. Theta synchronization, in these experiments, either developed spontaneously or it was elicited by injection of anticholinesterase (Physostigmine or Sintostigmine, i.p.) or by a series of short tail pinches. The relationship between hippocampal activity and glutamate release in the median raphe nucleus was characterized by comparison of the direction of changes in these two parameters in consecutive sampling periods. We found that as long as theta/non-theta ratio changed spontaneously or under the effect of anticholinesterase (n=7), the extracellular level of glutamate in the median raphe nucleus was elevated during periods dominated by desynchronized hippocampal activity as compared with those mostly containing long and/or frequently occurring theta segments. Such relationship was not observed in the adjacent reticular formation (n=4) and in the median raphe nucleus during sensory stimulation (n=2).

The present data complete those found earlier indicating that the desynchronizing serotonergic influence originating from the brainstem is maintained by a tonic excitatory input to the median raphe nucleus. Since the majority of glutamatergic afferents to the median raphe nucleus originates from the lateral habenula and the interpeduncular nucleus, known to connect limbic forebrain to the brainstem, theta associated changes in median raphe nucleus glutamate levels might reflect descending forebrain influences, suggesting therefore a feedback regulation of the hippocampal activity involving brainstem structures.

Key words: microdialysis, GABA, excitatory amino acids, serotonin, limbic forebrain, pontine reticular formation.

Rhythmic synchronized activity (theta) dominates the hippocampal electroencephalogram (EEG) of the rat during specific behaviours of waking and during rapid eye movement sleep.[35] Theta is also present under urethane anaesthesia where it can either occur spontaneously or can be elicited with slight (e.g., stroking the fur) or relatively strong (e.g., tail pinch) sensory stimulation.[5] It is generally accepted that switching between theta and non-theta activities is under control of several structures in the brainstem, in both preparations.[8,21,38] The present study focused on the control of the ascending serotonergic input to the hippocampus originating from the median raphe nucleus (MRN) that has long been implicated in the regulation of the hippocampal EEG.[2,21,38] While the exact role of this input in the behaving animal remains controversial,[30,36] in the urethane anaesthetized rat, where its effect on the hippocampus can be tested separate from other components of the behavioural arousal, MRN was shown to exert a pronounced desynchronizing influence on the hippocampal EEG.[2,21,38] It has been reported from several laboratories that electrical stimulation of the MRN disrupted rhythmic theta activity in the septum and hippocampus[2,21,38] while pharmacological suppression of the MRN activity by microinjection of procaine produced constant uninterrupted theta.[16,39] This effect was dependent on the activity of the population of MRN serotonergic neurons since the effect of MRN stimulation could be blocked by pretreatment with the 5-hydroxytryptamine (5-HT) inhibitor, p-chlorophenylalanine (PCPA),[2] and selective inhibition of serotonergic cells through their 5-HT1a autoreceptor by microinjecting 8-hydroxy-

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Abbreviations: AP-7, 2-amino-7-phosphoroseptanoic acid; EAA, excitatory amino acid; EEG, electroencephalogram; GAMS, γ-glutamyl-aminomethane sulfonic acid; 5-HT, 5-hydroxytryptamine; MRN, median raphe nucleus; NMDA, *N*-methyl-D-aspartate; 8-OH-DPAT, 8-hydroxy-dipropylaminotetraline; OPA, orto-phtalaldehyde; PCPA, p-chlorophenylalanine; RF, reticular formation.

dipropylaminotetraline (8-OH-DPAT) into the MRN induced the same changes as procaine.[16,39]

Although ascending raphe serotonergic systems have been implicated in a number of physiological functions, the control of activity of the serotonergic neurons themselves is poorly understood. The MRN is a target of numerous afferent projections[1,3,9,23,24] utilizing a great variety of neurotransmitters.[9,20,33] Many of these afferents are components of different two-way connections between raphe and the limbic system. In fact, the most prominent inputs to the midbrain raphe nuclei originate from the lateral habenula and the interpeduncular nucleus both known to relay descending limbic influences to the brainstem.[1,3,11,12,22,23,40] These projections, using excitatory amino acid (EAA) for transmission[3,11–13] and terminating on the raphe serotonergic and non-serotonergic elements are in a position to directly and indirectly modulate serotonergic output of the MRN.[13,22,26,40] In accordance with these data, it has recently been shown in urethane-anaesthetized rats that hippocampal theta can be elicited by antagonizing the EAA transmission in the MRN.[15] Local microinjections of competitive (AP-7) or non-competitive (dizocilpine maleate; MK-801) *N*-methyl-D-aspartate (NMDA) antagonists or the kainate/quisqualate receptor antagonist, γ-glutamyl-aminomethane sulfonic acid (GAMS), into the MRN produced hippocampal theta rhythm at short latencies and for long durations.[15] Furthermore, the effect of AP-7 was reversed by local application of NMDA, i.e. NMDA injected into the MRN reliably and transiently disrupted theta and restored desynchronized hippocampal activity.[15] MRN injection of AP-7 was also shown to reduce serotonin turnover in the hippocampus.[41] On the basis of these findings and available anatomical data, Kinney *et al.*[15] hypothesized that the activity of MRN serotonergic neurons is under control of a tonically-active glutamatergic drive most likely originating from the lateral habenula and/or interpeduncular nucleus.[1,11–13,22,23,40] If so, during theta activity, due to suppression of this tonic activation, serotonergic neurons in the MRN would become disfacilitated and the resulting decrease of their effect on the septum/hippocampus would further assist shifting the activity of the septum and hippocampus from a desynchronized non-rhythmic to synchronized theta rhythmic pattern. The aim of the present study was to find evidence for such negatively theta-associated tonic release of glutamate in the MRN.

EXPERIMENTAL PROCEDURES

Animals

Sprague–Dawley rats (Labor Animal BT, Budapest, Hungary) of either sex weighing 250–400 g were used. Rats were allowed food and water *ad libitum* prior to the beginning of the experiments. The experiments were performed under urethane anaesthesia (i.p., 1.2–1.5 g/kg).

Electrophysiological recordings

Hippocampal field activity was recorded with bipolar electrodes positioned in the dorsal hippocampi on both sides. With the rats mounted in a David-Kopf stereotaxic frame, two pairs of twisted insulated stainless steel wires (125 μm) separated by 1 mm at their tips were implanted (AP: −3.7; L: ±2.2; H: −3.5)[29], one in the CA1 region and the other below the hippocampal fissure, verified by the out-of-phase rhythmicity in the two recordings, and fixed with dental cement. Hippocampal EEG was amplified, filtered (1.5–70 Hz) and continuously recorded on a polygraph (Mingograph 81; Siemens-Elema) and were in a few cases also stored on magnetic tape (FM tape recorder; Schlumberger).

During baseline conditions, the hippocampal recordings showed desynchronized field activity mixed with spontaneously occurring segments of theta rhythmic EEG. In the first group of rats only the spontaneous activity of the hippocampus was monitored i.e. no attempt was made to modify the occurrence of theta activity other than by the level of urethane anaesthesia. In the second group of experiments, after control recordings, long-lasting theta activity was elicited by pharmacological manipulations (injection of Physostigmine, 0.1 μg i.v., or Sintostigmine, 20 μg, i.p.) or by sensory stimulation, i.e. a series of 20 s tail pinches applied every 2 min. Finally, in six rats, after control recordings, spontaneous theta was completely eliminated by i.p. injection of Nembutal in a dose of 45 mg/kg body weight.

Microdialysis procedure

Through a small hole stereotaxically drilled in the skull over the MRN (AP: −8; L:0) or the reticular formation (AP: −8; L: ±1.5), concentric microdialysis probes (membrane length: 3 mm, diameter: 0.2 mm, mol. wt cut-off=5000; see Ref. 10 for details) were slowly lowered into the brainstem (H: −9.5 in MRN; H: −8.5 in reticular formation, RF; tip of the probe). The probes were perfused (80 μl/h; Infusor B. Braun Melsungen) with artificial cerebrospinal fluid (NaCl 128.2 mM; $KHCO_3$ 1.758 mM; Ca-lactate 0.567 mM; $MgSO_4$ 0.996 mM; glucose 4.395 mM). Two to eight samples (20 min each) were collected in each experiment. Extracellular concentrations of glutamate and other amino acids (see Table 1) was determined from the dialysates by high-performance liquid chromatography using a Chrompack MicroSphere C18 reversed phase column (dimensions: 4.6 mm × 100 mm; particle size 3 μm). Precolumn derivatization of amino acids were carried out with orto-phtalaldehyde (OPA) in the presence of mercapto-ethanol (pH: 10.4). OPA (50 mg) was solved in borate buffer solution (1 M, 500 μl) containing KOH (pH 10.4), methanol (4000 μl) and mercapto-etOH (50 μl). The gradient profile of A-eluent (phosphoric acid 800 μl; tetrahydrofurane 2000 μl; tetraetilamine 100 μl in 500 ml solution; pH 7.0–7.1) and B-eluent (phosphoric acid 600 μl in 500 ml solution mixed in 3:7 with acetonitrile, pH 7.0–7.1) was 100%–0% (0 min), 85%–15% (10 min), 75%–25% (25 min), 50%–50% (35 min). The column was equilibrated between the samples for 10 min in 100% of B-eluent and for 10 min in 100% A-eluent. After every 10 samples standard solution was injected into the column. The derivatized amino acids were measured with fluorescence detector (Pharmacia AminoSys; excitatory filter: 305–395 nm; emission filter: 430–470 nm). The chromatograms were evaluated with PE Nelson 2000 software.

The identification of HPLC peaks was accomplished by using standard amino acid solution with known amino acid concentrations. The glutamate peak was further verified in five rats by application of 120 mM K in the perfusion fluid. A marked increase of glutamate peak was also observed *post mortem*, in these experiments.

Table 1. Extracellular concentration of different amino acids in the median raphe nucleus and the pontine reticular formation

	Median raphe nucleus (n=7)		Pontine reticular formation (n=4)	
	Amino acid concentration* (μM ± S.E.M.)‡	χ^2 test† (P-value)	Amino acid concentration* (μM ± S.E.M.)‡	χ^2 test† (P-value)
asp	0.523+0.079	>0.5	0.788+0.053	>0.7
glu	1.243+0.092	<0.001	1.068+0.079	<0.1
ser	2.992+0.336	>0.5	4.374+0.504	>0.9
gln	15.82+1.626	>0.5	6.954+0.704	<0.1
gly	3.617+0.490	>0.5	2.939+0.351	>0.9
thr	2.652+0.162	>0.5	1.397+0.108	>0.9
arg	0.523+0.037	>0.2	0.777+0.160	>0.9
ala	2.106+0.323	>0.5	2.58+0.222	>0.1
tau	1.583+0.175	>0.5	0.998+0.184	>0.9

*Amino acid concentrations represent the average of all samples.

†The χ^2 test refers to statistical analysis of the relationship between the direction of changes in theta percentage and corresponding amino acid concentrations. P-values indicate the significance level of the association exhibited by 2 × 2 contingency table (see Experimental Procedures).

‡Concentrations are expressed according to the system SI. For comparison with values reported in some other publications, these concentrations can be expressed in pmol/sample (i.e. pmol/26 μl) by multiplying the numbers in the Table by 26.6.

Histology

The position of the microdialysis probes was verified by *post mortem* microscopic examination of the rat's brains. At the end of each experiment, the rat was transcardially perfused with 200 ml saline and 200 ml of 10% solution of formaldehyde, under deep barbiturate anaesthesia. The brains were removed and stored in a 1:1 solution of 96% ethyl alcohol and 40% formaldehyde until sectioning. Sections of 60 μm made in the coronal plane using a Lancer Vibratome were mounted and stained with Cresyl Violet (Nissl's method).

Data analysis

The relationship between hippocampal EEG synchronization and glutamate release in the brainstem was evaluated by comparison of the changes in these parameters during consecutive observation periods. Each observation lasted 20 min during which one microdialysis sample was collected and the concurring hippocampal activity was recorded. The beginning and end of each theta and non-theta segment were marked on the polygraphic recordings and their percentage (theta% and non-theta%) of the total length of the observation was calculated. Glutamate levels were determined in Rate of Fluorescence Unit (RFU) and the concentration was calculated using standards with known glutamate concentrations. For between-experiment comparison, both glutamate levels and the amount of theta were normalized i.e. expressed as the percentage of the maximal values found in that particular experiment (glutamate% and theta%). Group values are presented as mean ± S.E.M. The observations were cross-classified according to the direction of changes in glutamate% and theta% to build 2 × 2 contingency tables and significant associations between the two variables was statistically tested using the χ^2 test.

RESULTS

The location of the microdialysis probe was histologically verified in the MRN in eight out of 12 experiments. In the other four rats, the probe was found in the reticular formation lateral (n=3) or antero-lateral (n=1) to the MRN (Fig. 1).

In all experiments, two patterns of the electrical activity of the hippocampus, one characterized by regular rhythmic oscillations (theta) and the other by desynchronization of the extracellular field potentials (non-theta), changed spontaneously without any experimental interventions. The length of spontaneously occurring theta segments showed large variations, from 5 s to 30–40 min. When summed over 20 min periods, theta segments comprised 3–100% of these observations, in different experiments. There were less variations, however, in the percentage of theta segments between the observation periods in individual experiments (12–79%; average: 29%). Therefore, in those rats showing small or no changes in theta/non-theta ratio over several observations the frequency of theta segments was manipulated, i.e. increased by sensory stimulation (to 29–79%; n=3) or by injection of anticholinesterase (to 54–83%; n=3) or completely eliminated by injection of Nembutal (n=6).

Figure 2 demonstrates two observations, from a representative experiment, with unequal levels of glutamate in the MRN and corresponding differences in the character of hippocampal activity. Hippocampal recording during the first 20 min period was dominated by desynchronized activity interrupted by short episodes of theta rhythmicity (Fig. 2A). The total length of spontaneously occurring theta segments was as low as 36 s (i.e. 3%) and the concentration of glutamate was 2.481 μM (65.994 pmol/20 min sample). In the other recording, taken 40 min later, the total length of theta segments was substantially higher (16.6 min, i.e. 83%) and the change was associated with a decrease of glutamate level to

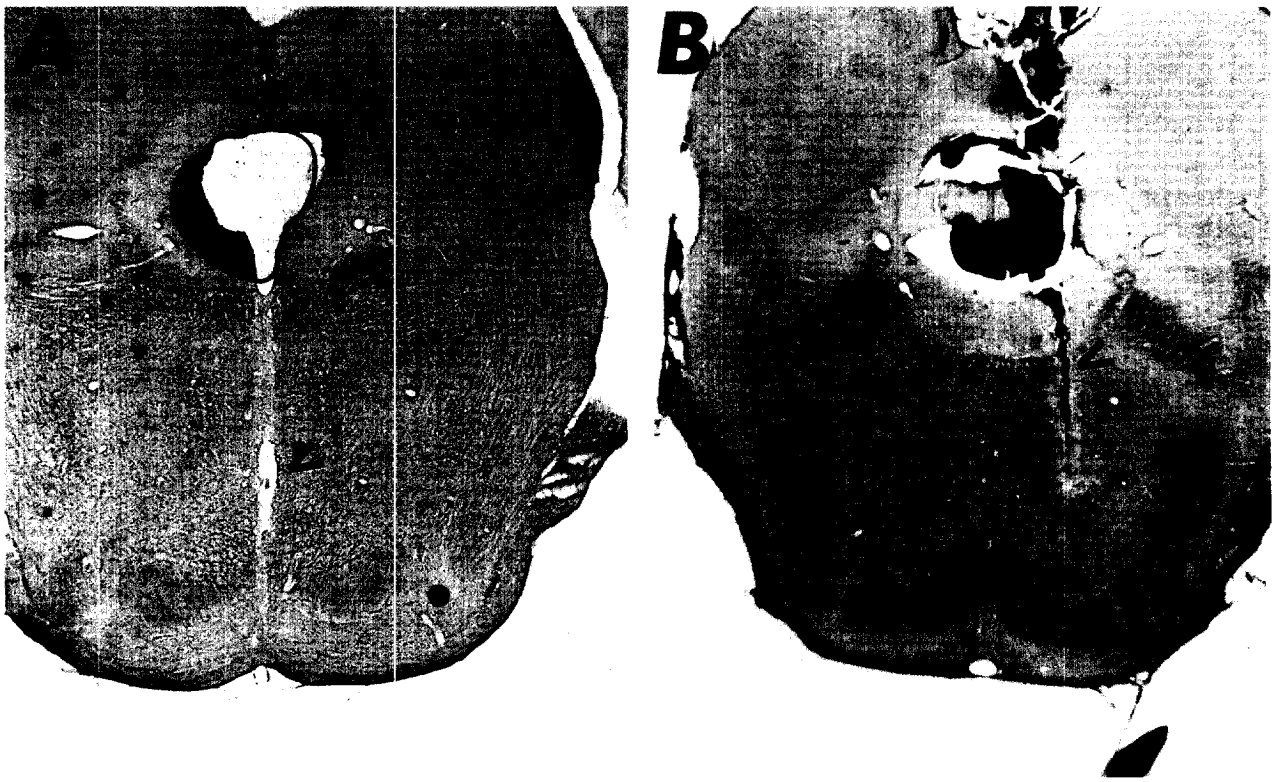

Fig. 1. Photomicrograph of coronal sections through the pons of two rats. Arrowheads show the location of the microdialysis probes in the median raphe nucleus (A) and in the pontine reticular formation (B).

1.613 μM (42.905 pmol/20 min sample) in the MRN (Fig. 2B).

The glutamate concentration in the MRN and the percentage of non-theta segments in the hippocampal EEG always shifted in the same direction, in consecutive samples, when the changes occurred either spontaneously or were elicited by i.p. injection of Physostigmine or Nembutal (Fig. 3A). The extent of these changes, however, appeared quite different for different sample pairs. Expressed as percentage of the maximum recorded in each particular experiment, variations in the occurrence of desynchronized EEG and the level of glutamate in the MRN were in many samples highly correlated (see overlapping data points and connecting lines in Fig. 3A). In other cases, however, large variations in one parameter was associated with relatively small changes in the other indicating a non-linear relationship between the two. The scatter plot in Fig. 4A summarizing the results of 24 observations from seven experiments also demonstrates that higher glutamate levels were associated with less amount of theta activity. The MRN glutamate concentration and hippocampal theta activity changed in opposite directions in each pair of consecutive observations (significance tested by χ^2 test, $n=7$, $P<0.001$). It should be noted, however, that due to the differences in the extent of variations in the two parameters, the clear correspondence between the direction of changes in individual experiments notwithstanding, there was a relatively low linear correlation between the two variables, in the whole sample.

In two experiments, in which the manipulation applied to increase theta activity involved sensory stimulation, glutamate level in the MRN did not show such correlation with hippocampal activity (Fig. 3B, expt nos 12 and 7). In one experiment (expt no. 7), in which both spontaneous and tail pinch-elicited theta were tested, the two parameters changed in the same direction during the first 80 min (i.e. four samples with spontaneous theta segments; see in Fig. 3A) but appeared dissociated after a series of tail pinches (see continued, in Fig. 3B). Glutamate level in the samples taken during sensory stimulation (marked by arrow in Fig. 3B) and during the next 20 min dropped in parallel with an increase in the occurrence of desynchronized hippocampal activity. In the other rat (expt no. 12, Fig. 3B), tail pinches applied every 2 min during the second observation period were also followed by opposite changes in the two parameters for 40 min and turned again in the same direction after an injection of Nembutal.

In four control experiments the microdialysis probe was placed in the brainstem outside the MRN. In three rats (expt nos 8–10, Fig. 3B), in which dialysis samples were collected from the pontine reticular formation lateral to the MRN (Fig. 1B), variations in the concentration of glutamate followed the direction of changes in theta activity (i.e. changed

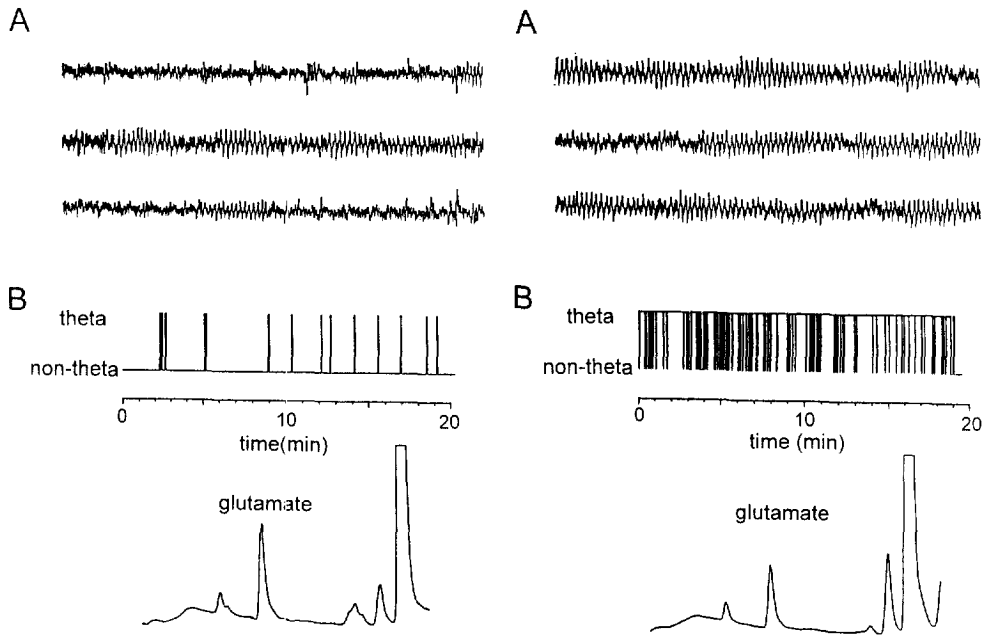

Fig. 2. Spontaneous hippocampal EEG and corresponding extracellular glutamate concentration in the median raphe nucleus during two observation periods dominated with non-theta (A) or theta (B) activity. The two observations, made in the same rat, were separated by 40 min. Top traces: sample recordings of hippocampal EEG; middle traces: "hippocampal thetagram" i.e. diagrammatic representation of the switching between theta (higher level) and non-theta (lower level) states during the entire 20 min observation period; bottom traces: part of the chromatogram with the deflection corresponding to glutamate concentration in the dialysate.

against the direction of variations in non-theta segments, as shown in Fig. 3), with the exception of the last sample in expt no. 10 taken after injection of Nembutal. Thus, the correlation between theta occurrence and glutamate levels in the reticular formation was opposite to those observed in the MRN but weaker (χ^2 test, n=3, P<0.1). In one rat with the probe located anteriolateral to the MRN, the concentration of glutamate showed no correlation with the activity of the hippocampus (expt no. 11, Fig. 3B).

In addition to glutamate, clear peaks could be identified on the chromatograms representing other amino acids, such as aspartate, serine, glutamine, glycine, threonine, arginine, alanine, and taurine. The molar concentration of these amino acids in the MRN (n=8) and the pontine RF (n=4) are shown in Table 1. The extent of variations in the extracellular concentrations of different amino acids was not uniform (see different S.E.M. values in Table 1). Neither showed, however, a consistent state-dependent change when samples collected during theta and non-theta periods were compared using the χ^2 test.

DISCUSSION

The present study demonstrates a significant relationship between spontaneous changes in the electrical activity of the hippocampus and the extracellular level of glutamate in the MRN of the urethane anaesthetized rat. The extracellular level of glutamate was elevated in the MRN during periods dominated by desynchronized hippocampal activity as compared with those mostly containing long and/or frequently-occurring theta segments. Similar to that found during spontaneous changes in the theta/non-theta ratio, increased glutamate concentration was also measured after complete elimination of theta by switching to barbiturate anaesthesia whereas higher theta occurrence due to injection of anticholinesterase agents was associated with a suppression of glutamate release. Such relationship did not appear to exist, however, when hippocampal theta was elicited by sensory stimulation. On the contrary, during tail pinches applied in two rats MRN glutamate level increased relative to the control non-theta segments. In control experiments (n=3), with the dialysis probe placed into the reticular formation, this latter "reversed" pattern was observed during both spontaneous and sensory-evoked theta segments.

Potential methodological limitations of the study

The primary aim of these experiments was to complete previous pharmacological data suggesting that glutamatergic influences on the MRN serotonergic neurons play an important role in regulation of the hippocampal EEG[15] and to test in particular whether such suggestion is supported by spontaneous, state-dependent variations in extracellular

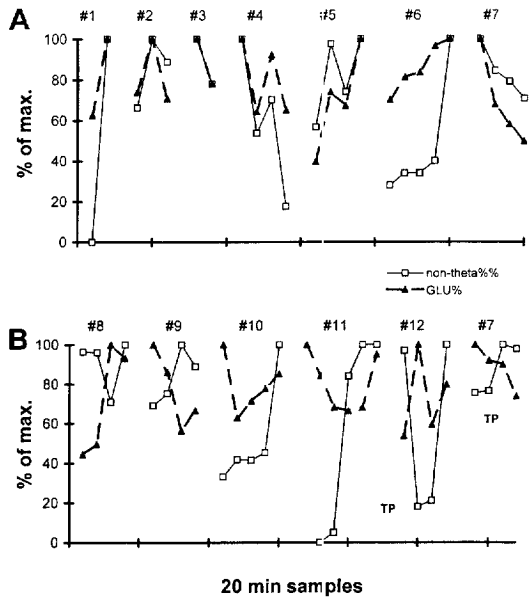

Fig. 3. Concomitant changes in the percentage of non-theta segments in hippocampal activity (solid lines) and corresponding extracellular glutamate concentration (dashed lines) in the median raphe nucleus and pontine reticular formation. Each dot corresponds to one 20-min observation and the dots representing consecutive observations in the same experiment are connected. Both parameters are expressed as percentage of the maximum measured in the same rat. (A) Experiments (nos 1–7) in which the dialysis probes were located in the MRN and the theta/non-theta segments changed spontaneously or by injection of barbiturate or anticholinesterase agents, i.v. (B) Control experiments in which the probe was located in the pontine RF (nos 8–11) or in which theta was elicited by tail pinch (TP, nos 12 and 7). Note parallel changes in the two parameters in A and opposite changes in B.

glutamate concentration in the MRN. It should be noted, however, that changes of extracellular glutamate concentration could derive from different sources and microdialysis method cannot by itself differentiate glutamate of synaptic and metabolic origin.[4,7,14,31] In fact, our data suggest that to some extent metabolic release indeed contributed to the concentrations we measured in the perfusates. It was noted that the decrease in glutamate concentration during theta was not proportional to the total amount of theta in the sampling period as might have been expected were the variations in glutamate exclusively determined by changes in synaptic release. Due to unspecificity of sampling the extracellular space and to the poor temporal resolution of the microdialysis method the dynamics of these processes can only be given a very coarse estimate, as e.g., detecting the direction of changes in every 20 min.

On the other hand, none of the other amino acids changed in the extracellular space during theta activity (see Table 1) which supports the specificity and a probable synaptic dominance of non-theta related extracellular glutamate increase and discards several explanations that could be offered for the present findings based on state-dependent metabolic alterations in the nervous tissue. For example, there was no increase in taurine indicating that glutamate was not released as osmolyte due to a volume control-related mechanism[14,32] and the stability of glutamine made it unlikely that modifications in the glutamine-glutamate shuttle[7,14] played a major role. It can be concluded in general terms that, whatever the origin of glutamate in the MRN is, significant decrease of the extracellular level of glutamate is the result of synaptic and/or metabolic alterations induced by changes of the firing pattern of the neurons[18,19] involved in theta regulation within the MRN and/or the afferents influencing the neuronal circuits of the MRN.[1,11–13,22,23,40] For further conclusions concerning the relative role of different mechanisms microdialysis data should be evaluated together with the results of electrophysiological and pharmacological studies.

Possible role of glutamatergic control of median raphe nucleus serotonergic neurons in regulation of hippocampal theta activity

It is well established that activation of the serotonergic input to the septum/hippocampus desynchronizes hippocampal EEG[2,21,38] while suppression of the serotonergic neurons in the MRN results in long-standing theta activity.[2,16,39] Since hippocampal theta could also be elicited by antagonizing the glutamate transmission in the MRN, it was hypothesized[15] that the activity of MRN serotonergic neurons was under control of a tonically active glutamatergic drive which could this way be involved in switching between and/or maintaining non-theta and theta activities. When active, this input to the MRN could participate in sustaining the tonic desynchronizing effect of the raphe-hippocampal system while its suppression would disfacilitate serotonergic neurons in the MRN allowing for theta synchronization in the septum and hippocampus. The character of natural variations of the extracellular level of glutamate in the MRN of the urethane-anaesthetized rat, examined in the present experiments, were found supportive of this hypothesis. Thus, the present observations are complementary with previous pharmacological findings of Kinney *et al.*[15] which also support the validity of methods used in this study.

The majority of EAAergic afferents of the MRN originates from the lateral habenula and the interpeduncular nucleus.[1,11–13,22,23,40] The habenular complex has traditionally been considered as the key element of the dorsal diencephalic conduction system "serving a pivotal role in funnelling information from limbic forebrain to the limbic midbrain area".[24,34,40] More recent anatomical reports identified the raphe nuclei as the primary targets of the habenulo-midbrain projections composed of a direct pathway from the lateral habenula and an indirect one from the medial habenula relayed via the interpeduncular

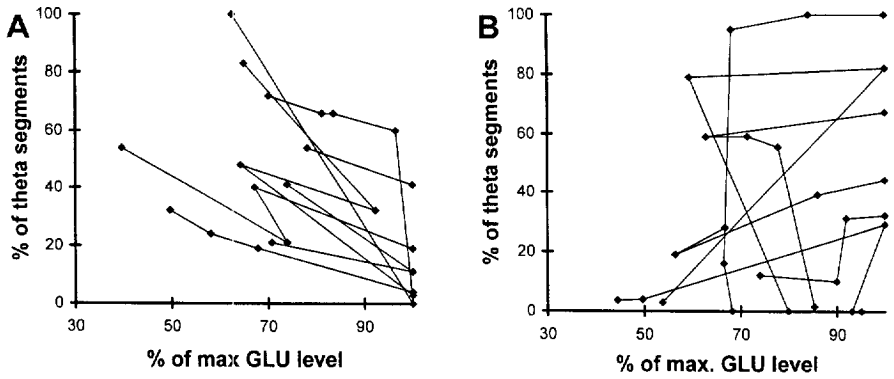

Fig. 4. Relationship between the occurrence of theta activity in the hippocampus and glutamate concentration in the median raphe nucleus and pontine reticular formation. Experiments shown in A and B are the same as those in Fig. 3A and B. Connected dots indicate measurements in the same animal. Note that scatterplot in A (but not B) reveals a marked negative correlation between theta percentage and MRN glutamate levels (see similar direction of all connecting lines) although the linear correlation between the two parameters, for the whole sample, is low.

nucleus.[3,9,11,22] Since habenular afferents in turn originate from forebrain sites involved in theta generation, such as the septum and the nucleus of the diagonal band of Broca,[24,28,34] this system appears to be in a position to convey state-dependent descending influence to the MRN and participate in a feedback regulation of the hippocampal activity.

Although electrophysiological data concerning the firing characteristics of serotonergic neurons related to theta under urethane are not available, indirect evidence obtained by selective suppression of their activity[2,15,21,38,39] indicates that serotonergic neurons are more active during non-theta states. The results of the present study is then consistent with an excitatory action on the MRN serotonergic neurons of glutamate released during spontaneous theta/non-theta recordings although an inhibitory effect is also conceivable during sensory induced theta states. Direct application of glutamate has an excitatory effect on the raphe serotonergic neurons.[20,22,27] MRN, however, contains a population of GABAergic cells[25] and it was suggested that habenular afferents may exert their influence in part through GABAergic interneurons.[13,26,27,40] Further experiments are necessary to clarify whether final excitatory or inhibitory effect on the MRN output is determined by the balance between direct EAAergic activation of serotonergic cells and their interneuron-mediated inhibition by common EAAergic drive(s) or by the action of different afferents terminating on different types of MRN units.

Behzadi *et al.*[3] reported that neurons in other regions of the limbic forebrain and diencephalon were also labelled by injection of D-[^{3}H]aspartate into the MRN indicating additional EAAergic afferents to the raphe. Although these projections appear relatively weak as compared with those originating from the lateral habenula and the interpeduncular nucleus their contribution to the MRN glutamate level is also possible. Some of these structures, e.g., cingulate cortex, supramammillary nucleus,[17] posterior hypothalamus, play key roles in controlling theta frequency and exhibit strong theta-related activity and therefore EAA release due to this input is expected to change in a direction opposite to that seen during the present spontaneous theta/non-theta recordings. Thus, affecting a second source of EAA in the MRN, theta-active afferents might produce an increase of glutamate during certain theta states e.g., triggered by strong sensory stimulation (tail pinches). It is worth noticing that theta-associated increase of glutamate concentration was also observed in the pontine reticular formation. Pontine reticular neurons are strongly activated during theta states in the freely moving[37] rat and direct stimulation of the nucleus pontis oralis elicits theta in the hippocampus and related structures under urethane.[21,38] Assuming that serotonergic neurons are silenced during theta, afferents from these structures could primarily affect GABAergic interneurons of the MRN.

Another alternative for the involvement of GABAergic interneurons is that state-dependent modulation of the release of glutamate takes place at the level of the MRN through a presynaptic mechanism. In this case, EAAergic afferents would convey a tonic drive to the MRN but the transmission would be selectively suppressed during theta by a different input. This effect may also originate from theta-related structures and might involve local GABAergic interneurons. Regarding this possibility, presence of GABA-B receptors have been demonstrated in the MRN[6] and theta-related discharge of putative non-serotonergic neurons in the midbrain raphe nuclei was reported in freely behaving as well as urethane-anaesthetized rats.[18,19]

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