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Modulation of hippocampal theta rhythm by the opioid system of the pedunculopontine tegmental nucleus

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Abstract. The pedunculopontine tegmental nucleus (PPN) belongs to the brainstem system which synchronizes hippocampal activity. Theta relevant intra-PPN circuitry involves its cholinergic, GABA-ergic and glutamatergic neurons and Substance P as neuromodulator. Evidence that PPN opioid elements also modulate the hippocampal theta is provided here. In urethane-anesthetized rats a unilateral microinjection of morphine (MF) (1.5 and 5 μg) increased the maximal peak power of tail pinch-induced theta. The higher dose also increased the corresponding frequency. When the theta was evoked by intra-PPN injection of carbachol (10 μg), the addition of MF (5 μg) prolonged theta latency and shortened the duration of the theta. These effects of MF were blocked by naloxone (5 μg). The results obtained suggest that the PPN opioid system can enhance or suppress the hippocampal theta depending on the actual level of PPN activation.

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Key words: hippocampal theta rhythm, PPN, morphine, naloxone, carbachol

INTRODUCTION

Theta rhythm, nearly sinusoidal, large amplitude, synchronous EEG field activity that could be recorded from the hippocampal formation and neighboring temporal cortex (e.g. Błaszczyk et al. 1996, Buzsaki 2002, Chrobak and Buzsaki 1998) has attracted attention, due to its proposed involvement in memory formation and learning (for review see: Axmacher et al. 2006, Kirk and Mackay 2003, Lengyel et al. 2005), and because it is a good model to study the mechanism and functional significance of the central synchronizing activity (Buzsaki and Draguhn 2004, Ward 2003).

In rodents, the hippocampal theta is present in waking animals during voluntary movements or attentive immobility (Kramis et al. 1975, Oddie and Bland 1998), it accompanies the paradoxical (REM) phase of sleep (Karashima et al. 2004, Leung 1998) and survives under urethane anesthesia (Kramis et al. 1975). In humans, EEG synchronization in the theta band is often observed during learning and memory tasks (Barbeau et al. 2005, Molle et al. 2002), and virtual navigation tasks (Ekstrom et al. 2005). Short hippocampal theta episodes were recorded during REM sleep in humans (Cantero et al. 2003).

Major synchronizing inputs to the hippocampus, which are involved in theta generation, originate in the brainstem reticular nuclei. The most vital being nucleus reticularis pontis oralis (RPO) (Kinney et al. 1998, Nunez et al. 1991, Vertes 1981, Vertes et al. 1993) and pedunculopontine tegmental nucleus (PPN) (Kinney et al. 1998, Nowacka et al. 2002, Vertes et al. 1993). Pathways originating in the rostral pons ascend *via* the posterior hypothalamic and supramammillary nuclei to the medial septum/vertical limb of the diagonal band of Broca, which distributes them to the limbic regions including the hippocampal formation (Bland 2000).

The pivotal role of the PPN in theta regulation was demonstrated by experimental manipulations of its transmitter systems and by PPN inactivation. Cholinergic stimulation of the PPN by means of carbachol (CA) microinjections elicited theta activity (Kinney et al. 1998, Vertes et al. 1993), while inactivation of the PPN by procaine microinjection suppressed the sensory-induced theta (Nowacka et al. 2002) in rats. The cholinergic PPN neurons, whose axons leave the structure (Inglis and Winn 1995, Semba and Fibiger 1992, Woolf and Butcher 1989), may be considered as main PPN effector elements building the brainstem theta circuitry involving also the RPO (Grofova and Keane 1991, Woolf and Butcher 1989). Within the PPN, cholinergic activity may be regulated by GABA-ergic and glutamatergic elements, as intra-PPN bicuculline, a $GABA_A$ antagonist, enhanced and muscimol, a $GABA_A$ agonist, suppressed sensory-elicited theta in urethane anesthetized rats (Nowacka and Trojniar 2000). Intra-PPN MK-801, a glutamate NMDA receptor antagonist, increased theta activity, while intra-PPN glutamate acid prevented the induction of theta oscillations in the same experimental model (Nowacka and Trojniar 2000).

Besides these main transmitter systems, a number of neuromodulatory peptides and/or their receptors were found in the PPN of the rat. These include adrenergic (Hou et al. 2002), serotonergic (Steininger et al. 1997), dopaminergic and orexin- (Baldo et al. 2003), and tachykinin-containing elements (Kohlmeier et al. 2002, Standaert et al. 1986, Szeidemann et al. 1995). The latter were found to inhibit the theta (Leszkowicz and Trojniar 2005).

Another neuromodulatory system of the PPN, which might contribute to theta regulation is the opioid system. Opioid μ receptors have been found in the PPN (Capece et al. 1998, Corrigall et al. 1999). They are known to mediate REM inhibition (Shaw et al. 2005), a sleep stage accompanied by the hippocampal theta in rodents. Opioid REM-inhibition is connected with the opioid-mediated reduction of acetylcholine (ACh) release in the medial pontine reticular formation regions (the PPN targets) (Lydic et al. 1993), which contribute to REM generation (Mortazavi et al. 1999). It has been proven that opioids when given intraperitoneally (i.p.) can suppress hippocampal theta rhythm (Zheng and Khanna 1999) and attenuate cortical EEG in the theta band (Sala et al. 1995).

The above data concerning the opioid system, REM and ACh release, and the fact that cholinergic activation of the PPN induces the theta suggested that opioids involvement in theta regulation was plausible at the level of the PPN. In the present study we provide evidence that activation of μ-opioid receptors by intra-PPN morphine (MF) administration indeed modulates hippocampal theta rhythm. This study is a continuation of our previous work (Leszkowicz and Trojniar 2005, Nowacka and Trojniar 2000, Nowacka et al. 2002) aimed at identifying PPN neuronal network engaged in the regulation of hippocampal theta rhythm.

METHODS

Subjects

Experiments were performed on 41 male Wistar rats, 4–6 months old, weighing about 300–500 g. The rats were housed in a vivarium, which was maintained in a 12-h light-dark cycle (lights on 6 AM) at 22 ± 1°C and provided with food and water *ad libitum*. The animals were divided into groups receiving intra-PPN injections of: morphine in a dose of 1.5 μg (MF1.5 group, *n*=8) or 5 μg (MF5 group, *n*=6), 10 μg of carbachol (CA group, *n*=6), carbachol (10 μg) with morphine (5 μg) in one injection (CA+MF group, *n*=7), carbachol (10 μg) with morphine (5 μg) and naloxone (NAL; 5 μg) in one injection (CA+MF+NAL group, *n*=6), distilled water (solvent of the drugs; H_2O group, *n*=8). All the drugs and their mixtures were dissolved in 0.5 μl of distilled water. The principles for the care and use of laboratory animals in research, as outlined by the Local Ethical Committee, were strictly followed. All the protocols were reviewed and approved by the Committee. All efforts were made to minimize both animals' discomfort and the number of animals used.

Drugs

Morphine (morphine hydrochloride; Polfa, Kutno S.A., Poland), an opioid agonist, in doses of 1.5 μg and 5 μg, and a nonselective opioid antagonist, naloxone (naloxone hydrochloride; Sigma Chemical c.o., St Louis) in a dose of 5 μg were used to test for their influence on theta rhythm. Carbachol, a cholinergic agonist (RBI, USA) in a dose of 10 μg was used to evoke the theta. The drugs were dissolved in distilled water on the day of the experiment and injected directly into the PPN.

Procedure

Surgery and hippocampal EEG recordings were performed under deep urethane anesthesia (Urethane, Sigma-Aldrich, Chemie Gmbh, Steinheim, Germany; 1.2–1.6 g/kg, i.p.). The details of the experimental procedure were previously described (Leszkowicz and Trojniar 2005). Briefly, bilateral hippocampal recording electrodes were located in the stratum moleculare of the upper blade of the dentate gyrus. Stereotaxic coordinates depended on the size of an animal, AP: –3.6 to –3.8 mm, L: ±2.0 to ±2.1 mm, D: –3.0 to –3.7 mm relative to the bregma; according to the atlas by Paxinos and Watson (1998). Reference and ground electrodes were positioned over the olfactory bulbs, where electric activity is minimal. A 10-μl Hamilton microsyringe (Aldrich, Milwaukee, WI, USA) placed over the PPN was used for drug administration. PPN stereotaxic coordinates depended on the size of the animal, AP: –7.8 to –8.1 mm, L: ±1.7 mm, D: –7.0 to –7.3 mm relative to the bregma; according to the atlas by Paxinos and Watson (1998).

The hippocampal EEG signal was recorded (sampling rate 240 Hz) using a Medicor electroencephalograph as a preamplifier (bandpass 0–70 Hz) with EEG DigiTrack software (Elmiko, Warsaw, Poland). The animals were maintained at a level of anesthesia at which spontaneous theta rhythm was not present in the hippocampal EEG, but could be elicited by a tail pinch. The tail pinch was produced with a plastic clamp (the same one for all the animals) positioned in the vicinity of the rat's tail base. Every tail pinch lasted 1 min. Before the cannula insertion three to four pinches were applied every 10 min to check for theta presence. Only animals with theta amplitude of at least 400 μV in one or both hippocampi were included in the experiment. The rats were divided into two groups. In one group the influence of the opioid system was tested on the model of tail pinch-elicited theta and in the other group of CA-elicited theta.

SENSORY STIMULATION (*TAIL PINCH*)

Once the control theta was elicited successfully, 0.5 μl of the solvent was microinjected into the PPN over 3 min and the tail pinch was applied 10 min later (checking for possible vehicle effect). Then, the cannula was gently removed, water in the microsyringe was replaced (except for the control group) by MF (MF1.5 and MF5 groups). Next, the cannula was slowly lowered into the same place. Again, a 10-min period of adjustment was allowed following the insertion, after which the presence of the theta was checked with a tail pinch. Then, the drug (0.5 μl) was injected into the PPN over the period of 3 min and subsequently a tail pinch was reapplied every 10 min over the period of 1 hour. The EEG was recorded continuously.

PHARMACOLOGICAL STIMULATION (*CA*)

Ten minutes after the control tail-pinch induction of the theta, 0.5 μl of solution containing CA (CA group) or CA with MF in one injection (CA+MF group), or

CA with MF and with NAL in one injection (CA+MF+NAL group) were administered into the PPN over the period of 3 min.

The hippocampal EEG was recorded continuously for about 60–90 min until CA-induced theta disappeared from the EEG.

Data analysis

The spectral analysis of the hippocampal EEG in theta frequency band was performed off-line with the discrete Fourier transform (DFT, resolution 0.01 Hz). It was calculated by Chirp-Z algorithm on 6–10 artifact-free 5-s epochs. They were chosen from 1-min records of the sensory-induced theta (3.01–6 Hz), on 5 artifact-free 5-s epochs taken at the beginning and the end of CA-induced theta, as well as on 3 artifact-free 10-s epochs taken every minute of the CA-evoked, well developed theta (2.5–6 Hz).

The following theta records were analyzed: one preceding the cannula insertion into the PPN (pre-injection baseline), 10 min post-solvent (control of the vehicle effect), and 10, 20, 30, 40, 50, and 60 min after MF administration in the case of the sensory-induced theta; or according to the scheme indicated above in the case of CA-induced theta.

To quantify the drug influence on the theta two power spectrum measures: the maximal peak power (P_{max}) in the theta band and the frequency corresponding to P_{max} (F_{max}) were assessed. To eliminate an inter-subject variability both P_{max} and F_{max} were normalized. In the case of the sensory-induced theta, P_{max} in each rat was expressed as a percentage of the pre-injection baseline value taken as 100%. F_{max} was normalized to 4 Hz, because a preliminary analysis of theta P_{max} revealed that the mean (± SD) F_{max} in all the rats in the pre-injection conditions was 4 ± 0.07 Hz and that its distribution was normal. In the case of the pharmacologically-induced theta both P_{max} and F_{max} in each rat (from the CA+MF and CA+MF+NAL groups) were expressed as a percentage of the mean P_{max} or F_{max} in the CA group. The latency for CA-induced theta and its duration were also measured. That minute post-injection in which spontaneous, large amplitude irregular activity (LIA) was replaced by theta oscillations in the EEG record (visual inspection) and a peak in the theta band (2.5–6 Hz) was dominant in the power spectra of all five 5-s epochs was taken as the beginning of the theta. That minute post-injection in which theta oscillations disappeared from the EEG record (visual inspection) and in which the theta peak was no longer dominant in the power spectra of all five 5-s epochs was taken as the end of the theta.

The effects of the intracerebral injections on P_{max} and F_{max} were statistically analyzed with either two-way analysis of variance (ANOVA) with "experimental condition" (pre-injection, 10 min post-solvent, 10, 20, 30, 40, 50, and 60 min post-drug) and "drug" (1.5 μg MF, 5 μg MF, H_2O) as factors followed by the *post-hoc* Tukey's test (at $P<0.05$) for parametric data, or with Kruskal-Wallis test with a factor "drug" (the CA, CA+MF, CA+MF+NAL groups) followed by the *post-hoc* Mann-Whitney test for non-parametric data. Data distribution was checked for its skewness (>0.03, non-parametric data). All the measurements were subjected to the analysis: i.e. P_{max} and F_{max} values from all the 5-s epochs in each tail pinch-elicited theta episode × number of animals or each theta latency/duration × number of animals (df2 in the case of sensory- and pharmacologically-induced theta, respectively). If P_{max} or F_{max} values differed significantly from both the pre-injection and post-solvent control it was considered as an effect of the drug. Following McNaughton and others (1995) suggestion only differences of $F_{max} \geq 0.4$ Hz (mean) were considered as functionally meaningful. The effects of the intracerebral injections on theta latency and duration of the theta were statistically analyzed with the one-way ANOVA with a factor "drug" (the CA, CA+MF, CA+MF+NAL groups). This was followed by the *post-hoc* Tukey's test (at $P<0.05$).

Comparisons of P_{max}, F_{max}, theta latency and duration for hippocampi ipsi- and contralateral to the injection were performed using the Independent-Samples Student's *t*-test. In the cases when the differences in the quality (amplitude) of the control hippocampal theta induced by sensory stimulation in the two hemispheres were found, the effect of the intracerebral injection was analyzed only in that hippocampus (ipsi- or contralateral to the injection), which fulfilled the criterion of the theta amplitude ≥ 400 μV in this control conditions.

Histology

At the end of each experiment, the animal was given an overdose of the anesthetic. Then, electrolytic lesions (anodal current of 200 μA/20 s) were performed through the hippocampal electrodes to visualize their tips locations. Next, the rat was intracardially

perfused with 0.9% saline (200 ml) followed by 10% formalin (200 ml). The brain was removed, fixed in 10% formalin and sectioned at 60 μm. The placement of the hippocampal electrodes and microinjection cannulas tips in the PPN were then confirmed histologically. Additionally, to visualize the spread of the fluid around the injection cannula 2% (in saline) alcian blue 8GX (Fluka, Switzerland) in a volume of 0.5 μl was injected into the PPN. The diffusion area was then measured under a light microscope (Nikon Eclipse E600) in a sample rat.

List of abbreviations

PPN	pedunculopontine tegmental nucleus
RPO	nucleus reticularis pontis oralis
LDT	laterodorsal tegmental nucleus
LIA	large amplitude irregular activity
REM	paradoxical phase of sleep
ACh	acetylcholine
CA	carbachol
MF	morphine
NAL	naloxone
P_{max}	maximal peak power in the theta band
F_{max}	frequency corresponding to P_{max}

RESULTS

Histological verification

Out of 41 animals tested 38 had proper localization of the cannula tips in the PPN (Figs 1, 2; an example cannula trace is presented in Fig. 3). In 3 rats (injected with CA, or CA+MF, or CA+MF+NAL) the cannulas were misplaced and localized below or above the PPN (Fig. 2). These animals were used as control for anatomical specificity of the observed effects. All three misplaced injections were ineffective and excluded from statistical analysis.

The hippocampal electrodes (not shown) from which theta rhythm was of an amplitude ≥400 μV

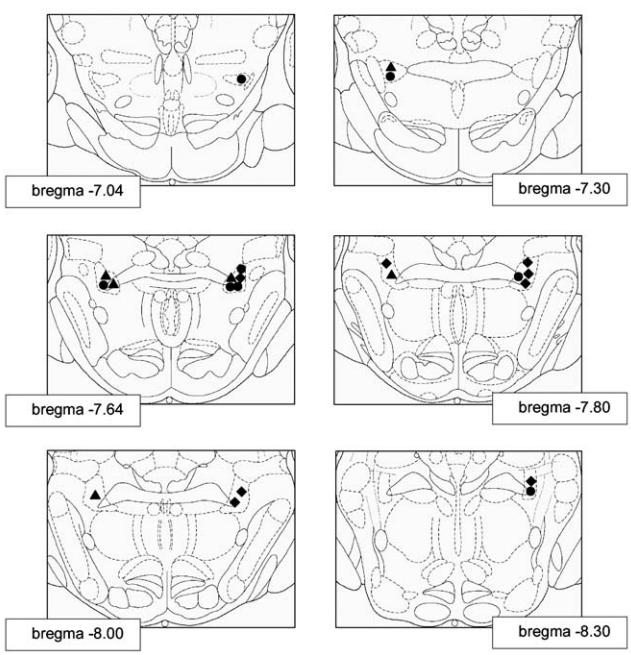

Fig. 1. Localization of the microinjections sites in the pedunculopontine tegmental nucleus in the sensory-stimulated groups superimposed on plates taken from the atlas by Paxinos and Watson (1998) (modified). Explanations: (•) water-injected group; (♦) 1.5 μg morphine-injected group; (▲) 5 μg morphine-injected group. All the cannulas were placed within the PPN (the left or right) mostly in the vicinity of the superior cerebellar peduncule.

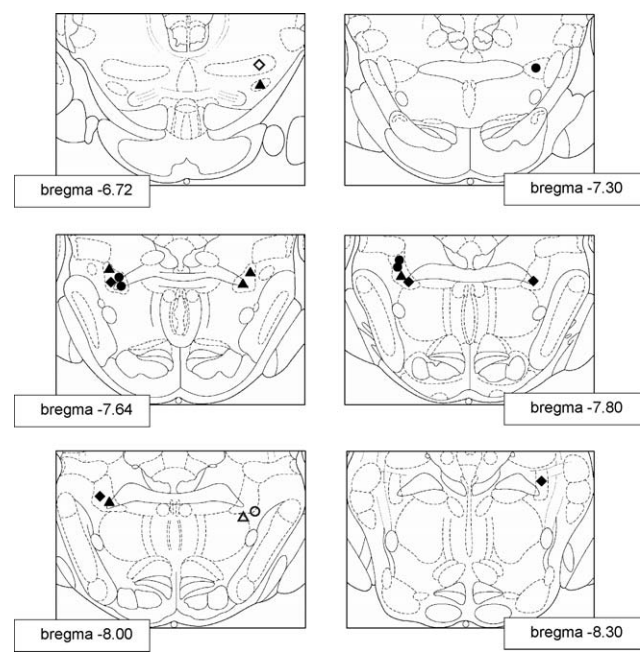

Fig. 2. Localization of microinjections sites in pharmacologically-stimulated groups superimposed on plates taken from the atlas by Paxinos and Watson (1998) (modified). Explanations: filled marks – cannulas localized within the pedunculopontine tegmental nucleus (PPN); open marks – cannulas outside the PPN (ineffective); (•) carbachol-injected group; (▲) carbachol with morphine-injected group; (♦) carbachol with morphine and naloxone-injected group.

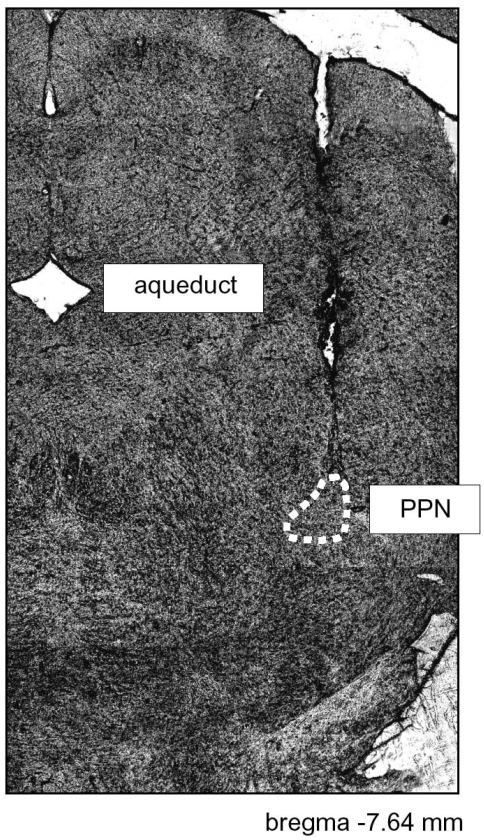

Fig. 3. The trace of the cannula track in an example rat from the morphine-injected group (after morphine administration; Nikon Eclipse light microscope, magnification 40×).

were localized in the stratum moleculare of the upper blade of the dentate gyrus (either bilaterally or unilaterally). In the cases when the theta amplitude was ≤400 μV the electrodes were found in the stratum radiatum of CA1. The latter were excluded from the analysis.

As found in a sample rat injected with the alcian blue dye in the PPN, the spread of the injection was approximately 0.6/0.4/0.8 mm along the AP/L/D axes.

Effect of morphine on sensory-elicited theta

In the MF1.5 group, hippocampi ipsilateral to the injection site were analyzed in 6 rats and contralateral ones in 5 animals. In the MF5 group, hippocampi ipsi- and contralateral were analyzed in 6 and 5 subjects, respectively. In the H_2O group, both hippocampi were analyzed in 7 subjects.

The sensory stimulation evoked theta rhythm bilaterally both in the control conditions, i.e. before any injection, after water administration, and following 1.5 and 5 μg MF. Switch from the spontaneous LIA into theta oscillations during the tail pinch was evident upon visual inspection of the EEG records and in the power spectra (a peak in the theta frequency range) in the analyzed groups (example rats, Figs 4, 5).

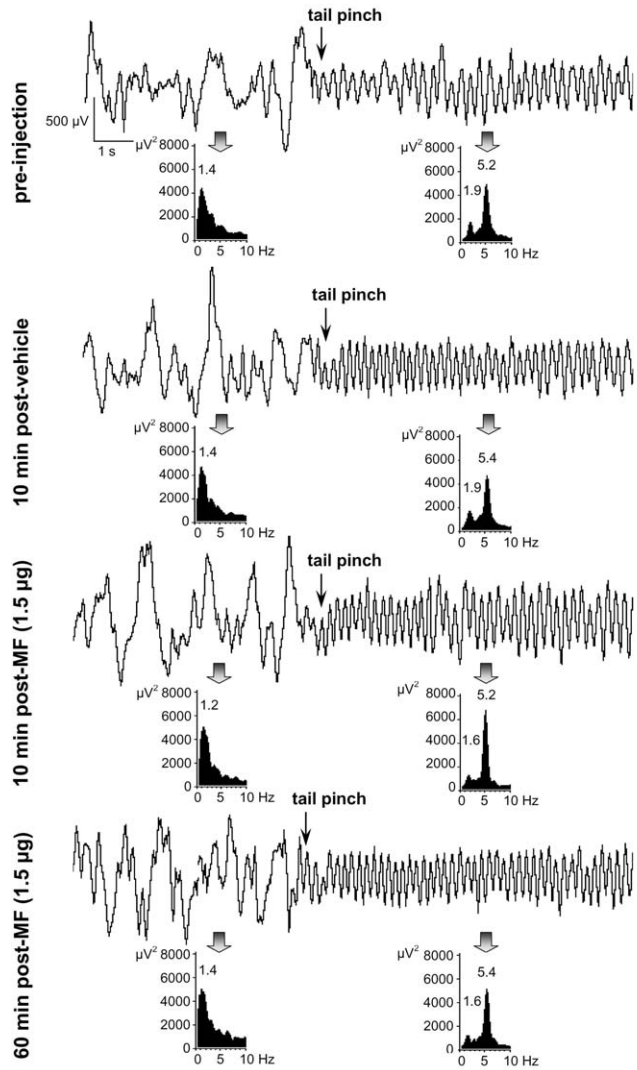

Fig. 4. The course of the experiment in a typical rat receiving intra-pedunculopontine tegmental nucleus (PPN) injection of 1.5 μg of morphine (MF) (hippocampus ipsilateral to the injection). EEG records show the essential moments of the experiment: transitions from the spontaneous large amplitude irregular EEG activity to the tail pinch-induced hippocampal theta rhythm in the pre-injection conditions, 10 min post-vehicle (post-water), 10 and 60 min post-MF. The power spectrograms below the EEG records were obtained from 5-s samples of EEG taken from two periods: the one before and the one during the tail pinch, as indicated by solid arrows. The only effect of intra-PPN MF was an enhancement of power at theta frequency 10 min post-injection.

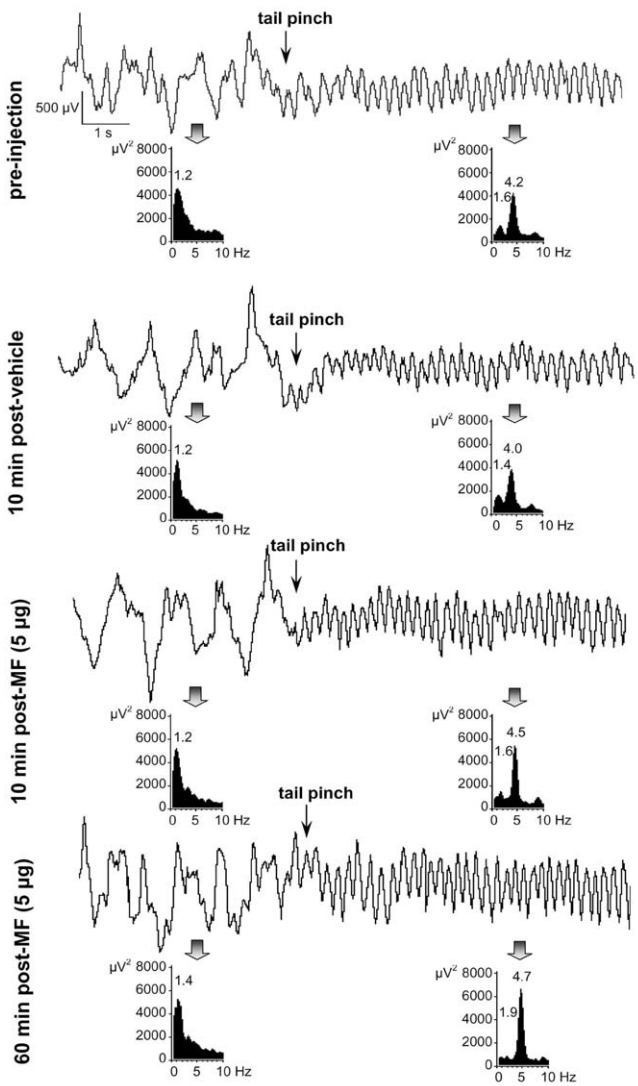

Fig. 5. The course of the experiment in a typical rat receiving intra-pedunculopontine tegmental nucleus (PPN) injection of 5 μg of morphine (MF) (hippocampus ipsilateral to the injection). EEG records show the essential moments of the experiment: transitions from the spontaneous large amplitude irregular EEG activity to the tail pinch-induced hippocampal theta rhythm in the pre-injection conditions, 10 min post-vehicle (post-water), 10 and 60 min post-MF. Explanations as in Fig. 4. Intra-PPN MF caused an enhancement of power and frequency at the theta band lasting 10–60 min post-injection.

Analysis of theta P_{max} and F_{max} revealed that MF promoted theta oscillations in a dose-dependent manner. The dose-dependent increase in theta P_{max} and F_{max} was proven statistically.

P_{max} value depended bilaterally on the injected drug (1.5 and 5 μg MF or water) ($F_{2,1429}$=47.74, P<0.001 and $F_{2,1321}$=35.86, P<0.001, in the ipsi- and contralateral hippocampus, respectively) and on the time relative to the injection ipsilaterally ($F_{7,1429}$=4.71, P<0.001). Figure 6 (upper panels) presents changes in P_{max} after MF administration in comparison with the control group receiving water. Both doses of MF significantly increased theta P_{max} in the hippocampus ipsilateral to the injection 10 min after drug administration (to 128.8 ± 6.7% and 134.0 ± 9.6% of the pre-injection value following 1.5 μg MF and 5 μg MF, respectively; P<0.05). In the MF1.5 group P_{max} returned to the baseline in 20 min (103.1 ± 8.0%). In the MF5 group it continued to rise till the end of the experiment (to 178.9 ± 18.7% at the 60th min post-injection; P<0.05).

In the hippocampus contralateral to the injection, P_{max} was increased from 20 to 60 min after the administration of 5 μg MF (to 131.3 ± 10.6% and 140.6 ± 13.1% of the pre-injection value, respectively; P<0.05). There was no change in P_{max} in MF1.5 group on the contralateral side.

Theta P_{max} was higher in the MF5 than the MF1.5 group from 20 to 60 min post-injections, both in the ipsilateral (at the 20th min: 135.2 ± 10.4% and 103.1 ± 8.0% of the pre-injection values, respectively; P<0.05; at the 60th min: 178.9 ± 18.7% and 105.6 ± 5.7%, respectively; P<0.05) and contralateral hippocampus (at the 20th min: 131.3 ± 10.6% and 88.7 ± 8.1%, respectively; P<0.05; at the 60th min 140.6 ± 13.1% and 93.6 ± 6.8%, respectively; P<0.05).

Inter-hemispheric comparisons revealed a higher P_{max} on the ipsi- than contralateral side 10 min following 1.5 μg MF (128.8 ± 6.7% and 107.3 ± 7.1% of the control, respectively; P<0.05). No other inter-hemispheric differences were found.

F_{max} value depended bilaterally on the injected drug (1.5 and 5 μg MF, or water) ($F_{2,1429}$=171.54, P<0.001 and $F_{2,1321}$=119.52, P<0.001, in the ipsi- and contralateral hippocampus, respectively) and on the time relative to the injection ($F_{7,1429}$=9.59, P<0.001 and $F_{7,1321}$=8.58, P<0.001, on the ipsi- and contralateral side, respectively). Figure 6 (lower panels) presents changes in F_{max} after MF administration in comparison with the control group receiving water. No significant effect of 1.5 μg MF on theta F_{max} was observed. Administration of 5 μg MF led to F_{max} increase in both hippocampi. F_{max} rose in the MF5 group 30 min post-injection by 0.5 Hz (to 4.4 ± 0.0 Hz compared with the control group 30 min post-water; P<0.05) on the ipsilateral side and stayed at this level until the 60th min

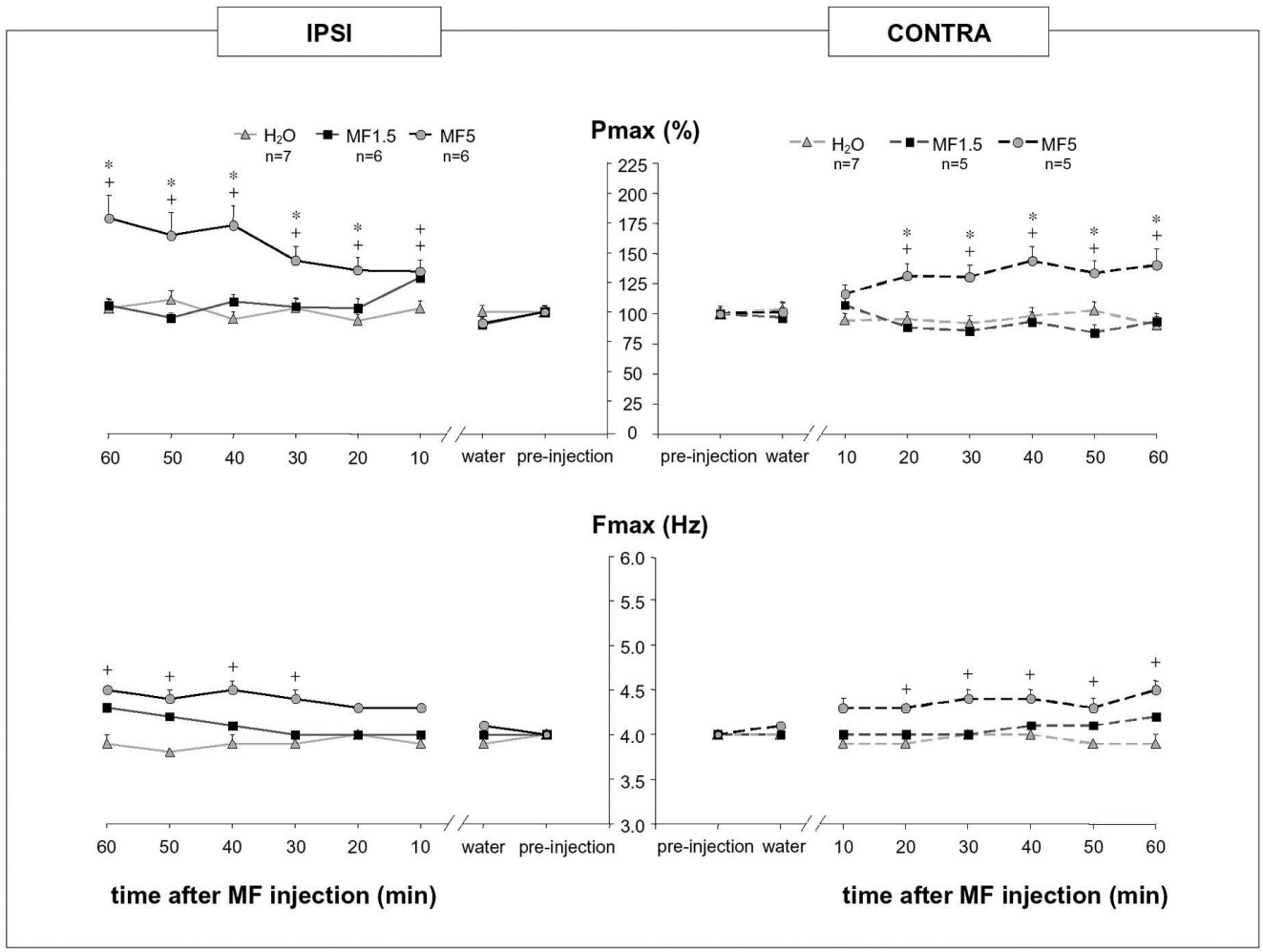

Fig. 6. Effect of unilateral injections of morphine in a dose of 1.5 μg (MF1.5) or 5 μg (MF5) into the PPN on theta maximal peak power (P_{max}, upper graphs) and the corresponding theta frequency (F_{max}, lower graphs) in the hippocampus ipsi- and contralateral to the injection side. Theta rhythm was induced by a tail pinch. Data are presented as means ± SE. *P<0.05, as compared with the control H_2O group; ^{+}P<0.05, comparison between the MF1.5 and MF5 groups (*post-hoc* Tukey's test). Note a dose-dependent effect of MF. MF in a dose of 1.5 μg increased theta P_{max} only 10 min post-injection and only on the ipsilateral side, while 5 μg MF caused bilateral elevation of P_{max}, which persisted over 60 min post-injection. Only the latter dose increased bilaterally also F_{max}.

post-injection when it was higher by 0.6 Hz (4.5 ± 0.1 Hz) than the control (P<0.05). On the contralateral side, F_{max} increased at the 20th min after 5 μg MF by 0.4 Hz (to 4.3 ± 0.3 Hz compared with the control group 20 min post-water, P<0.05), and continued at this level until the 60th min post-injection when it was higher by 0.6 Hz (4.5 ± 0.1 Hz) than the control (P<0.05).

Differences in F_{max} between the MF1.5 or MF5 group were insignificant. No inter-hemispheric differences were observed. At no time was the effect of vehicle administration observed.

Effect of morphine on carbachol-elicited theta

In the CA, CA+MF and CA+MF+NAL groups, hippocampi ipsilateral and contralateral to the injection site were analyzed in 5 rats each (n=5 in each group).

CA injection to the PPN induced theta activity in both hippocampi in the CA, CA+MF and CA+MF+NAL groups. Transitions from LIA to theta oscillations were evident in the EEG records and in the power spectra (a peak at 2.5–6 Hz frequency range; example rats, Fig. 7). Theta oscillations appeared bilaterally during the injection in 2 rats

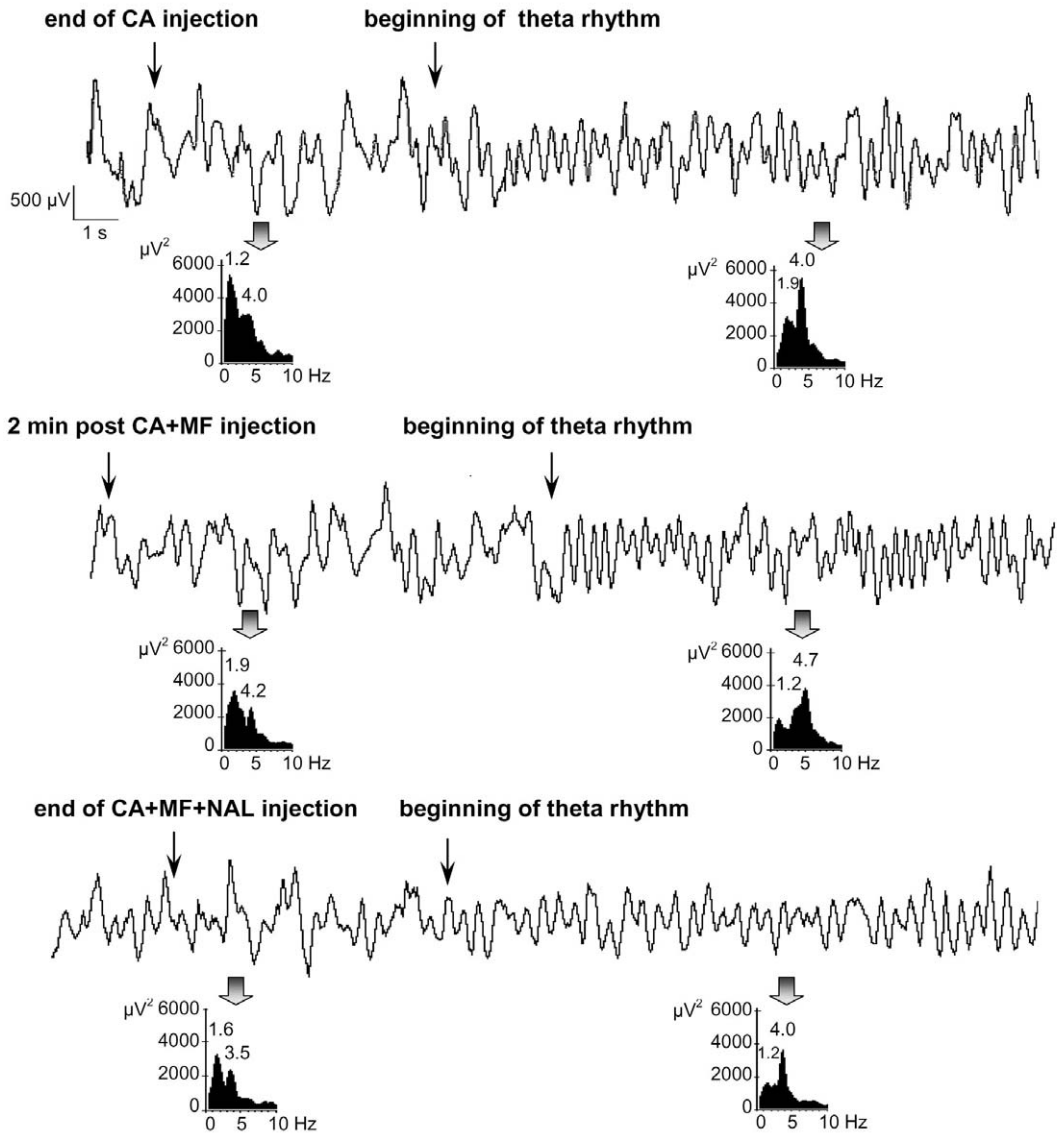

Fig. 7. The course of the experiments in typical rats receiving intra-PPN injections of carbachol (CA), carbachol with morphine (CA+MF), or carbachol with morphine and naloxone (CA+MF+NAL) (hippocampus ipsilateral to the injection). EEG records show the transitions from the spontaneous irregular EEG activity to theta oscillations produced by CA injections. The power spectrograms below the EEG records were obtained from 5-s post-injection samples of EEG taken during the irregular EEG activity and during theta oscillations, as indicated by solid arrows. Note the dominance of slow EEG activity in the power spectrograms before the end of the injection and appearance of a peak in the theta frequency range (2.5–6 Hz) post-injection. Post-(CA+MF) theta oscillations appeared later than post-CA alone (bilaterally). NAL blocked this effect.

from the CA and 4 from the CA+MF+NAL group, 3 min after the injection in 3 rats from the CA and 1 from the CA+MF+NAL group. In rats from the CA+MF group the theta became visible 2–9 min post-injection on the ipsilateral and 4–12 min post-injection on the contralateral side, except for 1 rat, when the oscillations appeared 3 s post-administration in the ipsilateral hippocampus. The longest theta episodes lasted bilaterally about 60 min in the CA, 38–40 min in the CA+MF and 48–51 min in the CA+MF+NAL group.

Figure 8 presents latency and duration of the theta in the experimental groups. Latency for theta depended on the injected drug (CA, CA+MF or

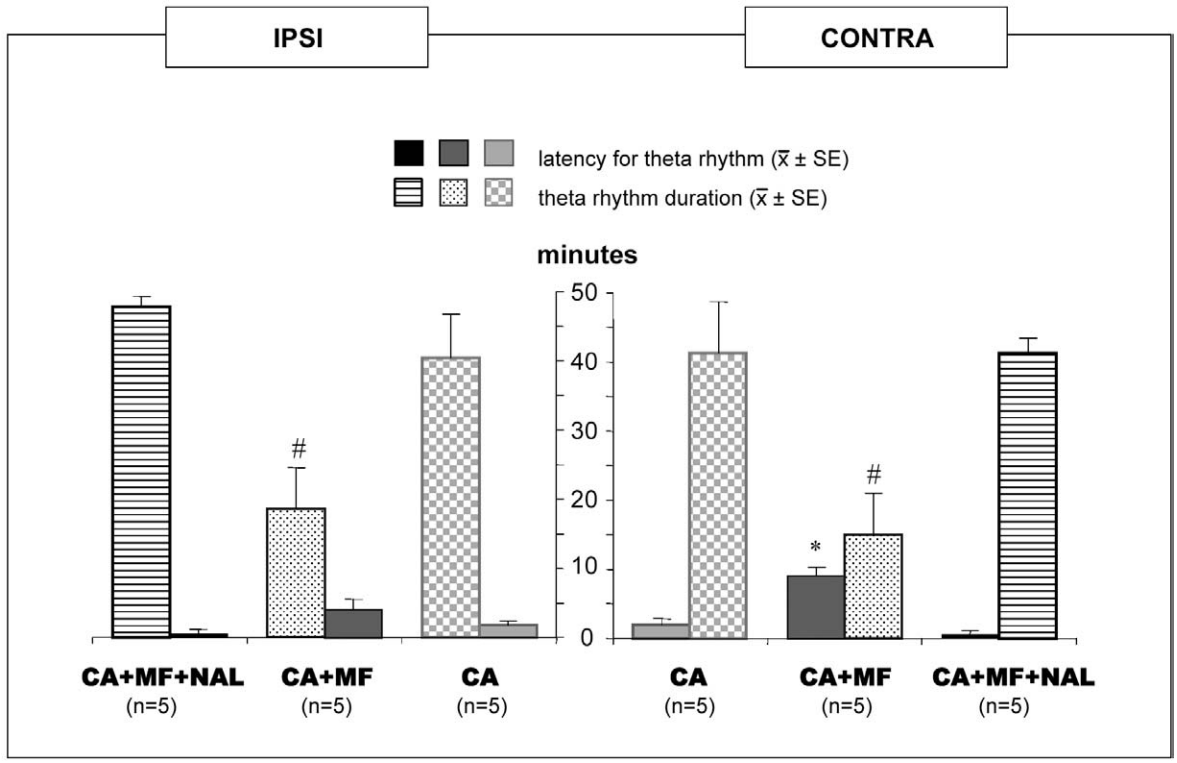

Fig. 8. Effect of unilateral injections of carbachol (CA), carbachol with morphine (CA+MF), or carbachol with morphine and naloxone (CA+MF+NAL) into the pedunculopontine tegmental nucleus on latency for theta rhythm and theta rhythm duration in the hippocampus ipsi- and contralateral to the injection side. Data are presented as means ± SE. **P*<0.05 as compared to the latency in both CA and CA+MF+NAL groups; #*P*<0.05 as compared to the duration of the theta in both CA and CA+MF+NAL groups (*post-hoc* Tukey's test). All three injections produced theta oscillations. MF caused bilateral suppression of the theta manifested by increased latency for theta (significant in the contralateral hippocampus) and a shorter theta duration (significant bilaterally). NAL blocked MF effect.

CA+MF+NAL) only contralaterally ($F_{2,14}$=21.24, *P*<0.001). Theta duration was, however, affected bilaterally ($F_{2,14}$=8.68, *P*<0.01 and $F_{2,14}$=7.5, *P*<0.01, in the ipsi- and contralateral hippocampus, respectively). CA alone produced the theta with a mean latency of 2.0 ± 0.8 min, addition of MF to CA injection prolonged the latency to 9.0 ± 1.4 min (*P*<0.05) (contralaterally). NAL blocked MF effect. In the CA+MF+NAL group theta oscillations appeared with a latency of 0.6 ± 0.6 min (*P*<0.05 compared to the CA+MF group) on the contralateral side. The latency changes on the ipsilateral side were parallel, however they did not reach significance level.

Theta durations were significantly different among these three groups in both hippocampi. In the hippocampus ipsilateral to the injection CA alone produced the theta of a mean duration of 40.4 ± 6.4 min (CA group). MF significantly shortened theta episodes to 18.6 ± 6.1 min (*P*<0.05). NAL blocked MF-evoked theta suppression (47.8 ± 1.5 min in the CA+MF+NAL group; *P*<0.05 compared to the CA+MF group). On the contralateral side, MF shortened theta episodes from 41.4 ± 7.3 min in the CA group to 15.0 ± 6.0 min in the CA+MF group (*P*<0.05). NAL blocked MF effect (41.4 ± 2.1 min in the CA+MF+NAL group; *P*<0.05 compared to CA+MF).

P_{max} in the CA and CA+MF groups did not differ significantly in either hippocampus (Fig. 9, upper panel). The same concerns F_{max} (Fig. 9, lower panel). Similarly, NAL changed significantly neither F_{max} nor P_{max}, except for P_{max} in the ipsilateral hippocampus (309.9 ± 77.2%, *P*<0.01 in comparison with the CA group and *P*<0.05 compared to the CA+MF group) (Fig. 9, upper panel).

Injections of CA, CA+MF, or CA+MF+NAL outside the PPN did not produce the theta (3 cases shown in Fig. 2).

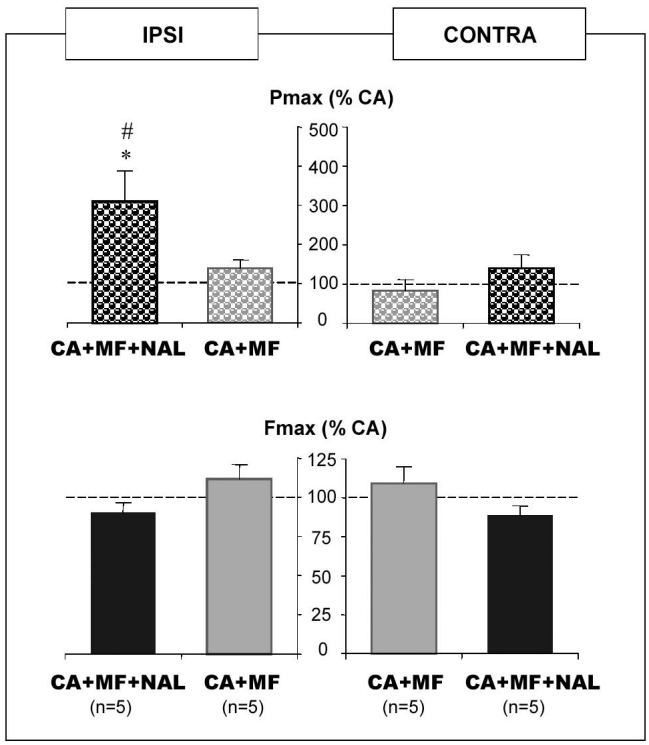

Fig. 9. Influence of unilateral injections into the pedunculopontine tegmental nucleus of carbachol with morphine (CA+MF) or carbachol with morphine and naloxone (CA+MF+NAL) on theta maximal peak power (P_{max}; upper graphs) and the corresponding frequency (F_{max}; lower graphs) in comparison with the injection of carbachol (CA) alone (dashed lines; P_{max} and F_{max} in the CA group taken as 100%) in the hippocampus ipsi- and contralateral to the injection side. Data are presented as means ± SE. *P<0.01, #P<0.05 as compared with the CA or CA+MF group, respectively (*post-hoc* Mann-Whitney test).

DISCUSSION

The principal finding of this study is that unilateral intra-PPN administration of an opioid agonist, MF, can modulate hippocampal theta rhythm in a state-dependent manner. MF effect depends on MF dose and on the nature of the evoked theta. Since the latter may determine the level of the overall PPN activation, MF effect could be related to that level. MF promoted the theta which had been induced by sensory stimulation (tail-pinch, moderate activation) and suppressed the theta evoked by direct PPN cholinergic stimulation (intra-PPN CA, strong activation). Theta enhancement was manifested as an increase in theta maximal power and, following 5 but not 1.5 μg MF, in the corresponding frequency. The rise of theta maximal power was more pronounced following 5 μg MF (long-lasting and bilateral) than 1.5 μg MF (short-lasting and ipsilateral only). MF-related theta suppression was manifested as a lengthening of latency for theta and shortening theta duration, the effect blocked by the opioid antagonist, NAL.

Although literature on opioid-cholinergic (Lydic et al. 1993, Mortazavi et al. 1999) and cholinergic-theta relationships (Kinney et al. 1998, Vertes et al. 1993) suggests opioid engagement in the theta regulation, the observed dual effect of MF on theta rhythm was unexpected. We supposed that MF would rather suppress the theta as it reduces cholinergic transmission in the pons structures related to REM sleep (e.g. Keifer et al. 1992, Mortazavi et al. 1999), and type 2 theta studied in the present work is cholinergic-sensitive, i.e. cholinergic agonists promote (Kinney et al. 1998, Vertes et al. 1993) and antagonists block (Kramis et al. 1975) the rhythm. The only paper which reports different effects of MF on ACh release *in vivo* is that by Lydic and coauthors (1993), where MF reduced ACh transmission in the pons induced by electrical stimulation of cholinergic structures [PPN and the laterodorsal tegmental nucleus (LDT)] leaving the basal, unstimulated level of ACh intact.

The role of ACh neurons, the main PPN outputs during the theta, is still disputable. There are seemingly contradictory data. On the one hand, some reports suggest an increase in ACh transmission during the theta. For example, activity of cholinergic PPN neurons is highest during wakefulness (accompanied with type 1 and, possibly type 2 theta) (Oddie and Bland 1998, Whishaw and Vanderwolf 1973), lower during REM (accompanied by type 2 theta) (Vanderwolf et al. 1977), and lowest during slow wave sleep (no theta) (Datta and Siwek 2002). During REM, ACh level in some cholinoceptive targets of the PPN is elevated (Lydic et al. 1993). Moreover, cholinergic activation of the RPO, also a cholinergic target of the PPN, by direct CA injections induced the theta (Kinney et al. 1998, Vertes et al. 1993). In addition, electric stimulation of the PPN elevated ACh level in a PPN target in the pontine reticular formation, the gigantocellular tegmental field, in cats (an equivalent of the RPO in rats) (Lydic and Baghdoyan 1993). Such a PPN stimulation increased the firing of 41% RPO type I cells, which are active during the theta (Nunez et al. 1991). The effect was blocked by intra-RPO atropine, a cholinergic antagonist (Nunez et al. 2002). On the other hand, some data suggest suppression of PPN cholinergic system during the theta. For example, intra-PPN CA injections evoked the theta (Kinney et al. 1998, Vertes et al. 1993, present study), while in *in vitro*

experiments ACh inhibited presumed cholinergic neurons of the PPN/LDT (Leonard and Llinas 1994). Cholinergic autoinhibition of the PPN is suggested by the study where extracellular ACh level in a PPN target, the ventral tegmental area, was dramatically increased by intra-PPN scopolamine (M receptors antagonist) (Chen et al. 2006), and muscarinic receptors are known to be located on PPN cholinergic cells (Vilaro et al. 1994).

These apparent contradictions can be resolved only after careful mapping of the PPN circuitry and all possible ways of the regulation of its theta-relevant (supposedly cholinergic) outputs. PPN cholinergic cell population is heterogenous and two subpopulations of different morphological and electrophysiological characteristics were described: small neurons with short spike duration, a high input resistance and high firing frequency, and medium to large neurons with long spike duration, a low input resistance and low firing frequency (Takakusaki et al. 1997). Different classes of muscarinic receptors were detected on cholinergic PPN cells: M2, M3, M4 (Vilaro et al. 1994), from which M2 can mediate cholinergic autoinhibition (Forster and Blaha 2003, Leonard and Llinas 1994, Roth et al. 1996) and M3 an increase in ACh release (e.g. Flores et al. 1996). Opioid μ receptors, whose activation results in inhibition of a neurotransmitter release (Ingram 2000), were detected in the PPN (Capece et al. 1998, Corrigall et al. 1999). Similar to other brain structures they can be located on cholinergic cells (e.g. Svingos et al. 2001) and GABA-ergic axon terminals (e.g. Gutierrez 2003, Milner and Drake 2001).

The above facts allow us to assume that there may be two functionally different populations of ACh projection neurons in the PPN, whose activity could be directly regulated by excitatory (including M3) and inhibitory (including M2 and μ-opioid) somatodendritic receptors, as well as by other major PPN neurotransmitter systems, i.e. glutamatergic and GABA-ergic interneurons (Childs and Gale 1983, Clements and Grant 1990, Ford et al. 1995). The role of μ-opioid receptors in this circuitry would be to modulate the activity of the ACh output neurons directly or indirectly by suppression of GABA-ergic inhibitory tone, thus influencing the release of ACh, GABA and glutamate on the ACh projection cells. Depending on the actual level of activation of the PPN network and the degree to which cholinergic autoinhibitory processes occur, μ receptors activation would result in theta inhibition, in conditions of overactivation (together with M2-mediated autoinhibition) or theta promotion in the cases of moderate activation.

CONCLUSION

The opioid system of the PPN modulates hippocampal theta rhythm in a bimodal fashion. The direction of this modulation (enhancement or suppression) seems to depend on the actual level of the activity of PPN cholinergic output neurons.

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