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Differential effect of procaine injection into the rostral and caudal part of the nucleus pontis oralis on hippocampal theta rhythm in urethane-anesthetized rats

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The nucleus reticularis pontis oralis (RPO) is a reticular structure important for the regulation of paradoxical sleep (PS). However, the data concerning the relation between the RPO and the main tonic indicator of PS, hippocampal theta rhythm, are contradictory: although electrical or cholinergic stimulation of the RPO evoked well-synchronized theta activity, the electrolytic lesion of the structure had no effect on theta. In our experiment, the effect of procaine injections into different parts of the RPO on the electrical activity of the hippocampus, as well as on tail pinch-elicited hippocampal theta rhythm was assessed in urethanized rats. Power spectral analysis was performed using a Fast Fourier Transform routine in 1-Hz and 3-Hz bands between 0.6 and 12 Hz frequency. We have found that unilateral procaine inactivation of neurons in the caudal part of the RPO blocked the sensory-elicited theta rhythm. The same injection into the rostral RPO either had no effect or evoked long-lasting episodes of theta rhythm without sensory stimulation. These results suggest functional diversity of the parts of the RPO in mechanisms underlying production of hippocampal theta.

Key words: nucleus reticularis pontis oralis, procaine, hippocampal theta rhythm

INTRODUCTION

The rhythmical slow activity in the hippocampus (RSA or theta activity) is the most distinct extracellular signal that can be recorded in the mammalian brain during waking and paradoxical sleep (PS) (Vanderwolf 1969, Winson 1974, Kramis et al. 1975, Robinson et al. 1977, Vertes 1981, Bland 1986, Vinogradova 1995) and is concomitant with cortical activation (Maloney et al. 1997, Leung 1998, Steriade 1998). Theta rhythm is regulated by many structures in the brainstem (Vertes 1982, Vertes and Kocsis 1997, Bland and Oddie 1998, Vertes et al. 2004) among which the nucleus reticularis pontis oralis (RPO) of the rostral pontine is the most important source of tonic activation transferred to the midbrain and forebrain. The first evidence for the RPO involvement in theta generation was obtained by Vertes in 1981. He found that electrical stimulation of the RPO could elicit theta rhythm in urethanized rats at relatively low current applied. Similar results were obtained by others (Macadar et al. 1974, Klemm 1972) and now electrical stimulation is a standard method for theta induction (Kirk and McNaughton 1991, Nunez et al. 1991, Kocsis and Vertes 1997, Bland and Oddie 1998). Another method of theta elicitation is cholinergic stimulation of the RPO with carbachol microinjections, which evokes well-synchronized theta rhythm in hippocampus (Vertes 1981, Vertes et al. 1993, Kinney et al. 1998) with a shorter latency in comparison to the stimulation of the other reticular nuclei such as the nucleus pedunculopontine (PPN) or nucleus reticularis tegmenti (RTG). Theta rhythm can be also elicited in urethane-anesthetized rats by tail pinch sensory stimulation. While many types of anesthesia (e.g., barbiturate) block theta rhythm completely (Kramis et al. 1975) or at least partially (Kekovic et al. 2010), under the urethane anesthesia it is still possible to induce a particular type of theta, the so-called theta II, based on cholinergic transmission. This type of theta can be recorded during attentive immobility in

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waking (Vanderwolf 1969, Kramis et al. 1975, Sainsbury and Partlo 1993) and also in PS as the main tonic indicator of this phase of sleep in animals (Sano et al. 1973, Vertes 1984).

A large body of evidence shows the crucial role of the RPO in generation of paradoxical sleep. Carbachol injections into the RPO induced not only theta rhythm but also PS (George et al. 1964, Vanni-Mercier et al. 1989, Yamamoto et al. 1990, Bourgin et al. 1995, Garzon et al. 1997, Horner and Kubin 1999, Marks and Birabil 2001), the same effect was also observed after neostigmine (acetylcholine esteraze inhibitor) (Baghdoyan et al. 1984, Lydic et al. 2002). In earlier experiments it was found that damages localized in the RPO region suppressed PS (Gutierrez-Rivas et al. 1978, de Andres et al. 1985), the same effect was seen recently after tetrodotoxin blockade of the RPO (Sanford et al. 2005). RPO neurons increase their activity during PS (Siegel et al. 1977) as well as during hippocampal theta rhythm, especially in the rostral part of the nucleus (Hanada et al. 1999).

However, other experiments did not confirm essential role of the RPO in PS generation or in theta induction. The PS duration was not changed after the lesion localized in the medial part of the cat's RPO (Jouvet 1994). After RPO lesion theta rhythm was also unaffected (Faris and Sainsbury 1990). Furthermore, carbachol injected into the RPO did not reinforce PS (Deurveilher et al. 1997) and microinjection of cholinomimetics into this structure activates waking rather than PS (Pollock and Mistlberger 2005). Horner and Kubin (1999) found in the RPO places ineffective for theta generation after carbachol injection.

The main reason of this discrepancy could be the functional differentiaton of RPO neurons. The electrophysiological experiments on cats indicate that neurons in the RPO differ in their activity: part of them could be classified as PS-off (which are suppressed during PS), and part as PS-on neurons (which are more active during PS) (Dergacheva et al. 2004). Additionally, Nunez and coworkers (1991) found that there are neurons within the RPO that can react differently (rhythmically or non-rhythmically) to electrical stimulation of this structure. It is possible that this functional differentiation reflects anatomical division of the RPO into the rostral and caudal part. It was found that the most effective sites for carbachol elicitation of PS are localized in the caudal part of the RPO and in the neighboring nucleus reticularis pontis caudalis (RPC) (Bourgin et al. 1995, Deurveilher et al. 1997). In comparison to the sites localized near the junction of RPO/RPC, more rostral injections were ineffective for PS elicitation (Gnadt and Pegram 1986). To our knowledge, in relation to theta rhythm this anatomical differentiation was not described. A simple and frequently used method of estimating the importance of a particular structure in theta generation is its procainic blockade and testing the possibility of theta induction in such conditions (Dickson et al. 1994, Oddie et al. 1994, Gołębiewski et al. 1999, Bocian and Konopacki 2001, Nowacka et al. 2002, Orzeł-Gryglewska et al. 2006).

Microinjection of procaine temporarily inhibits neurons (and axons passing through the given structure) by blocking the Na^+ channels in the region of diffusion. Our first experiments (published in an abstract form, Nowacka and Trojniar 2003) indicated that the effects of such blockade of RPO nucleus are different depending on the injection place. In the present study we attempted to verify whether induction of the theta rhythm with sensory stimulation is possible after a partial procainic blockade of different RPO regions. Procaine was infused into the rostral or caudal RPO and electrical activity of the hippocampus was examined in rats under urethane anesthesia.

METHODS

Subjects

The experiments were performed on 39 male Wistar rats, weighing 300-350 g. The rats were maintained in a 12-h light-dark cycle (lights on at 6 A.M.) at 22±1°C and provided with food and water ad libitum. The experiments were evaluated and approved by the Local Ethical Committee of the Medical University of Gdańsk. All effort was made to minimize both animals' discomfort and the number of animals used.

Experimental procedure

Surgery and EEG recordings were performed under deep urethane anesthesia (Urethane, Sigma-Aldrich, Germany, 1.5 g/kg, i.p.) whose level was controlled by monitoring the frequency of breathing. The animals were implanted with bilateral recording electrodes in dentate gyrus of dorsal hippocampus, as theta obtained from this area is of high amplitude (Orzeł-Grylewska et al. 2006, Kasicki et al. 2009). The following Paxinos and

Watson (1998) stereotaxic coordinates (skull leveled) were used for implantation: AP – 3.8 mm, L – 2.4 mm and D – 3.2 mm. The electrodes were made of stainless steel wire of a 0.2 mm dia. insulated on the entire length except for the flat-cut tip. Stainless steel skull screws positioned over the olfactory bulb served as reference and ground electrodes. All screws and electrodes were fixed to the skull surface with dental acrylic. A small hole was drilled over the RPO to allow the subsequent insertion of an injection cannula; stereotaxic coordinates for the rostral RPO (rostral RPO group, n=25) were: AP – 7.3-7.6 mm, L – 0.9-1.2 mm and D – 8.3 mm, and for the caudal RPO (caudal RPO group, n=14): AP – 8.3-8.7 mm, L – 0.9-1.2 mm and D – 8.5 mm. As the injection cannula, the needle (0.4 mm dia., with the flat-cut tip) of a 10 µl Hamilton syringe was used. The syringe was placed in the stereotaxic holder connected with a microinfusion pump (Kopf Instruments, USA).

EEG was recorded from the hippocampal electrodes during the whole experiment using EEG DigiTrack computer system (ELMIKO, Warsaw, Poland) to amplify and register the signal (bandpass 0-70 Hz, sampling rate 240 Hz) on a computer's hard drive. The animals were maintained at the level of anesthesia at which spontaneous theta rhythm was not present in the hippocampal EEG but could be elicited by sensory stimulation (tail pinch lasting 1 min, produced with a plastic clamp positioned in the rat's tail base). Sensory stimulation was applied 2 – 4 times in the pre-injection conditions and after cannula insertion to the RPO to make sure whether theta rhythm can be still elicited by tail pinch. Only those animals in which the theta amplitude was at least 300 µV in one or both hippocampi were included in the experiment. Theta rhythm elicited during sensory stimulation represented control signal.

After the completion of the control recordings, the intra-RPO injection procedure started. Unilateral microinjections (0.5 µl for 3-5 min) of procaine hydrochloride (Polpharma, Starogard Gdański, Poland) in 20% aqueous solution were applied to the rostral and caudal part of the RPO. Hippocampal EEG was recorded continuously and the tail pinch was administered immediately after the procaine injection and 10, 20, 30, 40 and 50 min post-injection. Sensory stimulation was not applied in animals in which after procaine spontaneous theta episodes were seen in the recording.

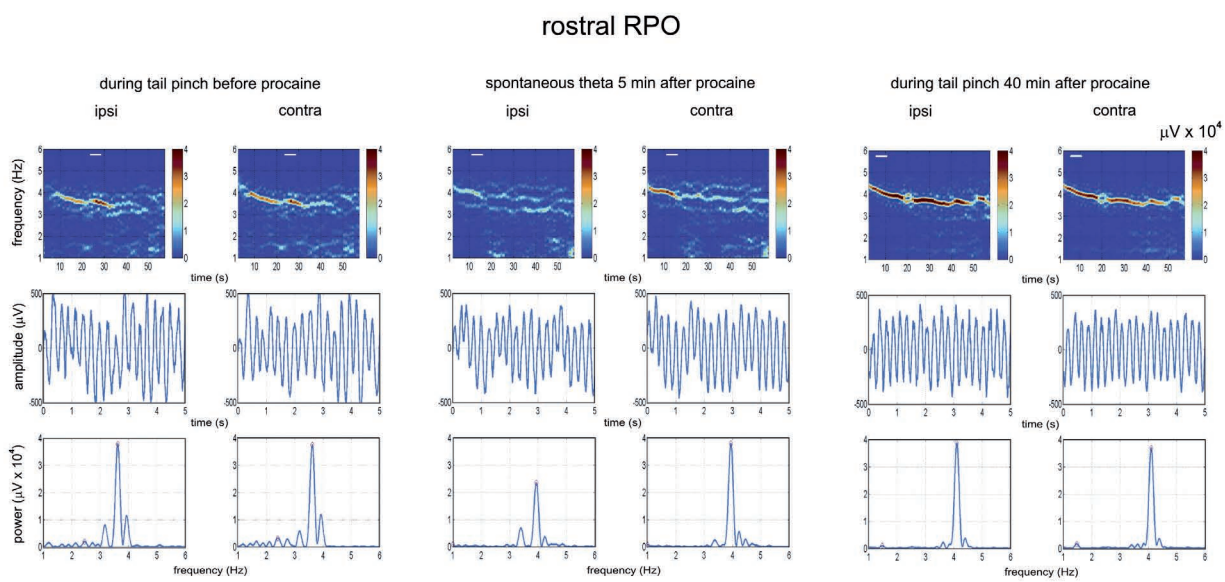

Fig. 1. Effect of unilateral procaine microinjection into the rostral part of the RPO on hippocampal EEG in the hemisphere ipsi- and contralateral to the injection site in a representative rat. Explanations: upper row - spectrograms for 60-s epochs of hippocampal EEG; middle row - 5-s hippocampal EEG samples taken from 60-s epochs indicated by a white short line on the corresponding spectrograms; bottom row - power spectra corresponding to the hippocampal EEG samples shown in the middle row. Left panel - tail pinch induced theta in control conditions, middle panel - spontaneous theta rhythm after procaine injection; right panel - tail pinch after the end of procaine effect. After rostral microinjections of procaine the theta rhythm appeared without sensory stimulation (in this particular rat 5 min after the injection, middle panel).

Data analysis

If theta rhythm appeared in hippocampal EEG as a result of procaine microinjection, latency and duration of such episodes were measured and mean values (±SD) were calculated for the entire group of animals. If theta rhythm did not appear within the first 10 min after the microinjection, tail pinch was applied as in control conditions, and if theta appeared immediately after sensory stimulation, the duration of such episode was counted starting from the moment when the tail pinch was over. The beginning of theta episode was recognized as the minute post-injection in which large amplitude irregular slow activity (<3 Hz) of hippocampus was replaced by theta synchronous oscillations (more than 50% of 1-min epoch, visual inspection with analysis of peak power in 5-s samples). By analogy, the minute post-injection in which theta oscillation disappeared and the theta peak was no longer dominant, was considered the end of the theta episode. The spectral analysis of the hippocampal EEG signal was performed off-line with SPIKE 2 (Cambridge, UK) computer application. The Fast Fourier Transformation (FFT, resolution 0.01 Hz) was calculated for 10 – 12 artifact-free 5-s samples randomly chosen from the control recording (pre-injection baseline - large irregular slow activity with predominant delta frequency present before tail pinch and procaine injection) and from theta episodes that appeared after injection or were elicited by tail pinch. We assessed mean power in 1 Hz as well as in 3 Hz frequency bands and statistically analyzed this parameter in 3 Hz bands in samples taken after procaine (till the 60th min post-injection) in comparison to the recordings before procaine. To eliminate inter-subject variability, power was expressed as a percentage of the pre-injection baseline value taken as 100%, for each frequency band separately. For a statistical analysis of the results, Student's *t*-test for independent samples was applied.

Histology

After the completion of the experiment, a small electrolytic lesion (anodal current of 100 µA/15 s) was performed through the hippocampal electrodes to visualize the tips locations. The rats were then treated with an overdose of anesthetic and intracardially perfused with 0.9% saline followed by a 10% solution of formalin. The brains were subsequently removed from the skulls and stored in 10% formalin. After fixation, brain sections of 60 µm thickness were cut using frozen tissue technique and the recording electrodes and injection cannulas placements were determined.

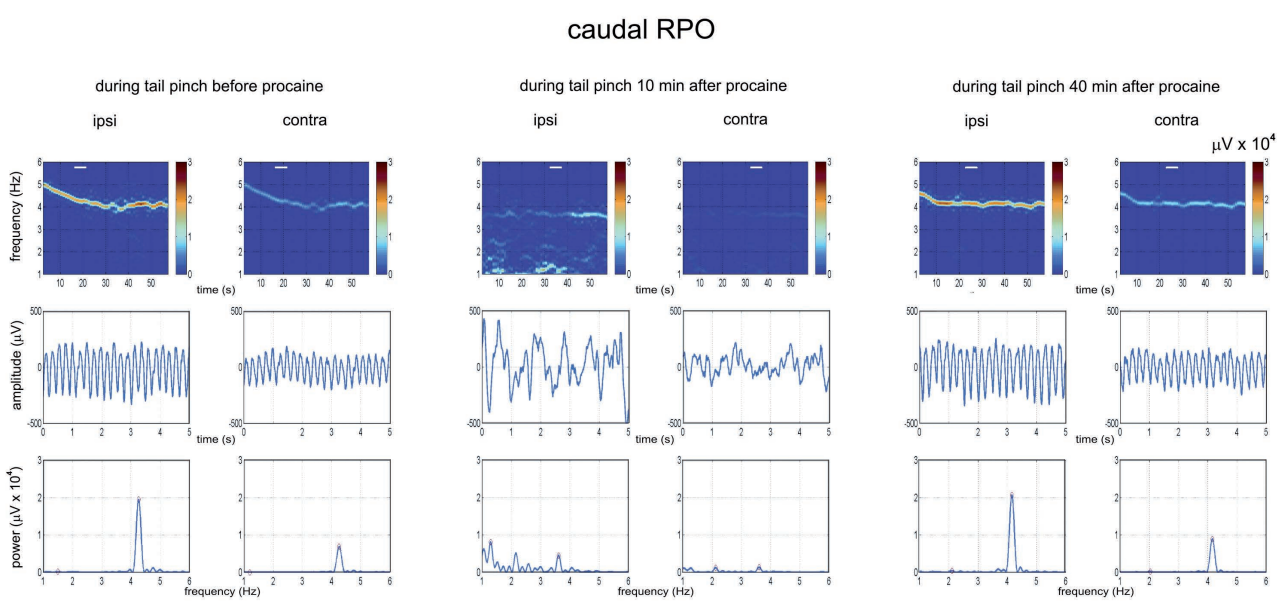

Fig. 2. Effect of unilateral procaine microinjection into the caudal part of the RPO on hippocampal EEG in the hemisphere ipsi- and contralateral to the injection site in a representative rat. Explanations as in Fig 1. Note the lack of the theta rhythm and a decrease in theta peak power 10 min after procaine injection (middle panel) and the return of the sensory-induced theta 40 min after the injection (right panel).

RESULTS

Effect of procaine on the hippocampal activity

In control conditions (before procaine injection) the tail pinch always changed the hippocampal activity from the large amplitude slow irregular activity (with dominant frequency at the delta band, between 1-3 Hz frequency) to synchronous theta rhythm (with the peak power at 4-5 Hz) lasting till the end of the stimulation, i.e. 1 min (Fig. 1 and 2, the left part of the figure). The episodes of the theta rhythm started immediately after the application of tail pinch or with 1-2 s latency.

The unilateral microinjection of procaine into the rostral part of the RPO (rostral PROC group) evoked episodes of theta rhythm, which appeared bilaterally without sensory stimulation; the effect was observed in 14 animals out of 25 examined in the experiment. In the remaining animals from the rostral PROC group the theta rhythm was produced by tail pinch stimulation as easily as in control conditions. The Fig. 1 presents samples of EEG recording and the corresponding power spectra from a rat in which theta rhythm was evoked by procaine injection without tail pinch application. The theta episode appeared in the 5th min after the injection, was still present 10 min later and lasted for 25 min. After the end of the theta episode evoked by procaine, at the end of the experiment, theta could be evoked also with the sensory stimulation (in that particular animal 40 min post-injection). The mean latency of the spontaneous theta rhythm calculated for 14 rats was 8.3±2.5 min and the mean duration was 34.4±3.12 min. There was no difference between the hemispheres in those parameters.

The unilateral injection of procaine into the caudal RPO suppressed the theta response to tail pinch. Fig. 2 presents changes in a representative rat from this group: theta rhythm is absent in the EEG recording during tail pinch stimulation and the corresponding power spectra show desynchronization of EEG signal (central part of the figure). The blocking effect of procaine started about 5-7 min after the injection and ended 25-30 min later; 40 min after the injection theta could be elicited again with the sensory stimulation (Fig. 2, right part).

In the Fig. 3 we presented power distribution in 1-Hz bands (mean±SD for each band) after procaine in rats receiving the injection into the rostral and caudal parts of the RPO nucleus. In rats from the rostral PROC group, after procaine injection, the hippocampal signal power at the frequency of 4 Hz (frequency belonging to the theta range) increased ipsilaterally to the injection up to 300% and contralaterally to 200% of the control value with concomitant reduction of power at the frequency below 3 Hz. Relatively smaller increase in power at the frequencies of 3 and 5 Hz was also visible (of about 75% ipsi- and 40-30% contralaterally to the injection site). Such changes were not observed in caudal PROC group: instead, procaine blockade of the caudal RPO suppressed the possibility

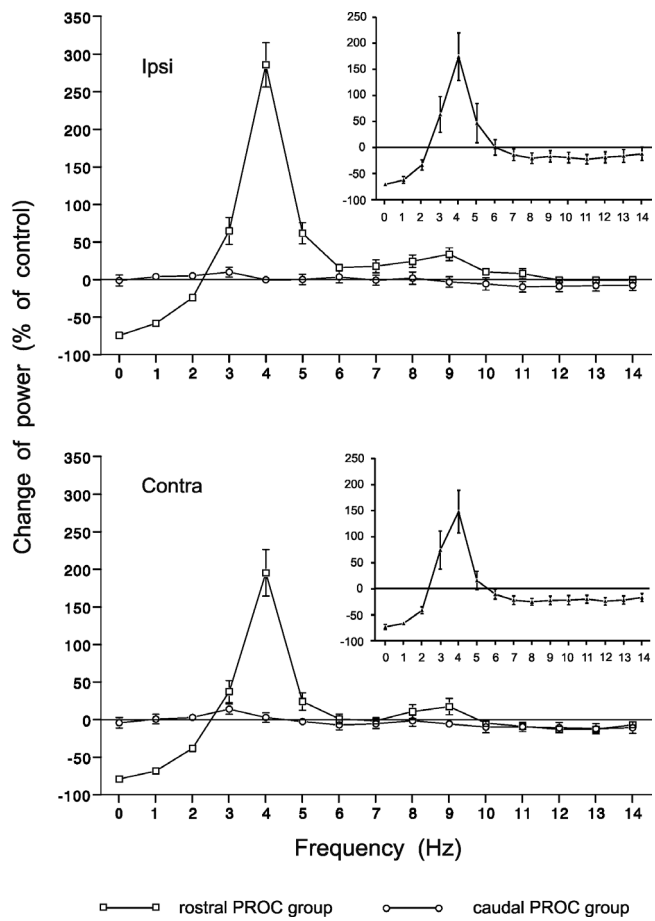

Fig. 3. Hippocampal power distribution in 1-Hz frequency bands (changes in % of baseline control - large irregular slow activity before injection or tail pinch, mean ± SD for each frequency band) after procaine injection into the rostral and caudal part of RPO nucleus. Procaine-induced theta rhythm after rostral injection is seen as an increase of power at the frequency of 4 Hz (line with squares). Procaine injected into the caudal RPO does not produce any changes in hippocampal EEG (line with circles). For a comparison, power distribution after tail pinch sensory stimulation is presented in the smaller graphs.

of the sensory elicitation of theta rhythm. For comparison, the distribution of power during the theta rhythm induced with sensory stimulation is presented in the small graphs on the right side of Fig. 3. It is worth noticing that the power of theta evoked by sensory stimulation is not as high as the power of this rhythm evoked by procaine microinjection.

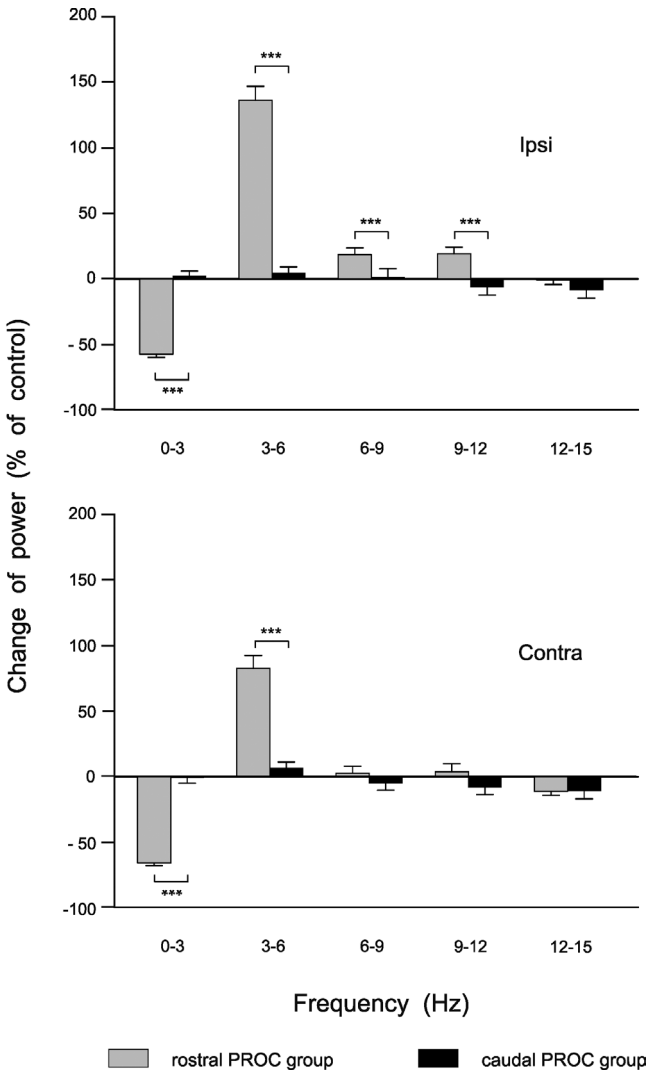

Fig. 4. A comparison of the hippocampal power in 3-Hz frequency bands (changes in % of baseline control - large irregular slow activity before injection or tail pinch) after procaine injection into the rostral (n=14) and caudal (n=14) part of RPO nucleus. The increase of power during procaine-induced theta rhythm at 3-6 Hz band with the concomitant reduction of power at 0-3 Hz is seen in the group of rats with rostral RPO injection of procaine.
The data are presented as mean ± SD. Asterisks indicate the differences between groups (Student's *t*-test for independent samples, *** - $P \leq 0.0001$.

The differences between the rostral and caudal PROC groups were statistically analyzed in five of 3-Hz frequency bands, at the range of 0.6 to 15 Hz (Fig. 4) with the use of Student's *t*-test for independent samples. Statistically significant increase in the power was found bilaterally at 3-6 Hz frequency band in animals receiving procaine injection into the rostral part of the RPO. It was 136.6±10.8% of control value ipsi- and 82.5±9.8% contralaterally to the injection side in this group versus 4.4±4.7% ipsi- and 6.2±4.6% contralaterally in the group with the caudal injection. Smaller, but also significant increase in power (not exceeding 20% of control) was seen in the rostral PROC group ipsilaterally at 6-9 and 9-12 Hz frequencies. At the lowest frequency band (0.6 to 3 Hz) the decrease in power to 57.4±9.8% and 66.4±2.0% of control value (ipsi- and contralaterally, respectively) was found in animals with rostral procaine injection; in caudal PROC group, the changes did not exceed 2% on either side.

Location of the injection sites

The hippocampal electrodes were localized in the stratum moleculare of the upper blade of the dentate gyrus. In the cases when the theta amplitude was not satisfying (<300μV) the tips of electrodes were positioned below or under moleculare layer and the recordings were excluded from the analysis.

In 39 animals injected with procaine, the cannulas' tips were localized within the RPO at different rostro-caudal levels (Fig. 5). Animals with injection placed outside the RPO, were excluded from the analysis, regardless of the effect of procaine injection. In 25 rats, the cannula was placed in the rostral part of the RPO (from -7.30 to -7.64 mm) and in the remaining 14 in the caudal part (from -8.30 to -8.72 mm). For the clarity of results, the places in the rostral RPO (Fig. 5A, B) from which the procaine injection induced the hippocampal theta rhythm, are presented on the right side of the figure (gray circles), and the other locations, where the procaine injection did not result in the theta induction but theta could be elicited by tail pinch after procaine, are presented on the left side of the figure (black circles). We did not find anatomical differences between the sites at which procaine did and did not, induce spontaneous theta rhythm.

After caudal injections of procaine (Fig.5 C, D) episodes of theta rhythm did not occur spontaneously and they could not be elicited by tail pinch.

DISCUSSION

In the present study we have demonstrated different effects of procainic blockade of the RPO on the hippocampal activity: inactivation of the rostral part of the structure induced theta episodes even without the sensory stimulation, whereas the same inactivation of the caudal part made the theta induction impossible.

From the works reviewed in the Introduction it is known that among the structures in the pons, the RPO, together with the PPN, plays an important role in the generation of the paradoxical sleep as well as theta rhythm, which is the main indicator of PS. The aim of our experiments was to find which part of the RPO is necessary for theta production, presuming that it might be the caudal RPO, as this part was found critical for PS (Bourgin et al. 1995, Marks and Birabil 2001, Marks et al. 2008). Indeed, after procainic inactivation of the caudal RPO we could not obtain hippocampal theta rhythm with sensory stimulation in any animal with injection centered at the level between -8.30 and -7.72 of Paxinos and Watson atlas (1998). Different results, and particularly interesting to us, were obtained after procaine injection into the rostral part of the

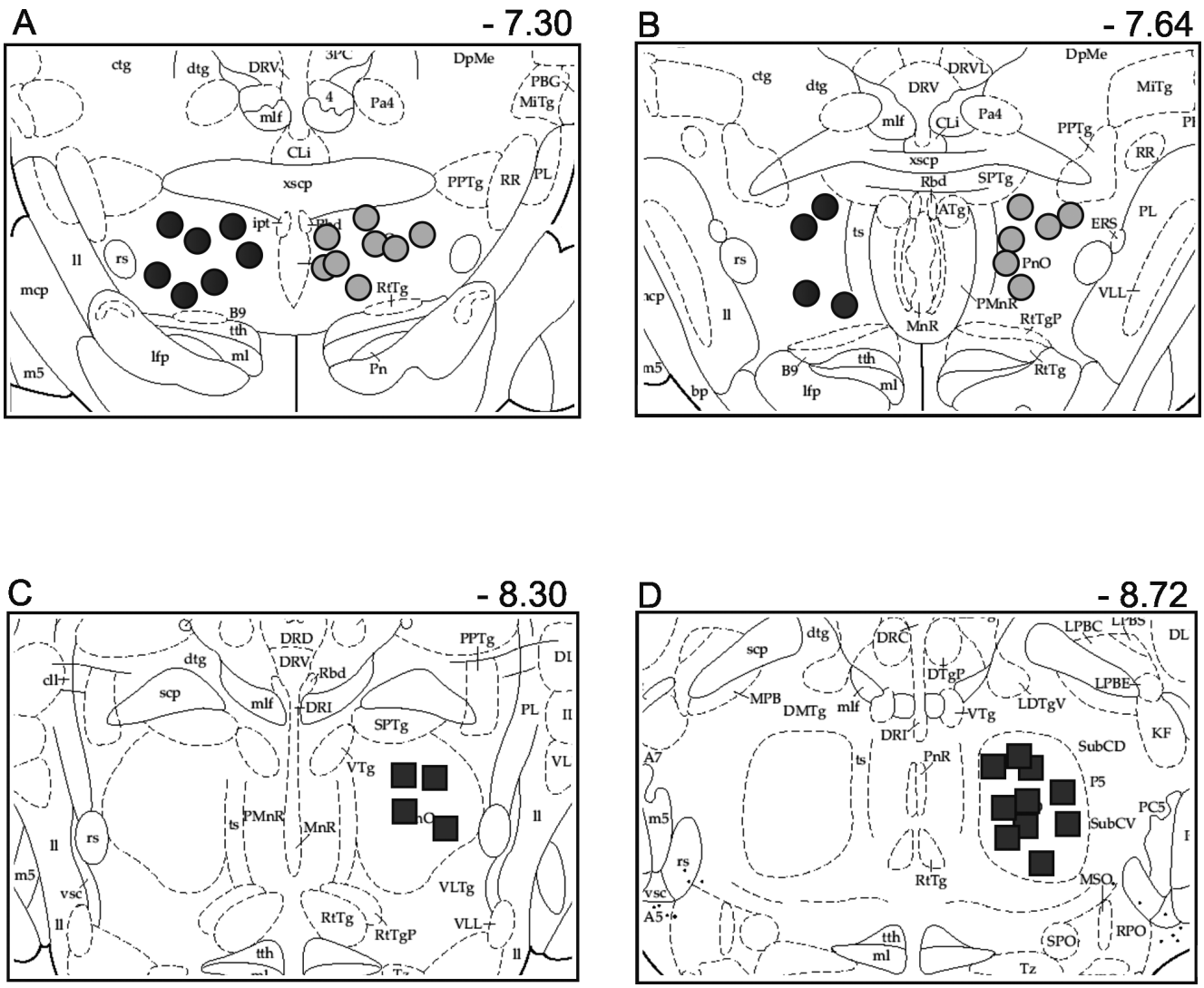

Fig. 5. Localization of procaine microinjection sites in rostral (A, B) and caudal (C, D) part of RPO superimposed on plates adapted from the atlas by Paxinos and Watson (1998). Numbers to the right indicate distance from bregma along A/P axis. Explanations: gray circles – localization of cannulas within the rostral part of the RPO in rats with procaine-induced theta rhythm (n=14); black circles - in rats with procaine and the sensory-induced theta rhythm (n=11); black squares - cannulas within the caudal part of RPO in animals without theta rhythm after procaine injections (n=14).

RPO: such blockade released theta rhythm without tail pinch. As it is seen in the atlas, the RPO in the rat is rather a big structure, visible on the plates between -7.3 mm and -8.8 mm (with reference to bregma) and at a depth about 7.8 mm to 8.8 mm from the skull surface. It means that the nucleus is 1.5 mm long and about 1.0 mm in diameter. As we have observed using alcian blue, diffusion area after 0.5 µl procaine solution in the RPO during 1.5 h experiment is about 0.5-0.6 mm. It seems, that the effect consisting in either theta induction or blockade after procaine injection is anatomically specific, connected either to rostral or caudal part of the RPO, respectively. Medially, the RPO borders with the median and paramedian raphe, ventrally – with the reticulotegmental nucleus of the pons, and laterally with the rubrospinal tract. The laterodorsal part of the RPO neighbors the PPN, which is placed more rostrally and a little higher than RPO.

At its anterior and dorsal part, the RPO spreads from the decussation of the superior cerebellar peduncle to the medial pons and it is continuation of the nucleus reticularis pontis caudalis (RPC), the only difference between these two structures is lack of giant cells in the RPO (Siegel 2000). In the recent years, many authors investigate the relationship between the RPO and RPC with regard to paradoxical sleep in rats (Sanford et al. 2003) and cats (Xi et al. 1999, 2004). The function of the RPO and RPC in PS regulation is explored for instance with the use of GABAergic and cholinergic stimulation of those areas (Marks et al. 2008, Fenik and Kubin 2009).

In our experiments, we chose the stereotaxic coordinates for injection cannula in the most rostral and the most caudal position within the RPO borders in order to search for the best places for theta induction or blockade. It seems that the sensory pathways for theta induction that ascend from medulla oblongata and reach the pons, midbrain and forebrain, were blocked by procaine injection into the caudal but not rostral RPO. These tracts appear to bypass the rostral RPO because procainic injection into this region did not block theta rhythm generation (Fig. 4). Such pathways probably course more dorsally, which could explain our earlier experiments in which we could not obtain the tail pinch theta after procainic blockade of the PPN (Nowacka et al. 2002). Moreover, our present results indicate that neurons or axons passing through the rostral RPO serve as a brake or inhibitory mechanism for the theta rhythm and their role varies with the state of the animal, e.g., the level of anesthesia. Elimination of this mechanism with procaine elicits spontaneous theta episodes, a finding that we see as a key outcome from our study.

What is the transmitter basis of that brake, we can only speculate. RPO is a large structure and we cannot exclude the possibility that it contains a population of GABAergic or glycinergic neurons which act as RPO's internal inhibitory mechanism. Such neurons could be a part of the GABAergic neuron system recently described by Liang and Marks (2009). The authors demonstrate that GABAergic cells form a longitudinal column in the brainstem that extends from the level of the rostral PPN to the most caudal part of the reticular formation of the pons.

CONCLUSION

Our results indicate a functional diversity within the RPO in relation to theta rhythm regulation. The caudal part contains cells or pathways necessary for theta generation in response to sensory stimulation, whereas the rostral part of the RPO contains neurons or axonal pathways that mediate endogenous suppression of hippocampal theta rhythm in urethane-anesthetized rats.

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