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ROLE OF THE DORSAL PARAGIGANTOCELLULAR RETICULAR NUCLEUS IN PARADOXICAL (RAPID EYE MOVEMENT) SLEEP GENERATION: A COMBINED ELECTROPHYSIOLOGICAL AND ANATOMICAL STUDY IN THE RAT

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Abstract—It is well known that noradrenergic locus coeruleus neurons decrease their activity during slow wave sleep and are quiescent during paradoxical sleep. It was recently proposed that their inactivation during paradoxical sleep is due to a tonic GABAergic inhibition arising from neurons located into the dorsal paragigantocellular reticular nucleus (DPGi). However, the discharge profile of DPGi neurons across the sleep–waking cycle as well as their connections with brain areas involved in paradoxical sleep regulation remain to be described.

Here we show, for the first time in the unanesthetized rat that the DPGi contained a subtype of neurons with a tonic and sustained firing activation specifically during paradoxical sleep (PS-on neurons). Noteworthy, their firing rate increase anticipated for few seconds the beginning of the paradoxical sleep bout. By using anterograde tract-tracing, we further showed that the DPGi, in addition to locus coeruleus, directly projected to other areas containing wake-promoting neurons such as the serotonergic neurons of the dorsal raphe nucleus and hypocretinergic neurons of the posterior hypothalamus. Finally, the DPGi sent efferents to the ventrolateral part of the periaqueductal gray matter known to contain paradoxical sleep-suppressing neurons.

Taken together, our original results suggest that the PS-on neurons of the DPGi may have their major role in simultaneous inhibitory control over the wake-promoting neurons and the permissive ventrolateral part of the periaqueductal gray matter as a means of influencing vigilance states and especially PS generation.

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Abbreviations: AS, asymmetry index; CTb, cholera toxin b subunit; DAB, 3,3′-diaminobenzidine-4HCl; DPGi, dorsal paragigantocellular reticular nucleus; DpMe, deep mesencephalic reticular nucleus; DRN, dorsal raphe nucleus; EEG, electroencephalogram; EMG, electromyogram; EOG, electrooculogram; Fg, Fluorogold; ISI, interspike interval; LC, locus coeruleus; PBST, 0.1 M PB, containing 0.9% NaCl and 0.3% Triton X-100; PBST-Az, 0.1 M PB, containing 0.9% NaCl, 0.3% Triton X-100, and 0.1% sodium azide; PHA-L, *Phaseolus vulgaris* leucoagglutinin; PS, paradoxical sleep; PSB, Pontamine Sky Blue; REM, rapid eye movement; SWS, slow wave sleep; TH, tyrosine hydroxylase; vlPAG, ventrolateral part of the periaqueductal gray matter; VLPO, ventrolateral preoptic nucleus; W, wake.

Key words: unanesthetized head-restrained rat, electrophysiology, tract-tracing, locus coeruleus, rapid eye movements, monoamines.

Paradoxical sleep (PS or rapid eye movement (REM)-sleep) is a sleep stage characterized by a paradoxical association of a low amplitude fast electroencephalogram (EEG) with muscle atonia and REMs. Since its discovery in the 1950s (Aserinsky and Kleitman, 1953; Jouvet et al., 1959), numerous studies had focused on brain areas involved in PS onset and maintenance. However, the neuronal network responsible for PS regulation remains to be fully identified.

According to the classical "reciprocal interaction" models (Hobson et al., 1975; Sakai, 1985), the inactivation of monoaminergic wake (W)-promoting neurons within the brainstem, essentially serotonergic neurons of the dorsal raphe nucleus (DRN) and noradrenergic neurons of the locus coeruleus (LC), is a requirement for PS onset. Indeed, it has long been known that the activity of LC neurons is closely related to behavioral states since they decrease their firing during slow wave sleep (SWS) and become virtually quiescent during PS (Aston-Jones and Bloom, 1981). GABAergic neurons within the ventrolateral preoptic nucleus (VLPO) likely contribute, through reciprocal inhibitory interactions, to the firing decrease of W-promoting neurons during SWS (Sherin et al., 1996; Gallopin et al., 2000; reviews in Saper et al., 2001; Fort et al., 2004). Regarding PS, recent congruent data demonstrated that the firing cessation is due to a PS-selective tonic GABAergic inhibition. On one hand, it has been shown using microdialysis that the amount of GABA is greatly increased in the LC during PS compared with SWS or waking (Nitz and Siegel, 1997). On the other hand, using extracellular single-unit recordings in head-restrained unanesthetized rats, Gervasoni et al. (1998) showed that iontophoretic application of bicuculline, a specific $GABA_A$-receptor antagonist, during PS, restores a firing activity in LC neurons similar to that of waking. These data highly suggest that GABA is maximally released within the LC from inputs activated specifically during PS. To identify these inputs, we combined injections in the LC of cholera toxin b subunit (CTb) as a retrograde tracer with the immunodetection of Fos protein in control rats, rats selectively deprived of PS during 3 days, and rats allowed to recover from such deprivation to obtain a long-lasting PS hypersomnia (Verret et al., 2005, 2006). We showed that after PS recovery, the largest number of

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CTb/Fos double-labeled cells was found in the dorsal paragigantocellular reticular nucleus (DPGi), suggesting that this medullary nucleus contains GABAergic neurons responsible for the tonic inhibition of LC neurons during PS. Supporting this hypothesis, it has been previously shown in anesthetized rats that the electrical or chemical stimulation of the DPGi area induced a firing inhibition of LC neurons. Furthermore, this inhibition is GABAergic in nature since it is blocked by LC application of bicuculline (Ennis and Aston-Jones, 1989). Finally, the electrical stimulation of the DPGi area is followed by an increase in PS quantities that is blocked by concomitant LC application of picrotoxin (a specific $GABA_A$-receptor antagonist, Kaur et al., 2001). Despite the large body of experimental data supporting the contribution of DPGi to the PS-specific inhibition of LC neurons, the presence in this nucleus of neurons activated specifically during PS (PS-on neurons) remains to be directly demonstrated *in vivo*. Furthermore, determining the anatomical place of the DPGi within the complex neuronal network involved in PS should put complementary insight regarding its contribution to the vigilance regulation.

To fill this gap and to precisely determine the role of the DPGi in PS mechanisms, we performed first, extracellular recordings of DPGi neurons across the sleep–waking cycle by using the head-restrained rat model (Souliere et al., 2000) and second, an anterograde tract-tracing study with *Phaseolus vulgaris* leucoagglutinin (PHA-L) to identify the brain areas receiving direct inputs from the DPGi.

EXPERIMENTAL PROCEDURES

Electrophysiology

The head-restrained rat method. The procedure (fixation of the head-restraining system and chronic implantation for the polygraphic recordings) has been previously described in detail (Boissard et al., 2002; Gervasoni et al., 1998). All experiments were conducted in agreement with the Guide for the Care and Use of Laboratory Animals (NIH Publication 80-23; authorization no. 03-505 of the French Ministry of Agriculture) and every effort was made to minimize the number of animals used and their suffering. Briefly, male Sprague–Dawley rats (280–320 g, $n=9$; IFFA Credo, L'arbreslie, France) were anesthetized with chloral hydrate (400 mg/kg, i.p) and mounted conventionally in a stereotaxic frame (David Kopf, CA, USA). Five electrodes were fixed in the skull bilaterally above the frontal (Bregma +4 mm AP and ±2 mm L) and parietal (Bregma −3 mm AP and ±3 mm L) cortices, and unilaterally above occipital (Bregma −9 mm AP and −3 mm L) cortex to monitor the EEG. Two wire electrodes were inserted into the neck muscles and two electrodes were inserted behind each ocular globe to monitor electromyogram (EMG) and electrooculogram (EOG), respectively. The head-restraining system was then put in place. After recovery (2 days), the rats were habituated to the restraining and recording system for 8–10 days. At the end of the training, they could stay calm for 5–6 h daily sessions during which active waking (with movements, AW), quiet waking (without movement, W), SWS and PS were routinely observed. After the habituation process and before the first single-unit recording session, rats were anesthetized with chloral hydrate (320 mg/kg, i.p., additional doses as needed), and a 4 mm hole was drilled over the DPGi. Daily recording sessions were typically performed over a maximum of 7–10 days, each session lasting ~4–6 h. The brain surface was cleaned under local lidocaine anesthesia at the beginning of each daily recording session.

Recording and analysis of neuronal activity. Extracellular recordings of DPGi neurons were performed using single-barrel glass micropipettes (external tip diameter, 2–3 μm) filled with Pontamine Sky Blue (PSB, 2% in sodium acetate 0.5 M, pH 7.5) or PHA-L (2.5% in 0.01 M phosphate-buffered saline; Vector Laboratories, Burlingame, CA, USA). Electrode impedances measured at 10 Hz ranged between 7 and 15 MΩ. Filtered (AC, 0.3–10 kHz) and unfiltered (DC) electrode signals were amplified (P16; Grass Instruments, RI, USA) and fed to storage oscilloscopes (2211 Tektronix, OR, USA) and an audio monitor (AM8, Grass). Single-unit activity (signal-to-noise ratio of at least 3:1) was isolated with an amplitude spike discriminator (Neurolog Spike Trigger, Digitimer Ltd., UK), collected and stored on a personal computer via a Cambridge Electronic Design (Cambridge, UK) interface using the Spike 2 software, in parallel with analog-to-digital samplings of amplified (Alvar, Reega, Paris, France) polygraphic signals (EEG and EMG; sample rate, 500 and 250 Hz, respectively), the AC trace at a rate of 15 kHz and a video acquisition of the rat behavior. Neurons from the DPGi were identified 1) by their stereotaxic location (bregma, anteroposterior, −11.4 to −12 mm; lateral, 0.4–0.6 mm; ventral, 6.8–8 mm); 2) *on line* by comparison with the unit recordings in adjacent brain areas during the electrode penetration through the cerebellum (dorsally), the gigantocellular reticular nucleus (ventrally), medial vestibular and prepositus hypoglossi nuclei (dorso-laterally) and the hypoglossal motor nucleus (caudally to the DPGi); and 3) *off line* after injection of PSB or PHA-L for localization of the recording sites. The mean discharge rates of individual neurons during W, SWS and PS were calculated by averaging spike counts made for at least 30 s continuous recordings in one given vigilance state. The effects of behavioral states on the discharge of each class of recorded neurons were assessed with one-way analysis of variance followed by a Tukey's multiple comparison test. A P value inferior to 0.05 was considered statistically significant. The discharge pattern of neurons was appreciated by first-order interspike interval (ISI) histograms (ISHs), displaying the distribution of intervals between consecutive spikes, that were built with 500 bins of 1 ms width. For each vigilance state, an asymmetry index (AS) was defined as the ratio of the mode (the most frequent ISI) to the mean ISI (the reciprocal of the mean firing rate). Thus, an AS near the unit reveals a relatively regular discharge pattern, whereas the more the index differs from the unit, the more irregular is the spike train. For PS-on neurons, the onset of the firing increase and decrease was defined as the time when the firing rate during three consecutive 1-s bins was at least 2 S.D. greater or smaller than the mean rate during 30–60 s before the state transition (corresponding to a 30 s window). Finally, to measure the spike duration, action potentials were averaged 20–30 times. All data are expressed as mean±S.E.M. and the significance level for all statistical analyses was set at $P<0.05$.

Neuroanatomical experiments

Surgery. PHA-L injections into the DPGi. Male Sprague–Dawley rats (280–320 g, IFFA Credo; $n=7$) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted conventionally in a stereotaxic frame (David Kopf, Epinay-sur-Seine, France) with ear bars and a head holder. The bone was exposed and cleaned and a hole was made under the DPGi stereotaxic coordinates. A single-barrel glass micropipette (external tip diameter: 2–3 μm) filled with PHA-L (2.5%) was lowered into the brain with a piezoelectric micromanipulator. The localization of the DPGi was assessed by the recording of neuronal activity throughout the electrode penetration: 500 μm ventral to the last neurons recorded in the cerebellum, 500 μm dorsal to the first neurons recorded in the gigantocellular nucleus and 500 μm rostral to the hypoglossal