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The effect of $GABA_A$ antagonist bicuculline on dorsal raphe nucleus and frontal cortex extracellular serotonin: A window on SWS and REM sleep modulation

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Abstract

We investigated the effects of the perfusion of γ-aminobutyric $acid_A$ antagonist bicuculline in the dorsal raphe nucleus, on brain 5-hydroxytryptamine level and on sleep. Perfusion of 25 and 50 μM bicuculline into the dorsal raphe nucleus dose-dependently increased dorsal raphe nucleus 5-hydroxytryptamine level during sleep and wakefulness. Frontal cortex 5-hydroxytryptamine level was not affected by either 25 or 50 μM perfusion.

25 μM bicuculline produced only minimal effects on sleep. 50 μM decreased rapid eye movement sleep, slow wave sleep 1 and 2 and increased waking.

Sleep changes leveled out towards the end of the bicuculline perfusion despite serotonin levels were still elevated. This suggests that an adaptation mechanism may take place in order to counteract the high serotonergic output, producing uncoupling between serotonin level and behavioural state. The results support the notion that γ-aminobutyric acid is a strong modulator of dorsal raphe nucleus serotonergic neurons, and that this modulation is important in the regulation of slow wave sleep, rapid eye movement sleep and waking.

Keywords: Sleep–waking; Brainstem; Rat; Microdialysis; HPLC-EC

1. Introduction

The role of the serotonergic system, and most importantly the dorsal raphe nucleus (DRN) in sleep regulation has been debated during the last 40 years (see reviews: Portas et al., 2000; Ursin, 2002).

One line of reasoning favours the possibility that DRN 5-hydroxytryptamine (5-HT, serotonin) neurons are important in maintaining waking (W) and inhibiting rapid eye movement (REM) sleep (serotonergic modulation of REM sleep; McCarley et al., 1995). This is mainly based on 3 lines of evidence: i) serotonergic neurons in the DRN show a state-related firing (higher in W, lower in slow wave sleep (SWS), and lowest in REM sleep) (Fornal et al., 1994; Lydic et al., 1987; McGinty

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; DRN, dorsal raphe nucleus; ECD, electrochemical detection; EDTA, ethylenediaminetetraacetic acid; EEG, electroencephalogram; EMG, electromyogram; FC, frontal cortex; GABA, gamma amino butyric acid; HPLC, high performance liquid chromatography; I.P., intraperitoneally; IPSP, inhibitory postsynaptic potentials; LDT, laterodorsal tegmental nucleus; PAG, periaqueductal grey; PPT, pedunculopontine tegmental nucleus; PRF, pontine reticular formation; REM sleep, rapid eye movement sleep; S.C., subcutaneously; SWS, slow wave sleep; VLPO, ventrolateral preoptic area; W, waking.

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and Harper, 1976; Trulson and Jacobs, 1979), ii) extracellular levels of 5-HT within the DRN (Portas et al., 1996, 1998; Portas and McCarley, 1994) and in the serotonergic projection areas (Auerbach et al., 1989; Horner et al., 1997; Orosco et al., 1995; Strecker et al., 1999; Wilkinson et al., 1991) similarly vary with state, iii) inhibition of REM promoting neurons by 5-HT change according to the DRN neurons firing (W>SWS>REM) (e.g. suppress REM sleep) (Horner et al., 1997). In line with this hypothesis, the modulation of the activity of the DRN serotonergic neurons would directly affect sleep and waking (e.g. high serotonin levels are associated with waking, low serotonin levels are associated with sleep). An autoinhibitory mechanism has been postulated (Wang and Aghajanian, 1978) on the evidence that stimulation of the 5-HT receptors present on the soma (autoreceptors) decreases DRN neuronal firing (e.g. Evrard et al., 1999; Fornal et al., 1994; Sprouse and Aghajanian, 1987) and DRN 5-HT level (Portas et al., 1996). A concomitant increase of REM sleep has consistently been observed (Bjorvatn et al., 1997; Portas et al., 1996) supporting the role of serotonin in the modulation of REM sleep (McCarley et al., 1995). Consistent with these findings systemic administration of the 5-HT_{1A} antagonist p-MPPI dose-dependently decreases REM sleep (Sørensen et al., 2000). However, recent experiments in our laboratory showed that microdialysis perfusion of p-MPPI into DRN produces only minor (Sørensen et al., 2001) or no effects on REM sleep despite a dramatic increase of 5-HT levels. (Ursin et al., unpublished observations).

Another important mechanism suggested for modulation of DRN serotonergic neurons is provided by the γ-aminobutyric acid (GABA) ergic input. Iontophoretically applied GABA into DRN in anaesthetised animals inhibits activity of these neurons and this effect can be reversed by application of antagonists (Gallagher and Aghajanian, 1976). Several GABAergic projections reach the DRN from widespread areas of the brain, including the ventrolateral preoptic area (VLPO), the lateral hypothalamic area, the ventral tegmental area, pontine reticular formation (PRF) (Gervasoni et al., 2000) and the DRN itself. Microdialysis perfusion of $GABA_A$ agonists (e.g. muscimol) and antagonists (e.g. bicuculline, GABAzine) into the DRN, respectively, decreased and increased 5-HT level (Tao et al., 1996; Tao and Auerbach, 2000, 2003). The $GABA_B$ agonist baclofen perfused in DRN decreased the DRN 5-HT levels, the antagonist failed to have any effect (Abellán et al., 2000; Tao et al., 1996; Tao and Auerbach, 2000). These data are consistent with the hypothesis that GABA is a source of inhibition of the DRN 5-HT cells.

Only a few studies have directly investigated the role of the GABAergic system in relation to DRN activity and sleep, and the results are not entirely consistent. Microiontophoretic application of bicuculline in DRN across the sleep–waking cycle increased the 5-HT neuron firing rate during SWS in cats, but failed to change the firing of the neurons during REM sleep and waking (Levine and Jacobs, 1992). In contrast, extracellular level of GABA were found to be significantly higher during REM sleep compared to other behavioural states (Nitz and Siegel, 1997), indicating that GABAergic cells, within or projecting to the DRN, are selectively active during REM sleep. Several workers have identified small neurons within the DRN that are more active during artificially induced REM sleep (Shiromani et al., 1995; Yamuy et al., 1995). These neurons have later been proved to be GABAergic (Torterolo et al., 2000). Microdialysis or microinjection application of GABA agonists into DRN leads to increased amounts of REM sleep, while antagonists decreased REM sleep in cats (Nitz and Siegel, 1997; Sakai and Crochet, 2001). However, in rats, bicuculline markedly increased the firing rate of DRN neurons both during waking, SWS and REM sleep (Gervasoni et al., 2000). Hence, the effect of GABA appears to be relevant throughout the sleep–wake cycle.

The aim of this study is to clarify the discrepancies related to the effect of GABA modulation on sleep and waking while simultaneously monitoring extracellular 5-HT. We hypothesise that bicuculline perfusion in DRN should increase 5-HT release in DRN and its projection sites (including REM sleep promoting areas), reduce REM sleep (McCarley et al., 1995) and possibly, affect waking and SWS. Serotonin was monitored at the site of serotonergic cells (DRN) and at a representative projection site, the frontal cortex (FC).

2. Experimental procedures

2.1. Ethical evaluation

The experiments described in this article have been approved by the local responsible laboratory animal science specialist under surveillance of the Norwegian Animal Research Authority and registered by the Authority. Norway has signed and ratified The European Convention for the protection of Vertebrate Animals used for experimental and other scientific purposes. All efforts were made to minimize the number of animal used and their suffering.

2.2. Experimental animals and surgery

Twenty-one male Sprague–Dawley (MolTac:SD) rats (Taconic M and B, Copenhagen, Denmark) weighing 200–350 g at surgery were used in these experiments. The animals were housed individually in conventional macrolone III cages. They were exposed to a 12:12 h light/dark schedule with lights on at 06:00 h. The ambient temperature averaged 22±1 °C and the relative humidity was 40–60%.

The animals had free access to food (rodent low protein diet, B&K Universal AS, Norway) and water ad libitum. To induce surgical anaesthesia animals were injected subcutaneously (s.c.) with a mixture of fentanyl, 0.05 mg/ml, fluanizone, 2.5 mg/ml, and midazolam, 1.25 mg/ml, (Hypnorm, Janssen; Dormicum, Roche). The animals received 0,05 ml/100 g of both Hypnorm and Dormicum initially, and then 0,033 ml/100 g each hour to sustain anaesthesia. The final dose ranged from 0,19 ml–0,3 ml depending on weight of the animal and length of surgery. The animals were implanted with stainless steel screw electrodes for bilateral fronto-frontal and fronto-parietal electroencephalogram (EEG) recording and silver wires in the neck muscle for electromyogram (EMG) recording (Ursin and Larsen, 1983). The frontal screw electrodes were placed

epidurally 1–2 mm anterior to bregma and 1–2 mm lateral to the midline, and the parietal screw electrodes were placed 2 mm anterior to lambda and 2 mm lateral to the midline. The rats were also implanted with two intracerebral guide cannulas (CMA 12/guide, CMA Microdialysis, Sweden) allowing easy insertion of microdialysis probes into DRN (AP = − 7.8, ML = 0.0, DV = 7.0) and frontal cortex (AP = +3.2, ML = −2.5, DV = −5.0) (Casanovas and Artigas, 1996). The frontal cortex was chosen to evaluate extracellular 5-HT level at a projection site (presumably reflecting serotonin levels present in other projection sites like lateral dorsal tegmentum (LDT) and ponto pedunculo tegmentum (PPT)). All DRN 5-HT projection areas investigated so far have shown the same state-related 5-HT levels (W > SWS > REM) (for review see Portas et al., 2000). Simultaneous placement of microdialysis probes in the rat DRN and LDT/PPT areas is impossible due to the small size and the proximity of the structures.

2.3. Postoperative animal care and recording conditions

Following surgery all animals received analgesic doses of buprenorphinum (Temgesic, Reckitt and Colman) (0.15 ml s.c.) twice a day for three days. At least two weeks were allowed for recovery and adaptation prior to recording. For EEG and EMG recording the animals remained in their home cages which were placed into sound attenuated recording chambers (430 × 280 × 620 mm) with light (15-W electric bulb) and ventilation. Food and water were accessible ad libitum. During recording, the temperature inside the chambers was 24–28 °C. Free movement of the animal was allowed using a flexible recording cable linked to a combined fluid and electrical swivel (Alice King Chatham, Medical Arts, USA) fixed to a movable arm outside the chamber. A servomotor was connected to the swivel to allow easy rotation of the cable. The microdialysis probes (CMA 12, CMA/Microdialysis, Sweden) had a diameter of 500 μm and a membrane length of 1 mm (DRN) and 3 mm (frontal cortex), and were inserted the day before the first experiment day to allow time for equilibration of the extracellular environment (at 15:00 h).

2.4. Sleep scoring

Fronto-frontal EEG, fronto-parietal EEG and neck muscle EMG signals were recorded using a paper velocity of 10 mm/s on a Grass polygraph and Embla system with Somnologica 2 (Flaga, Iceland). Scoring of the behavioural states (W, SWS-1, SWS-2 and REM sleep) was done manually in 10 s epochs using the Somnologica software according to the criteria given by Ursin and Larsen (1983) and Neckelmann and Ursin (1993). Transition sleep was not scored.

2.5. Experimental design

Each animal served as its own control and received all three perfusions on three consecutive days. On day 1 the DRN probe was perfused with artificial cerebral spinal fluid (aCSF = 147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$, and pH 7.4) and on day 2 and 3 the DRN probe was perfused with 25 or 50 μM bicuculline. The order of drug dose was reversed in half of the animals to correct for any leftover effect from the previous day. Only aCSF was perfused in the frontal cortex probe during the three experimental days. To avoid glia proliferation in the probe sites (Hamberger et al., 1983; Imperato and Di Chiara, 1985), experiments were conducted on consecutive days instead of allowing wash out between the experimental conditions.

2.6. Experimental procedure

The experiments started at 08:00 h by perfusing the FC and DRN probes with aCSF on day 1. On day 2 and 3 bicuculline was perfused in DRN, whereas FC was perfused again with aCSF. Flow rates of 0.8 μl/min were maintained by microdialysis pumps (CMA 100, Sweden). Microdialysis sampling of about 10-min stage-specific epochs started 2 h later (at 10:00 h) to allow for equilibration of the chemical environment around the probe. EEG and EMG signals were recorded for 6 h starting at 09:00 h, ending at 15:00 h. During this period the animals were left undisturbed. Microdialysis samples were collected manually (≥ 7 μl of dialysate per sample) during EEG/EMG defined behavioural states; W, SWS and REM sleep. For the purpose of microdialysis sampling SWS was not subdivided into SWS-1 or SWS-2, due to the difficulty of obtaining pure samples. Due to short duration of each REM sleep epoch in the rat, several epochs of this sleep stage were added together to achieve 10-min samples. In order to get enough microdialysis samples, sampling was sometimes extended until 18:00 h. This period of perfusion extension varied between the animals, but the perfusion never exceeded 10 h (from 08:00 to 18:00 h). Sometime during the last recording period animals were kept awake by gentle sensory stimulation (e.g. knocking on Plexiglas door and gentle handling) in order to obtain enough waking samples. For each animal, 4–6 samples were collected during waking and SWS and 1–3 during REM sleep, except in a few animals where no REM sleep samples were obtained.

2.7. Drugs

(−)-Bicuculline Methiodide, 1(*S*), 9I with molecule formula $C_{20}H_{17}NO_6 \times CH_3I$, usually called Bicuculline Methiodide was obtained from Research Biochemicals Incorporated (RBI), USA. 25 and 50 μM bicuculline solutions were made by dissolving bicuculline in aCSF. One pilot animal received bicuculline as 30 and 100 μM. We chose the dose of 50 μM over 100 μM because of the side effects observed after using the higher dose.

2.8. High-performance liquid chromatography procedure

Microdialysis samples (7 μl of dialysate) were injected into a High Performance Liquid Chromatography (HPLC) System (BAS, USA). Separation was achieved using a microbore column (3 μm, ODS, 100 × 1 mm). The HPLC system was coupled to an electrochemical detector (ECD) (Unijet LC-4C amperometric detector, BAS, USA). The potential applied to the glassy carbon electrode was 550 mV with respect to the reference electrode. The sensitivity was set to 1 nA full scale. Mobile

phase consisted of 0.62 mM ethylenediaminetetraacetic acid (EDTA), 0.65 mM sodium octyl sulphate, 10 mM sodium chloride, 0.08 M sodiumacetate, 11% acetonitrile. pH was adjusted to 4.5 with acetic acid. Under these conditions 5-HT retention time was 4–6 min. Chromatographic data were recorded and peak areas determined with an automatic integration system (BAS ChromGraph 1.5.01). Concentration of 5-HT in the samples was evaluated by converting peak area units into fmols using an external standard calibration curve method. Putative 5-HT peaks were identified by comparing retention times of 5-HT standards (5-HT, Sigma Chemicals) to retention times of sample peaks. The technique of "spiking" was also used to confirm the retention time identification. In this technique a known amount of 5-HT was added to a dialysate sample and analyzed. The resulting chromatogram showed that the peak identified by retention time as 5-HT was increased and that the increase was proportional to the known amount of 5-HT used for spiking. The detection limit for 5-HT in the dialysate was approximately 0.2 fmol/sample.

2.9. Histology

Following the termination of the experiment the animals were anaesthetized with 2 ml Mebumal (50 mg/ml pentobarbital, Svaneapoteket, Bergen, Norway) intraperitoneally (i.p.), and the brains were perfused via the ascending aorta with saline followed by 10% formaldehyde in 0.1 M phosphate buffer. The brains were left in situ overnight at 4 °C, and then blocked and placed in 20% sucrose (w/v) in 0.1 M phosphate buffer until equilibration. To verify the placement of the probes, the brains were cut at a thickness of 40 μm on a freezing microtome. Sections were stained with Giemsa and observed under microscope. Only data from animals with probes verified to be located within DRN and FC were considered for statistical analysis.

2.10. Statistics

Data were analyzed with Statsoft Statistica (5.0). Sleep data from 13 animals were obtained. All animals received both 25 and 50 μM bicuculline apart from one pilot animal that received 30 and 100 μM bicuculline. The data from the 30 μM dose were treated statistically together with the 25 μM dose, while the 100 μM data were not considered in the statistics. The sleep data were calculated in 2-h epochs and analyzed with 3-way ANOVA (3 treatments × 4 stages × 3 2-h periods). Significant effects were further investigated with *t*-tests for dependent samples. Significance was accepted at $p<0.05$.

DRN 5-HT data were obtained from 11 animals and FC 5-HT data from 7 animals. The statistical analysis of results from the two sites was run separately to be able to utilize all the DRN data. Mean values from each sleep stage in each animal were used. In the overall ANOVA run for the DRN 5-HT values, missing 5-HT values were substituted by the mean value for the relevant experiment and sleep stage (e.g. the aCSF values in one animal, and the values for 50 μM in two animals). This was done in order to utilize the main body of the data from all animals. In one animal the 50 μM data were excluded, since 5-HT values were more than two standard deviations above the mean of the other animals. In the overall ANOVA for the FC 5-HT values, missing values were substituted with means where only one value were missing. All 5-HT data are presented as mean level ± S.E.M. and were analyzed with 2-way ANOVA (3 treatments × 3 stages). Significant effects were further investigated with *t*-tests for dependent samples. Significance was accepted at $p<0.05$.

Sleep data analysis was carried out on 13 animals out of 21 used because of some technical problems: 3 animals died during or following surgery, 2 had probe or flow problems, 3 had the probe implanted outside the DRN, 2 were disturbed during sleep recording. Additional chromatographic and flow problems reduced the number of animals for DRN to 11 and for FC to 7.

3. Results

All results in this study came from animals verified by histology to have their microdialysis probes located within the DRN and frontal cortex.

3.1. 5-HT level results

3.1.1. Dorsal raphe nucleus

DRN 5-HT data were obtained in 11 animals. There was an overall increase in 5-HT levels in DRN following bicuculline perfusion: ANOVA (3 treatments × 3 stages) ($F_{(2,16)}=3.84$, $p=0.043$). DRN 5-HT levels following 25 μM bicuculline perfusion did not show any significant differences, although there was a strong trend towards increased levels both in W and SWS (post hoc *t*-test $p=0.056$ and $p=0.083$, respectively).

DRN 5-HT levels following 50 μM bicuculline perfusion were significantly higher during W ($p<0.05$), SWS ($p<0.05$) and REM sleep ($p<0.01$) compared to aCSF perfusion (see Table 1).

There was a significant effect of stage, indicating stage related differences in DRN 5-HT levels (ANOVA ($F_{(2,16)}=4.17$, $p=0.035$). During perfusion of aCSF, 5-HT levels were higher

Table 1
Extracellular 5-HT levels presented in absolute values, fmol/sample in DRN and frontal cortex (FC)

	DRN			FC		
	aCSF	25 μM bic	50 μM bic	aCSF	25 μM bic	50 μM bic
W	1.96±0.33	4.31±1.46	6.57±2.07*	3.39±1.74	1.73±0.39	4.12±2.67
SWS	1.38±0.23†	5.72±2.56	6.22±2.53*	0.97±0.23	1.54±0.48	4.91±3.61
REM	1.40±0.21†	3.45±1.42	4.07±0.81**	1.50±0.47	1.45±0.37	4.82±3.07

Values are expressed as means ± S.E.M. *$p<0.05$, **$p<0.01$ significantly different from baseline. †$p<0.05$, significantly different from waking.

during W than SWS ($p<0.05$) and REM sleep ($p<0.05$). During 50 μM bicuculline perfusion 5-HT levels were significantly higher during W compared to REM sleep ($p<0.05$). Other stage differences were not significant. 5-HT levels were still high at the end of the experiment (Fig. 2).

3.1.2. Frontal cortex

Frontal cortex 5-HT data were obtained in 7 animals. There was no overall effect of DRN bicuculline perfusion (either 25 or 50 μM) on frontal cortex 5-HT levels: ANOVA (3 treatments × 3 stages) ($F_{(2,6)}=0.84$, $p=0.48$) (see Table 1), although high variability was observed. Some animals responded by doubling the 5-HT levels during the 25 μM dose and one animal increased the 5-HT level by over 1000% during 50 μM perfusion. There were no stage related differences (ANOVA $F_{(2,6)}=0.26$, $p=0.78$) (see Table 1).

3.2. Sleep results

Sleep data were obtained in 13 animals. There was no overall effect of bicuculline perfusion (overall ANOVA, 3 treatments × 4 sleep stages × 3 2-h periods; $F_{(2,18)}=0.40$, $p=0.67$), but a highly significant interaction between treatment and sleep stage was found: ($F_{(6,54)}=11.43$, $p<0.001$). Post hoc *t*-tests showed that 25 μM bicuculline perfusion did not change total amount of W, SWS-1 and REM sleep whereas total SWS-2 was reduced ($p<0.01$). 50 μM bicuculline perfusion increased the total amount of W ($p<0.01$) and decreased the total amounts of SWS-1 ($p<0.05$), SWS-2 ($p<0.01$) and REM sleep ($p<0.05$). There was also an interaction between treatment, sleep stage and 2-h period ($F_{12,108)}=5.66$, $p<0.001$). Post hoc *t* –tests showed that following 25 μM bicuculline perfusion, W increased ($p<0.05$) and REM sleep decreased ($p<0.01$) during the second

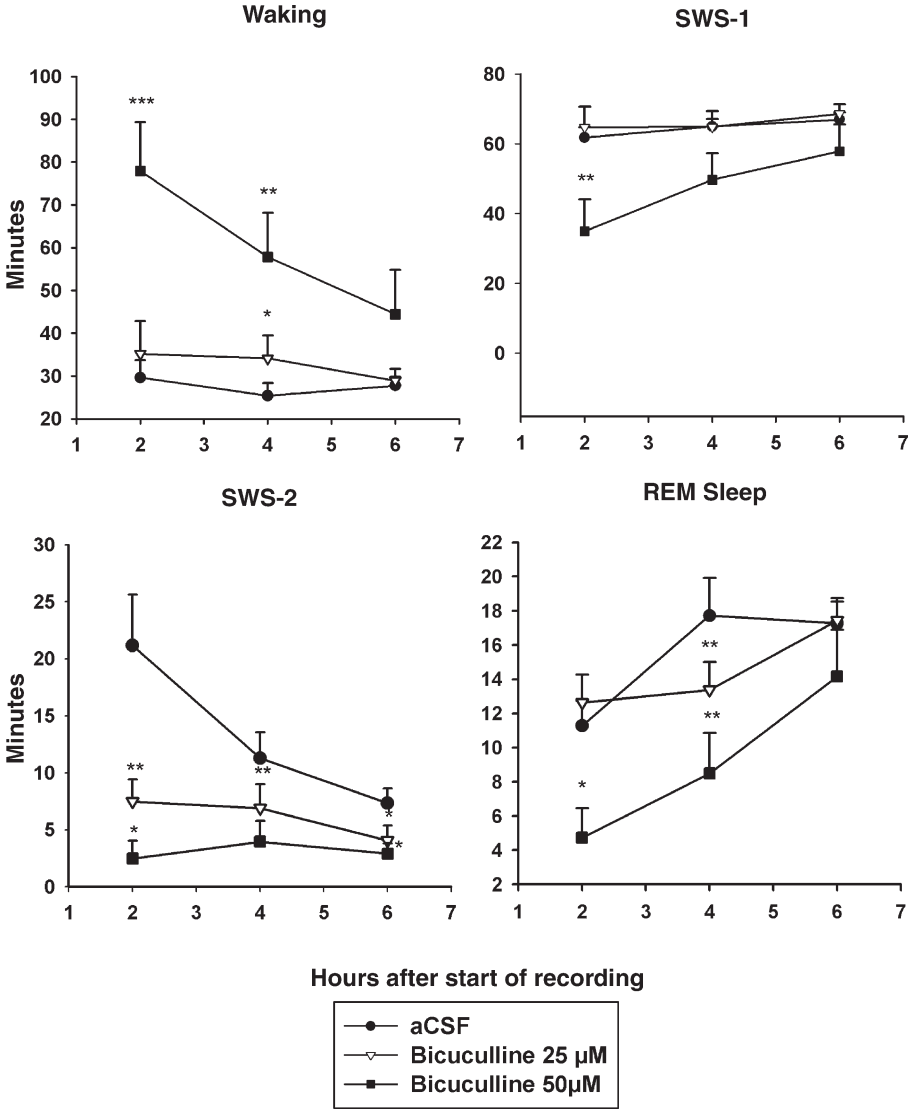

Fig. 1. Sleep and waking are shown as 2-h recording periods. Data are expressed as mean values ± S.E.M. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (post hoc paired *t*-test). The 25 μM dose consistently reduced only the total amount of SWS-2. This reduction was evident throughout all three 2-h periods of sleep recording. During the second 2-h period REM sleep was decreased and W was increased. The 50 μM dose of bicuculline dramatically decreased REM sleep during the 6-h recording, as well as SWS-1 and SWS-2, while total amount of W was increased. These effects were more evident during the first and second 2-h periods.

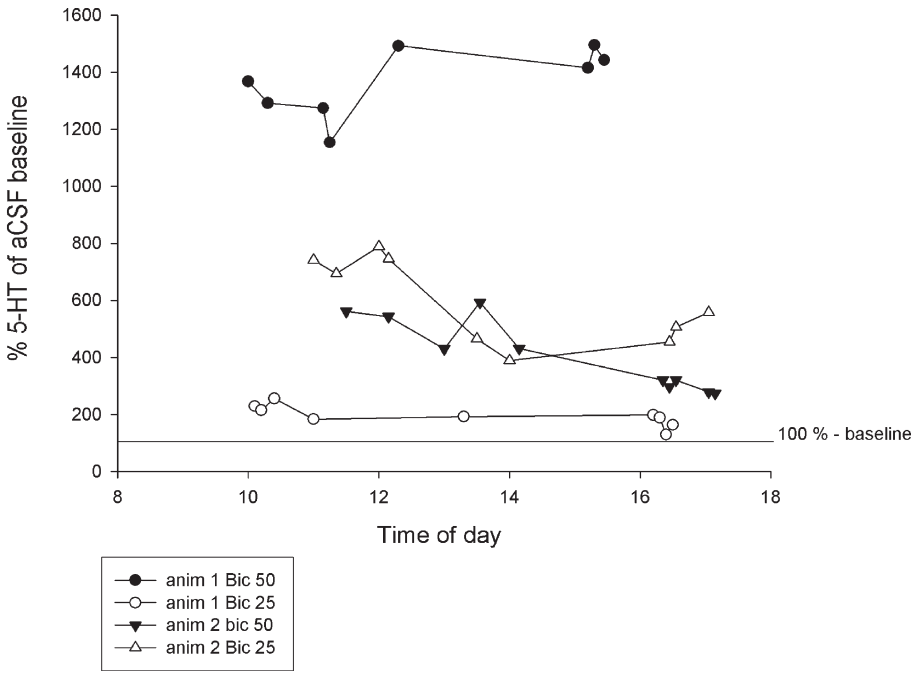

Fig. 2. Examples of 5-HT extracellular levels in DRN during perfusion of 25 and 50 μM bicuculline in two selected animals. Values are given as percent of aCSF baseline for W and SWS. The figure shows that the levels remain high throughout the perfusion (from 08:00 to 18:00 h). Note that at the end of sleep recording time (15:00 h), extracellular 5-HT levels are still well above baseline levels.

2-h period. SWS-2 was reduced during all three 2-h periods ($p<0.01$, $p<0.01$, $p<0.05$, respectively) (see Fig. 1). Following 50 μM bicuculline, W increased during the two first 2-h periods ($p<0.001$, $p<0.01$) and REM sleep decreased ($p<0.05$, $p<0.01$). SWS-1 decreased during the first 2-h period ($p<0.01$). SWS-2 was reduced during the first and third 2-h periods (both $p<0.05$) (see Fig. 1). Unlike 5-HT levels, sleep changes levelled off towards the end of the perfusion (see Fig. 2).

4. Discussion

To our knowledge, this is the first in vivo study investigating simultaneously the effect of DRN perfusion of a $GABA_A$ antagonist on DRN and FC 5-HT extracellular level and sleep. Our findings support the hypothesis that the DRN serotonergic neurons are under inhibitory influence of GABA and that this inhibition is associated with a decrease in both SWS and REM sleep.

During aCSF perfusion, DRN 5-HT showed the usual state-dependent fluctuations [W>SWS>REM, e.g. (Portas et al., 1998)]. Perfusion of bicuculline dose-dependently affected 5-HT levels in the DRN. At the lower dose (25 μM) 5-HT levels in DRN showed a trend towards increase. The higher dose (50 μM) significantly increased 5-HT level during all stages. These findings were associated with a decrease of SWS-2 (25 μM dose), a dramatic decrease of REM as well as SWS-1 and SWS-2, and an increase of W (50 μM dose). This may imply that GABA not only modulate serotonergic neurons in REM sleep as earlier suggested by Nitz and Siegel (1997), but also in other behavioural states. A study by Gervasoni et al. (2000) supports this possibility. They demonstrated the existence of a tonic GABAergic inhibition of 5-HT cells during quiet W. This inhibition would be further intensified during SWS and REM sleep. This also suggests that the GABAergic input is the main regulator of the DRN serotonergic firing during SWS and REM sleep (Nitz and Siegel, 1997; Gervasoni et al., 2000).

Fluctuation of the level of 5-HT in the DRN are usually paralleled by fluctuations in the projection areas (Auerbach et al., 1989; Horner et al., 1997; Orosco et al., 1995; Strecker et al., 1999; Wilkinson et al., 1991). Hence, we expected to observe an increase of 5-HT in FC after bicuculline. According to the hypothesis of the serotonergic modulation of sleep (McCarley et al., 1995), an increased level of 5-HT in REM sleep promoting areas (e.g. LDT and PPT) should inhibit the onset of that state. We could not directly measure 5-HT in LDT or PPT but used a representative projection area, the frontal cortex. FC 5-HT levels did not change under bicuculline influence (neither 25 nor 50 μM dose).

This lack of response, leave us to ponder about three possibilities:

1) DRN 5-HT changes are only local, perhaps as a consequence of dendritic release. In such case, any sleep change cannot be explained on the basis of the serotonergic hypothesis of sleep modulation (McCarley et al., 1995).
2) It is possible that when DRN 5-HT reaches ceiling levels other modulatory mechanisms may become dominant in the modulation of sleep. This possibility is further discussed later.
3) We may have failed to detect 5-HT changes in FC.

The latter possibility may deserve some thought since two previous studies have shown an effect of DRN bicuculline application on forebrain 5-HT levels (Kalen et al., 1989; Tao et

al., 1996). Kalen et al. (1989) showed that striatum 5-HT levels increased by more than 70% after DRN microinjection of bicuculline (300 μl 2 mM), whereas Tao et al. (1996) identified a 100% rise in nucleus accumbens 5-HT level following DRN microdialysis infusion of 100 μM bicuculline. Perfusion of 30 μM bicuculline also significantly raised nucleus accumbens 5-HT level (Tao et al., 1996).

We were not able to replicate our earlier findings that aCSF FC 5-HT levels decrease during SWS and REM sleep compared to waking (Portas et al., 1998). The reason for this discrepancy is not completely clear. We used a different sampling site in the present study, which may have implications with respect to the density of serotonergic terminals.

The decrease in REM sleep and SWS, and the increase in W observed in this study, is consistent with previous findings showing that $GABA_A$ blockers increase DRN firing and 5-HT release (Gervasoni et al., 2000; Tao et al., 1996), decrease REM sleep (Nitz and Siegel, 1997; Sakai and Crochet, 2001) and SWS-2 (Sakai and Crochet, 2001) and increase W (Sakai and Crochet, 2001). Nitz and Siegel (1997) also showed that GABA levels are significantly higher in DRN during REM sleep than during W or SWS. The infusion of muscimol, a $GABA_A$ agonist, increases REM sleep while the $GABA_A$ blocker picrotoxin selectively decreases REM sleep. Sakai and collaborators showed that microdialysis perfusion of bicuculline (at doses of 50 and 100 μM) in cat DRN produces a decrease of REM sleep and SWS-2 in addition to an increase in W (Sakai and Crochet, 2001).

Some of the sleep changes observed in our study (high dose) levelled off towards the end of the recording (after about 4 h). However, DRN 5-HT levels remained high above baseline levels throughout the perfusion (up to 9 h in most animals, see Fig. 2). The reason for the discrepancy between the short-lived effects of bicuculline on sleep compared to the long-lasting effects on 5-HT level is not fully understood. The continuous high 5-HT levels indicate that the local $5\text{-}HT_{1A}$ autoreceptor affinity is unchanged. The circadian variation in sleep is well known and is obvious in our control group: SWS is high in the morning while REM sleep is more pronounced in the afternoon. It is known that 5-HT modulates the circadian clock (i.e. Jovanovska and Prosser, 2002; for review, see Morin, 1999). However, the sleep changes following bicuculline, with low SWS in the morning but high REM sleep in the afternoon cannot simply be explained as a 5-HTergic phase shift. Another possibility is that an adaptation mechanism in the sleep regulatory system may counteract the bicuculline-related increase of serotonergic output (ceiling effect), eventually producing an uncoupling of 5-HT levels and behavioural state. Uncoupling between firing and state after bicuculline blockade of $GABA_A$ receptors has also been observed by Gervasoni et al. (2000). Moreover, a high dose of bicuculline (1 mM) perfused in median raphe nucleus has been shown to uncouple the 5-HT output from the theta waves firing in the hippocampus (Kocsis et al., abstract, 2003). Finally, our data from another study suggest an uncoupling between extracellular 5-HT release and behavioural state after $5\text{-}HT_{1A}$ receptor blockade, with highly increased DRN 5-HT values without concomitant sleep changes (Ursin et al., unpublished observations). Thus, the putative uncoupling following the serotonergic ceiling effect make us speculate that other neuromodulators may take over in the regulation of sleep and waking.

In conclusion, the findings in this study argue in favour of a GABAergic modulation of the serotonergic DRN cells. Blockage of $GABA_A$ receptors in DRN by microdialysis perfusion of bicuculline increased 5-HT levels. The increase of DRN 5-HT output was associated both with reduction of SWS and REM sleep and increase of W. These findings support the hypothesis of the serotoninergic modulation of REM sleep (McCarley et al., 1995), and SWS (Gallopin et al., 2000). However, the possibility of a 5-HT ceiling effect and the consequent interference of other neuromodulators in such circumstances should be further investigated.

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