

Changes in rectal temperature and ECoG spectral power of sensorimotor cortex elicited in conscious rabbits by i.c.v. injection of GABA, GABA_A and GABA_B agonists and antagonists

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1 In order to ascertain whether both GABA_A and GABA_B, or only GABA_B receptors, directly modulate thermoregulation in conscious rabbits, GABA_A/GABA_B agonist and antagonist agents were injected intracerebroventricularly in conscious rabbits while monitoring changes in rectal temperature (RT), gross motor behaviour (GMB) and electrocorticogram (ECoG) power spectra (ps) from sensorimotor cortices.

2 GABA (48 μ mol), nipecotic acid (50 nmol), THIP (60 nmol), muscimol (18 nmol) and baclofen (8 nmol) induced hypothermia ($-\Delta$ RT_{max} values of 1.70 ± 0.1 , 1.4 ± 0.2 , 1.0 ± 0.4 , 1.1 ± 0.2 and $1.6 \pm 0.3^\circ\text{C}$, respectively), accompanied by inhibition of GMB and ECoG synchronization. THIP increased ps at δ frequency band (1.1–3.3 Hz), while GABA, nipecotic acid, muscimol and baclofen did the same at both δ and θ (4.6–6.5 Hz) frequency bands. ECoG ps changes were concomitant or even preceded hypothermia.

3 Bicuculline (1.8 nmol) induced hyperthermia (Δ RT_{max} $1.2 \pm 0.5^\circ\text{C}$) and slight excitation of GMB, while CGP35348 (1.2 μ mol) did not affect RT nor GMB. Both compounds did not affect ECoG ps.

4 Bicuculline potentiated muscimol-induced hypothermia, inhibition of GMB and synchronization of ECoG, while CGP35348 fully antagonized these effects.

5 In conclusion, the present results, while confirming the prevailing role of GABA_B, also outline a direct involvement of GABA_A receptors in the central mechanisms of thermoregulation. Ascending inhibition towards discrete cortical areas controlling muscular activity and thermogenesis may result from GABA receptor activation in neurones proximal to the ventricles, thus contributing to hypothermia, although hypothermia-induced reduction of neuronal activity of these cortical areas cannot be ruled out.

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Abbreviations: CGP35348, (3-aminopropyl)(diethoxymethyl)phosphinic acid; CSF, cerebrospinal fluid; ECoG, electrocorticogram; GABA, γ -aminobutyric acid; GMB, gross motor behaviour; muscimol, 5-aminomethyl-3-hydroxyisoxazole; NA, noradrenaline; nipecotic acid, (\pm)-3-piperidinecarboxylic acid; POAH, preoptic region of the rostral hypothalamus; *R*(-)-baclofen, (*R*)-4-amino-3-(4-chlorophenyl)butanoic acid; RT, rectal temperature; SMC, sensorimotor cortex; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol

Introduction

Regulation of body temperature in mammals is driven by a hierarchy of neural structures among which hypothalamic nuclei are considered of primary importance (for a review, see Boulant, 2000). Effector areas for specific thermoregulatory responses are located throughout the brain stem and spinal cord. The preoptic region of the rostral hypothalamus (POAH) acts as a coordinating centre and strongly influences each of the lower effector areas. The POAH contains neurons that are sensitive to subtle changes in hypothalamic or core temperature. Preoptic thermosensitive neurons also receive a wealth of somatosensory input from the skin and spinal thermoreceptors. In this way, POAH neurons compare and integrate central and peripheral thermal informations. As a result of

sensory integration and control over lower effector areas, the POAH elicits thermoregulatory responses that are most appropriate for both internal and environmental thermal conditions. The central processing of afferent signals from thermoreceptors into the appropriate thermoregulatory output entails complex interneuronal communications, which involve several neurotransmitters. Considerable experimental evidence supports the involvement of both central GABAergic and taurineric systems in thermoregulation (Serrano *et al.*, 1985; Yakimova *et al.*, 1996; Jha *et al.*, 2001; Frosini *et al.*, 2003a, b). Moreover, a recent study performed in the conscious rabbit has demonstrated that heat stress modifies brain metabolism of taurine and GABA, so that increases in cerebrospinal fluid (CSF) concentrations of these amino acids were induced (Frosini *et al.*, 2000). This rise was interpreted as a measure for counteracting the hyperthermia promoted by exposure to heat.

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Central or systemic injection of either GABA or agonists of either GABA_A as well as GABA_B receptors usually induces hypothermia, whereas the injection of antagonists of either class of receptors induces hyperthermia (Serrano *et al.*, 1985; Jackson & Nutt, 1991). The mechanism by which GABA interacts with the neuronal network responsible for thermoregulatory control is not fully understood, and the question whether GABA-ergic drugs affect body temperature by acting directly or indirectly on thermoregulatory processes is still debated. It has been hypothesized that the hypo- or hyperthermic effect of GABA-ergic compounds is related to changes in the temperature sensitivity of warm sensitive hypothalamic neurones, and mediated through GABA_B receptors (Yakimova *et al.*, 1996; Pierau *et al.*, 1997). This is confirmed by the fact that mice lacking the B(1) subunit of the GABA_B receptors do not become hypothermic when GABA_B agonists are applied (Schuler *et al.*, 2001) and exhibit basal rectal temperature (RT) values significantly lower than the wild type (Queva *et al.*, 2003). Presently, it is still unclear as to whether and how GABA_A receptors are involved in GABA-mediated changes of body temperature. Experiments performed *in vitro* have suggested that they act in an antagonistic fashion with GABA_B receptors in order to modulate thermoregulatory responses (Yakimova *et al.*, 1996). This hypothesis, however, has not been supported by data *in vivo* obtained with use of pharmacological models.

Hypothalamic preoptic neurones share considerable GABA-ergic inputs (Lu *et al.*, 2000; Jha *et al.*, 2001), and are strongly interconnected with sensorimotor cortex (SMC) neurons. The activity of SMC neurones supports muscular thermogenesis, and the degree of activation/inhibition is reflected in ECoG spectral power recorded from this area. The power frequency spectrum distribution, in fact, is considered as a drug-specific 'fingerprint', and can be used to understand and/or predict the receptor which is activated by that drug (Bagetta *et al.*, 1990; Ferger *et al.*, 1994; Tortella *et al.*, 1997). Consequently, in the present study, ECoG spectral power from SMC was monitored in order to assess the ECoG 'finger-print' linked to a specific GABA-subtype receptor activation/inhibition, and then to correlate the changes in body temperature to the activation of a specific subclass of GABA receptors. The objective of this study was to investigate whether both GABA_A and GABA_B, or only GABA_B receptors, directly modulate the mechanisms that drive body temperature in conscious rabbits. To this end, GABA_A/GABA_B agonist and antagonist agents were injected i.c.v. at doses that elicited comparable effects on RT. The effect of the above-mentioned compounds on gross motor behaviour (GMB) has also been monitored. The pharmacological modulation of central GABA-ergic transmission resulted in consistent changes in RT and GMB, which correlated to changes in ECoG power spectra. The present results, while confirming the prevailing role of GABA_B, also outline a direct involvement of GABA_A receptors in the central mechanisms of thermoregulation.

Methods

Animals

All the experiments were performed in strict compliance with the recommendations of the EEC (86/609/CEE) for the care

and use of laboratory animals, and were approved by the Animal Care and Ethics Committee of the University of Siena, Italy.

The study was carried out on adult male New Zealand albino rabbits (Charles River, Calco, Como, Italy) weighing 2.0–2.5 kg, and kept in large individual cages under a 12 h : 12 h day–night cycle at 20°C ambient temperature (T_a). Drinking water and conventional laboratory rabbit food were available *ad libitum*. Nine groups of 4–6 rabbits each have been used throughout.

Surgery

At least 3 weeks before the experiment, animals were implanted with a cannula for intracerebroventricular (i.c.v.) injection at the level of the lateral ventricle (1 mm anterior to bregma and 2.8 mm lateral to sagittal suture, 6 mm depth) and with two bipolar stainless-steel electrodes bilaterally onto the left and right SMC (7 mm anterior to bregma, 2 mm lateral to sagittal suture, 1 mm depth) for ECoG recording. Anaesthesia was induced by i.m. injection of xylazine chloride (10 mg kg⁻¹, Rompun® vet., Bayer AG, Germany) and ketamine hydrochloride (35 mg kg⁻¹, Ketavet®, Parke Davis/Warner-Lambert, U.S.A.). Under sterile conditions, the dorsal structure of the skull was exposed by a midline skin incision and, after drilling the holes near the bregma according to stereotaxic coordinates, a thin stainless-steel cannula and two bipolar electrodes were inserted. Spontaneous CSF outflow indicated the correct i.c.v. placement of the cannula. The cannula and the electrodes were fixed to the skull with stainless-steel screws and dental acrylic cement and the skin incision was sutured.

After surgery, rabbits were injected for at least 5 days with the following drugs: prednisolone acetate (Novosterol®, Vetem S.p.A., Italy), 10 mg day⁻¹ i.m.; enrofloxacin, (Bay-tril®, Bayer AG, Germany) 25 mg day⁻¹ i.m. The animals were allowed to recover for at least 15 days. During this time, they were accustomed to the test environment.

Drugs, doses and i.c.v. injection

The following compounds were used: GABA, nipecotic acid (GABA uptake inhibitor), muscimol (GABA_A agonist), bicuculline (GABA_A antagonist) from Sigma Chemical Co, St Louis, MO, U.S.A.; R(–)-baclofen (GABA_B agonist), 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP, GABA_A agonist) from RBI, Netik, MA, U.S.A. (3-aminopropyl) (diethoxymethyl)phosphinic acid (CGP35348, GABA_B antagonist) synthesized by CIBA GEIGY (Basel, CH, Switzerland), was a generous gift from Dr Carla Ghelardini (Department of Pharmacology, University of Florence, Italy).

GABA and muscimol elicit comparable effects when injected at 48 µmol and 18 nmol, respectively. As to the other compounds employed, doses that induced comparable effects on RT (see below in Analysis of data section) were selected by performing dose–response curves.

All the compounds were easily dissolved in double-distilled pyrogen-free water for clinical use prior to use. The resulting solutions were injected i.c.v. in a 10–20 µl volume, with an Agla micrometer-operated syringe (Burroughs Wellcome and Co., London, U.K.).

Temperature and ECoG recording

RT was measured every 5 min by means of a thermocouple thermometer (Columbus, OH, U.S.A.) connected to a personal computer running an Iso-thermex programme (Columbus, Ohio, U.S.A.).

ECoG signal was recorded by connecting the bipolar stainless-steel electrodes to a 14-channel OTE Biomedica (Florence, Italy) E14d model polygraph (low-pass filter and high-pass filter set at 0.5 and 20 Hz, respectively) and equipped with a real-time spectrum analyser (antialiasing filter 18 db oct⁻¹, time constant 1 s), which allowed calculation of the power variable (μV^2) of the following frequency bands (Hz): 1.1, 1.6, 2.3, 3.3 (δ band), 4.6, 6.5 (θ band), 9.2, 13 (α band) and 18.4 (β band). The absolute power spectra of ECoG signals were calculated on 5 s epochs and at least three consecutive spectra were then averaged.

Experimental protocol

As soon as the rabbits had recovered from the surgical operation, in order to reduce the artefacts arising from moving, they were habituated to remain quiet in the restraining cage that holds the neck of the animal while allowing the movement of the trunk and posterior legs. All experiments started between 08:30 and 09:30 h to avoid the bias on measurements caused by diurnal rhythms. Conscious rabbits, placed in the restraining cage, were kept for all the duration of the experiment in a sound-attenuated, electrically shielded and thermostatted ($T_a = 20^\circ\text{C}$) chamber. A light source was present laterally to the rabbit. Under these conditions, the rabbits remained quiet with open eyes and the head held up, quickly reacting to a standard slight sound stimulation by turning their head to the source of the sound. This behaviour was elicited throughout the recording period both before and after drug treatment, in order to keep the animal aroused during ECoG recording. RT and ECoG recordings started at least 60 min before drug administration and lasted approximately 7 h thereafter. No animal was used more often than once a week. RT was recorded every 5 min, while ECoG every 15 min during the first hour after i.c.v. injection and every 30 min thereafter; both parameters were again monitored at 24 h after treatment. At the peak of RT change which followed i.c.v. drug injection, GMB was monitored. The time of peak hypothermia was that wherein two subsequent readings of RT were equal or the second one was slightly higher. Shortly thereafter, animals were taken out from the thermostatted chamber and removed from the restraining cage by an observer attending the experimental session and not aware of the treatment, positioned on a table and scored for GMB according to the following arbitrary scale (Frosini *et al.*, 2003b): 0, no effect; –, sedation and slight motor incoordination; —, sedation and motor incoordination; ——, sedation and long-lasting motor incoordination; ———, sedation, severe and long-lasting motor incoordination and loss of righting reflex; +, alertness; ++, alertness and increased panting rate (50–75 breaths min⁻¹); +++, alertness and elevated panting rate (75–100 breaths min⁻¹); + + + +, alertness, sustained panting rate (breath min⁻¹ > 100) and episodic convulsive attacks. GMB scoring procedure took place in a 5–10 min period.

To isolate any experimental artefacts arising from animal restraint, manipulation or i.c.v. injection, all rabbits used in the present study were injected with 10 μl pyrogen-free water (vehicle) and RT, ECoG and GMB were monitored as previously described. Only those animals that did not exhibit significant changes in RT during the entire observation period were used in the subsequent experiments. Two out of a group of 50 became hyperthermic and were discarded. Among all the rabbits used in this study, six were randomly selected to form the control group.

Analysis of data

RT data The values are expressed as mean \pm s.e.m., and are reported as changes in RT (ΔRT). The area under the RT experimental curve (AUC) was calculated by a combined linear logarithmic trapezoidal method using Graphpad-Prism II program (GraphPad Software Inc., San Diego, CA, U.S.A.). The comparison between $\text{AUC}_{(0-24\text{ h})}$ of control (vehicle-injected) vs treated rabbits was performed using ANOVA followed by *post-hoc* Dunnett's test. A *P*-value < 0.05 was considered significant. Doses of GABA_A/GABA_B agonists, which elicited RT effects comparable to those induced by 48 μmol GABA, were selected for the subsequent experiments. Their $\text{AUC}_{(0-24\text{ h})}$'s were not significantly different.

ECoG data ECoG drug-induced changes were evaluated by using the method described by Sebban *et al.* (2002). Since ECoG power densities tend to decrease over time (Lancel *et al.*, 1998), they were normalized by calculating the ratio between mean power at a defined frequency band post-drug treatment and mean power at the same frequency band post-vehicle treatment, according to the equation:

$$\begin{aligned} \text{variation of spectral power (\%)} \\ = \frac{\text{ECoG power following drug}}{\text{ECoG power following vehicle}} \times 100 \end{aligned}$$

Thus, drug-induced changes in ECoG power were expressed as the percentage of ECoG power recorded in control animals (i.e. six rabbits injected only with pyrogen-free water). ECoG power was recorded during a 15 s period of constant immobility, as determined by close inspection of the animal. For each time interval following i.c.v. injection, at least three spectra were averaged. The time course of changes in ECoG power at the various frequency bands considered was plotted and the AUC (0–24 h after treatment) relative to each frequency band was calculated by using a combined linear logarithmic trapezoidal method (Graphpad-Prism II program, GraphPad Software Inc., San Diego, CA, U.S.A.). Data are reported as mean \pm s.e.m. values. The comparison between $\text{AUC}_{(0-24\text{ h})}$'s was performed by using ANOVA followed by *post hoc* Dunnett's test. A *P*-value < 0.05 was considered significant.

Results

Effects of i.c.v. injection of vehicle, GABA, GABA_A/GABA_B agonists and antagonists on RT and GMB

Pyrogen-free water To isolate any experimental artifacts arising from animal restraint, manipulation or i.c.v. injection,

a group of six rabbits was treated by i.c.v. injection of 10 μ l pyrogen-free water, while monitoring RT. The basal values were $39.0 \pm 0.1^\circ\text{C}$. Pre- and postinjection RT values showed no statistically significant differences. GMB was not affected by this treatment.

GABA, nipecotic acid, THIP, muscimol and R(-)baclofen GABA, when injected i.c.v. in μ mol amounts, causes a dose-related hypothermia (Sgaragli *et al.*, 1978). As reported in Figure 1 (panel a), a 48 μ mol dose of GABA induced hypothermia peaking with a $-\Delta\text{RTmax}$ value of $1.6 \pm 0.2^\circ\text{C}$ ($n=6$) at 120 min. Subsequently, RT increased regularly, regaining the basal values at 300 min and then remaining almost stable until the end of the experimental session. GABA was shown to affect GMB causing sedation and motor incoordination (arbitrary scale ---), which, however, were no more evident at 300 min. Nipecotic acid caused a dose-related hypothermia (see Table 1). At the dose of 50 nmol, it reduced RT with a $-\Delta\text{RTmax}$ value of $1.4 \pm 0.2^\circ\text{C}$ ($n=6$), at 120 min (Figure 1, panel a) with a return to pretreatment values at 300 min; it caused depression of GMB comparable to that elicited by GABA. THIP-induced

hypothermia was dose-related (see Table 1), its effect becoming evident from a 30 nmol dose onwards. This dose caused a $-\Delta\text{RTmax}$ of $0.6 \pm 0.2^\circ\text{C}$ ($n=4$) at 120 min ($P<0.01$ vs vehicle). At a 60 nmol dose, it induced a long-lasting hypothermia with a $-\Delta\text{RTmax}$ value of $1.1 \pm 0.4^\circ\text{C}$ ($n=4$) at 180 min (Figure 1, panel b). Subsequently, RT returned to basal values at 360 min. During the hypothermic phase, sedation and motor incoordination (arbitrary scale ---) were observed.

Muscimol, when injected i.c.v. in nmol amounts, cause a dose-related hypothermia (Sgaragli *et al.*, 1978). At the dose of 18 nmol, it induced a hypothermic response which was polyphasic in nature, RT sharply decreasing with a $-\Delta\text{RTmax}$ of 1.0 ± 0.3 ($n=5$) at 105 min, then rising and finally decreasing again with a $-\Delta\text{RTmax}$ value of $0.7 \pm 0.3^\circ\text{C}$ at 330 min. At the end of the experimental session, rabbits were still hypothermic. After 24 h, all muscimol-treated animals were normothermic. Muscimol deeply affected GMB, causing sedation and long-lasting motor incoordination (arbitrary scale ---), which were still evident 24 h after treatment. R(-)baclofen at 1 nmol dose caused hypothermia (Table 1) with a $-\Delta\text{RTmax}$ value of $0.6 \pm 0.2^\circ\text{C}$ ($n=4$) at 110 min ($P<0.01$ vs vehicle). At a dose of 8 nmol, it caused a progressive decrease of RT with a $-\Delta\text{RTmax}$ value of $1.2 \pm 0.3^\circ\text{C}$ ($n=6$, Figure 1, panel b) at 105 min. RT regained the basal values at 300 min. After R(-)baclofen treatment, GMB was affected in a muscimol-like fashion. Rabbits exhibited sedation and long-lasting motor incoordination (arbitrary scale ---). At the end of the experimental session, however, GMB had regained a normal pattern.

Coadministration of bicuculline + muscimol and CGP35348 + muscimol Coadministration of bicuculline (0.2 nmol) with muscimol (18 nmol) caused a decrease in RT (Figure 2, panel a), which was polyphasic, similarly to what happened with muscimol alone. RT, in fact, sharply decreased ($-\Delta\text{RTmax}$ value of $1.2 \pm 0.3^\circ\text{C}$ at 150 min, $n=4$), then rose and finally decreased again with a $-\Delta\text{RTmax}$ value of $1.0 \pm 0.2^\circ\text{C}$ at 240 min. At the end of the experimental session, RT was still below basal values. As reported in Table 1, however, relative $\text{AUC}_{(0-24\text{h})}$ was not different from that related to an 18 nmol muscimol-alone treatment. On the contrary, at the dose of 1.8 nmol, bicuculline significantly potentiated muscimol-induced hypothermia (Figure 2, panel a). RT, in fact, decreased regularly, attaining a $-\Delta\text{RTmax}$ value of $1.6 \pm 0.1^\circ\text{C}$ at 240 min. The recovery of RT to baseline values was reached only at the end of the experimental session. The relative $\text{AUC}_{(0-24\text{h})}$ was significantly higher ($P<0.05$, $n=4$) than that of muscimol alone. Bicuculline was also able to potentiate muscimol-induced depression of GMB, sedation being severe enough and accompanied by a long-lasting motor incoordination and loss of righting reflex (arbitrary scale ---). Coadministration of CGP35348 (0.4 μ mol) and muscimol (18 nmol) did not entirely antagonize muscimol-induced hypothermia, as reported in Figure 2, panel b ($\text{AUC}_{(0-24\text{h})}$ vs vehicle, $P<0.05$, $n=4$). Conversely, at the dose of 1.2 μ mol, it fully antagonized muscimol-induced hypothermia ($\text{AUC}_{(0-24\text{h})}$ vs vehicle, NS) and depression of GMB.

Bicuculline and CGP35348 When bicuculline and CGP35348 were administered separately at the highest dose employed in the previous experimental session, that is,

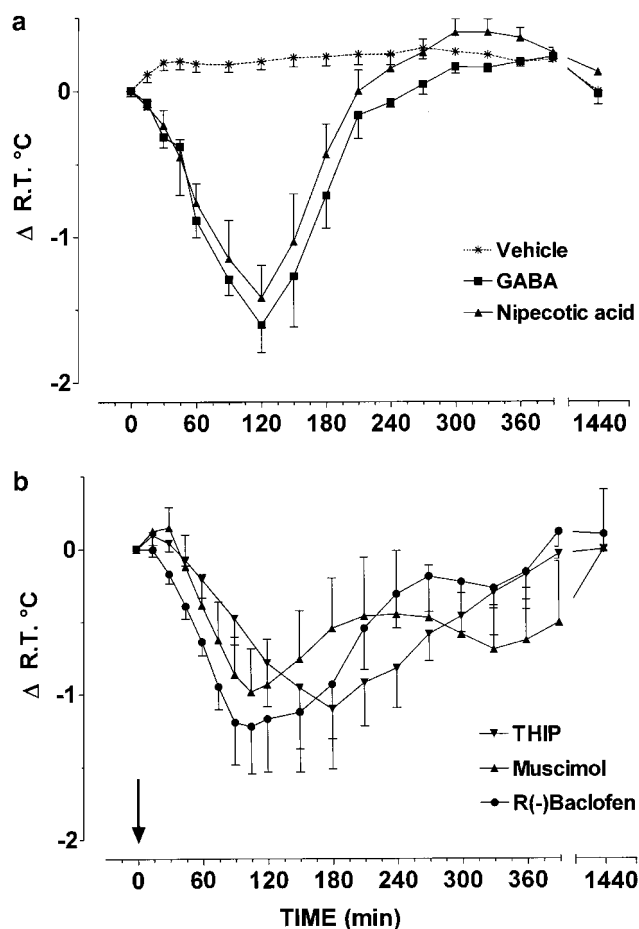


Figure 1 Time course of the effects elicited by i.c.v. injection of pyrogen-free water (vehicle, $n=6$), GABA (48 μ mol, $n=6$) and nipecotic acid (50 nmol, $n=6$) (panel a); THIP (60 nmol, $n=4$), muscimol (18 nmol, $n=5$) and R(-)baclofen (8 nmol, $n=6$) (panel b) on rabbit RT. Data are reported as mean \pm s.e.m. of RT changes. The arrow indicates time of i.c.v. injection.

Table 1 Effects of i.c.v. injection of different doses of GABA, GABA_A/GABA_B agonist/antagonists on RT and ECoG spectrum power in conscious rabbits

Compound	Dose (n)	ΔRt_{max} (°C)	ECoG power spectra
Vehicle	—(6)	+0.3±0.1	
GABA	48 µmol (6)	-1.7±0.1**	↑ δ , θ
Nipecotic acid (GABA uptake inhibitor)	5 nmol (4)	-0.2±0.2 NS	
	50 nmol (6)	-1.4±0.2** #	↑ δ , θ
THIP (GABA _A agonist)	10 nmol (4)	-0.3±0.1 NS	
	30 nmol (4)	-0.6±0.2**	
	60 nmol (4)	-1.0±0.4** #	↑ δ
Muscimol (GABA _A agonist)	18 nmol (5)	-1.0±0.3** #	↑ δ , θ
R(-)baclofen (GABA _B agonist)	1 nmol (4)	-0.6±0.2**	
	8 nmol (6)	-1.2±0.3** #	↑ δ , θ
CGP35348 (GABA _B antagonist)	12 µmol (4)	+0.1±0.2 NS	n.e.
Bicuculline (GABA _A antagonist)	1.8 nmol (4)	1.1±0.5*	n.e.
CGP35348 + muscimol GABA _B antagonist + GABA _A agonist	0.4 µmol + 18 nmol (4)	-0.4±0.2*	
	1.2 µmol + 18 nmol (4)	-0.1±0.3 NS †	n.e.
Bicuculline + muscimol (GABA _A antagonist + GABA _A agonist)	0.2 nmol + 18 nmol (4)	-1.2±0.3**,\$	
	1.8 nmol + 18 nmol (4)	-1.8±0.2**,\$ †	↑ δ , θ ** ↑ δ †

n.e.: no effect. ↑: increase. δ : 1.1–3.3 Hz frequency band. θ : 4.6–9.2 Hz frequency band. RT data are reported as mean ± s.e.m. The comparison between AUC_(0–24 h) obtained after different treatments was performed using ANOVA followed by *post hoc* Dunnett's test.

* $P < 0.05$ vs vehicle; ** $P < 0.001$ vs vehicle; #: NS vs 48 µmol GABA, \$NS vs 18 nmol muscimol, † $P < 0.05$ vs 18 nmol muscimol.

1.8 nmol and 1.2 µmol, respectively, only bicuculline induced a hyperthermic response (Figure 2, panel c) with a ΔRt_{max} value of $1.1 \pm 0.5^\circ\text{C}$ ($n = 4$) attained at the end of the experimental session (390 min). GMB was also markedly affected by this treatment, giving rise to alertness and elevated panting rate (arbitrary scale + + +) and occasional episodes of kicking. At the end of the experimental session, however, GMB showed a normal pattern. CGP35348 did not induce significant changes on both RT (Figure 2, panel c) and GMB.

Effects of i.c.v. injection of vehicle, GABA, GABA_A/GABA_B agonists and antagonists on ECoG pattern and related power spectra

ECoG of conscious, alert and restrained rabbits consisted of low-voltage (<100 µV) and fast-activity waves. This pattern, as well as the related ECoG power spectrum, were not modified by the i.c.v. administration of 10 µl pyrogen-free water.

ECoG was monitored either after i.c.v. injection of doses of GABA_A/GABA_B agonists tested, which elicited comparable effects on RT, or after the highest dose of GABA_A and GABA_B antagonists, which were proven to potentiate or antagonize muscimol effects, namely bicuculline and CGP35348, respectively.

ECoG following GABA, nipecotic acid, THIP, muscimol and R(-)baclofen underwent synchronization with large and slow waves of up to 500 µV amplitude. In particular, GABA and nipecotic acid (see Figure 3) specifically increased the energy power spectrum at both δ (1.1–3.3 Hz) and θ (4.6–6.5 Hz) frequency bands. The mean percent increase of ECoG energy power following GABA and nipecotic acid treatment as AUC_(0–24 h) values at δ and θ frequency bands were 180 ± 11 and 156 ± 6 , 176 ± 21 and 145 ± 7 , respectively. The energy power at α and β spectrum regions, however, were unaffected.

THIP was able to significantly increase the energy power only at δ frequency band, this increase amounting to $233 \pm 31\%$. Muscimol and R(-)baclofen gave rise to a huge increase of the energy power at both δ and θ frequency bands, similarly to that observed after GABA. In particular, δ frequency band values after muscimol averaged $372 \pm 94\%$ over control values, and were significantly higher than those promoted by both R(-)baclofen and GABA. Either CGP35348 or bicuculline did not modify the ECoG pattern and relative power spectrum (Figure 3). When bicuculline was coadministered with muscimol, it potentiated muscimol-induced synchronization, giving rise to a large and slow ECoG activity, with waves up to 750 µV amplitude. Furthermore, bicuculline significantly potentiated the increase at 2.3 and 3.3 Hz frequency bands (δ region) elicited by muscimol, while leaving the θ region frequency bands unchanged.

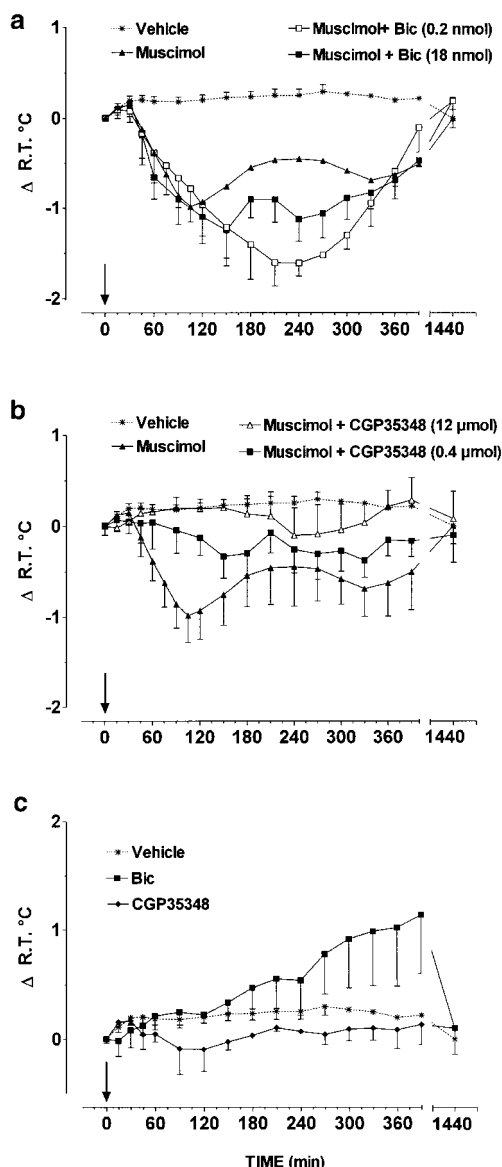


Figure 2 Time course of the effects elicited by i.c.v. injection of pyrogen-free water (vehicle, $n=6$), muscimol alone (18 nmol, $n=5$, data from Figure 1), muscimol+0.2 nmol bicuculline ($n=4$), muscimol+1.8 nmol bicuculline ($n=4$) (panel a); pyrogen-free water (vehicle, $n=6$), muscimol+0.4 μ mol CGP35348 ($n=4$), muscimol+1.2 μ mol CGP35348 ($n=4$) (panel b); bicuculline (1.8 nmol, $n=4$) and CGP35348 (1.2 μ mol, $n=4$) (panel c). Data are reported as mean \pm s.e.m. of RT changes. To improve the clarity, the s.e.m. of RT values of muscimol in panel a are not depicted. The arrow indicates time of i.c.v. injection.

CGP35348, when coadministered with muscimol, reversed muscimol-induced effect on ECoG pattern, which was similar to that recorded under control conditions, and suppressed muscimol-induced ECoG power spectrum increase at δ and θ regions.

As shown in Figure 4, ECoG power spectrum changes at the peak of hypothermia induced by GABA, THIP, muscimol, *R*(-)-baclofen and nipecotic acid were consistent with AUC_(0-24h) changes reported in Figure 3. When RT was at the peak of the hypothermic response, in fact, ECoG power spectra after GABA, muscimol, nipecotic acid and *R*(-)-baclofen injection

exhibited a significant increase in δ and θ region, while after THIP injection there was only an increase at δ region. Following the coadministration of muscimol + bicuculline, at the peak of hypothermia, ECoG power spectrum exhibited a marked increase at both δ and θ regions. This increase, however, was similar to that induced by muscimol alone, with the exception of the 1.6 Hz frequency band, which was much less enhanced.

In order to assess a temporal relationship between neuronal activity at SMC and RT changes, the time courses of both muscimol- and GABA-induced increase in ECoG activity and hypothermia are reported in Figures 5 and 6, respectively. With either treatment, the energy power increase at δ region was sustained throughout the experimental session, while that at θ region occurred mostly in the 0–180 min period after injection. In the case of muscimol, the time courses of both hypothermia and ECoG increase at δ region were almost synchronous, the maximum of the effects being achieved at the same time; the ECoG power increase at 2.3 and 3.3 Hz of the δ region and at 4.6 and 6.5 Hz frequency bands of θ regions, however, anticipated the peak of hypothermia. In the case of GABA, ECoG power increase at δ region and hypothermia occurred almost synchronously in the 0–60 min period after injection. Afterward, however, while ECoG power underwent a slight decrease subsequent to the maximum attained at around 60 min, RT continued to diminish, peaking at 120 min. Furthermore, the increase in energy power at θ region was mostly limited to 0–180 min period after injection. The energy power at δ region (with the exception of 3.3 Hz frequency band) normalized later than RT, while that of the θ region returned to basal values along with RT. With both treatments, α and β regions were not affected (data not shown).

Discussion

The present findings show that the i.c.v. injection of drugs acting at either GABA_A or GABA_B receptors resulted in consistent changes in RT, accompanied by changes in ECoG pattern and relative power spectra (see Table 1). The compounds tested were injected at different amounts so that comparable effects on RT were obtained; this, however, required the use of doses that differed by 2–3 orders of magnitude. To explain this discrepancy, it is worth outlining that GABA is quickly removed from the synaptic cleft into neurones and glial cells by a high-affinity, high-efficacy-uptake system (Iversen & Kelly, 1975) and, consequently, it exerts a spatio-temporally confined action. Conversely, GABA analogues, owing to their low degree of conformational flexibility, are poor substrates for the uptake system and cause more sustained and widespread effects also at doses considerably lower than those of GABA (Lancel *et al.*, 1998).

Neural pathways mediating the generation and/or maintenance of either sleep or body temperature reside within the preoptic/anterior hypothalamus (Szymusiak, 1995). These two functions may affect each other, for example, body temperature rises during wakefulness and decreases during sleep (McGinty & Szymusiak, 1990), while body warming and body cooling induce sleep and wakefulness, respectively (Szymusiak & Satinoff, 1984; Nakao *et al.*, 1995). The GABA-ergic drugs employed here are known to promote non-REM sleep with high low-frequency activity in the EEG. The animals used in

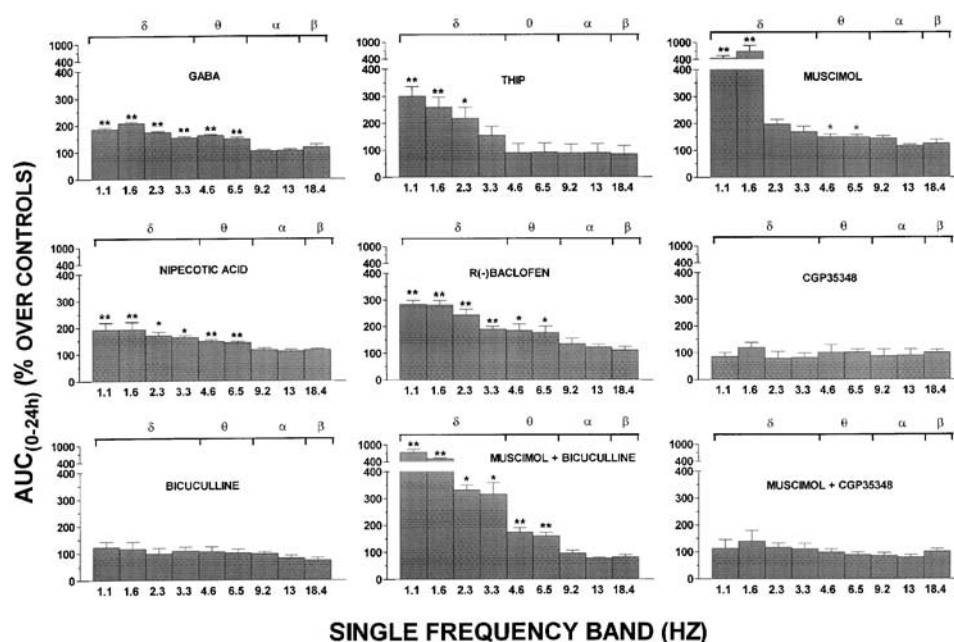


Figure 3 Changes in ECoG power spectrum induced by i.c.v. injection of GABA, GABA_A/GABA_B agonists and antagonists. Values of energy power at each frequency band were firstly normalized by calculating the ratio of mean spectral power obtained following the injection of drug vs the mean spectral power obtained following administration of vehicle. The time course of changes in ECoG power (expressed as % over controls) at each frequency band gave areas under the curve (AUC_(0-24h)) after the injection) reported as mean \pm s.e.m. values ($n=4-6$). The comparison between AUC_(0-24h) post-drug and AUC_(0-24h) post-vehicle was performed using ANOVA followed by *post hoc* Dunnett's test. A P -value <0.05 was considered significant. ** $P<0.01$; * $P<0.05$.

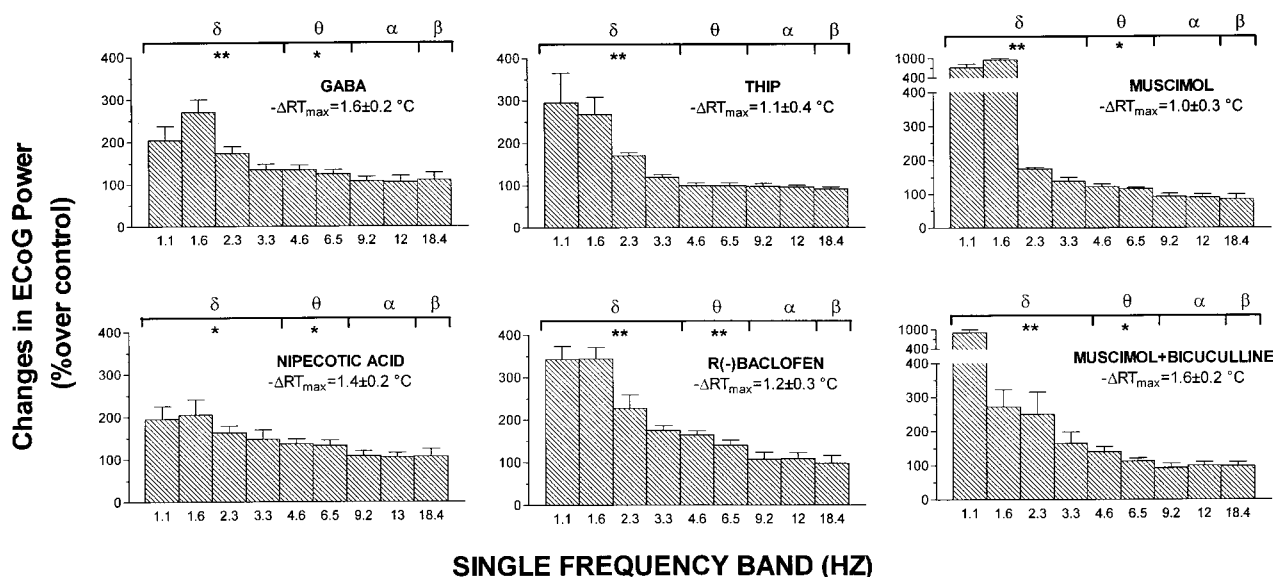


Figure 4 Changes in ECoG power spectrum following drug treatments which induced hypothermia. Energy power values at each frequency band were normalized as the ratio of mean spectral power post-drug at the peak of hypothermia vs the mean spectral power obtained following administration of vehicle at the same frequency. Data are reported as mean \pm s.e.m. values ($n=4-6$). The comparison between ECoG changes of different drugs was performed using ANOVA followed by *post hoc* Dunnett's test. A P -value <0.05 was considered significant. ** $P<0.01$; * $P<0.05$.

the present study were kept in an environment (such as the presence of a light source laterally to the rabbit and the periodic stimulation with a standard sound every 15 min and just before the ECoG recording), which should have guaranteed their persistence in an arousal condition. Rabbits, in fact, reacted quickly to the noise by turning their head to the source of the sound, suggesting a wakefulness condition. Further-

more, the ECoG signal helped to monitor not only the absence of a non-REM sleep condition during recording, but also the lack of a condition resembling absence epilepsy, which is characterized by an epileptiform-like hypersynchronous low-frequency ECoG activity while animals stay immobile. Thus, it is conceivable that the effects on RT of the GABA-ergic drugs employed in this study did not depend on a primary effect on

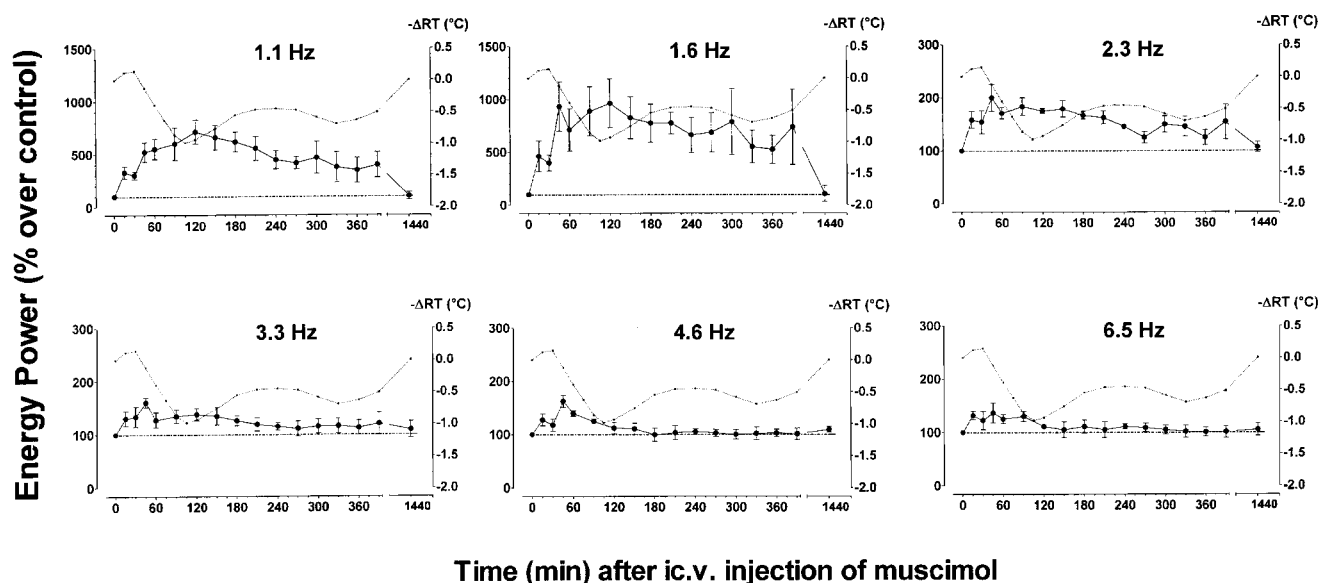


Figure 5 Time course of the effects elicited by muscimol (18 nmol, $n=5$) on ECoG energy power at single frequency bands and on RT. Data are reported as mean \pm s.e.m. ECoG values were normalized by expressing them as the percent value of the average ECoG power density at the same frequency band, recorded after i.c.v. injection of the vehicle during the same period of time (horizontal line; for further details, see Methods).

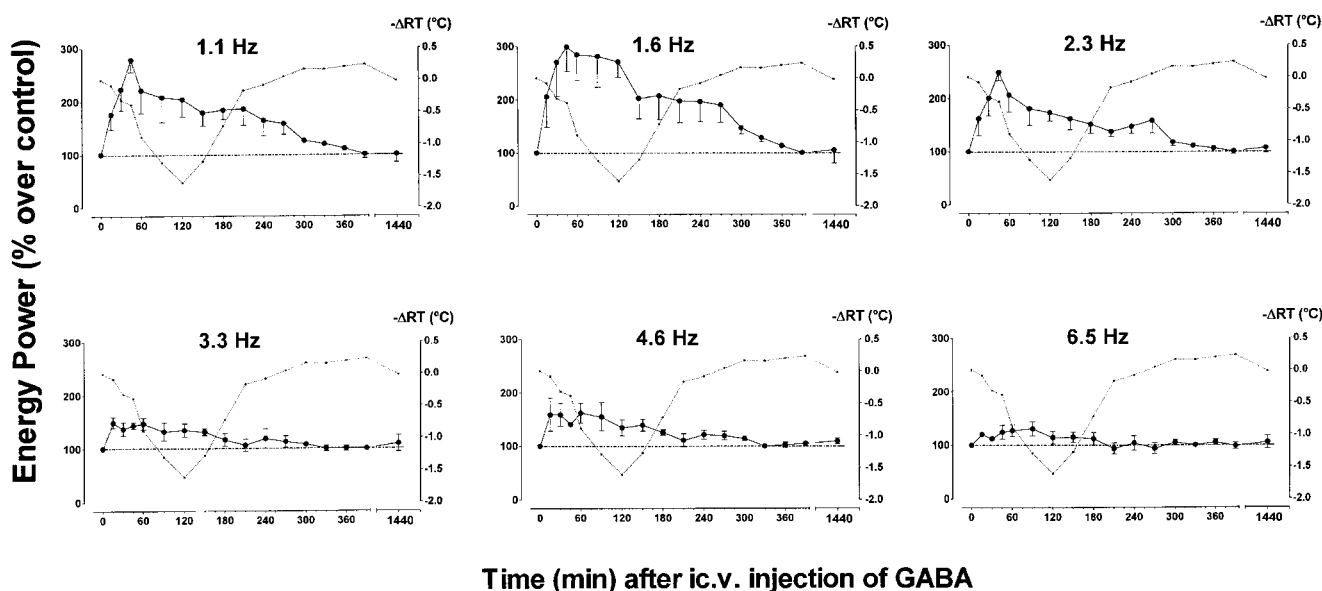


Figure 6 Time course of the effects elicited by GABA (48 μ mol, $n=6$) on ECoG energy power at single frequency bands and on RT. Data are reported as mean \pm s.e.m. ECoG values were normalized by expressing them as the percent value of the average ECoG power density at the same frequency band, recorded after i.c.v. injection of the vehicle during the same period of time (horizontal line; for further details, see Methods).

vigilance. Neuropharmacological studies have reported that microinjection of agonists and antagonists of adreno- (Mallick & Alam, 1992; Mallick & Joseph, 1997; 1998), cholin- (Mallick & Joseph, 1997; 1998) and GABA receptors (Gray *et al.*, 1987; Osborne *et al.*, 1994; Jha *et al.*, 2001) into the mPOAH influence both the sleep–wakefulness pattern and body temperature. However, it has also been found that the tonic influence of the medial preoptic area on sleep–wakefulness cycle is independent of the simultaneous changes in body temperature (Mallick & Alam, 1991). Moreover, Jha *et al.*

(2001) were not able to establish a cause and effect relationship between changes in sleep–wakefulness cycle and body temperature induced by the GABA_A antagonist picrotoxin in freely moving rats. Nevertheless, while changes in body temperature may affect EEG (Mallick & Alam, 1991), changes in EEG have been reported to alter the activities of both the medial preoptic-anterior hypothalamic area (Mallick *et al.*, 1983) and brain stem neurons (Graham *et al.*, 1989), either in unanaesthetized or anaesthetized preparations. Previous reports have shown that EEG alternates between synchronization

and desynchronization, concomitantly with changes in body temperature. However, it is also worth outlining how difficult it is to ascribe neuronal firing rate changes to changes in body temperature by distinguishing them from those secondarily due to changes of EEG or *vice versa*. Nevertheless, it is conceivable that changes in neuronal activities at SMC observed in the present study were primarily due to effects on neural pathways governing body temperature, since the alterations in the ECoG were sustained throughout the period of hypothermia (see Figures 3 and 4). Further support to this hypothesis comes from the fact that the time window in which maximal changes of ECoG power of each frequency band occurred anticipated peak hypothermia. This can be explained by considering that the ensemble of rhythmic changes observed on ECoG or EEG recordings (synchronization) and, inversely, the disappearance of such rhythms (desynchronization) are 'on line' phenomena, while changes in RT involve the activation of more complex pathways such as the activation of thermoeffectors (panting rate and vasodilatation) towards heat dissipation, which need a long time period to be effective. The alterations in ECoG and related power spectra following the injection of GABAergic drugs correlate clearly with changes in RT, dependently on the subtype of GABA receptor activated. In the present study, the i.c.v. injection of the selective GABA_A agonist THIP caused hypothermia and synchronization of the ECoG activity, which resulted in an increase of energy power at δ frequencies. THIP has been shown to increase δ activity in man and rats during non-REM sleep (Faulhaber *et al.*, 1997; Lancel, 1997). The i.c.v. injection of the selective GABA_B agonist *R*(-)-baclofen caused a deep hypothermia along with synchronization and increase of ECoG power spectrum at δ and θ regions. Thus, while the activation of GABA_A receptors resulted in the increase at only the δ region, that of GABA_B receptors involved, additionally, the θ band of frequencies, in accordance with data reported in the literature for rabbit (Massotti, 1985) and in a genetic rat model of absence epilepsy (Richards *et al.*, 2000). When injected i.c.v. in nanomolar amounts, muscimol was able to increase ECoG power both at δ and θ regions of frequencies, along with synchronization of the signal. These data match previous findings by Lancel *et al.* (1996). The 'GABA_B-agonist profile' of muscimol was also confirmed by the finding that CGP35348 (GABA_B antagonist) fully antagonized its effects on both ECoG and body temperature. Muscimol, however, is reported to bind to both GABA_A and GABA_B receptors, but with nanomolar and micromolar affinity, respectively (Bowery *et al.*, 1985). It is possible that GABA_B receptors are the most abundant GABA receptor subtype present in neurones of the periventricular area, or those most involved functionally in the effects recorded.

ECoG spectral power changes induced by centrally injected GABA may be related to GABA_B-receptor activation, since it enhanced ECoG power density at both δ and θ frequency bands. This observation provides additional information on which of the two subtypes of GABA receptors drives the specific ECoG-related changes induced by GABA and, at the same time, the GABA hypothermogenic effect. Nipepicotic acid is reported to increase extracellular GABA levels in the brain of many mammalian species (Timmerman & Westerink, 1997), thus enhancing and elongating the action of endogenous GABA. In the present study, when GABA levels in the synaptic cleft were increased by i.c.v. injection of nipepicotic

acid, an ECoG profile overlapping that of GABA_B activation (i.e. an increase of the power at δ and θ regions) was observed. This finding gives support to the hypothesis that GABA effects on both ECoG and RT were mediated by GABA_B receptors.

The first indication that the GABA effect on RT might be receptor specific came from the experiments of Serrano *et al.* (1985), showing that GABA-induced hypothermia is not blocked by bicuculline, thus suggesting that GABA effect is not primarily mediated by the activation of GABA_A receptors. This was also confirmed by the results of the present study demonstrating a direct involvement of GABA_B receptors in GABA-induced hypothermia. Yakimova *et al.* (1996), in an attempt to correlate the effects of GABAergic substances to changes in temperature regulation, have hypothesized that changes in temperature sensitivity of hypothalamic neurones rather than an effect on their tonic activity generate hypo- or hyperthermia. Jha *et al.* (2001), by studying the effects of GABA on individual thermosensitive neurones in the anaesthetized rat, have suggested that GABA itself possesses a direct inhibitory action on cold-sensitive neurones, while it has an indirect noradrenaline (NA)-mediated excitatory action on the warm-sensitive neurones. The activation of the former type of neurones initiates phenomena that reduce body temperature, while that of the latter triggers processes that increase body temperature. Presently, the role and the interaction of GABA_A and GABA_B pre- and postsynaptic receptors in this neuronal network are still under investigation. Sancibrian *et al.* (1991), by intraperitoneally injecting GABA, muscimol and *R*(-)-baclofen, concluded that GABA_A and GABA_B receptors have opposite effects on body temperature. Furthermore, since bicuculline was shown to potentiate both GABA- and baclofen-induced hypothermia, these authors proposed that GABA_A receptors might act by inhibiting the effects induced by the activation of GABA_B receptors. Also, in the present study, bicuculline was able to potentiate muscimol-induced hypothermia. However, the above-mentioned hypothesis seems unlikely, since THIP induced hypothermia through GABA_A-receptors activation, thus suggesting that this subtype of GABA receptors can directly participate in body temperature regulation by triggering the pathways that lead to a decrease of RT. The potentiating effect of bicuculline can be explained by considering the existences of two independent neurochemical systems for the genesis of hyperthermia colocalized within the POAH, one prostaglandin E₂- and the other GABA-mediated. The latter pathway projects to areas outside the POAH involved in the autonomic changes that induce hyperthermia (Osborne *et al.*, 1994). It is reasonable that, in the present study, bicuculline, when coadministered with muscimol, inhibited this system, thus potentiating muscimol-induced hypothermia. Furthermore, Sancibrian *et al.* (1991) hypothesized that hypothermia promoted by the A subtype of GABA receptors is mediated by other endobiotics such as prostaglandin(s), since indomethacin was able to antagonize muscimol-, but not GABA-induced hypothermia. This observation, however, can be explained when considering that in glioma C6 cells muscimol activates phospholipase A₂, which, in turn, increases the release of arachidonic acid and prostaglandin D₂ (Majewska & Chuang, 1985); prostaglandin D₂ can induce a dose-related hypothermia when injected into POAH of the rat (Ueno *et al.*, 1982). Thus, it is conceivable that muscimol-induced hypothermia involves complex interactions between GABA-ergic neurones

and glial cells; this phenomenon can also be at the basis of the biphasic hypothermic effect of muscimol itself, observed at the doses up to 18 nmol and lower, as shown in the present study. Also Jha *et al.* (2001) have postulated an indirect effect of GABA_A agonists on body temperature. They suggested that GABA_A agonists and GABA itself act on presynaptic GABA_A heteroreceptors, causing a release of NA, which, in turn, excites warm sensitive neurons, thus resulting in an increase of heat dissipation and, subsequently, hypothermia. The results of the present study, while confirming the direct and predominant involvement of GABA_B receptors in temperature regulation of conscious rabbit, do not substantiate the indirect involvement of GABA_A receptors towards hypothermia. THIP, in fact, modified ECoG power spectrum (i.e. increased the power only at δ frequency bands) in a fashion characteristic of direct GABA_A activation (Lancel & Faulhaber, 1996), while inducing hypothermia. Furthermore, the involvement of NA in THIP-induced hypothermia seems unlikely, since the activation of NA receptors causes a decrease in EEG power (Sebban *et al.*, 1999a, b) and not an increase, as observed in the present study.

GABA and GABA_A/GABA_B agonists were shown here to elicit hypothermia accompanied by depression of GMB and inhibition of muscle tone. The myorelaxant effect, ascribed to their central, direct action as already shown (Sgaragli *et al.*, 1978; Turski *et al.*, 1990), was accompanied by synchroniza-

tion of the ECoG signal recorded from SMC. Motor functions depend on the activity of clusters of neurons organized in networks inside SMC, detectable as changes of the ongoing EEG/ECoG signals. It is well established that GABAergic inhibition plays a pivotal role in synchronizing the firing rate of pyramidal neurons in the hippocampus and the cortex, thereby coordinating neuronal interactions in columnary cortical activity (Costa, 1998). Thus, the muscle-relaxant properties of GABA and GABA_A/GABA_B agonists might depend on the inhibition of neuronal activity at SMC. Consequently, the hypothermogenic effect elicited by these compounds might be partially due – besides their effects on hypothalamic nuclei of thermoregulation – to the inhibition of shivering and basal muscular thermogenesis.

In conclusion, GABA_A and GABA_B receptors appear directly involved in the effects on body temperature promoted by GABA-ergic agents; moreover, the participation of ECoG activity of SMC in thermoregulation can be envisaged as an additional intracentral thermoeffector pathway.

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