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Role of inhibitory amino acids in control of hypoglossal motor outflow to genioglossus muscle in naturally sleeping rats

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The hypoglossal motor nucleus innervates the genioglossus (GG) muscle of the tongue, a muscle that helps maintain an open airway for effective breathing. Rapid-eye-movement (REM) sleep, however, recruits powerful neural mechanisms that can abolish GG activity even during strong reflex stimulation such as by hypercapnia, effects that can predispose to sleep-related breathing problems in humans. We have developed an animal model to chronically manipulate neurotransmission at the hypoglossal motor nucleus using *in vivo* microdialysis in freely behaving rats. This study tests the hypothesis that glycine receptor antagonism at the hypoglossal motor nucleus, either alone or in combination with $GABA_A$ receptor antagonism, will prevent suppression of GG activity in natural REM sleep during room air and CO_2-stimulated breathing. Rats were implanted with electroencephalogram and neck muscle electrodes to record sleep–wake states, and GG and diaphragm electrodes for respiratory muscle recording. Microdialysis probes were implanted into the hypoglossal motor nucleus for perfusion of artificial cerebrospinal fluid (ACSF) and strychnine (glycine receptor antagonist, 0.1 mM) either alone or combined with bicuculline ($GABA_A$ antagonist, 0.1 mM) during room air and CO_2-stimulated breathing. Compared to ACSF controls, glycine receptor antagonism at the hypoglossal motor nucleus increased respiratory-related GG activity in room air ($P = 0.010$) but not hypercapnia ($P = 0.221$). This stimulating effect of strychnine in room air did not depend on the prevailing sleep–wake state ($P = 0.625$) indicating removal of a non-specific background inhibitory glycinergic tone. Nevertheless, GG activity remained minimal in those REM sleep periods without phasic twitches in GG muscle, with GG suppression from non-REM (NREM) sleep being > 85 % whether ACSF or strychnine was at the hypoglossal motor nucleus or the inspired gas was room air or 7 % CO_2. While GG activity was minimal in these REM sleep periods, there was a small but measurable increase in GG activity after strychnine ($P < 0.05$). GG activity was also minimal, and effectively abolished, in the REM sleep periods without GG twitches with combined glycine and $GABA_A$ receptor antagonism at the hypoglossal motor nucleus. We conclude that these data in freely behaving rats confirm that inhibitory glycine and $GABA_A$ receptor mechanisms are present at the hypoglossal motor nucleus and are tonically active, but that such inhibitory mechanisms make only a small contribution to the marked suppression of GG activity and reflex responses observed in periods of natural REM sleep.

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Hypoglossal motoneurons innervate the genioglossus (GG) muscle of the tongue. The GG is an important pharyngeal muscle that helps maintain an open upper airway for effective breathing (Remmers *et al.* 1978). GG activity is significantly altered by changes in sleep–wake states. In particular, periods of rapid-eye-movement (REM) sleep are associated with marked suppression of GG activity both in animals (Megirian *et al.* 1985; Horner *et al.* 2002) and humans (Sauerland & Harper, 1976; Wiegand *et al.* 1991). Such major suppression of GG activity occurs in periods of REM sleep even if hypoglossal motoneurons are stimulated by strong reflex drives such as hypercapnia (Megirian *et al.* 1985; Parisi *et al.* 1987; Horner *et al.* 2002) or local application of excitatory neurotransmitters such as serotonin (Jelev *et al.* 2001). In humans, such suppression of GG activity in REM sleep can predispose to hypoventilation and obstructive sleep apnoea (Remmers *et al.* 1978), a common and serious breathing disorder affecting ~4 % of adults (Young *et al.* 1993). However, although REM sleep can recruit powerful neural mechanisms that can markedly suppress motor outflow to GG muscle, the neurotransmitters mediating

this effect have not been determined. Determining these mechanisms is relevant not only to understanding the basic neural control of the pharyngeal musculature as a function of sleep–wake states, but also to the development of new clinical strategies aimed at increasing pharyngeal muscle activity to reduce the severity of obstructive sleep apnoea (Horner, 2001; Veasey, 2001).

Glycine and γ-aminobutyric acid (GABA) are the main inhibitory neurotransmitters in the central nervous system. Glycine mediates a major component of the suppression of lumbar and massetter motoneuron activity in natural REM sleep (Soja *et al.* 1987, 1991; Chase *et al.* 1989) and in the pharmacological model of REM sleep produced in decerebrate or anaesthetized animals by microinjection of carbachol into the pontine reticular formation (Morales *et al.* 1987; Kohlmeier *et al.* 1997). *In vitro* studies have characterized inhibitory glycine and $GABA_A$ inputs to hypoglossal motoneurons (Umemiya & Berger, 1995; Singer *et al.* 1998; O'Brien & Berger, 1999; Donato & Nistri, 2000). Some of these inputs arise from the medullary reticular formation and may comprise a component of the inhibitory neural circuitry modulating motoneurons in REM sleep (Chase *et al.* 1984; Chase & Morales, 1994; Yamuy *et al.* 1999; Berger, 2000). In support of this, iontophoresis of the glycine receptor antagonist strychnine onto hypoglossal motoneurons abolishes inhibitory post-synaptic potentials recorded during the REM sleep-like state elicited by carbachol in decerebrate or anaesthetized cats (Yamuy *et al.* 1999; Fung *et al.* 2000), with preliminary data also confirming the presence of inhibitory potentials on hypoglossal motoneurons in cats during natural REM sleep (Fung *et al.* 2001). Likewise, increased glycine and GABA release occurs at both the hypoglossal motor nucleus and the lumbar ventral horn following cholinergic pontine stimulation in decerebrate cats (Kodama *et al.* 2003). Nevertheless, microinjection of glycine or $GABA_A$ receptor antagonists into the hypoglossal motor nucleus failed to reduce suppression of hypoglossal nerve activity in the carbachol REM sleep model in decerebrate cats (Kubin *et al.* 1993).

In support of a role of inhibitory amino acids in modulating hypoglossal motor output and reflex responses *in vivo*, glycine and $GABA_A$ receptor agonists at the hypoglossal motor nucleus produce marked suppression of GG muscle activity during room air and CO_2-stimulated breathing in anaesthetized rats, with antagonism of these receptors increasing GG activity and reversing this suppression (Morrison *et al.* 2002; Liu *et al.* 2003). We have also confirmed the presence of functional inhibitory $GABA_A$ receptors at the hypoglossal motor nucleus in freely behaving rats, as evidenced by increased GG activity in non-REM (NREM) sleep with $GABA_A$ receptor antagonism (Morrison *et al.* 2003). However, GG activity remained minimal in periods of REM sleep even during hypercapnic respiratory stimulation, showing that mechanisms other than $GABA_A$ pathways were involved in mediating this REM sleep suppression of GG activity (Morrison *et al.* 2003). Accordingly, the present study tests the hypothesis that glycine receptor antagonism at the hypoglossal motor nucleus will prevent suppression of GG activity in REM sleep during room air and CO_2-stimulated breathing. In addition, since glycine and GABA are released together within the hypoglossal motor nucleus, with individual hypoglossal motoneurons containing receptors for both neurotransmitters (O'Brien & Berger, 1999) we also determined if co-application of glycine and $GABA_A$ receptor antagonists to the hypoglossal motor nucleus would produce additional release of GG suppression in REM sleep.

METHODS

All procedures conformed to the recommendations of the Canadian Council on Animal Care and the University of Toronto Animal Care Committee approved the experimental protocols. Rats were individually housed in environmentally enriched cages with free access to food and water and were maintained on a 12 h–12 h light–dark cycle (lights on 07:00 h).

Anaesthesia and surgical procedures

Studies were performed in 25 male Wistar rats (Charles River, mean body weight 284 g, range, 240–333 g). Sterile surgery was performed under anaesthesia induced with intraperitoneal (I.P.) ketamine (85 mg kg^{-1}) and xylazine (15 mg kg^{-1}) as previously described (Morrison *et al.* 2003). Rats were also injected with saline (3 ml, 0.9%, I.P.) for fluid loading, atropine sulphate (1 mg kg^{-1}, I.P.) to reduce airway secretions, and buprenorphine (0.03 mg kg^{-1}, I.P.) to control potential post-operative pain. An anaesthesia mask (Freedman, 1992) was placed over the snout and the rats spontaneously breathed a 50:50 mixture of air and oxygen. Any additional anaesthesia was given by inhalation (isoflurane, typically 0.5–1%). Effective anaesthesia was judged by abolition of the pedal withdrawal and corneal blink reflexes.

With the rats supine, the ventral surface of GG was exposed via a submental incision and dissection of the overlying geniohyoid and mylohyoid muscles. Two insulated, multi-stranded stainless steel wires (AS631; Cooner Wire, Chatsworth, CA, USA) were then implanted on either side of the midline (~4 mm apart) into GG muscle and secured with sutures and tissue glue. Section of the medial branches of the hypoglossal nerves has shown that GG activity is recorded with such electrodes (Morrison *et al.* 2002). To record diaphragm activity, two insulated, multi-stranded stainless steel wires (AS636: Cooner Wire) were sutured onto the costal diaphragm via an abdominal approach. To assist in electrode placements the GG and diaphragm signals were monitored on chart and loudspeaker during surgery. To ensure that the GG electrodes were positioned correctly, a standard test voltage (1.0 V) was applied to these electrodes to confirm tongue protrusion. The GG and diaphragm wires were tunnelled subcutaneously to an incision in the neck and the submental and abdominal incisions were closed with absorbable sutures.

Rats were placed in a stereotaxic apparatus (Kopf Model 962, Tujunga, CA, USA). An alignment tool (Kopf Model 944) was

used to achieve the flat skull position for consistent positioning between rats. Electrodes were implanted to record the neck electromyogram (EMG) and cortical electroencephalogram (EEG) (Morrison *et al.* 2003). A small hole was drilled at the junction of the interparietal and occipital bones to insert the microdialysis guides (CMA/11, Acton, MA, USA). The guides were lowered 14.0 ± 0.14 mm (mean ± S.E.M.) posterior to bregma (range, 12.4–15.2 mm), 0.3 ± 0.02 mm lateral to the midline (range, 0–0.33 mm) and aimed 3 mm above the right hypoglossal motor nucleus using a stereotaxic atlas (Paxinos & Watson, 1998). At the end of surgery, all the electrodes were connected to pins and inserted into a miniature plug (STC-89PI-220ABS, Carleton University, Ottawa, ON, CA, USA). The plug and microdialysis guides are affixed to the skull with dental acrylic and anchor screws.

After surgery, the rats were transferred to a clean cage and kept warm under a heating lamp until full recovery as judged by normal locomotor activity, grooming, drinking and eating. Rats were given soft food for the first day after surgery. All rats recovered fully and were housed individually for an average of 6.9 ± 0.3 days (range, 5–9 days) before the experiments.

Recording procedures

For recordings, a lightweight shielded cable was connected to the plug on the rat's head. The cable was attached to a counterbalanced swivel that permitted free movement. For habituation the rats were placed in the recording chamber (MD-1500, BAS Inc., West Lafayette, IN, USA) the day before the experiments with fresh bedding, food and water. The recording chamber was placed inside an electrically insulated, sound attenuated cubicle (EPC-010, BRS/LVE, Laurel, MD, USA). A video camera allowed continuous visual monitoring without disrupting the rat.

Air or CO_2 mixtures (see below) were delivered to the chamber at ~5 l min^{-1} after humidification over a water reservoir (Horner *et al.* 2002; Morrison *et al.* 2003). CO_2 levels were measured continuously (Beckman LB-2) at a flow rate of 500 ml min^{-1} with sampled air returned to the chamber. A thermohygrometer probe (Model 37950–10, Cole-Parmer Instruments, Vernon Hills, IL, USA) continuously monitored temperature and relative humidity which averaged 24.9 ± 0.05 °C (range, 22.5–27.1 °C) and 33.4 ± 0.2 % (range, 26.5–42.1 %) respectively.

The electrical signals were amplified and filtered (Super-Z head-stage amplifiers and BMA-400 amplifiers/filters, CWE Inc., Ardmore, PA, USA). The EEG was filtered between 1 and 100 Hz, while the GG, neck and diaphragm EMGs were filtered between 100 and 1000 Hz. The electrocardiogram was removed from the diaphragm EMG using an oscilloscope and electronic blanker (Model SB-1, CWE Inc.). The moving-time averages (Model MA-821, CWE Inc., time constant = 200 ms) of the EMGs were also obtained (Model MA 821, CWE Inc.). All signals were recorded on chart paper (Grass model 78D polygraph) and computer (Spike 2 software, 1401 interface, CED Ltd., Cambridge, UK). The moving-time average of the GG, diaphragm and neck EMGs were digitized at 50 Hz and the EEG at 500 Hz. The raw EMGs were sampled by computer at 250 Hz as these signals were only used for display purposes with all the EMG analyses performed on the moving averages.

Microdialysis

On the evening prior to the experiments the rats were gently restrained while the internal dummy cannula was removed from the guide and the microdialysis probe (CMA/11 14/01) was inserted. The probes projected 3 mm from the tip of the guide and were 240 μm in diameter with a 1 mm cuprophane membrane and a 6000 Da cut-off. In each rat, a burst of GG activity was observed on chart and loudspeaker when the probe was initially inserted and penetrated the hypoglossal motor nucleus, and this proved useful as a preliminary confirmation of probe placement (Jelev *et al.* 2001; Morrison *et al.* 2003). This burst of GG activity during probe insertion was transient and lasted 3.35 ± 0.37 min, and did not occur in the neck or diaphragm signals (Jelev *et al.* 2001; Morrison *et al.* 2003). The microdialysis probes were connected to FEP Teflon tubing (inside diameter = 0.12 mm) in turn connected to 1.0 ml plastic syringes via a zero dead-space switch (Uniswitch, BAS, West Lafayette, IN, USA). The probes were continually flushed with artificial cerebrospinal fluid (ACSF) at a flow rate of 2.1 μl min^{-1} using a syringe pump and controller (MD-1001 and MD-1020, BAS Inc.). With the length of tubing used for the experiments, the lag time for fluid to travel from the switch to the tip of the probe was 10 min 34 s. The composition of ACSF (mM) was NaCl (125), KCl (3), KH_2PO_4 (1), $CaCl_2$ (2) $MgSO_4$ (1), $NaHCO_3$ (25) and D-glucose (30). The ACSF was bubbled with CO_2 to a pH of 7.36 ± 0.01. The experiments began the morning after the insertion of the microdialysis probes, and were typically performed between 07:00 and 20:00 h, i.e. during the time of day that rats normally sleep.

Protocol

Study 1. Glycine receptor antagonism. During microdialysis perfusion of ACSF (control) into the hypoglossal motor nucleus recordings were made with the animals breathing room air and 7 % inspired CO_2 (actual delivered CO_2 = 6.8 ± 0.1 %). Hypercapnia provided robust GG stimulation in NREM sleep thereby allowing the changes from NREM to REM sleep to be observed readily (Horner *et al.* 2002; Morrison *et al.* 2003) especially during baseline conditions when GG activity is normally low (e.g. Figs 2 and 3; and Jelev *et al.* 2001; Horner *et al.* 2002; Morrison *et al.* 2003). A CO_2 concentration of 7 % was chosen as this stimulus is sufficient to overcome the vagal inhibition of GG muscle which is particularly strong in the intact rat (Bailey *et al.* 2001; Horner *et al.* 2002). After at least two full sleep cycles (i.e. periods containing all of wakefulness, NREM and REM sleep) had been obtained with ACSF at the hypoglossal motor nucleus during room air, and two further full sleep cycles had been obtained during CO_2-stimulated breathing, the perfusion medium was then switched to strychnine (glycine receptor antagonist, strychnine hydrochloride, FW = 370.9, Sigma). Strychnine was applied at a dose of 0.1 mM, and at a pH of 7.55 ± 0.03, i.e. a pH similar to that of the ACSF controls (see above). With strychnine, recordings were also made with the rats breathing room air and breathing 7 % inspired CO_2 (actual delivered CO_2 = 6.8 ± 0.1 %) again for at least two full sleep cycles for each inspired gas mixture. At least 1 h elapsed after the switch to strychnine before the data were analysed.

Study 2. Combined glycine and $GABA_A$ receptor antagonism. In a separate study recordings were made with the animals breathing room air and 7 % inspired CO_2 (actual delivered CO_2 = 6.9 ± 0.1 %) during microdialysis perfusion of ACSF (pH 7.39 ± 0.03) into the hypoglossal motor nucleus. Again, after at least two full sleep cycles had been recorded for each gas mixture, the perfusion medium was switched to combined strychnine and bicuculline ($GABA_A$receptor antagonist, (−)-bicuculline methiodide, FW = 509.3, Sigma). Bicuculline was applied at a dose of 0.1 mM whereas strychnine was applied at a dose of 0.01 mM (n = 3 rats) or 0.1 mM (n = 4). Initial studies in anaesthetised rats showed that

0.1 mM bicuculline reverses the inhibition of hypoglossal motor output produced by $GABA_A$ receptor stimulation with muscimol (Liu *et al.* 2003). Given that the results were similar irrespective of whether the 0.01 or 0.1 mM strychnine was co-applied with bicuculline, the data were pooled and analysed together. The pH of the combined bicuculline and strychnine solution was 7.67 ± 0.05. In the presence of combined bicuculline and strychnine at the hypoglossal motor nucleus, recordings were also made with the rats breathing room air and breathing 7 % inspired CO_2(actual delivered CO_2 = 6.8 ± 0.1 %), again for at least two full sleep cycles for each gas mixture.

Analyses

EMG signals. The data were analysed in consecutive 5 s time bins. The GG, diaphragm and neck EMG signals were analysed from the respective moving-time average signals (above electrical zero) and were quantified in arbitrary units. Electrical zero was the voltage recorded with the amplifier inputs grounded. The GG and diaphragm signals were analysed breath-by-breath, which corresponded to approximately 7–12 breaths for each 5 s epoch. For each breath, GG activity was quantified as tonic activity (i.e. basal activity in expiration), peak and phasic respiratory activity (i.e. peak inspiratory activity–tonic activity), and average values for these measures of GG activity were calculated. The measure of tonic GG activity will include a component due to noise. Average diaphragm amplitudes (i.e. phasic respiratory diaphragm activity) and respiratory rates were also calculated for the breaths in each 5 s bin. Diaphragm minute activity was also calculated as the product of diaphragm amplitude and respiratory rate. Neck muscle activity was quantified as the mean signal over each 5 s period. These average values of GG, diaphragm and neck EMGs for each 5 s period were then taken to a spreadsheet for later alignment with the data corresponding to the prevailing sleep–wake state, level of respiratory stimulation and drug at the hypoglossal motor nucleus.

EEG. The EEG was sampled at 500 Hz and then analysed on overlapping segments of 1024 samples, windowed using a raised cosine (Hamming) function and subjected to a fast Fourier transform to yield the power spectrum. The window was advanced in steps of 512 samples, and the mean power spectrum of the EEG signal over each 5 s epoch was calculated. The power contained within six frequency bands was recorded as absolute power and also as a percentage of the total power of the signal. The band limits were δ_2 (0.5–2 Hz), δ_1 (2–4 Hz), θ (4–7.5 Hz), α (7.5–13.5 Hz), β_1 (13.5–20 Hz) and β_2 (20–30 Hz).

Measurements across sleep–wake states. The moving-time averages of the GG, neck and diaphragm EMGs were analysed in established periods of wakefulness, NREM and REM sleep identified by EEG and EMG criteria (Morrison *et al.* 2003). Measurements were made during 30 s periods of quiet wakefulness (i.e. in the absence of behaviours such as eating, drinking or grooming) and compared to preceding periods of NREM and REM sleep that occurred closest in time to each other. Accordingly, the periods of NREM that were analysed occurred immediately before the REM periods and were therefore designated $NREM_{Pre\text{-}REM}$. However, neuronal systems that contribute to the generation and maintenance of REM sleep can alter their discharge patterns during NREM periods prior to the onset of EEG defined REM sleep (Trulson & Jacobs, 1979; Aston-Jones & Bloom, 1981; el Mansari *et al.* 1989). This effect may have minimized the chance of detecting a potential contribution of inhibitory glycinergic and/or $GABA_A$ mechanisms to suppression of GG activity in REM sleep if such mechanisms were already recruited prior to REM sleep onset. Accordingly, GG activity was also measured in additional NREM periods that were separated in time 81.8 ± 14.0 s from the $NREM_{Pre\text{-}REM}$ and subsequent REM periods. Furthermore, since previous studies (Richard & Harper, 1991; Jelev *et al.* 2001; Morrison *et al.* 2003) have shown that REM sleep is characterized by periods both with and without phasic bursts of GG activity (i.e. periods of $REM_{PHASIC\ GG}$ and $REM_{TONIC\ GG}$ respectively), these periods were both analysed and the proportion of the whole REM sleep time spent in these periods with and without phasic GG twitches was also calculated. Periods of $REM_{PHASIC\ GG}$ were identified from the moving-time average signal when bursts of GG activity occupied >25 % of the analysed period and achieved a level >25 % of GG activity in REM sleep (Morrison*et al.* 2003); these phasic bursts were quantified as peak GG activity. To prevent potential bias in the selection of periods with and without phasic GG twitches in REM sleep, analyses were also performed on GG activity measured throughout the whole of the REM periods (i.e. encompassing all the 5 s epochs within REM sleep). Finally, to determine the time course of the GG responses to the onset of REM sleep, the average GG activity in each 5 s epoch for 1 min periods immediately prior to and following the onset of EEG defined REM sleep were also calculated.

Averaging data within and between rats. Each rat served as its own control with all interventions performed in one experiment, therefore allowing for consistent effects of experimental condition (e.g. ACSF and strychnine, or inspired CO_2 level) to be observed across sleep–wake states within and between rats. Two to six periods of each sleep–wake state, each of 30 s duration, were analysed for each experimental condition in each rat. Then for each animal a grand mean was calculated for each variable, for each sleep–wake state, at each CO_2 level and for each drug delivered to the hypoglossal motor nucleus.

Responses to serotonin at the hypoglossal motor nucleus

In all rats we applied serotonin (10 mM, creatinine sulfate complex, FW = 387.4, Sigma) to the hypoglossal motor nucleus at the end of the experiments to confirm an intact nucleus that was able to respond to manipulation of neurotransmission. Serotonin at the hypoglossal motor nucleus produces marked GG muscle activation in rats (Jelev *et al.* 2001).

Function of GG electrodes and histology

At the conclusion of the studies, the rats were re-anaesthetized with ketamine and xylazine (85 and 15 mg kg^{-1} respectively as described above), and documentation of tongue protrusion in response to electrical stimulation of the GG wires was performed. In all rats the electrodes were in place from the beginning to the end of the study.

To mark the lesion site left by the microdialysis probe, potassium permanganate (1 %) was microinjected for 10 min at 2.1 μl min^{-1} via a microdialysis probe with the membrane cut at the tip (Sun *et al.* 2000). The rats were then overdosed with urethane (0.5 g) and perfused intracardially with 40 ml of 0.9 % saline followed by 20 ml of 10 % formalin. The brains were then removed and fixed in 10 % formalin. The medullary regions were blocked, transferred to 30 % sucrose and cut in 50 μm coronal sections with a cryostat (Leica, CM 1850, Nussloch, Germany). Each section with the lesion site was mounted and stained with Neutral Red. Microdialysis sites were localised and marked on brain maps (Paxinos & Watson, 1998).

Statistical analysis

Variables were compared across all sleep–wake states with ACSF and strychnine at the hypoglossal motor nucleus during room air and CO_2-stimulated breathing. To also determine whether there was an effect of strychnine on GG activity in REM sleep, three complementary analyses were performed: (a) analysis of selected periods of REM sleep distinguishing between epochs with and without phasic GG twitches, (b) analysis of the whole REM sleep period to avoid potential bias in the selection of periods with and without phasic GG twitches, and (c) analysis of the change in GG activity during transition from NREM to REM sleep. For all comparisons, differences were considered significant if the null hypothesis was rejected at $P < 0.05$ using a two-tailed test. Data were analysed using either analysis of variance with repeated measures (RM-ANOVA) or paired *t* tests. Where *post hoc* comparisons were performed after analysis of variance, the Student-Newman-Keuls test was used to determine significant differences. Analyses were performed using Sigmastat (SPSS Inc., Chicago, IL, USA). All data are expressed as means ± S.E.M.

Additional control studies in anaesthetized rats

Although previous studies showed that perfusate applied to one hypoglossal motor nucleus spreads bilaterally to encompass both hypoglossal motor nuclei (Morrison *et al.* 2003), and that 0.1 mM strychnine effectively reverses the inhibition of hypoglossal motor output produced by glycine (Morrison *et al.* 2002), further studies were performed to assess the efficacy of the interventions in this study. Additional interventions were performed in four rats that were anaesthetized with urethane (I.P., 0.5 g ml^{-1}, 1 g kg^{-1}), maintained with halothane (typically 0.2–2 %) and tracheotomized as previously described (Morrison *et al.* 2002). These additional studies were performed in anaesthetized animals as they necessitated recording simultaneously from both sides of the GG muscle as well as having microdialysis probes in both hypoglossal motor nuclei, instrumentation that was not suitable in the behaving animals.

In the first experiment ($n = 2$), a microdialysis probe was placed into one hypoglossal motor nucleus (left and right in separate experiments) and bipolar electrodes were placed into each side of GG muscle to separately record the left and right GG EMGs. To then determine if manipulation of one hypoglossal motor nucleus had effects on both the left and right GG EMGs (indicating spread of perfusate to both nuclei), we first microdialysed ACSF at 2.1 μl min^{-1} for at least 30 min and then determined the response of both left and right GG EMGs to application of glycine (1 mM) which is known to decrease GG EMG (Morrison *et al.* 2002).

In the second experiment ($n = 2$), two microdialysis probes were fixed side-by-side and lowered into the left and right hypoglossal motor nuclei (separation between microdialysis probe tips = 1.2 mm). GG EMG was also recorded on the left and right sides. ACSF was applied to one hypoglossal motor nucleus while 0.1 mM strychnine was applied to the contralateral nucleus for at least 1 h, i.e. the minimum amount of time strychnine was applied in the sleeping animals before any data were collected (see above). To then determine whether strychnine at one hypoglossal motor nucleus blocked the GG responses to increased glycine on the opposite side, the ACSF microdialysis was switched first to 1 mM and then 10 mM glycine for at least 60 min and 30 min respectively in the continued presence of strychnine on the contralateral side.

RESULTS

Of the 25 rats implanted with electrodes for recording sleep and breathing, full experiments were completed in 17: 10 rats for study 1 and seven rats for study 2. In the remaining eight rats, the experiments were incomplete due to problems such as an inability to sleep in at least one experimental condition or blocked microdialysis tubing.

Histology

Figure 1*A* shows an example of the lesion site made by the microdialysis probe in the hypoglossal motor nucleus. For studies 1 and 2 the locations of all lesion sites from all rats were within the hypoglossal motor nucleus or in the midline immediately adjacent to both nuclei (Fig. 1*B* and *C* respectively).

EMG recordings

Figure 2 shows an example of GG activities recorded across sleep–wake states during room air breathing. Note that despite prominent tonic GG activity recorded during wakefulness, a degree of respiratory-related GG activity is also present, as has been observed in previous studies (Horner *et al.* 2002; Morrison *et al.* 2003). Also note that GG activity is markedly decreased from wakefulness to NREM sleep, and effectively abolished in those periods of REM sleep without phasic GG twitches. Periods of REM sleep are also associated with phasic bursts of GG activity that did not have a consistent relationship to breathing.

Study 1. Glycine receptor antagonism at the hypoglossal motor nucleus

Examples of response. Figure 3 shows an example of the GG responses to strychnine at the hypoglossal motor nucleus in a naturally sleeping rat, both with and without reflex respiratory stimulation with increased CO_2. Note that in NREM sleep, strychnine produced clear increases in respiratory-related GG activity compared to ACSF controls during room air breathing (Fig. 3*C vs. A*) but less so in hypercapnia (Fig. 3*D vs. B*). However, major suppression of GG activity occurred in periods of REM sleep without phasic GG twitches regardless of the presence of strychnine at the hypoglossal motor nucleus or CO_2-stimulated breathing. Although the raw trace from this one animal shows that strychnine increased diaphragm amplitude and respiratory rate, this was not observed consistently and was not apparent in the group analysed data (see later section 'Specificity of responses').

Group data: effects of strychnine. Figure 4 shows the effects on GG activity of strychnine at the hypoglossal motor nucleus in the group of 10 rats. During room air breathing there was a significant stimulating effect of strychnine on respiratory-related GG activity compared to ACSF ($F_{1,9} = 10.57$, $P = 0.010$, two-way RM-ANOVA). This stimulating effect of strychnine did not depend upon the prevailing sleep–wake state ($F_{4,36} = 0.66$, $P = 0.625$),

i.e. occurred across all states of wakefulness and sleep. Nevertheless, there was a significant effect of sleep–wake state *per se* on respiratory-related GG activity ($F_{4,36} = 22.64$, $P < 0.001$) with activity in wakefulness being greater than that in NREM sleep and both $REM_{PHASIC\ GG}$ and $REM_{TONIC\ GG}$ sleep (all $P < 0.001$, *post hoc* tests). In sleep, GG activity was greater in NREM periods compared to $REM_{TONIC\ GG}$ ($P < 0.01$, *post hoc* tests).

In contrast to room air breathing, however, in hypercapnia there was no further increase in respiratory-related GG activity with strychnine at the hypoglossal motor nucleus compared to ACSF ($F_{1,9} = 1.73$, $P = 0.221$, two-way RM-ANOVA), i.e. similar to the example shown in Fig. 3. Nevertheless, there remained a significant effect of sleep–wake state *per se* on respiratory-related GG activity ($F_{4,36} = 27.28$, $P < 0.001$) with activity in wakefulness being greater than in NREM and both $REM_{PHASIC\ GG}$ and $REM_{TONIC\ GG}$ sleep (all $P < 0.001$, *post hoc* tests). As with room air breathing, in hypercapnia the sleeping levels of respiratory-related GG activity were also greater in NREM periods compared with $REM_{TONIC\ GG}$ ($P < 0.001$, *post hoc* tests).

The effects of strychnine on GG activity were restricted to increases in respiratory-related activity as levels of tonic GG activity were not affected during either room air or CO_2-stimulated breathing (both $F_{1,9} < 3.99$, both $P > 0.08$, two-way RM-ANOVA).

Effects of CO_2. There was a significant stimulating effect of CO_2 on respiratory-related GG activity in sleep with both ACSF and strychnine at the hypoglossal motor nucleus (both $F_{1,9} > 8.14$, $P < 0.02$, two-way RM-ANOVA).

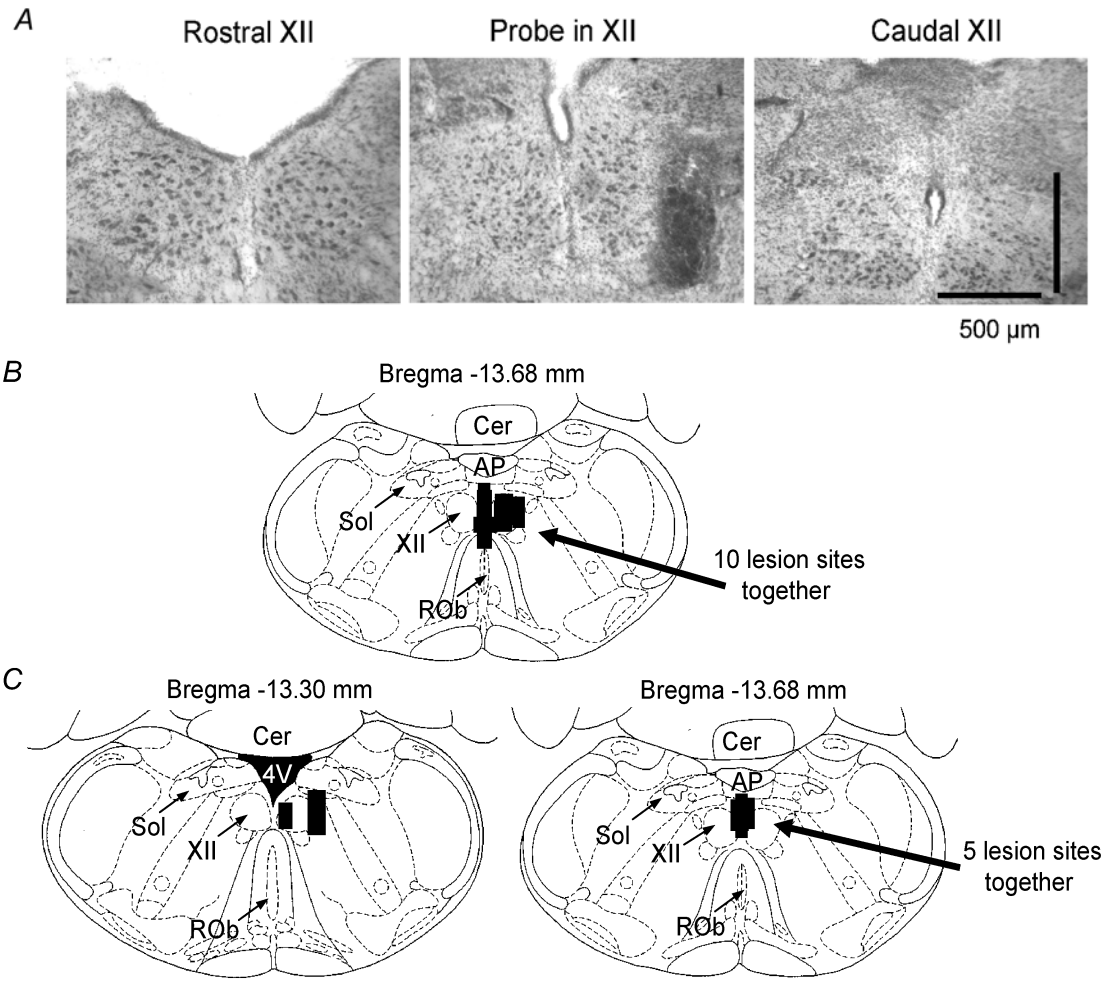

Figure 1. Location of microdialysis probes

A, example of a lesion site made by the microdialysis probe in the hypoglossal motor nucleus. The dark staining is produced by perfusing a microdialysis probe with the membrane cut at the tip with 1 % potassium permanganate to mark the lesion site. Intact cells in the hypoglossal motor nucleus can be seen on this section as well as those rostral (left) and caudal (right). *B* and *C*, the distribution of individual microdialysis sites from all rats for studies 1 and 2 respectively. The size of each bar represents the apparent size of the lesion from the histological sections. Abbreviations: AP, area postrema; Cer, cerebellum; 4V, fourth ventricle; Sol, nucleus tractus solitarius; XII, hypoglossal motor nucleus; ROb, raphe obscurus.

Nevertheless, there was still major suppression of GG activity from NREM to $REM_{TONIC\ GG}$ regardless of the presence of ACSF or strychnine at the hypoglossal motor nucleus during room air or hypercapnia ($P < 0.001$, *post hoc* tests). Indeed, the average percent reduction in respiratory-related GG activity from NREM to $REM_{TONIC\ GG}$ was 85.6–87.2 % with ACSF at the hypoglossal motor nucleus during room air or CO_2-stimulated breathing and was 87.0–91.6 % with strychnine.

Effects of strychnine on GG activity in REM sleep

The data in Figs 3 and 4 and the analyses described above showed that respiratory-related GG activity was minimal in periods of REM sleep without phasic GG twitches, regardless of whether ACSF or strychnine was at the hypoglossal motor nucleus, and irrespective of whether the animal breathed room air or 7 % CO_2. Nevertheless, despite GG activity being minimal in these periods of REM sleep without phasic GG twitches there was a measurable and statistically significant increase in respiratory-related GG activity after strychnine compared with ACSF ($F_{1,9} = 11.80$, $P = 0.007$, two-way RM-ANOVA) although this was of small magnitude (Fig. 4) and not seen in periods of REM sleep with phasic GG twitches ($F_{1,9} = 2.06$, $P = 0.185$).

The previous analyses, however, were performed on selected 30 s epochs of REM sleep with and without phasic GG twitches. To avoid potential bias in the selection of these periods, further analyses were performed on the levels of GG activity recorded over the entire REM episodes for both ACSF and strychnine (Fig. 5*A*). These REM episodes averaged 119.6 ± 13.5 and 136.8 ± 12.4 s with ACSF at the hypoglossal motor nucleus during room air and CO_2-stimulated breathing respectively, and 151.3 ± 13.8 and 117.5 ± 12.2 s with strychnine. The analysis of GG activity during the entire REM episodes confirmed that strychnine increased respiratory-related GG activity compared with ACSF ($F_{1,9} = 14.29$, $P = 0.004$, two-way RM-ANOVA,) and that this effect did not depend on room air or CO_2-stimulated breathing ($F_{1,9} = 0.42$, $P = 0.535$). Although there was a significant stimulating effect of strychnine on respiratory-related GG activity in REM sleep, the GG muscle did not respond significantly to CO_2 stimulation *per se* in REM ($F_{1,9} = 1.73$, $P = 0.221$), a result in agreement with previous studies in rats (Horner *et al.* 2002). Nevertheless, the level of CO_2 was effective as a respiratory stimulus as shown by the significant response of the diaphragm to CO_2 stimulation in REM sleep ($F_{1,9} = 128.93$, $P < 0.001$).

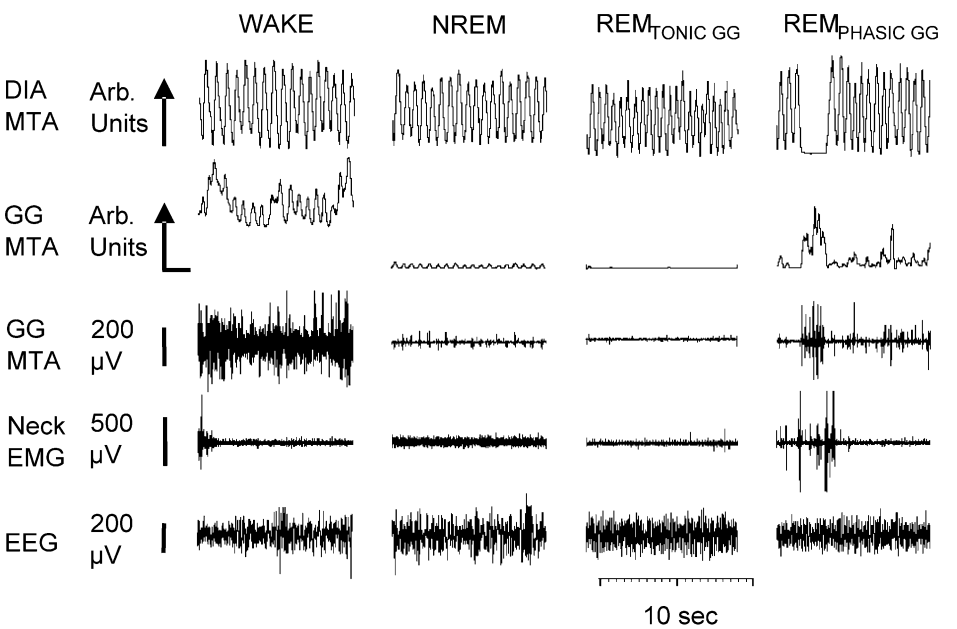

Figure 2. Typical GG activities recorded across sleep–wake states

Shown are the electroencephalogram (EEG), neck electromyogram (EMG), diaphragm (Dia) and genioglossus (GG) signals. The GG and Dia are displayed as their moving-time averages (MTA) in arbitrary (arb) units. The baseline of the integrator (i.e. electrical zero) is shown for the GG MTA. The arrow on the DIA and GG calibration bars denotes the direction of inspiration. Note that despite prominent tonic GG activity during room air breathing in wakefulness, a degree of phasic respiratory-related GG activity is also present. Note also that respiratory-related GG activity is markedly decreased from wakefulness to non-rapid-eye-movement (NREM) sleep, and effectively abolished in those periods of rapid-eye-movement (REM) sleep without phasic GG twitches, i.e. $REM_{TONIC\ GG}$. Periods of REM sleep are also associated with phasic bursts of GG activity (i.e. $REM_{PHASIC\ GG}$) that did not have a consistent relationship to breathing. Traces are taken during room air breathing and microdialysis perfusion of artificial cerebrospinal fluid into the hypoglossal motor nucleus.

Although strychnine did not increase absolute levels of phasic GG twitches in REM sleep, an increased proportion of the REM sleep episodes being spent with GG twitches could have accounted for the overall increase in GG activity when the REM sleep episodes were analysed as a whole (Fig 5*A*). Figure 5*B* shows the percentage of entire REM episodes associated with tonic and phasic GG twitches for both ACSF and strychnine at the hypoglossal motor nucleus. Strychnine increased the proportion of the REM sleep time accompanied by phasic GG twitches and caused corresponding decreases in the proportion spent without such twitches ($F_{1,9} = 22.09$, $P = 0.001$, two-way RM-ANOVA). This effect of strychnine did not depend upon room air or CO_2-stimulated breathing ($F_{1,9} = 0.83$, $P = 0.387$) and was also specific to the GG muscle as there was no effect of strychnine or CO_2 stimulation on the amounts of REM sleep accompanied by bursts of neck muscle activity (both $F_{1,9} < 2.40$, both $P > 0.156$).

GG activity at the transition from NREM to REM sleep

The previous analysis was performed on selected periods of stable NREM and REM sleep data separated in time. To allow characterization of GG suppression at the onset of REM sleep further analyses were performed for 1 min periods immediately preceding and following the onset of REM sleep as defined by EEG criteria. Figure 6*A* shows an

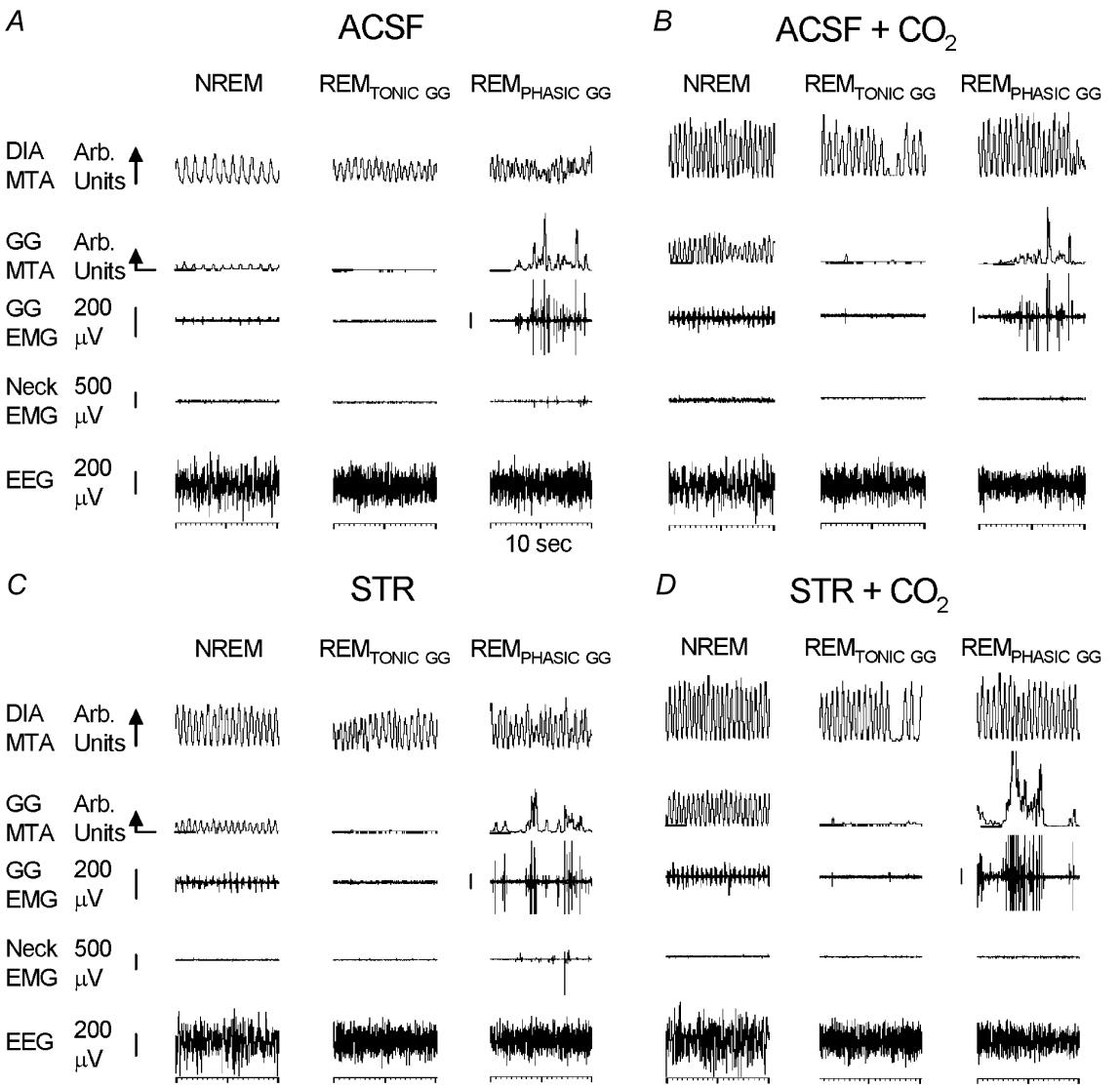

Figure 3. GG responses to strychnine at the hypoglossal motor nucleus in natural sleep

Sample traces from one rat showing typical sleep patterns and respiratory muscle activities with microdialysis perfusion of artificial cerebrospinal fluid (ACSF) and strychnine into the hypoglossal motor nucleus during room air and CO_2-stimulated breathing. In NREM sleep, strychnine produced increases in respiratory-related genioglossus (GG) activity compared to ACSF in room air (*C vs. A*) but less so with CO_2 (*D vs. B*). However, GG activity was minimal in periods of $REM_{TONIC\ GG}$ across all conditions. Abbreviations are as for Fig. 2.

example of the change in GG activity from NREM to REM sleep with strychnine at the hypoglossal motor nucleus. Note the major suppression of GG activity at the onset of REM sleep despite glycine receptor antagonism. Periods of phasic bursts of GG activity occur later in the REM episode.

Overall group data. Figure 6*B* shows group data for the change in GG activity from NREM to REM sleep with both ACSF and strychnine at the hypoglossal motor nucleus, during both room air and CO_2-stimulated breathing. Similar to the previous analyses, strychnine increased respiratory-related GG activity during NREM sleep in room air ($F_{1,9} = 4.82$, $P = 0.056$, two-way RM-ANOVA) but not hypercapnia ($F_{1,9} = 0.035$, $P = 0.856$). There was no statistical effect of time on GG activity in NREM sleep either in room air or hypercapnia, irrespective of whether ACSF or strychnine were present at the hypoglossal motor nucleus (both $F_{5,45} < 1.50$, both $P > 0.20$, two-way RM-ANOVAs). In $NREM_{Pre\text{-}REM}$ sleep, however, respiratory-related GG activity showed gradual suppression prior to the onset of EEG-defined REM sleep ($F_{11,99} > 4.06$, $P < 0.001$). However, for both room air and CO_2-stimulated breathing, this change in GG activity over time did not depend on the presence of ACSF or strychnine at the hypoglossal motor nucleus (both $F_{11,99} > 1.08$, $P > 0.387$), i.e. glycinergic mechanisms were not responsible for the decline in GG activity in NREM sleep immediately before the onset of EEG defined REM sleep.

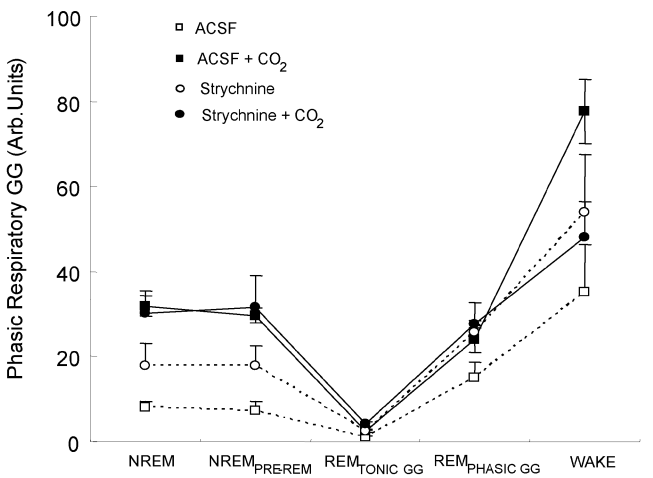

Figure 4. Group data showing GG responses to strychnine at the hypoglossal motor nucleus

Group data showing changes in respiratory-related GG activity across all sleep–wake states during microdialysis perfusion of ACSF and strychnine into the hypoglossal motor nucleus during both room air and CO_2-stimulated breathing. Note that although GG activity in $REM_{TONIC\ GG}$ was increased slightly by strychnine, the levels of GG activity in these periods of REM sleep remained minimal during both room air and CO_2-stimulated breathing. All values are means ± S.E.M.

In REM sleep there was also a significant effect of time on respiratory-related GG activity ($F_{11,99} = 2.57$, $P = 0.007$, two-way RM-ANOVA) consistent with $REM_{TONIC\ GG}$ being prominent at the onset of REM sleep and $REM_{PHASIC\ GG}$ occurring later into the REM episode (e.g. Fig. 6*A*). In hypercapnia this change in GG activity over time was not different between ACSF or strychnine at the hypoglossal motor nucleus ($F_{11,99} = 1.02$, $P = 0.437$). In room air, however, the effect of time on respiratory-related GG activity depended on the presence of ACSF or strychnine at the hypoglossal motor nucleus ($F_{11,99} = 2.17$, $P = 0.022$), with increases in GG activity with strychnine occurring earlier following the transition to REM sleep compared to ACSF (Fig. 6*B*). Further analysis showed that the latency to phasic bursts in GG activity in REM sleep was reduced by 14 ± 5 s with strychnine compared to ACSF ($F_{1,9} = 11.17$,

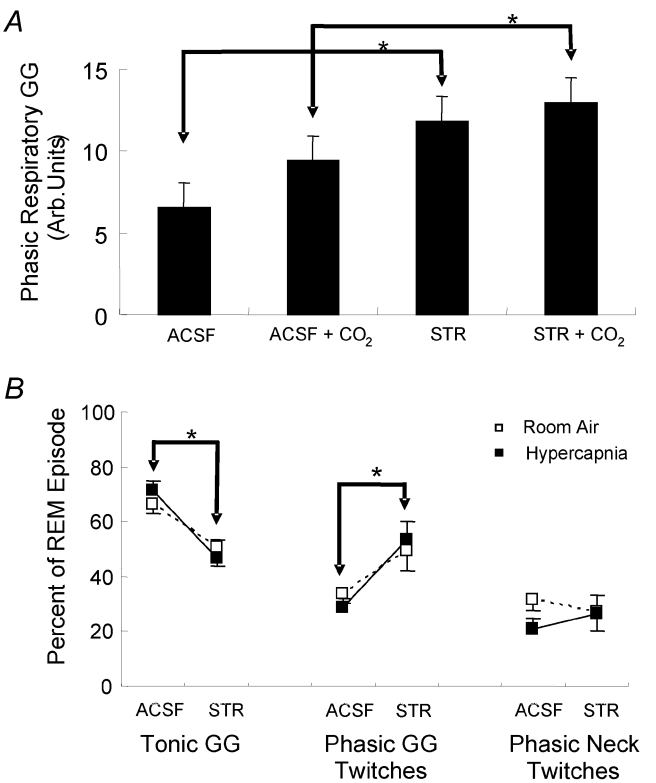

Figure 5. Effects of strychnine on GG activity measured over the entire REM episode and the percentage of REM sleep with periods of tonic activity and phasic GG twitches

A, group data showing GG activity measured throughout the entire REM episodes. GG activity was increased with strychnine (STR) at the hypoglossal motor nucleus in REM sleep, compared with ACSF, during both room air and CO_2-stimulated breathing. *B*, group data showing the changes in the proportion of the REM sleep time spent with and without phasic twitches in GG muscle with strychnine and ACSF at the hypoglossal motor nucleus during room air (open symbols) and CO_2-stimulated breathing (filled symbols). Responses are also shown for neck muscle activity. The percentage of REM sleep accompanied by phasic GG twitches was increased by strychnine compared with ACSF. All values are means ± S.E.M. * $P < 0.05$, other abbreviations are as for Fig. 2.

$P = 0.009$), consistent with an increase in phasic GG twitches in REM sleep after strychnine as observed in Fig. 5*B*.

Specificity of strychnine effects on GG activity

Figure 7 shows the grouped mean data for the effects of strychnine *vs.* ACSF at the hypoglossal motor nucleus on respiratory and sleep-related parameters during both room air and CO_2-stimulated breathing. Importantly, in both room air and hypercapnia there was no statistically significant effect of strychnine on respiratory rate, respiratory-related diaphragm activity, the ratio of high to low frequencies in the EEG (i.e. $\%\beta_2/\%\delta_1$) and neck muscle activity (all $F_{1,9} < 4.56$, $P > 0.06$, two-way RM-ANOVAs). The independent effects of sleep–wake states on respiratory variables (e.g. increased respiratory rates in REM) and sleep data (e.g. changes in EEG frequencies and neck EMG), and their response to CO_2 stimulation, were typical and as previously described (Jelev *et al.* 2001; Horner *et al.* 2002).

Study 2. Combined glycine and $GABA_A$ receptor antagonism

Figure 8 shows mean respiratory-related GG activity recorded across all sleep–wake states during room air and CO_2-stimulated breathing with combined glycine and $GABA_A$ receptor antagonism at the hypoglossal motor nucleus. As with strychnine alone (study 1), strychnine

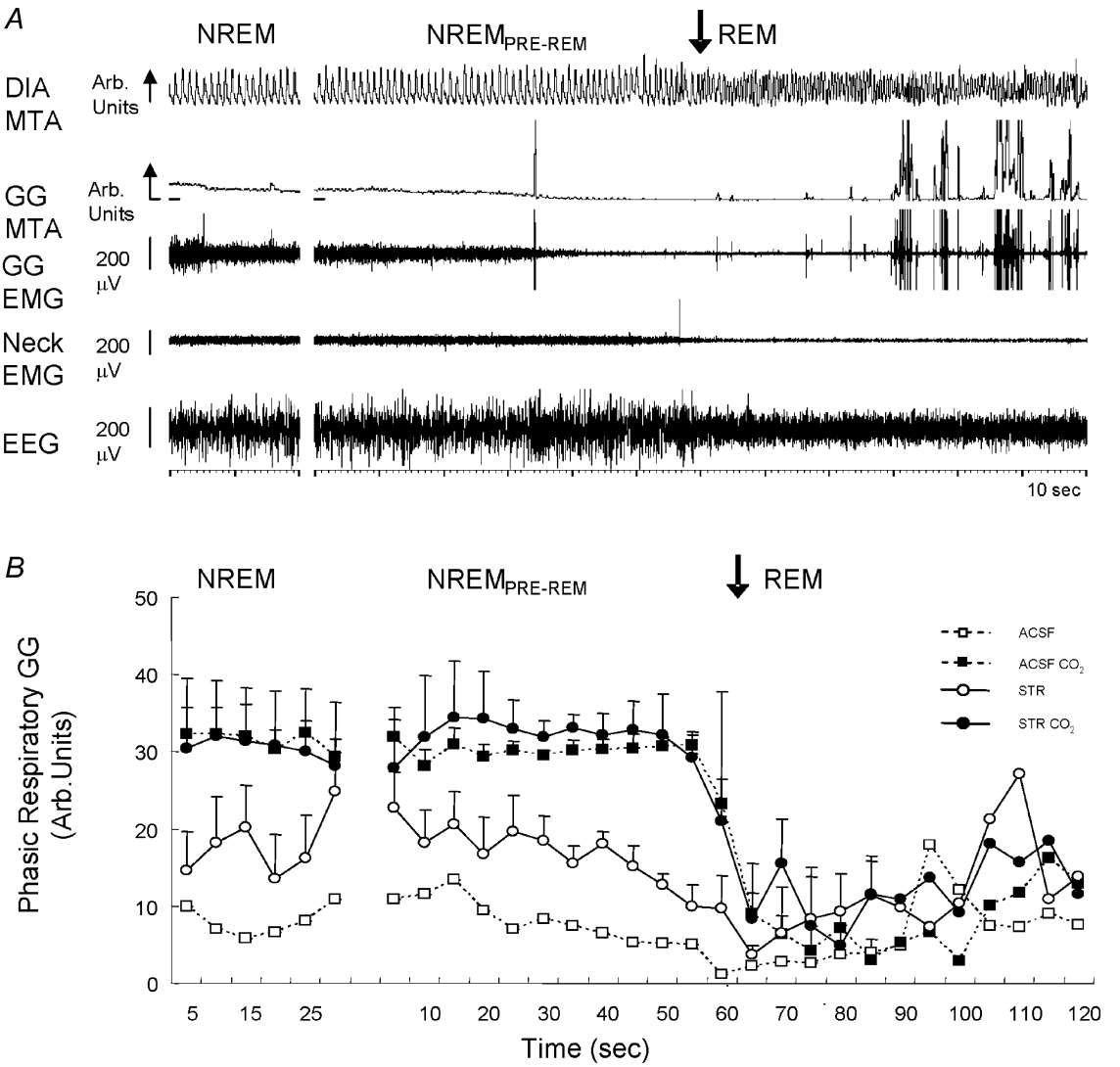

Figure 6. GG activity upon transition from NREM to REM sleep with strychnine at the hypoglossal motor nucleus

A, sample trace from one rat showing the abolition of GG activity at the onset of REM sleep despite strychnine at the hypoglossal motor nucleus. Note the onset of phasic twitches in GG muscle occurred later into the REM sleep episode. Abbreviations are as for Fig. 2. *B*, group data showing changes in respiratory-related GG activity from NREM to REM sleep during microdialysis perfusion of ACSF and strychnine into the hypoglossal motor nucleus during both room air and CO_2-stimulated breathing. GG activity in REM sleep was similar across all conditions. All values are means ± S.E.M. See text for further details.

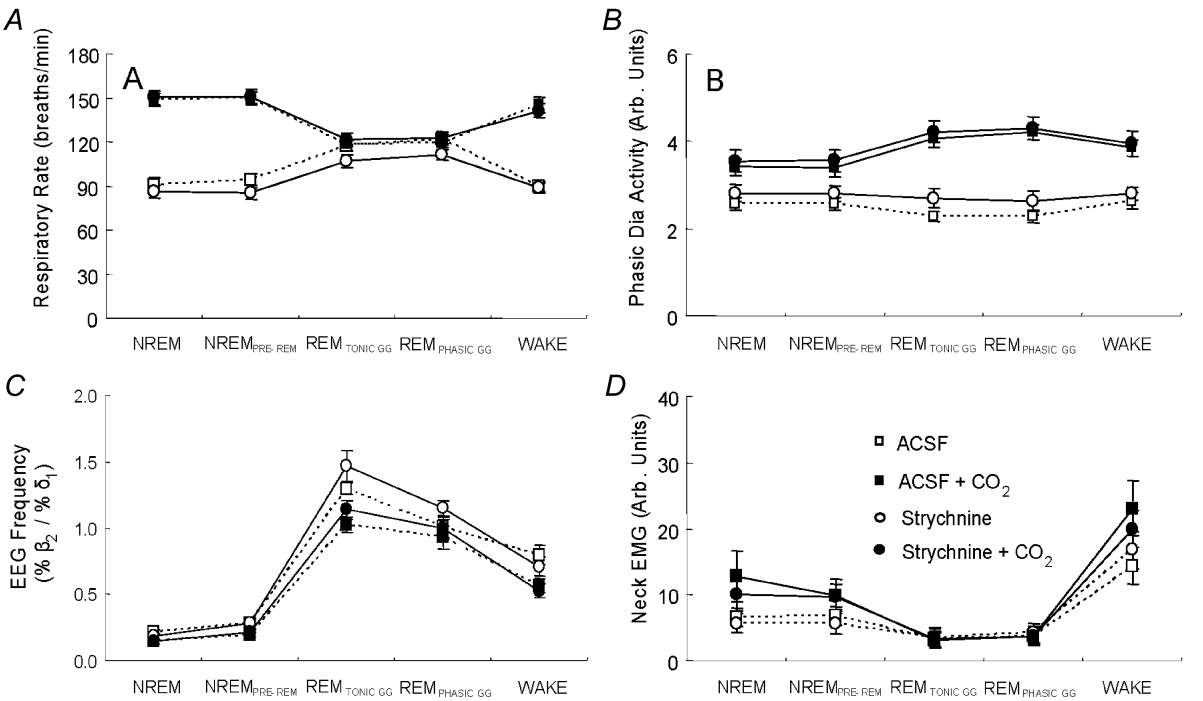

Figure 7. Group data showing specificity of responses to strychnine at the hypoglossal motor nucleus

Group data showing specificity of responses to strychnine at the hypoglossal motor nucleus. Responses of respiratory rate (*A*), phasic diaphragm (DIA) activity (*B*), the ratio of high to low frequencies in the EEG (*C*) and neck EMG (*D*) are shown across all sleep–wake states for microdialysis perfusion of ACSF and strychnine into the hypoglossal motor nucleus during both room air and CO_2-stimulated breathing. There were no effects of strychnine on any parameter either in room air or hypercapnia. All values are means ± S.E.M.

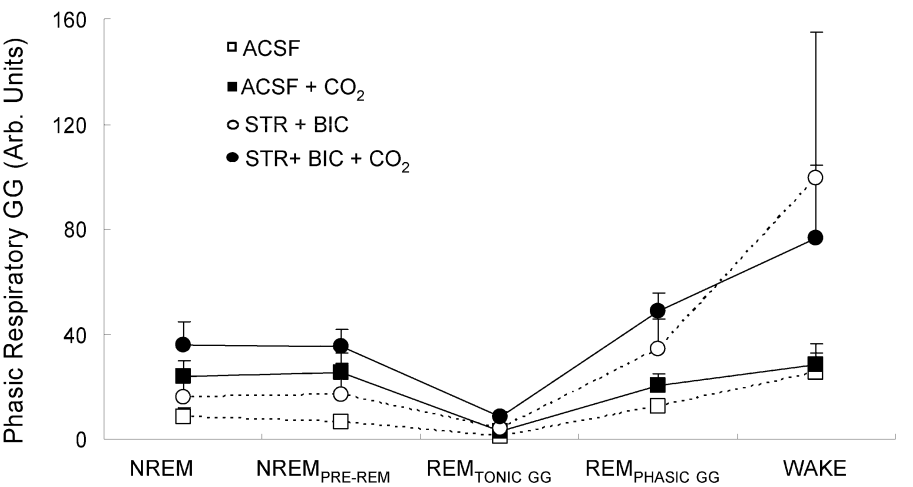

Figure 8. Effects of combined strychnine and bicuculline on GG activity across sleep–wake states

Group data showing changes in respiratory-related GG activity across all sleep–wake states during microdialysis perfusion of ACSF and combined strychnine and bicuculline into the hypoglossal motor nucleus during both room air and CO_2-stimulated breathing. Note that the levels of GG activity were minimal in the periods of REM sleep without phasic GG twitches during both room air and CO_2-stimulated breathing. All values are means ± S.E.M.

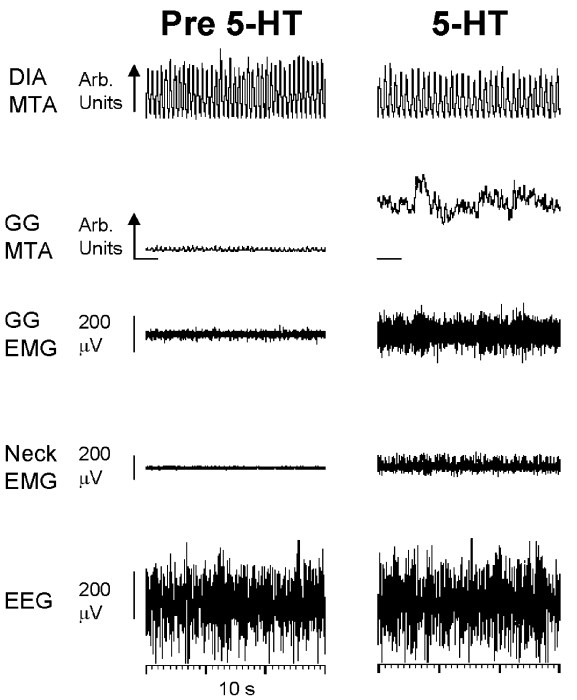

Figure 9. GG activation by serotonin at the hypoglossal motor nucleus

Example showing an increase in GG activity with microdialysis perfusion of serotonin (5-HT) into the hypoglossal motor nucleus. These traces from one rat were both obtained in NREM sleep. Abbreviations are as for Fig. 2.

plus bicuculline increased GG activity in NREM sleep compared with ACSF in room air but not hypercapnia ($P = 0.032$ and 0.244 respectively, *post hoc* tests). Nevertheless, respiratory-related GG activity was minimal in those periods of REM sleep without phasic GG twitches, irrespective of room air or CO_2-stimulated breathing and regardless of whether ACSF or combined strychnine and bicuculline was present at the hypoglossal motor nucleus (Fig. 8).

Stimulation of the hypoglossal motor nucleus with serotonin

At the end of each experiment the microdialysis probes were perfused with 10 mM serotonin to confirm an intact hypoglossal motor nucleus that was able to respond to manipulation of neurotransmission. Figure 9 shows an example of the increase in GG activity with serotonin to the hypoglossal motor nucleus. Similar responses were observed in all rats.

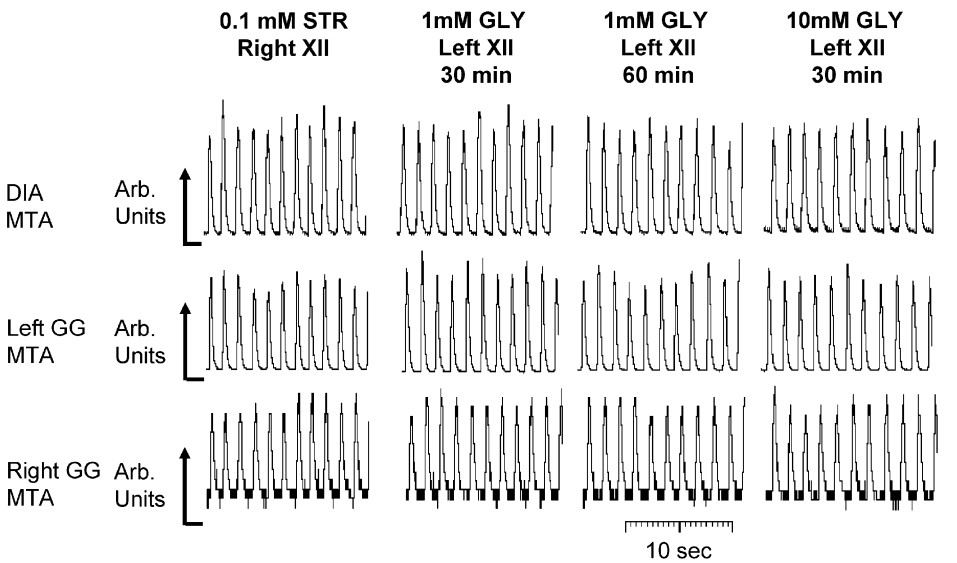

Figure 10. Strychnine at the hypoglossal motor nucleus prevents GG responses to glycine at both the ipsilateral and contralateral sides

Microdialysis perfusion of strychnine (STR) into the right hypoglossal motor nucleus (XII) prevented the suppression of both right and left GG activity in response to glycine (GLY) applied to the left motor nucleus. Abbreviations are as for Fig. 2.

Responses to glycine and strychnine in anaesthetized rats with bilateral GG recordings

Microdialysis perfusion of glycine into one hypoglossal motor nucleus decreased GG activity on both the ipsilateral and contralateral sides with a latency of 2 min 53 s ± 1 min 20 s and 5 min 21 s ± 3 min 8 s respectively. Measurements taken in steady-state 30 min after the switch from ACSF showed that GG activity had decreased on both the ipsilateral and contralateral sides by 25.6 ± 0.6 and 32.5 ± 7.5 % respectively.

Figure 10 shows that microdialysis perfusion of strychnine into the right hypoglossal motor nucleus prevented the suppression of both right and left GG activity in response to glycine applied to the contralateral hypoglossal motor nucleus, confirming that the blocking action of strychnine spread to the contralateral side. The same data were observed in the other rat.

DISCUSSION

Responses to strychnine

This study shows that delivery of the glycine receptor antagonist strychnine to the hypoglossal motor nucleus by *in vivo* microdialysis in freely behaving rats caused robust increases in GG activity in wakefulness and NREM sleep during room air breathing. The increased GG activity after strychnine is consistent with *in vitro* (Umemiya & Berger, 1995; Singer *et al.* 1998; O'Brien & Berger, 1999; Donato & Nistri, 2000) and *in vivo* studies (Takata & Ogata, 1980; Kubin *et al.* 1993; Yamuy *et al.* 1999; Morrison *et al.* 2002) showing inhibitory glycine receptors on hypoglossal motoneurons affecting GG activity. However, despite the clear effects produced in wakefulness and NREM sleep, strychnine was unable to prevent the major suppression of GG activity from NREM to REM sleep (Fig. 4). Indeed, regardless of inspired CO_2 concentration or strychnine at the hypoglossal motor nucleus, GG activity was minimal during those REM sleep periods without phasic twitches in GG muscle. This overall result, the first obtained in natural REM sleep, is compatible with the results obtained using the carbachol model of REM sleep in decerebrate animals (Kubin *et al.* 1993).

Nevertheless, despite the minimal GG activity in such periods of REM sleep, there was a small but measurable increase in GG activity elicited by strychnine in REM sleep (Figs 4 and 5). This effect was due to two main factors. Firstly, after strychnine there was a small but consistent increase in GG activity in those REM sleep periods without GG twitches (Fig. 4), consistent with suppression of glycinergic inhibitory potentials acting on hypoglossal motoneurons in REM sleep (Yamuy *et al.* 1999; Fung *et al.* 2000, 2001). Secondly, a significantly increased proportion of the REM sleep time was spent with GG twitches after strychnine (Fig. 5*B*), a result consistent with the decreased latency to GG twitches in REM sleep after strychnine. Importantly, it is unlikely that these increased amounts of GG twitches after strychnine were due to alterations in central REM sleep processes *per se* because the proportion of REM sleep with phasic neck muscle twitches was not altered by strychnine, showing that the responses were specific to GG muscle. Rather, the increased GG twitches in REM sleep may have been due to suppression of the aforementioned inhibitory glycinergic potentials by strychnine, thereby allowing increased expression of the concomitant flurries of transient excitatory events that are a characteristic feature of motor activity in natural REM sleep for hypoglossal (Richard & Harper, 1991; Morrison *et al.* 2003) as well as other motoneuron pools (Glenn *et al.* 1978; Chase & Morales, 1983; Orem, 1994), i.e. strychnine may have increased expression of GG twitches in REM sleep as these on-going transient excitatory events were now unopposed by the inhibitory potentials in REM sleep.

These observations in natural sleep may help to explain some of the discrepancies in previous experiments using the pharmacological model of REM sleep in reduced preparations. The absence of such phasic excitatory events after carbachol may explain why strychnine did not significantly reverse hypoglossal motor suppression in a previous study (Kubin *et al.* 1993); the carbachol state in that preparation being more like the tonic periods of REM sleep observed in this study, i.e. the periods in which strychnine exerted the smallest effects on GG activity. In contrast, phasic inhibitory glycinergic potentials have been recorded on hypoglossal motoneurons in the carbachol REM sleep model in anaesthetized or decerebrate cats (Yamuy *et al.* 1999), but in this case the transient inhibitory potentials occurred in response to sensory stimulation or spontaneously (Yamuy *et al.* 1999) and as such those effects may be more analogous to the periods of fluctuating neural activities and motor drives that accompany REM sleep in the naturally sleeping preparation.

Interventions at the hypoglossal motor nucleus

The delivery of agents to the hypoglossal motor nuclei was by unilateral microdialysis and this may have influenced our results since the nuclei are distributed bilaterally. However, most probe placements were at, or close to, the midline (Fig. 1) and given that these nuclei are anatomically adjacent, with extensive distribution of dendrites across the midline (Paxinos, 1995), it is highly unlikely that our interventions would have affected only one nucleus. In the conscious animals, however, this was not specifically tested by recording GG activity on separate sides of the tongue because GG activity was recorded across the whole muscle. Nevertheless, in two additional anaesthetized rats we recorded GG activity from both the left and right sides and showed that delivery of glycine into one hypoglossal motor nucleus inhibited GG activity to

similar degrees on both the ipsilateral and contralateral sides. These data confirm previous fluorescein experiments showing that perfusate applied to one hypoglossal motor nucleus spreads bilaterally to affect both nuclei (Morrison *et al.* 2003), and suggests that the strychnine delivered in this study for several hours would have affected both motor nuclei.

There are also several lines of evidence to suggest that the dose of strychnine was able to block significant glycine effects at the hypoglossal motor nuclei. Firstly, strychnine caused significant increases in GG activity in NREM sleep and wakefulness (e.g. causing a doubling of activity in NREM sleep during room air breathing, see Fig. 4). Such increased hypoglossal activity after strychnine was also observed under baseline conditions in a previous study where effective blocking doses were used (Kubin *et al.* 1993). We also showed in additional experiments that 0.1 mM strychnine applied to one hypoglossal motor nucleus blocked the inhibitory effects of both 1 and 10 mM glycine applied to the contralateral motor nucleus, as documented by no change in either the left or right GG EMG to glycine application (Fig. 10). This result suggested that the delivery of strychnine was able to block significant glycine effects at both the ipsilateral and contralateral hypoglossal motor nuclei. Although it is acknowledged that it is difficult to definitively determine if we blocked all the putative endogenous levels of glycine that may potentially accumulate in REM sleep, it is important to note that this blocking effect of continued delivery of strychnine occurred despite 10 mM glycine at the hypoglossal motor nucleus normally being able to cause almost complete abolition of GG activity (Morrison *et al.* 2002). Moreover, strychnine blocked the GG responses to this high dose of glycine despite the glycine being applied for prolonged periods of time (e.g. at least 30 min) unlike the normally short periods of REM sleep that typically last 2–2.5 min (see Results). Also, this blocking effect occurred even at the contralateral hypoglossal motor nucleus (Fig. 10). Overall these data suggest that it is unlikely that any putative release of glycine that may occur during the short bouts of natural REM sleep would be sufficient to offset the effects of prolonged and continued delivery of strychnine in this study. Accordingly, the most parsimonious conclusion from our data is that GG activity was minimal in periods of REM sleep without phasic GG twitches regardless of application of strychnine to the hypoglossal motor nucleus at a dose that was able to effectively increase GG activity in NREM sleep and wakefulness.

In this study it was also necessary to show that the hypoglossal motor nucleus was intact for the duration of the study. Although the significant increases in GG activity after strychnine in wakefulness and NREM sleep were partial evidence for this, at the end of each study the hypoglossal motor nucleus responded to application of 5-HT with a robust increase in GG activity. This response to 5-HT supported of our previous observations (Jelev *et al.* 2001) and indicated an intact hypoglossal motor nucleus for the duration of the study.

Sites of microdialysis were confined to the hypoglossal motor nucleus in all rats. It is a concern, however, that the drugs could diffuse away from the probe site and affect hypoglossal motor activity via non-specific effects on general muscle tone, overall respiratory drive and/or arousal state. For this reason, control measurements included the neck EMG (control postural muscle), diaphragm (control respiratory muscle) and the EEG (marker of arousal). However, none of these measurements were altered by strychnine in room air or hypercapnia, indicating a specific effect of the interventions at the hypoglossal motor nucleus on GG activity.

GG activity across sleep–wake states and effects of strychnine

There remained significant modulation of GG activity across sleep–wake states despite the overall increase in GG activity produced by strychnine at the hypoglossal motor nucleus. The higher GG activity in wakefulness after strychnine is likely to be due to removal of the background inhibitory glycinergic tone coupled with increased excitatory neurotransmitters associated with wakefulness, e.g. serotonin, thyrotropin releasing hormone, substance P and noradrenaline (Kubin *et al.* 1998). Progressive withdrawal of these neurotransmitters from wakefulness to NREM and then REM sleep could then contribute to reduced GG activity (Kubin *et al.* 1994; Woch *et al.* 1996; Lai *et al.* 2001) although the involvement of such mechanisms remains to be formally tested in natural sleep. Withdrawal of serotonin in sleep may also contribute to decreased GG activity via increasing the efficacy of any ongoing inhibitory glycinergic inputs to hypoglossal motoneurons (Umemiya & Berger, 1995). Antagonism of such inhibitory inputs is likely to be responsible for the increased GG activity observed across sleep–wake states, but the persistent major suppression of GG activity that remained in REM sleep after strychnine suggests that other mechanisms with significant influences on hypoglossal motoneurons were still operating. Of relevance, there is evidence for projections from the cholinergic neurons involved in REM sleep generation (Lydic & Baghdoyan, 1994) to the hypoglossal motor nucleus (Chamberlin *et al.* 1999). In neonatal rats acetylcholine elicits nicotinic-mediated excitation of hypoglossal motoneurons (Zaninetti *et al.* 1999; Chamberlin *et al.* 2002), but in older rats acetylcholine exerts an overall suppressant effect on hypoglossal motoneurons via pre-synaptic muscarinic receptors (Bellingham & Berger, 1996). Whether such cholinergic mechanisms at the hypoglossal motor nucleus

are responsible for a component of the suppression of GG activity in periods of natural REM sleep needs to be determined.

It is also possible, however, that mechanisms unrelated to sleep neural processes could also influence GG activity in REM sleep. For example, central respiratory drive and respiratory rate increase in REM sleep (Fig. 7; and Orem *et al.* 2000) and this could potentially reduce GG activity via decreased arterial partial pressure of CO_2 (P_{a,CO_2}). However, periods of minimal GG activity during REM sleep occurred during both room air and CO_2-stimulated breathing (Fig. 4). Moreover, since respiratory rates decline rather than increase during REM sleep in hypercapnia (Fig. 7*A*; and Horner*et al.* 2002), a REM-related decrease in P_{a,CO_2} is unlikely to explain the periods of major suppression of GG activity in the present study.

The observation that strychnine at the hypoglossal motor nucleus produced clear increases in GG activity in NREM sleep but only modest effects in REM sleep also has implications for therapeutic strategies aimed at increasing GG activity in humans via modulation of glycinergic mechanisms. The results of the present study suggest that such strategies may not be as effective in REM sleep over and above any effects observed in NREM. Of relevance, systemic administration of strychnine to a patient with obstructive sleep apnoea led to reduced severity of apnoeas and increased tensor palatini and GG muscle activities, but lesser responses were observed in GG, differentiation between NREM and REM sleep was not reported and further patients were not presented (Remmers *et al.* 1980).

Effects of strychnine on respiratory-related GG activity

Strychnine at the hypoglossal motor nucleus increased respiratory-related GG activity rather than tonic activity, an effect that did not depend on the prevailing sleep–wake states and may be caused by influences of strychnine on the respiratory control of hypoglossal motoneurons. Inspiratory hypoglossal motoneurons are inhibited toward end-inspiration (Withington-Wray *et al.* 1988; Woch & Kubin, 1995; Peever *et al.* 2001). If such inhibition were mediated, at least in part, by glycine (Shao & Feldman, 1997; Singer *et al.* 1998; O'Brien & Berger, 1999; Berger, 2000; Donato & Nistri, 2000) then strychnine could, by alleviating late inspiratory inhibition of hypoglossal motoneurons, increase inspiratory-related GG activity. Similarly, $GABA_A$ receptor antagonism at the hypoglossal motor nucleus increased respiratory-related GG activity in freely behaving rats (Morrison *et al.* 2003) potentially by the same mechanism. Likewise, effective blocking doses of strychnine and bicuculline at the hypoglossal motor nucleus also increased hypoglossal nerve activity in decerebrate cats (Kubin *et al.* 1993) while, conversely, glycine and $GABA_A$ receptor stimulation suppressed respiratory-related GG activity during both room air and CO_2-stimulated breathing in anaesthetized rats (Morrison *et al.* 2002; Liu *et al.* 2003).

Combined effects of glycine and $GABA_A$ receptor antagonism in REM sleep

GABA and glycine immunoreactive fibres are intermingled in the hypoglossal motor nucleus (Li *et al.* 1997). Glycine and GABA are also released together onto hypoglossal motoneurons, with individual motoneurons containing receptors for both transmitters (O'Brien & Berger, 1999). Therefore, additional experiments were performed with co-administration of glycine and $GABA_A$ receptor antagonists to the hypoglossal motor nucleus to determine whether larger changes in GG activity occurred in REM sleep with combined receptor blockade compared with glycine receptor antagonism alone. Such combined effects of glycine and $GABA_A$ receptor antagonism occur in spinal sensory transmission in REM sleep (Taepavarapruk *et al.* 2002). The results showed, however, that with combined glycine and $GABA_A$ receptor antagonism at the hypoglossal motor nucleus GG activity remained minimal, and was effectively abolished, in those REM sleep periods without GG twitches, i.e. similar to the results with glycine receptor antagonism alone (Fig. 4) and $GABA_A$ receptor antagonism alone (Morrison *et al.* 2003).

Overall these results suggest that the major suppression of GG activity that remains in REM sleep after administration of strychnine and bicuculline indicates that other mechanisms with major influences on hypoglossal motoneurons are also operating in REM sleep.

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