Identification of the Transmitter and Receptor Mechanisms Responsible for REM Sleep Paralysis

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During REM sleep the CNS is intensely active, but the skeletal motor system is paradoxically forced into a state of muscle paralysis. The mechanisms that trigger REM sleep paralysis are a matter of intense debate. Two competing theories argue that it is caused by either active inhibition or reduced excitation of somatic motoneuron activity. Here, we identify the transmitter and receptor mechanisms that function to silence skeletal muscles during REM sleep. We used behavioral, electrophysiological, receptor pharmacology and neuroanatomical approaches to determine how trigeminal motoneurons and masseter muscles are switched off during REM sleep in rats. We show that a powerful GABA and glycine drive triggers REM paralysis by switching off motoneuron activity. This drive inhibits motoneurons by targeting both metabotropic GABA_B and ionotropic GABA_A/glycine receptors. REM paralysis is only reversed when motoneurons are cut off from GABA_B, GABA_A and glycine receptor-mediated inhibition. Neither metabotropic nor ionotropic receptor mechanisms alone are sufficient for generating REM paralysis. These results demonstrate that multiple receptor mechanisms trigger REM sleep paralysis. Breakdown in normal REM inhibition may underlie common sleep motor pathologies such as REM sleep behavior disorder.

Introduction

Skeletal muscle paralysis (motor atonia) is a defining feature of normal REM sleep (Jouvet, 1967; Brooks and Peever, 2008a). It may function to prevent the dreaming brain from triggering unwanted and potentially dangerous sleep movements (Mahowald and Schenck, 2005; Brooks and Peever, 2011). Breakdown in REM sleep mechanisms is linked to common sleep disorders such as narcolepsy/cataplexy and REM sleep behavior disorder (RBD) (Mahowald and Schenck, 2005; Lu et al., 2006; Siegel, 2006; Burgess et al., 2010). The mechanisms responsible for REM sleep paralysis remain a matter of considerable debate (Brooks and Peever, 2008a; Chase, 2008).

Classic studies showed that skeletal motoneurons are hyperpolarized by glycine-mediated IPSPs during REM sleep (Nakamura et al., 1978; Soja et al., 1991), which led to the prevailing hypothesis that REM paralysis is single-handedly triggered by glycinergic inhibition of motoneurons (Chase et al., 1989; Chase and Morales, 2005). However, recent studies found that REM paralysis remained even after glycine receptors were blocked on motoneurons (Kubin et al., 1993; Morrison et al., 2003; Brooks and Peever, 2008b). Subsequently, it was hypothesized that REM paralysis is caused by loss of serotonergic and noradrenergic ex-

citation of motoneurons (Fenik et al., 2005), but motor paralysis could not be overridden by direct chemical stimulation (e.g., glutamate, noradrenaline, serotonin) of motoneurons (Jelev et al., 2001; Chan et al., 2006; Burgess et al., 2008). These findings indicate that REM paralysis is triggered by a powerful, yet unidentified, inhibitory mechanism.

Several lines of evidence indicate that both metabotropic GABA_B and ionotropic GABA_A/glycine receptor-mediated inhibition of skeletal motoneurons underlies REM sleep atonia. First, brainstem circuits that control REM sleep contain GABA and glycine neurons that project to and synapse on motoneurons (Holstege, 1996; Rampon et al., 1996; Morales et al., 2006). Second, somatic motoneurons themselves express GABA_B, GABA_A and glycine receptors, which when activated trigger cellular hyperpolarization (Lalley, 1986; Araki et al., 1988; Persohn et al., 1992; Okabe et al., 1994; Margeta-Mitrovic et al., 1999; O'Brien et al., 1999; Berger, 1999; Charles et al., 2003; O'Brien et al., 2004). Third, motoneurons are hyperpolarized by large-amplitude IPSPs during REM sleep (Nakamura et al., 1978). However, it is unknown whether REM sleep atonia is triggered by activation of both metabotropic GABA_B and ionotropic GABA_A/glycine receptors.

Here, we aimed to identify the transmitter and receptor mechanisms responsible for REM sleep paralysis. We studied trigeminal motoneurons and the masseter muscles they innervate because this motor system experiences typical REM paralysis and contributes to sleep pathologies such as RBD (Schenck and Mahowald, 2002; Burgess et al., 2008; Brooks and Peever, 2011). We found that inactivation of both metabotropic GABA_B and ionotropic GABA_A/glycine receptors prevented and indeed reversed REM paralysis. However, neither metabotropic nor ionotropic pathways alone are sufficient for inducing REM inhibition. REM

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paralysis is only reversed when motoneurons are cutoff from both metabotropic and ionotropic receptor-mediated inhibition. These results reshape our understanding of the transmitter and receptor mechanisms underlying REM sleep paralysis.

Materials and Methods

Animals

All procedures and experiments were approved by the University of Toronto's Animal Care Committee and were in accordance with the Canadian Council on Animal Care. Rats were housed individually and maintained on a 12:12 light/dark cycle (lights on at 0700 h) and both food and water were available *ad libitum*. Procedures and experimental protocols are similar in nature to our previously published work (Brooks and Peever, 2008b; Burgess et al., 2008).

Surgical preparation for sleep and microdialysis studies

Studies were performed using male Sprague Dawley rats (average weight: 391 ± 6 g). To implant electroencephalographic (EEG) and electromyographic (EMG) electrodes and a microdialysis probe, sterile surgery was performed under anesthesia induced with intraperitoneal ketamine (85 mg/kg) and xylazine (15 mg/kg). Isoflurane (0.5–2%) was also used to maintain depth of anesthesia, which was determined by absence of the pedal withdrawal and blink reflexes. Body temperature was monitored with a rectal probe (CWE) and maintained at $37 \pm 1^\circ\text{C}$.

Three insulated, multistranded stainless steel wire EMG electrodes (Cooner Wire) were implanted into the left and right masseter muscles. The wires were tunneled subcutaneously to an incision along the dorsal surface of the cranium. Three EMG electrode wires were also inserted into the nuchal muscle. Four stainless steel screws (JI Morris) attached to insulated 34 gauge wire (Cooner Wire) were implanted in the skull for recording cortical EEG (coordinates: 2 mm rostral and 2 mm to the left and right of bregma, and 3 mm caudal and 2 mm to the left and right of bregma).

To implant a microdialysis probe into the left trigeminal motor pool, a ~ 2 mm burr hole was made at 9.4 mm caudal and 1.8 mm lateral to bregma (Paxinos and Watson, 1998). A microdialysis guide cannula (CMA) was then lowered 8.2 mm below the skull surface by stereotaxic manipulation. Dental cement (1234, Lang Dental) secured the cannula in place and after the cement was dry, EEG and EMG electrodes were connected to pins (Allied Electronics) and inserted into a custom-made head-plug (Allied Electronics) that was affixed to the skull with dental cement.

After surgery rats were given a subcutaneous injection of ketoprofen (5 mg/kg) and 5% dextrose in 0.9% saline and kept warm by a heating pad. They were also given a dietary supplement (Nutri-Cal) and soft food for the 2 d following surgery. Rats recovered for at least 7–10 d before experiments began.

Experimental procedures for sleep and microdialysis studies

Recording environment. During experiments, animals were housed in a Rurn system (BAS), which is a movement-responsive caging system eliminating the need for a commutator or liquid swivel. This caging system was housed inside a sound-attenuated, ventilated, and illuminated (lights on: 110 lux) chamber.

Electrophysiological recordings. EEG and EMG activities were recorded by attaching a lightweight cable to the plug on the rat's head, which was connected to a Super-Z head-stage amplifier and BMA-400 AC/DC Bio-amplifier (CWE). EEG signals were amplified 1000 times and bandpass filtered between 1 and 100 Hz. EMG signals were amplified between 500 and 1000 times and bandpass filtered between 30 Hz and 30 kHz. All electrophysiological signals were digitized at 500 Hz (Spike 2 Software, 1401 Interface, CED) and monitored and stored on a computer.

Microdialysis probes. A microdialysis probe was used to perfuse candidate drugs into the trigeminal motor pool. The microdialysis probe (6K Da cutoff; membrane length and diameter: 1 mm by $250 \mu\text{m}$, CMA) was placed into the left trigeminal nucleus. The microdialysis probe was connected to Teflon tubing (inner diameter = 0.1 mm; Eicom), which was connected to a 1 ml gastight syringe via a liquid switch (BAS). The probe was continuously perfused with filtered (0.2 μm PVDF, Fisher Scientific)

artificial CSF (aCSF: 125 mM NaCl, 5 mM KCl, 1.25 mM KH_2PO_4 , 24 mM NaHCO_3 , 2.5 mM CaCl_2 , 1.25 mM MgSO_4 , 20 mM D-glucose) at a flow rate of 2 $\mu\text{l}/\text{min}$ using a syringe pump (BAS).

Drug preparation. All drugs were made immediately before each experiment and dissolved in aCSF. The following drugs were used to manipulate GABA and glycine receptors: CGP52432 (GABA_B antagonist; FW: 420.27; Tocris Bioscience), baclofen (GABA_B agonist; FW: 213.66; Tocris Bioscience), bicuculline (GABA_A antagonist; FW: 435.87; Tocris Bioscience) and strychnine (glycine antagonist; FW: 370.9; Sigma-Aldrich). The AMPA receptor agonist (α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid, FW: 186.17; Tocris Bioscience) was prepared in advance and stored in stock solutions at -20°C . All drugs were vortexed and filtered (0.22 μm PVDF, Fisher Scientific) before use.

Experimental protocols

Each experiment took 2 d to complete. On the first day at 0800–1000 h, animals were placed into a recording chamber and given at least 1 h to habituate before being connected to the recording tether. They were then given a minimum of 3 h to habituate to this before recordings began. Baseline recordings (without the microdialysis probe in place) were established on Day 1 of experiments, between 1300 and 1600 h. The microdialysis probe was inserted at 1700 h and aCSF perfused throughout the night. Probes were inserted the night before experiments began because previous studies demonstrate that probe insertion induces spontaneous neurotransmitter release and local neuronal activation (Di Chiara, 1990; Kodama et al., 1998). On the second day of experimentation, candidate drug treatments (see below, Studies 1 and 2) were perfused in random order between 0800–1800 h. Because baseline (i.e., 1300–1600 h) and drug treatment (i.e., 0800–1800 h) times were overlapping, potential effects of candidate drugs on REM sleep could be compared and determined. Each drug was applied onto the trigeminal nucleus for 2–4 h, which typically allowed sufficient time for rats to transition through three complete sleep cycles (i.e., wake to NREM to REM sleep). An aCSF washout period of at least 2 h followed every drug treatment.

Study 1: Does GABA_B receptor activation at the trigeminal motor pool underlie REM sleep paralysis? We addressed this question in two ways. First, we activated GABA_B receptors by perfusing baclofen (GABA_B receptor agonist) into the left trigeminal motor pool while monitoring left masseter muscle EMG activity. We did this to determine whether receptor activation could trigger motor paralysis. We used 0.5 mM baclofen because previous studies showed that this concentration can activate GABA_B receptors both *in vitro* and *in vivo* (Okabe et al., 1994; Ouyang et al., 2007; Matsuki et al., 2009).

Next, we wanted to determine whether there is an endogenous GABA_B-mediated drive onto trigeminal motoneurons during either sleep or waking and whether removal of this drive in REM sleep could prevent REM paralysis of masseter muscle. We antagonized GABA_B receptors in a dose-dependent manner by applying increasing concentrations of CGP52432 (0.01 mM, 0.05 mM, 0.1 mM, and 0.2 mM) at the trigeminal motor pool.

Study 2: Is REM sleep paralysis triggered by activation of metabotropic GABA_B and ionotropic GABA_A/glycine receptors? We hypothesize that REM paralysis is caused by activation of both metabotropic GABA_B and ionotropic GABA_A/glycine receptors because (1) GABA and glycine are released onto motoneurons during REM sleep (Chase et al., 1989; Morrison et al., 2003; Brooks and Peever, 2008b), and (2) motoneurons express all three receptor types (Araki et al., 1988; Persohn et al., 1992; Margeta-Mitrovic et al., 1999). To test this hypothesis, we simultaneously antagonized GABA_B, GABA_A and glycine receptors by perfusing 0.2 mM CGP52432, 0.1 mM bicuculline and 0.1 mM strychnine onto trigeminal motoneurons during sleep and waking. We used this concentration of CGP52432 because results from Study 1 showed it triggers potent increases in masseter tone when applied to motoneurons. This is supported by *in vitro* and *in vivo* studies showing GABA_B receptors are effectively antagonized 0.2 mM CGP52432 (Westerink et al., 1996; Fedele et al., 1997; Chéry and De Koninck, 2000). We applied 0.1 mM bicuculline/strychnine because we previously showed that such concentrations antagonize GABA_A and glycine receptor-mediated neurotransmission at

Figure 1. Drug interventions preferentially target trigeminal motoneurons. *a*, A histological example showing the tip of a probe tract (red circle) in the trigeminal motor pool (blue circle). Scale bar, 500 μ m. *b*, Locations of the 31 probe tracts in the trigeminal motor pool plotted on standardized brain maps. Red dots represent probe tip locations in the trigeminal motor pool. Although probes were only placed in the left motor pool, we plotted probe locations in both left and right motor pools in this figure for the sake of visual clarity. *c*, EEG and EMG traces (top) and group data (bottom) showing that inserting a probe into the left trigeminal motor pool only increases left masseter muscle activity (left EMG), right masseter (right EMG) activity is unaffected. *d*, EEG and EMG traces (top) and group data (bottom) showing that AMPA perfusion at the left motor pool only increases left masseter muscle activity. $*p < 0.05$. All values are mean $\pm$ SEM.

the trigeminal motor pool (Morrison et al., 2003; Brooks and Peever, 2008b).

Because we had concerns that drug perfusion (via microdialysis) for extended periods of time (i.e., 2–4 h) might spread to REM sleep circuits near the trigeminal motor pool or that inadequate receptor antagonism may not block GABA and glycine inhibition on motoneurons, we directly microinjected high concentrations of receptor antagonists (0.3 mM strychnine/bicuculline and 0.6 mM CGP52432) at the trigeminal motor pool only during REM sleep ($n = 31$ rats).

Microinjections were performed by placing injection probes (i.e., CMA/11 microdialysis probes with dialysis membranes removed) into a guide cannula situated in the left trigeminal motor pool. A 1 μ l Hamilton syringe was then attached to each probe by a 30 cm length of tubing and 0.2 μ l of a candidate drug was applied over a 15 s period. All injections

were confined to individual REM sleep episodes with each injection beginning at the transitioned from NREM into REM sleep. Drug effects on masseter atonia were collected and analyzed only for the REM period in which receptor antagonists were applied onto trigeminal motoneurons. This approach enabled us to determine how rapid and focal antagonism of GABA_B and GABA_A/glycine receptors at the trigeminal motor pool influences REM masseter atonia during a discrete REM episode.

Verification of microdialysis probe location

Two procedures were used to demonstrate that microdialysis probes were both functional and located in the left trigeminal motor pool. At the end of each experiment, 0.1 mM AMPA was perfused into the trigeminal nucleus. If the probe is functional and at the motor pool then glutamatergic activation of motoneurons should increase left masseter muscle

tone. We also used postmortem histological analysis to demonstrate that microdialysis probe lesion sites were physically located in the left trigeminal nucleus.

Histology

Under deep anesthesia (ketamine: 85 mg/kg and xylazine: 15 mg/kg, i.p.) rats were decapitated, brains removed and placed in chilled 4% paraformaldehyde (in 0.1 M PBS) for 24 h. Brains were cryoprotected in 30% sucrose (in 0.1 M PBS) for 48 h; they were then frozen in dry ice and transversely sectioned in 40 μ m slices using a microtome (Leica). Brain sections were mounted, dried and stained with Neutral Red. Tissue sections were viewed using a light microscope (Olympus) and the location of probe lesion tracts were plotted on standardized brain maps (Paxinos and Watson, 1998).

Data analysis

Behavioral state. We classified 3 behavioral states. Waking (W) was characterized by high-frequency, low-voltage EEG signals coupled with high levels of EMG activity. NREM sleep was characterized by high-amplitude, low-frequency EEG signals and minimal EMG activity. REM sleep was characterized by low-amplitude, high-frequency theta-like EEG activity and REM atonia interspersed by periodic muscle twitches. Sleep states were visually identified and analyzed in 5 s epochs using the Sleepscore v1.01 script (CED).

EMG analysis. Raw EMG signals were full-wave rectified, integrated and quantified in arbitrary units (a.u.). Average EMG activity for left and right masseter and neck muscle activity was quantified in 5 s epochs for each behavioral state. EMG activity was not analyzed during the first 30 min of drug perfusion because of the delivery latency from the syringe pump to the microdialysis probe. At least three episodes of each behavioral state (i.e., W, NREM, and REM) were analyzed for each experimental condition. In each rat, the average EMG activity was calculated for each behavioral state for each drug perfused into the trigeminal motor pool.

EMG analysis in REM sleep. REM sleep consists of both tonic and phasic motor events. The stereotypical periods of motor atonia occur during tonic REM sleep and the periodic muscle twitches that punctuate REM atonia occur during phasic REM sleep (i.e., during rapid eye-movements). Because the goal of this study was to determine the role for GABA_B inhibition in REM motor control, we used a previously established method for identifying and quantifying the phasic (i.e., muscle twitches) and tonic (i.e., REM atonia) periods of REM sleep. In each rat, REM atonia and muscle twitches were quantified for each REM episode during the baseline condition and for each drug perfused into the trigeminal motor pool (Brooks and Peever, 2008b; Burgess et al., 2008).

EEG spectral analysis. EEG spectral analysis was calculated using fast Fourier transformation of each 5 s epoch, yielding a power spectra

Figure 2. Drug manipulations do not affect REM-generating circuits. **a**, REM sleep paralysis is abolished by preventing GABA and glycine receptor-mediated inhibition of trigeminal motoneurons. EEG and EMG traces (i.e., masseter muscle) showing that blockade of GABA_B/GABA_A/glycine receptors on trigeminal motoneurons reversed and prevented masseter REM sleep paralysis. Neither REM sleep amounts (**b**) nor REM sleep EEG spectral power (**c**) were affected when ionotropic GABA_A/glycine and metabotropic GABA_B receptors were antagonized at the trigeminal motor pool. Perfusion of either 0.1 mM bicuculline/strychnine or 0.2 mM CGP52432 and 0.1 mM strychnine/bicuculline at the trigeminal nucleus had no effects on REM sleep amounts or EEG power, indicating that applied drugs did not spread to and influence REM-regulating circuits in the nearby sublaterodorsal nucleus. However, metabotropic GABA_B and ionotropic GABA_A/glycine receptor antagonism on trigeminal motoneurons had profound effects on masseter tone during REM sleep (**a**). All values are mean $\pm$ SEM.

Figure 3. GABA_B receptor activation on trigeminal motoneurons reduces masseter tone, but does not trigger muscle paralysis. **a**, EEG and EMG traces showing that GABA_B receptor agonism by baclofen (0.5 mM) perfusion at the left trigeminal motor pool markedly reduces (compared with baseline) left masseter tone (left EMG) during waking. Right masseter muscle tone is unaffected. **b**, Group data ($n = 5$) showing that compared with baseline baclofen-induced activation of GABA_B receptors on trigeminal motoneurons reduces waking masseter tone. However, this intervention does not reduce waking masseter tone to normal REM sleep levels. All values are mean $\pm$ SEM.

Figure 4. GABA_B receptor antagonism on trigeminal motoneurons does not prevent REM paralysis. *a*, EEG and EMG traces showing that GABA_B receptor blockade by CGP52432 perfusion (0.2 mM) at the trigeminal motor pool causes robust increases in masseter activity during both waking and NREM sleep, but it does not affect levels of masseter tone during REM sleep. *b*, Group data ($n = 7$) showing that CGP52432 perfusion (0.01–0.2 mM) heightens masseter EMG activity during waking and NREM sleep, but it does not prevent REM atonia. * $p < 0.004$. All values are mean $\pm$ SEM.

profile within four frequency bands. The band limits used were delta (δ): 0.48–4 Hz; theta (θ): 4.25–8 Hz; alpha (α): 8.25–15 Hz; beta (β): 15.25–35 Hz. A mean EEG spectrum profile was obtained for each epoch and then, to minimize nonspecific differences in absolute power between individuals, EEG power in each frequency bin was expressed as a percentage of the total EEG power in the epoch. The spectral profiles of each behavioral state were then compared between treatments.

Statistical analyses

All statistical analyses were performed using Sigmapstat (SPSS Inc.) and applied a critical two-tailed α value of $p < 0.05$. All comparisons made between baseline and drug treatments were determined using ANOVA with repeated measures (RM-ANOVA) and *post hoc* comparisons were performed using a Student–Newman–Keuls (SNK) test. Comparisons for the microinjection experiments (i.e., drug treatments versus aCSF) were made using *t* tests. All data are expressed as mean $\pm$ SEM.

Results

Drug manipulations affect trigeminal motoneuron behavior

Our first aim was to show that receptor manipulations target motoneurons in the trigeminal nucleus. First, we showed that all probes were located in the left trigeminal motor pool (Fig. 1*a,b*) and then we showed that drug manipulations only influenced the activity of the muscle (i.e., left masseter) innervated by these cells. Drug manipulations at the left trigeminal motor pool never influenced the activity of right masseter or neck muscles (Fig. 1*c,d*). Probe insertion into the

left motor pool caused an immediate, but transient (<1 min), activation of only left masseter muscle tone (paired *t* test, $t_{(4)} = 3.022$, $p = 0.039$; Fig. 1*c*). Neither right masseter (paired *t* test, $t_{(4)} = 0.152$, $p = 0.887$) nor neck muscle activity (paired *t* test, $t_{(4)} = 1.265$, $p = 0.275$; data not shown) were affected by this intervention, suggesting that these interventions selectively targeted trigeminal motoneurons in the left motor pool.

To confirm that applied drugs affect motoneurons in the targeted motor pool, we perfused AMPA at the end of each experiment. AMPA triggered a robust motoneuron excitation that resulted in rapid and forceful activation of only left masseter EMG tone (paired *t* test, $t_{(6)} = 3.231$, $p = 0.018$), neither right masseter (paired *t* test, $t_{(6)} = 1.082$, $p = 0.321$) nor neck muscle activity (paired *t* test, $t_{(6)} = 0.042$, $p = 0.968$; data not shown) were affected (Fig. 1*d*). This finding shows that applied drugs targeted motoneurons in only the left motor pool, which themselves remained viable throughout experiments. It also confirms that probes remained functional during the course of experimental interventions.

Drug manipulations have negligible effects on REM-generating circuits

Although drug application targets motoneurons, we wanted to verify that cells bordering the trigeminal motor pool remained unaffected. The sublaterodorsal nucleus (SLD) sits beside the trigeminal motor pool (0.1–0.2 mm dorsomedial) and controls REM sleep (Boissard et al., 2002; Lu et

al., 2006). Previous studies show that REM sleep is influenced by GABA_A receptor antagonism at the SLD (Boissard et al., 2002; Pollock and Mistlberger, 2003; Sanford et al., 2003). Importantly, we found that antagonism of GABA_A, GABA_B and glycine receptors at the trigeminal motor pool reversed REM masseter muscle paralysis (Fig. 2*a*), but it had no effect on REM sleep expression (REM sleep amount: baseline vs drug, SNK, $q = 0.215$, $p = 0.879$; Fig. 2*b*). Blockade of only GABA_A and glycine receptors also had no effect on REM sleep expression (REM amount: baseline vs drug, SNK, $q = 0.760$, $p = 0.853$; Fig. 2*b*). EEG spectral power during REM sleep was also unaffected by pharmacological interventions (RM ANOVA, $F_{(3,9)} = 1.336$, $p = 0.232$; Fig. 2*c*). These findings show that changes in REM sleep muscle tone are not caused by indirect modulation of REM-generating SLD circuits, rather they result from direct manipulation of motoneurons themselves. Our results therefore document the transmitter and receptor mechanisms responsible for controlling trigeminal motoneurons and masseter muscles in natural REM sleep.

Neither metabotropic GABA_B nor ionotropic GABA_A/glycine receptor-mediated inhibition themselves can trigger REM paralysis

It is unknown whether metabotropic GABA_B receptors modulate motoneuron physiology during natural motor behaviors. We

Figure 5. Inotropic GABA_A/glycine receptor-mediated inhibition functions to suppress REM muscle twitches. *a*, EMG and EEG traces illustrating how masseter muscle twitches during REM sleep are affected by antagonism of GABA_B (0.2 mM CGP52432), GABA_A/glycine (0.1 mM bicuculline/strychnine) and GABA_B/GABA_A/glycine (0.2 mM CGP52432 and 0.1 mM bicuculline/strychnine) receptors at the trigeminal motor pool. *b–d*, Group data ($n = 14$) demonstrating how metabotropic, ionotropic, and combined metabotropic/ionotropic receptor blockade on trigeminal motoneurons affects the duration (*b*), frequency (*c*) and amplitude (*d*) of REM muscle twitches. * $p < 0.05$. All values are mean $\pm$ SEM.

found that activating GABA_B receptors (by baclofen) on trigeminal motoneurons reduced waking masseter muscle tone by $78 \pm 5\%$ (baseline vs baclofen; paired t test, $t_{(4)} = 3.960$, $p = 0.017$; Fig. 3*a,b*), indicating that receptor activation influences motoneuron behavior. However, GABA_B receptor agonism did not trigger complete muscle paralysis since waking masseter tone remained twofold above normal REM sleep levels (baclofen during waking: 1.1 ± 0.1 a.u. vs baseline REM: 0.5 ± 0.05 a.u.; t test, $t_{(18)} = 4.431$, $p < 0.001$; Fig. 3*b*). This finding suggests that GABA_B receptor activation alone is incapable of inducing REM sleep muscle paralysis.

It is unknown whether metabotropic GABA_B receptor-mediated inhibition underlies the motoneuron hyperpolarization that causes REM paralysis (Okabe et al., 1994; Brooks and Peever, 2008b). Therefore, we determined whether REM motor paralysis could be prevented by antagonizing GABA_B receptors (using CGP52432) on motoneurons. CGP52432 application heightened masseter tone during both waking (RM ANOVA, $F_{(5,4)} = 3.147$, $p = 0.037$) and NREM sleep (RM ANOVA, $F_{(5,4)} = 3.052$, $p = 0.041$; Fig. 4), suggesting that an endogenous GABA drive functions to inhibit motoneurons during these states by a GABA_B receptor mechanism. But surprisingly, GABA_B receptor antagonism had no effect on masseter muscle tone during REM sleep (RM ANOVA, $F_{(5,4)} = 1.521$, $p = 0.234$). Specifically, it had no effect on either masseter paralysis (SNK, $q = 0.901$, $p = 0.528$) or REM muscle twitch activity (baseline vs CGP52432; duration: SNK, $q = 0.722$, $p = 0.613$; frequency: SNK, $q = 1.551$, $p = 0.280$; amplitude: SNK, $q = 1.852$, $p = 0.198$; Fig. 5), suggesting that GABA_B receptor-mediated inhibition alone plays a trivial role in REM motor control. This finding also suggests that a residual inhibitory drive must continue to hyperpolarize motoneurons and cause REM paralysis.

However, we show that this residual inhibition is not mediated by ionotropic GABA_A and glycine receptors because antag-

onizing them has no effect on REM atonia. We found that ionotropic receptor antagonism increased masseter tone during both waking (SNK, $q = 10.258$, $p < 0.001$) and NREM sleep (SNK, $q = 7.751$, $p < 0.05$), suggesting that motoneurons are inhibited during these states (Fig. 6). Receptor antagonism also triggered marked increases in the size (SNK, $q = 4.576$, $p = 0.007$) and frequency (SNK, $q = 5.483$, $p = 0.001$) of muscle twitches during REM sleep (Fig. 5), demonstrating that an inhibitory drive is present during REM sleep and that it functions to suppress muscle twitches. This finding is consistent with intracellular recordings, which show that motoneurons are maximally inhibited when REM muscle twitches occur (Chase and Morales, 1983; Brooks and Peever, 2008b). However, we show that GABA_A and glycine receptor antagonism on trigeminal motoneurons had no effect on REM masseter paralysis (SNK, $q = 2.147$, $p = 0.294$; Figs. 6, 7). In fact, removal of only GABA_A and glycine receptor-mediated inhibition still allowed the normal drop in basal muscle tone from NREM to REM sleep (Figs. 6*a*, 7*d*). These observations indicate that an additional, but unidentified mechanism, continues to inhibit motoneurons during REM sleep. To identify this mechanism we simultaneously antagonized both metabotropic GABA_B and ionotropic GABA_A/glycine receptors at the trigeminal motor pool during REM sleep.

Blockade of GABA_B, GABA_A, and glycine receptors prevents REM motor paralysis

Metabotropic and ionotropic receptor-mediated mechanisms can act synergistically to affect neuron function (Liu et al., 2000; Lee et al., 2002; Balasubramanian et al., 2004). Therefore, we hypothesized that REM motor inhibition may be driven by mechanisms that require both metabotropic GABA_B and ionotropic GABA_A/glycine receptors. To test this hypothesis we simultaneously antagonized GABA_B, GABA_A and glycine receptors

a GABA_A/Glycine Antagonism **b**

Figure 6. GABA_A and glycine receptor antagonism increases masseter tone during waking and NREM sleep, but it does not prevent REM atonia. **a**, An EMG and EEG trace showing the abrupt loss of masseter tone on entrance into REM despite continued antagonism of GABA_A/glycine receptors. **b**, Group data ($n = 14$) showing that bicuculline and strychnine perfusion (0.1 mM for each) onto trigeminal motoneurons increases masseter EMG activity during both waking and NREM sleep. However, this same intervention has no effect on basal levels of muscle tone during REM sleep. * $p < 0.001$. All values are mean $\pm$ SEM.

to determine whether this intervention could prevent REM paralysis. We found that receptor antagonism on trigeminal motoneurons not only increased masseter tone during waking and NREM sleep (waking: SNK, $q = 8.616$, $p < 0.001$; NREM: SNK, $q = 7.246$, $p < 0.05$; Fig. 8), it also triggered a potent activation of masseter tone during REM sleep (Figs. 7, 8). Specifically, we found that perfusion of CGP52432 (0.2 mM) and bicuculline/strychnine (0.1 mM for each) onto trigeminal motoneurons reversed motor paralysis by triggering a $105 \pm 30\%$ increase in basal levels of masseter tone during REM sleep (SNK, $q = 5.237$, $p = 0.004$; Figs. 7, 8). In fact, when all 3 receptors were antagonized masseter muscle tone increased to levels observed during normal NREM sleep (NREM baseline vs REM drug; paired t test, $t_{(13)} = 1.929$, $p = 0.076$; Fig. 7c). Despite the reversal of REM atonia brief periods of low masseter tone intermittently punctuated REM periods (Fig. 7a). This effect is in sharp contrast to blockade of only GABA_A and glycine receptors, which had no influence on REM atonia (Figs. 6, 7, 8). Together, these findings suggest that REM paralysis is triggered when motoneurons are inhibited by concomitant activation of both metabotropic GABA_B and ionotropic GABA_A/glycine receptors.

However, our results suggest that reduced motoneuron excitation also contributes to REM paralysis. Although trigeminal motoneurons were cutoff from GABA and glycine receptor-mediated inhibition, REM muscle tone still remained below normal waking levels (post-REM waking during baseline vs REM drug; paired t test, $t_{(13)} = 3542$, $p = 0.0036$; Fig. 7c). This observation infers that loss of wake-active excitatory drives—likely stemming from glutamate, noradrenaline, hypocretin/orexin and dopamine sources (Peever et al., 2003; Fenik et al., 2005; Burgess et al., 2008; Schwarz et al., 2008; Schwarz and Peever, 2011)—also functions to reduce motoneuron and muscle activity during REM sleep.

Activation of both metabotropic GABA_B and ionotropic GABA_A/glycine receptors on motoneurons is required for triggering REM sleep paralysis

In the preceding experiments we perfused (via reverse-microdialysis) GABA and glycine receptor antagonists onto trigeminal motoneurons for 2–4 h, which could cause receptor desensitization (Jones and Westbrook, 1995; Pitt et al., 2008; Bright et al., 2011) and thus insufficient receptor antagonism. To address this concern we increased antagonist concentrations (from 0.1 to 0.3 mM) and applied them in a single bolus (via microinjection, 0.2 μ l) only during REM sleep. Despite this additional precaution, we found that REM masseter paralysis remained completely intact when GABA_A and glycine receptors on motoneurons were antagonized (aCSF ($n = 6$) vs drug ($n = 12$); t test, $t_{(16)} = 1.593$, $p = 0.131$; Fig. 9). However, this intervention triggered potent increases in the frequency, duration and amplitude of REM sleep muscle twitches ($p < 0.05$ for each variable; data not shown). Because intracellular studies show that motoneurons are maximally hyperpolarized during REM muscle twitches (Chase and Morales, 1983) and because GABA_A and glycine receptor antagonism markedly increase twitch activity (Brooks and Peever, 2008b), we contend that these receptors were fully antagonized. We conclude that neither GABA_A nor glycine receptor-mediated inhibition is sufficient for generating REM paralysis.

Finally, we confirmed that motoneurons are only released from REM inhibition when GABA_B, GABA_A and glycine receptors are simultaneously antagonized. We did this by microinjecting CGP52432 (0.6 mM) and bicuculline/strychnine (0.3 mM of each) at the trigeminal motor pool only during individual REM sleep episodes. We found that REM paralysis was rapidly overridden when both metabotropic GABA_B and ionotropic GABA_A/glycine receptors were blocked on trigeminal motoneurons (aCSF ($n = 6$) vs drug ($n = 12$); t test, $t_{(17)} = 3.400$, $p = 0.003$; Fig. 9). This result is consistent with microdialysis experiments (Figs. 7, 8) and confirms our findings that REM paralysis is only reversed when motoneurons are cutoff from both metabotropic GABA_B and ionotropic GABA_A/glycine inhibition.

Discussion

Our results identify the transmitter and receptor mechanisms responsible for REM sleep paralysis. We show GABA and glycine inhibition causes motor paralysis by switching-off motoneurons during REM sleep. This drive inhibits motoneurons by activating both metabotropic GABA_B and ionotropic GABA_A/glycine receptors. REM motor inhibition is only prevented when motoneurons are cutoff from all sources of GABA and glycine transmission. No single form of receptor-mediated inhibition is capable of triggering REM paralysis. Current results therefore

advance our understanding of the synaptic mechanisms underlying REM sleep paralysis.

Technical considerations

Somatic motor pools contain both motoneurons and interneurons (Moriyama, 1987; Nozaki et al., 1993). Therefore, a potential technical caveat is that our drug manipulations at the trigeminal motor pool influenced REM atonia by affecting interneuron function. However, current and previous results (Brooks and Peever, 2008b; Burgess et al., 2008; Schwarz et al., 2008) suggest that interneuron activity was negligibly affected by experimental manipulations. For example, we showed that drug interventions at the left motor pool only influenced left masseter muscle tone, they never affected right masseter tone (Figs. 1, 3). If changes in REM sleep atonia were in fact mediated by interneurons then both left and right masseter muscle tone would be affected because interneurons synaptically control motoneurons in both left and right motor pools (Ter Horst et al., 1990; McDavid et al., 2006). Accordingly, we conclude that drug interventions at the trigeminal motor pool predominantly affect motoneurons and therefore suggest that GABA and glycine receptor manipulations impact REM sleep paralysis by directly impacting trigeminal motoneuron function.

Multiple mechanisms mediate REM sleep paralysis

Our findings are important because they refute the long-standing hypothesis that a one-transmitter, one-receptor phenomenon is responsible for REM paralysis (Chase, 2008; Soja, 2008). In fact, we find no evidence to support the theory that glycine inhibition is single-handedly responsible for REM sleep paralysis. Our results clearly show that both GABA and glycine transmission are critical for normal motor control in REM sleep. Specifically, we show that metabotropic GABA_B and ionotropic GABA_A/glycine receptor-mediated inhibition are required for generating REM motor paralysis.

However, our results support the original concept that REM motor paralysis is caused by hyperpolarization of motoneurons (Nakamura et al., 1978). In fact, we show that an extraordinarily powerful GABA and glycine drive onto motoneurons is switched-on specifically during REM sleep. This inhibitory drive is far more pervasive than the relatively weak drive present during NREM sleep, which is easily blocked by inactivating either GABA or glycine receptors (Morrison et al., 2003; Brooks and Peever, 2008b, 2011). By comparison, REM motor inhibition is only rendered ineffective when motoneurons are completely deprived of both metabotropic GABA_B and GABA_A/glycine receptor-mediated inhibition.

Metabotropic and ionotropic receptor activation could cause motoneuron inhibition and REM paralysis in a number of ways. Straightforward summation of long-lasting GABA_B and short-lasting GABA_A/glycine receptor-driven IPSPs could generate motor atonia by hyperpolarizing motoneurons.

Figure 7. Activation of both metabotropic GABA_B and ionotropic GABA_A/glycine receptors is required for REM sleep paralysis. *a*, EMG and EEG traces illustrating that masseter REM atonia remains intact when only GABA_A/glycine receptors are antagonized, but prevented and overridden when both GABA_B and GABA_A/glycine receptors are simultaneously antagonized on trigeminal motoneurons. *b*, Group data showing that bicuculline and strychnine (0.1 mM) applied onto trigeminal motoneurons during REM sleep cannot prevent REM inhibition ($n = 12$). However, blockade of both metabotropic GABA_B and ionotropic GABA_A/glycine receptors by perfusion of CGP52432 (0.2 mM), bicuculline and strychnine (0.1 mM) abolishes masseter REM atonia ($n = 13$). *c*, Antagonism of GABA_B, GABA_A and glycine receptors at the trigeminal motor pool elevates REM masseter tone to baseline NREM sleep levels. However, despite continued receptor antagonism REM masseter tone remains below normal (i.e., baseline) waking levels that occur immediately after REM episodes. This finding indicates that loss of motoneuron excitation helps to reinforce REM muscle paralysis. *d*, REM paralysis is still triggered even after antagonism of both GABA_A/glycine receptors. This graph shows the normal drop in masseter tone when NREM sleep is exited and REM sleep is entered despite continued ionotropic receptor blockade. * $p < 0.004$. All values are mean $\pm$ SEM.

However, motoneuron inhibition could also result from dynamic interactions between metabotropic and ionotropic receptors (Obrietan and van den Pol, 1998; O'Brien et al., 2004). Synergy between metabotropic and ionotropic receptor function is a well documented phenomenon in neuroscience, particularly for glutamatergic receptors. But dynamic interaction between GABA_B, GABA_A and glycine receptors is also evident. For example, coactivation of GABA_B and GABA_A receptors produces more hyperpolarization than predicted by summation alone (Li et al., 2010) and cross talk between GABA_B and ionotropic GABA_A/glycine receptors affects inhibition by second-messenger and receptor phosphorylation mechanisms (Kardos and Kovacs, 1991; Kardos et al., 1994; Barilà et al., 1999). It remains to be determined how GABA and glycine inhibition functions to hyperpolarize motoneurons during REM sleep.

Additional synaptic mechanisms could also influence motoneuron physiology and muscle tone during REM sleep. For example, activation of chloride channels of the cystic fibrosis transmembrane regulator (CFTR) results in motoneuron hyperpolarization *in vitro* (Morales et al., 2011). Modulation of the neuron-specific potassium chloride cotransporter-2 (KCC2), which enables chloride-mediated inhibition, also influences motoneuron inhibition and motor function (Hübner et al., 2001). In addition, activation of muscarinic receptors on motoneurons functions to suppress muscle tone (Liu et al., 2005). Because brainstem cholinergic neurons also regulate REM sleep (Kodama et al., 2003; McCarley, 2004; Jones, 2008) and innervate somatic motor pools then such mechanisms also could mediate REM atonia (Lydic et al., 1989). Whether any or all of these mecha-

a GABA_B/GABA_A/Glycine Antagonism

Figure 8. Antagonism of GABA_B, GABA_A, and glycine increases basal masseter tone during waking, NREM and REM sleep. **a**, An EMG and EEG trace showing that REM masseter atonia is not triggered on entrance into REM despite when both metabotropic and ionotropic receptors are antagonized. This is in marked contrast to the complete loss of masseter tone that occurs on entrance into REM sleep when only GABA_A/glycine receptors are blocked. **b**, Group data ($n = 13$) showing that CGP52432 (0.2 mM) and bicuculline/strychnine perfusion (0.1 mM for each) at the trigeminal motor pool significantly increases basal levels of masseter EMG activity not only during waking and NREM sleep, but also during REM sleep. * $p < 0.001$. All values are mean $\pm$ SEM.

nisms control motoneuron behavior and REM paralysis remains to be determined.

REM paralysis requires both GABA and glycine inhibition

Our results reconcile a longstanding debate in biology. Two competing theories have argued that REM paralysis results from either increased glycinergic inhibition or decreased monoaminergic excitation of motoneurons. Early experiments showed that some motoneurons are hyperpolarized by glycine-sensitive IPSPs during REM sleep (Chase et al., 1989; Soja et al., 1991). However, subsequent pharmacological studies found that REM muscle paralysis was unaffected by direct antagonism of glycine receptors on motoneurons (Kubin et al., 1993; Morrison et al., 2003; Brooks and Peever, 2008b). More recently we found that REM atonia was unperturbed by the loss of function of glycine receptors in transgenic mice. Specifically, we showed that impaired glycine receptor function triggered robust REM sleep behaviors in mutant mice; however, these behaviors did not result from loss of REM sleep paralysis, but instead were caused by excessive muscle twitch activity during REM sleep (Brooks and Peever, 2011). Together, these observations refuted the claim that REM paralysis is caused by a glycine-dependent mechanism, but supported the hypothesis that it is caused by loss of motoneuron excitation. However, artificially restoring excitatory drives

Figure 9. REM atonia is triggered by activation of both metabotropic GABA_B and ionotropic GABA_A/glycine receptors on trigeminal motoneurons. **a**, EMG and EEG traces showing that REM atonia is reversed when GABA_B, GABA_A and glycine receptors are antagonized at the trigeminal motor pool; however, atonia remains intact when only GABA_A and glycine receptors are blocked. **b**, Group data showing that even high concentrations of bicuculline and strychnine (0.3 mM) applied onto trigeminal motoneurons during REM sleep cannot prevent REM inhibition ($n = 12$). However, blockade of both metabotropic GABA_B and ionotropic GABA_A/glycine receptors by microinjection of CGP52432 (0.6 mM), bicuculline and strychnine (0.2 mM) abolishes masseter REM atonia ($n = 13$). * $p < 0.003$. All values are mean $\pm$ SEM.

onto motoneurons by exogenous neurotransmitter application (e.g., glutamate, noradrenaline, serotonin) failed to reverse REM paralysis (Jeleu et al., 2001; Chan et al., 2006; Brooks and Peever, 2008b; Burgess et al., 2008). This finding provided strong support for the concept that a powerful inhibitory mechanism acts to switch-off motoneurons during REM sleep.

Our current results show that motoneuron inhibition is indeed the driving force behind REM paralysis. However, we also show that reduced motoneuron excitation acts to reinforce muscle paralysis during REM sleep. Although preventing GABA_B and GABA_A/glycine receptor function reversed REM paralysis, it did not restore it to waking levels. This finding suggests that motoneuron inhibition forces motoneurons and muscles to remain silent during REM sleep, but that reduced motoneuron excitation also contributes to REM atonia.

Current and previous work clearly indicate that REM paralysis results from a balance between increased motoneuron inhibition and reduced motoneuron excitation. Biochemical studies show that noradrenaline/serotonin release decreases, whereas, GABA/glycine release increases within spinal/cranial motor pools during drug-induced REM sleep (Kubin et al., 1994; Lai et al., 2001; Kodama et al., 2003). Reduced excitatory drive arising from glutamate, noradrenaline, dopamine and hypocretin neurons functions to weaken motoneuron activity and reinforce REM atonia (Lai et al., 2001; Kodama et al., 2003; Peever et al., 2003; Chan et al., 2006; Burgess et al., 2008; Schwarz and Peever, 2010, 2011). It is unknown how the inhibitory and excitatory neuro-circuits establish the balance between motoneuron inhibition and disfacilitation during REM sleep. But, breakdown of either side of this system could tip the normal balance of control and result in REM motor disruption such as in REM sleep behavior disorder.

Neuro-circuits underlying REM paralysis

Potential neuro-circuits responsible for REM paralysis have been identified and mapped-out. Cells in the ventromedial medulla (VMM) and the SLD region function to promote REM paralysis (Holmes and Jones, 1994; Boissard et al., 2002, 2003; Lu et al.,

2006). Immunohistochemical studies show that cells in these regions contain GABA and glycine (Holstege, 1996; Li et al., 1996). Electrophysiological and lesion studies show these cells are REM-active and destroying them disturbs motor function during REM sleep (Schenkel and Siegel, 1989; Siegel et al., 1991; Maloney et al., 1999; Boissard et al., 2002, 2003; Lu et al., 2006; Vetrivelan et al., 2009). In addition, chemical and electrical stimulation of these REM regulating regions also triggers muscle paralysis in anesthetized animals (Lai and Siegel, 1988, 1991). Although the VMM and SLD both promote REM motor inhibition, it is unclear how they communicate with each another to initiate and maintain REM paralysis.

Our data reshape our understanding of REM motor control and we propose a new model that incorporates both past and present data. We suggest that REM-on GABA and glycine neurons in VMM regions trigger REM paralysis by directly inhibiting motoneurons during REM sleep (Lai and Siegel, 1988; Lu et al., 2006; Vetrivelan et al., 2009). REM paralysis can also be initiated by REM-on glutamate neurons in the SLD region (Lu et al., 2006; Clément et al., 2011; Luppi et al., 2011). These cells indirectly trigger motor atonia by activating GABA/glycine interneurons, which in turn inhibit motoneurons. However, REM sleep paralysis is ultimately triggered when GABA and glycine co-release hyperpolarizes motoneurons by simultaneously activating both metabotropic GABA_B and ionotropic GABA_A/glycine receptors.

Understanding the mechanisms mediating REM sleep paralysis is clinically important because they could explain the nature of REM sleep disorders such as RBD, sleep paralysis and cataplexy/narcolepsy. RBD results from loss of typical REM atonia, which allows pathological motor activation and dream enactment, which often lead to serious injuries (Mahowald and Schenck, 2005; Peever, 2011). Conversely, sleep paralysis and cataplexy result when REM atonia intrudes into wakefulness thus preventing normal behavior and movement (Siegel, 2006; Peever, 2011). Determining the mechanistic nature of REM sleep paralysis will improve our understanding and treatment of such disorders.

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