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Intrathecal administration of a gap junction decoupler, an inhibitor of Na^+–K^+–$2Cl^-$ cotransporter 1, or a $GABA_A$ receptor agonist attenuates mechanical pain hypersensitivity induced by REM sleep deprivation in the rat

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ABSTRACT

We studied the hypothesis that some of the spinal mechanisms that are involved in neuropathic hypersensitivity play a role in hypersensitivity induced by REM sleep deprivation (REMSD). Rats with a chronic intrathecal (i.t.) catheter had REMSD of 48 h duration that induced hypersensitivity to mechanical stimulation. After REMSD, the animals were treated i.t. with carbenoxolone (a gap junction decoupler), bumetanide (a blocker of Na^+–K^+–$2Cl^-$ cotransporter 1 or NKCC1), muscimol (a $GABA_A$ receptor agonist), or pretreated intraperitoneally with minocycline (an inhibitor of microglia activation). Previously, all these treatments attenuated neuropathic hypersensitivity. Following REMSD, carbenoxolone, bumetanide and muscimol had a strong antihypersensitivity effect, whereas pretreatment with minocycline failed to prevent development of hypersensitivity. The results suggest that among spinal pain facilitatory mechanisms that are common to REMSD and neuropathy are NKCC1 blocker- and gap junction decoupler-reversible mechanisms. Moreover, there is a net pain inhibitory effect by spinal administration of an exogenous $GABA_A$ receptor agonist following REMSD as shown earlier in neuropathy. In contrast, activation of spinal microglia may not be as important for the development of hypersensitivity induced by REMSD as following nerve injury.

1. Introduction

There is considerable amount of evidence indicating that sleep deprivation can induce pain and hyperalgesia both in clinical and experimental conditions (Lautenbacher et al., 2006). In experimental animals, sleep deprivation is frequently induced by the flower pot technique that leads to deprivation of rapid eye movement (REM) sleep (Morden et al., 1967) and pain hypersensitivity (e.g., Damasceno et al., 2009; Hicks et al., 1978; Onen et al., 2000; Wei et al., 2008). Spinal mechanisms may contribute to pain hypersensitivity induced by REM sleep deprivation (REMSD). Moreover, some of the spinal mechanisms that underlie REMSD-induced pain hypersensitivity may be, at least partly, the same that contribute to pain hypersensitivity in nerve-injured animals. This is indicated by finding that REMSD is followed by facilitation of spinal withdrawal responses elicited by noxious stimulation. Moreover, pain hypersensitivity induced by REMSD has been reduced by intrathecal (i.t.) administration of a glutamatergic receptor antagonist or a nitric oxide synthase inhibitor at a dose that failed to influence pain behavior in healthy controls (Wei et al., 2007). Potential neural substrates for mediating the influence of REMSD to the spinal pain circuitry are the brainstem structures involved in control of both sleep (McCarley, 2007) and pain (Pertovaara and Almeida, 2006) and that have efferent projections to the spinal cord; among such brainstem structures are, for example, the noradrenergic locus coeruleus and the serotoninergic raphe nuclei.

Among spinal mechanisms contributing to injury-induced pain hypersensitivity is neuroinflammation, in which microglia and release of cytokines or other inflammatory mediators play a significant role (Hansson, 2010; McMahon et al., 2006). Pronociceptive molecules released by activated microglia include growth factors, such as brain-derived neurotrophic factor (BDNF). In addition to protection of neurons (Suter et al., 2007), BDNF, through action on the spinal TrkB receptor, is known to promote pain hypersensitivity (Wang et al., 2009). Moreover, activation of glial cells, particularly astrocytes, has been associated with their coupling to adjacent astrocytes or neurons that may promote spread of excitation (Alvarez-Maubecin et al., 2000). It is not yet known whether activation of spinal microglia or coupling of spinal astrocytes contributes to pain hypersensitivity induced by REMSD.

Transmembrane gradient for chloride ions influences the reversal potential for chloride. The reversal potential for chloride determines whether opening of chloride channels, e.g. by GABA acting on the $GABA_A$ receptor, induces hyper- or depolarization of the neuron (De Koninck,

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2007; Price et al., 2009). When intracellular chloride concentration is high as it normally is in primary somatosensory neurons, GABA induces depolarization of their central terminals that is considered to contribute to presynaptic inhibition of the sensory signal (Rudomin and Schmidt, 1999; Willis, 1999). When intracellular chloride concentration is low as it normally is in sensory interneurons, GABA induces hyperpolarization of the sensory interneuron. Inwardly directed Na^+–K^+–Cl^- cotransporter 1 (NKCC1) contributes to high intracellular Cl^- concentration in primary sensory neurons, whereas outwardly directed K^+–Cl^- cotransporter 2 (KCC2) contributes to low intracellular Cl^- concentration in interneurons (Russell, 2000). Earlier studies have shown that increase in BDNF (Rivera et al., 2002) and peripheral nerve injury or inflammation (Coull et al., 2003; Cramer et al., 2008; Miletic and Miletic, 2008; Zhang et al., 2008) are among factors that induce down-regulation of KCC2 in the spinal dorsal horn. After down-regulation of KCC2, GABA acting on the $GABA_A$ receptor may produce excitatory rather than inhibitory action on the pain-relay neuron (Coull et al., 2003). On the other hand, nerve injury or inflammation has increased phosphorylation, membrane mobilization and expression of NKCC1 in the spinal cord (Cramer et al., 2008; Galan and Cervero, 2005). It has been proposed that the net effect following increased activity of NKCC1 in central terminals of primary afferent nerve fibers is their excessive depolarization and generation of action potentials in pain pathways rather than presynaptic inhibition of the sensory signal; this type of mechanism presumably contributes to activation of pain pathways by touch (Cervero and Laird, 1996). In line with this proposal, a blocker of NKCC1, bumetanide, has attenuated inflammatory and neuropathic hypersensitivity (Cramer et al., 2008; Granados-Soto et al., 2005; Pitcher et al., 2007; Valencia-de Ita et al., 2006). It still remains to be studied whether a blocker of NKCC1 has an antihypersensitivity effect also following REMSD.

In the present study, we attempted to determine whether coupling of glial cells, activation of microglia, NKCC1 or a change in the GABAergic regulation of the chloride channel in the spinal cord plays a role in REMSD-induced pain hypersensitivity. For this purpose, pain behavior was assessed in REM sleep-deprived animals and healthy controls that were treated with compounds that influence glial cell coupling, activation of microglia, NKCC1 or the $GABA_A$ receptor.

2. Materials and methods

2.1. Experimental animals

The experiments were performed in adult, male Hannover–Wistar (HW) rats (weight: 150–200 g; CAS, Shanghai, China). All experiments were approved by the institutional ethics committee and all experimental procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

2.2. Techniques for microinjection

For intrathecal (i.t.) drug injections a catheter (PE-10) was administered into the lumbar level of the spinal cord under pentobarbital anesthesia (50 mg/kg i.p.) as described in detail elsewhere Størkson et al., 1996). Following recovery from anesthesia, the correct placing of the catheter was verified by administering lidocaine (4%, 7–10 μl followed by a 15 μl of saline for flushing) with a 50 μl Hamilton syringe. Only those rats that had no motor impairment before lidocaine injection but had a bilateral paralysis of hind limbs following i.t. administration of lidocaine were studied further. For i.t. administration, the drugs were microinjected with a 50 μl Hamilton microsyringe in a volume of 5 μl followed by a saline flush in a volume of 15 μl.

2.3. REM sleep deprivation procedure

The pedestal-over-water or flower pot technique of REM sleep deprivation was modified from the method described earlier (Morden et al., 1967). Briefly, rat was placed on top of platform surrounded by water. The base of the cage was submerged in 4 cm of water. The platform was 7.5 cm in diameter and 7.5 cm high. REM sleep deprivation was performed for 48 h. The rat was allowed to recover from sleep deprivation for at least one week before next testing.

Under control conditions, the animals were living in similar cages (one animal/cage) as during sleep deprivation, except that there was no flower pot or water in the cage.

2.4. Behavioral testing

To assess mechanical hypersensitivity, the frequency of the withdrawal response to the application of monofilaments (von Frey hairs) to the hind paw was examined. Nine hairs with forces of 1–60 g (Stoelting, Wood Dale, IL) were applied five times at a frequency of approximately of 0.5 Hz. Hairs were tested in ascending order of force. A visible lifting of the stimulated hind limb was considered a withdrawal response. The focus was on mechanical sensitivity, since our earlier study indicated that REM sleep deprivation has a more pronounced effect on mechanical than heat sensitivity (Wei et al., 2007). Moreover, central mechanisms that were studied in our experiments play an important role in hypersensitivity to mechanical stimulation (Treede et al., 1992).

2.5. Motor performance test

To exclude the possibility that the drug-induced effects on pain behavior were due to motor rather than sensory action, the potential motor impairment by the studied compounds was assessed in a Rotarod test. In the test, the animals were placed on a revolving drum (a constant speed of 26 rounds/min) of a Rotarod device (Ugo Basile, Varese, Italy). The latency until the animal dropped from the drum was determined with a stop watch. Before any drug testing, the rats were habituated to the Rotarod test during two previous days. The maximum observation period was 1 min after which the animal that was still on the drum was removed. The Rotarod test was repeated three times at 1 min intervals and the longest latency for each rat in each condition was used in further calculations.

2.6. Drugs

Carbenoxolone (a gap junction decoupler), bumetanide (a blocker of Na^+–K^+–$2Cl^-$ cotransporter 1), muscimol (a $GABA_A$ receptor agonist), and minocycline (an inhibitor of microglia activation) were purchased from Sigma-Aldrich (St. Louis, MO). Drugs were dissolved in saline. Physiological saline was used as control. Minocycline was administered intraperitoneally, while other compounds were administered intrathecally.

2.7. Course of the study

In a preliminary test, in which the hypersensitivity effect induced by REMSD *per se* was assessed, pain behavior was assessed 24 h and 48 h following REM sleep deprivation. Drug effects on pain-related behavior were assessed in two experimental conditions: 1) 48 h after REM sleep deprivation (testing started immediately after the end of the sleep deprivation), 2) control conditions without REM sleep deprivation. The monofilament test was performed prior to 5, 15, 30 and 60 min after intrathecal administration of each drug dose or vehicle control, except when testing minocycline (see further discussion). While the experiments were not formally blinded, it should be pointed out that the experimenter assessing pain behavior

was not aware of the nature, known pharmacological effects of the studied compounds, nor on the working hypothesis or expected results. Moreover, it should be emphasized that in the studied rat strain (unlike in most mouse strains), the limb withdrawal response evoked by monofilaments is brisk and easily detectable leaving little, if any possibility for interpretations, while the REMSD- and drug-induced effects in this study were quite large (up to 300–400% change in the response frequency).

Drug doses were selected based on literature and our preliminary experiments. The i.t. doses of carbenoxolone were 1 μg and 10 μg (Qin et al., 2006; Lan et al., 2007; Roh et al., 2010). The i.t. doses of bumetanide were 30 μg and 90 μg (Granados-Soto et al., 2005). The i.t. doses of muscimol were 0.1 μg and 0.3 μg (Hwang and Yaksh, 1997). In the vehicle session, physiological saline was administered intrathecally at a volume of 5 μl. When assessing the pre-emptive antihypersensitivity effect of minocycline, minocycline was administered intraperitoneally (i.p.) at a dose of 40 mg/kg/day (Raghavendra et al., 2003) and the administration was performed twice (minocycline was administered once before and once 24 h after the start of the REMSD of 48 h duration); thus, the last dose of minocycline was given 24 h before assessment of pain behavior both in the REMSD and control groups.

Each animal participated in 3–5 testing sessions on pain behavior. If animals were not deprived of sleep, the minimum interval between two drug testing sessions in one animal was 3 days. The minimum interval between two REM sleep deprivation sessions in one animal was 1 week. The order of testing different drugs, drug doses and experimental conditions was counterbalanced to avoid serial effects. When the same animal was tested more than once, it was verified that the pre-drug response of the animal was not different from the corresponding pre-drug response in the preceding test session. Since the duration of action by minocycline is considerably longer than that by other studied drugs, no further drug testing was performed after assessing the effect by minocycline treatment.

Rotarod test for assessment of motor performance was performed in a separate group of control animals. Each animal participated in all Rotarod testing sessions that were performed at 3 day intervals. To avoid serial effects, the order of testing different compounds was varied between the animals.

2.8. Statistical analysis of data

Results are presented as mean ± S.E.M. Statistical analysis was performed using two way analysis of variance (2-w-ANOVA) followed by a *t*-test with a Bonferroni correction for multiple comparisons or the Student's *t*-test (comparisons between two groups). Results of the Rotarod test were evaluated using Kruskall–Wallis test followed by Dunn's test. $P<0.05$ was considered to represent a significant difference. For comparisons of the efficacy of drug treatments in control versus sleep-deprived animals, the areas under the curves depicting the withdrawal response rates as a function of stimulus force were calculated in different conditions using Prism 4 software (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Induction of mechanical pain hypersensitivity by REMSD

REMSD *per se* induced a highly significant mechanical hypersensitivity that increased with an increase in the duration of REMSD from 24 h to 48 h ($F_{2,189}=382$, $P<0.0001$; Fig. 1).

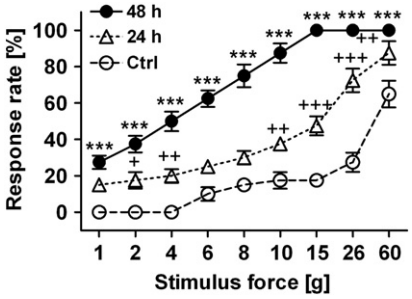

Fig. 1. Mechanical sensitivity before (Ctrl) and 24 h or 48 h after rapid eye movement sleep deprivation (REMSD). The graph shows the limb withdrawal response rate to repeated monofilament stimulation at different forces. Increase in the response rate indicates hypersensitivity. Error bars represent S.E.M. ($n=8$). $+P<0.05$, $++P<0.01$, $+++/***P<0.005$ (*t*-test with a Bonferroni correction for multiple comparisons; reference: the corresponding Ctrl-value).

3.2. Influence by i.t. administration of carbenoxolone on mechanical hypersensitivity induced by REMSD

I.t. administration of carbenoxolone, a gap junction decoupler, produced a dose-related (1 and 10 μg) mechanical antihypersensitivity effect in animals with REMSD of 48 h duration (2-w-ANOVA: $F_{2,135}=90.8$, $P<0.0001$; Fig. 2A). While the influence of carbenoxolone on pain-related behavior was not as prominent in controls as in sleep-deprived animals, i.t. treatment with carbenoxolone (1 and 10 μg) did produce a significant attenuation of mechanically evoked withdrawal responses also in control animals according to 2-w-ANOVA ($F_{2,135}=7.57$, $P=0.0008$; Fig. 2B). Post hoc testing (*t*-test with a Bonferroni correction for multiple comparisons) indicated that carbenoxolone reduced REMSD-induced hypersensitivity already at the low dose of 1 μg (Fig. 2A), whereas post hoc testing failed to find significant reduction of pain behavior of controls even at a dose of 10 μg (Fig. 2B). In controls, 10 μg of carbenoxolone reduced the total response (as revealed by area under the curve) to 54% of the corresponding response in saline-treated animals, whereas following REMSD, carbenoxolone at the dose of 10 μg reduced the mechanically evoked total response to 24% of the corresponding response in saline-treated animals. In both sleep-deprived and control animals, the maximum suppression of pain-related behavior by carbenoxolone was obtained 30 min after its i.t. administration (Fig. 2C).

3.3. Influence by i.t. administration of bumetanide on mechanical hypersensitivity induced by REMSD

I.t. administration of bumetanide, a blocker of Na^+–K^+–$2Cl^-$ cotransporter 1, suppressed in a dose-related fashion (30 and 90 μg) mechanical hypersensitivity in animals with 48 h of REMSD (2-w-ANOVA: $F_{2,135}=97.2$, $P<0.0001$; Fig. 3A). Bumetanide (30 and 90 μg, i.t.) suppressed mechanically induced pain behavior also in control animals (2-w-ANOVA: $F_{2,135}=8.8$, $P=0.0003$; Fig. 3B), although the pain suppressive effect was not as prominent in controls as following REMSD. Post hoc testing indicated that bumetanide attenuated pain behavior in the REMSD group already at the dose of 30 μg (Fig. 3A), but in controls only at the dose of 90 μg (Fig. 3B). In controls, bumetanide at the dose of 90 μg reduced the total response (as revealed by area under the curve) to 64% of the corresponding response in saline-treated controls, whereas following REMSD, bumetanide at the dose of 90 μg reduced the total response to 39% of the corresponding response in saline-treated animals. Both in the REMSD and control groups the maximum suppression of pain-related behavior was obtained 30 min following i.t. administration of bumetanide (Fig. 3C).

3.4. Influence by i.t. administration of muscimol on mechanical hypersensitivity induced by REMSD

I.t. administration of muscimol, a $GABA_A$ receptor agonist, produced a dose-related (0.1 and 0.3 μg) mechanical antihypersensitivity effect in animals with REMSD of 48 h duration (2-w-ANOVA:

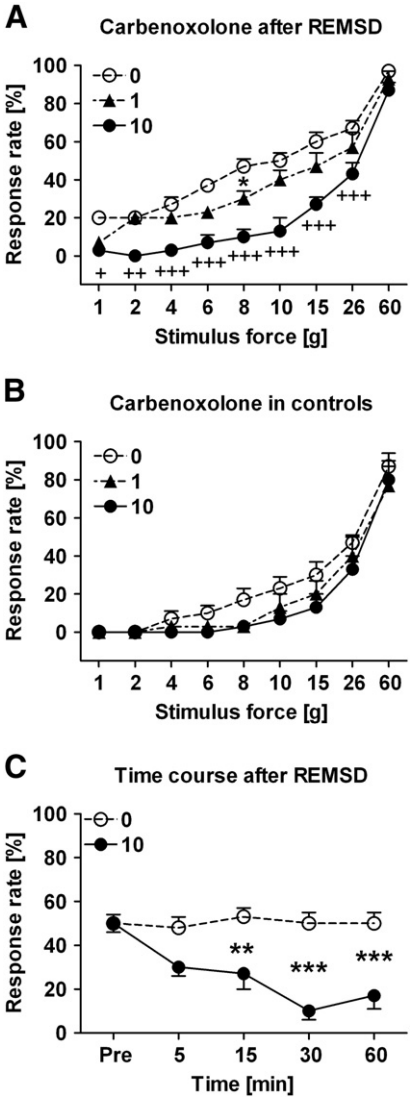

Fig. 2. Influence by i.t. treatment with carbenoxolone (a gap junction decoupler) on mechanical sensitivity 48 h after REMSD (A and C), or in control conditions (B). 0 represents vehicle control, 1 and 10 represent carbenoxolone doses in μg. Graphs A and B show pain behavior 30 min after i.t. treatment. In C, the response to a monofilament force of 8 g is shown. Error bars represent S.E.M. ($n=6$). */+$P<0.05$, **/++$P<0.01$, ***/+++$P<0.005$ (t-test with a Bonferroni correction for multiple comparisons; reference: the corresponding value in the vehicle group).

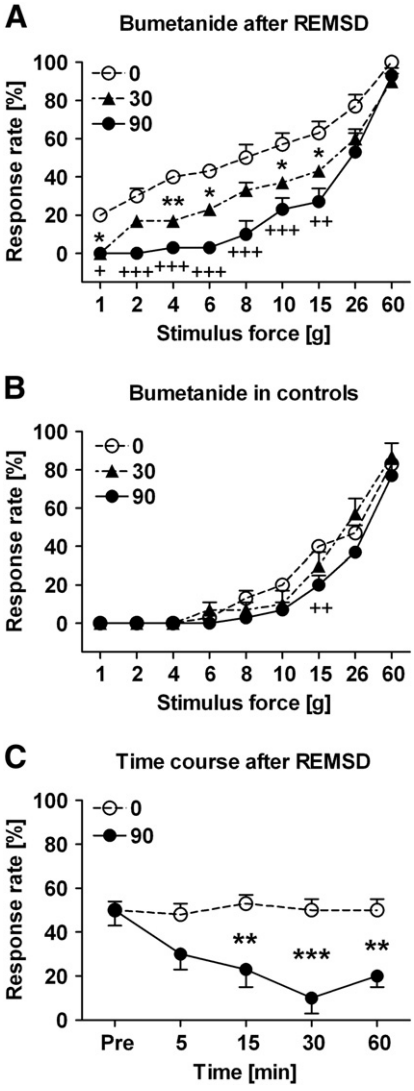

Fig. 3. Influence by i.t. treatment with bumetanide (a blocker of NKCC1) on mechanical sensitivity 48 h after REMSD (A and C), or in control conditions (B). 0 represents vehicle control, 30 and 90 represent bumetanide doses in μg. Graphs A and B show pain behavior 30 min after i.t. treatment. In C, the response to a monofilament force of 8 g is shown. Error bars represent S.E.M. ($n=6$). */+$P<0.05$, **/++$P<0.01$, ***/+++$P<0.005$ (t-test with a Bonferroni correction for multiple comparisons; reference: the corresponding value in the vehicle group).

$F_{2,135}=158.8$, $P<0.0001$; Fig. 4A). Muscimol (0.1 and 0.3 μg) produced a dose-related attenuation of mechanically induced pain behavior also in control animals (2-w-ANOVA: $F_{2,135}=15.0$, $P<0.0001$; Fig. 4B). Post hoc testing indicated that muscimol attenuated pain behavior in the REMSD group already at the dose of 0.1 μg (Fig. 4A), but in controls only at the dose of 0.3 μg (Fig. 4B). In controls, muscimol at the dose of 0.3 μg reduced the total response (as revealed by area under curve) to 45% of that in saline-treated controls, whereas following REMSD, muscimol at the dose of 0.3 μg reduced the total response to 30% of the corresponding response in saline-treated animals. In both control and sleep-deprived conditions, the maximum effect by muscimol was obtained 30 min after its i.t. administration (Fig. 4C).

3.5. Influence by i.p. pretreatment with minocycline on mechanical hypersensitivity induced by REMSD

I.p. pretreatment with minocycline, an inhibitor of microglia activation (40 mg/kg×2 at 24 h intervals; the latter dose was administered 24 before assessment of pain behavior and before the end of REMSD), failed to suppress mechanically induced pain behavior in the REMSD group (2-w-ANOVA: $F_{1,126}=3.57$, $P=0.061$; Fig. 5A) or in controls (2-w-ANOVA: $F_{1,168}=0.24$; Fig. 5B).

3.6. Influence by i.t. treatment with carbenoxolone, bumetanide or muscimol on motor performance

In general, drug treatments failed to produce any apparent changes in behavior of the experimental animals, except for the changes in the monofilament-induced limb withdrawal response. To exclude the possibility that suppression of pain behavior by the studied compounds was due to motor impairment rather than suppression of sensory signals, a Rotarod test was performed in control animals. Motor performance was not impaired by i.t. administration of carbenoxolone (10 μg), bumetanide (90 μg) or muscimol (0.3 μg) as indicated by the finding that all rats treated with these three compounds as well as saline-treated controls reached the cut-off value in the test (Fig. 6). Influence of pentobarbitone at a low sedative dose (20 mg/kg i.p.) was

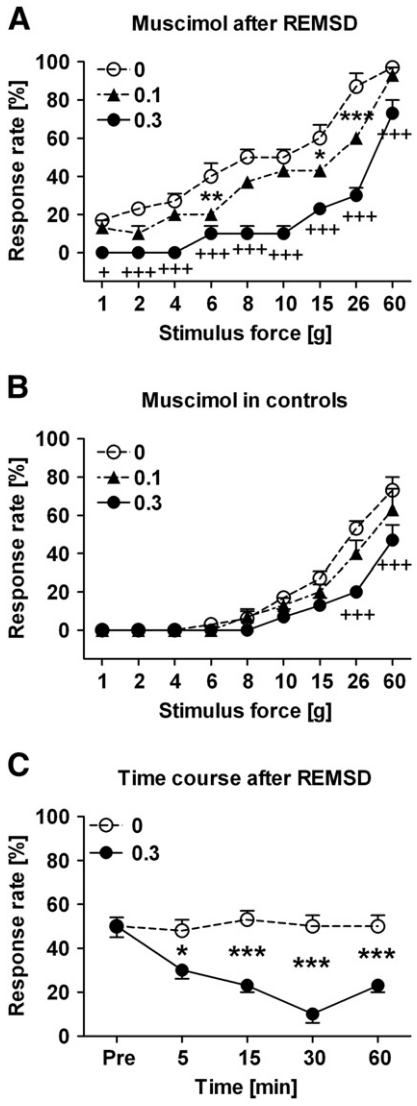

Fig. 4. Influence by i.t. treatment with muscimol (a $GABA_A$ receptor agonist) on mechanical sensitivity 48 h after REMSD (A and C), or in control conditions (B). 0 represents vehicle control, 0.1 and 0.3 represent muscimol doses in μg. Graphs A and B show pain behavior 30 min after i.t. treatment. In C, the response to a monofilament force of 8 g is shown. Error bars represent S.E.M. ($n=6$). */+$P<0.05$, **/++$P<0.01$, ***/+++$P<0.005$ (*t*-test with a Bonferroni correction for multiple comparisons; reference: the corresponding value in the vehicle group).

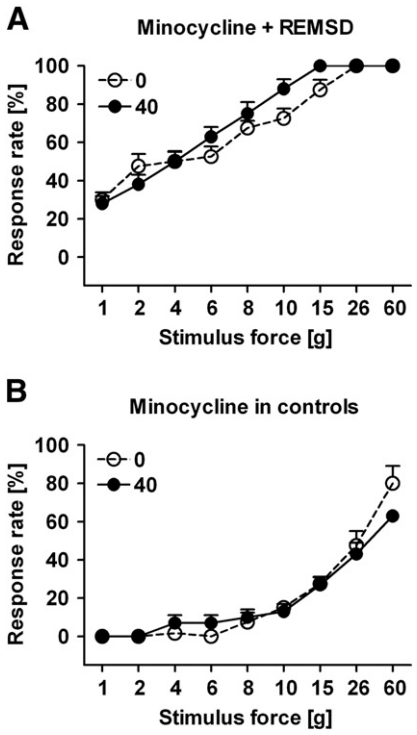

Fig. 5. Influence by i.p. pretreatment with minocycline (an inhibitor of microglia activation) on mechanical sensitivity that was assessed 48 h after REMSD (A) or in control conditions (B). 0 represents vehicle control, 40 represents the minocycline dose in mg/kg that was administered twice: once immediately prior to the start of REMSD of 48 h duration and once 24 h after the start of REMSD. Pain behavior was assessed 24 h after administration of the last dose of minocycline. Error bars represent S.E.M. ($n=6$).

tested to assess sensitivity of the Rotarod test; pentobarbitone-treated animals had a significant motor impairment as indicated by a decrease in the drop latency from the Rotarod drum (Fig. 6).

4. Discussion

In line with earlier results (e.g., Onen et al., 2000; Wei et al., 2007; 2008), REMSD of 48 h produced strong mechanical hypersensitivity. In the present study, pain-related behavior induced by mechanical stimulation was reduced in a dose-related fashion by i.t. treatment with carbenoxolone (a gap junction decoupler), bumetanide (a blocker of NKCC1), or muscimol (a $GABA_A$ receptor agonist). Importantly, while a minor or moderate reduction of pain-related behavior was observed in control animals, the relative suppression (in percent) of pain-related behavior by all of these three compounds was considerably stronger in sleep-deprived than control animals. Pretreatment with systemically administered minocycline (an inhibitor of microglia activation) at a dose that has reduced nerve injury-induced hypersensitivity (Raghavendra et al., 2003), however, failed to reduce mechanical hypersensitivity induced by REMSD in the present study.

4.1. Attenuation of pain-related behavior by carbenoxolone

Earlier studies have shown that spinal or medullary administration of carbenoxolone that decouples gap junctions has attenuated development of pain hypersensitivity in animals with nerve injury or inflammation (Chiang et al., 2010; Lan et al., 2007; Qin et al., 2006; Roh et al., 2010; Spataro et al., 2004). These findings are in line with

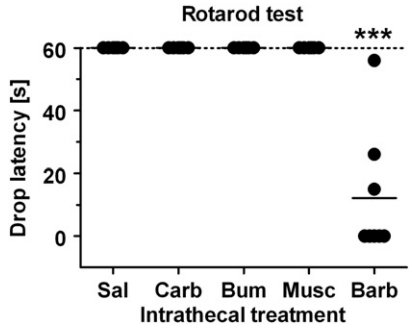

Fig. 6. Influence by i.t. treatment with carbenoxolone (Carb; 10 μg), bumetanide (Bum; 90 μg) or muscimol (Musc; 0.3 μg) on motor performance in control animals as revealed by the Rotarod test. Drop latency of 60 s (shown by the dotted horizontal line) represents the cut-off value. Drugs were delivered i.t., except for pentobarbitone (Barb) that was administered intraperitoneally at a dose of 20 mg/kg to assess sensitivity of the assay. Drug deliveries were performed 30 min before the Rotarod test. Each symbol represents one animal. Sal represents a saline control group. ***$P<0.005$ (non-parametric Dunn's test; reference: Sal group).

the hypothesis that among spinal mechanisms contributing to pain hypersensitivity in neuropathic and inflamed animals are gap junctions between astrocytes or between astrocytes and neurons (Alvarez-Maubecin et al., 2000). The strong antihypersensitivity effect by i.t. administration of carbenoxolone in sleep-deprived animals of the present study suggests that gap junctions in the spinal cord may have a role in pain hypersensitivity induced by REMSD, too. It is noteworthy that according to post hoc tests, carbenoxolone attenuated pain behavior after REMSD at a dose that failed to influence pain behavior in controls. This finding indicates that carbenoxolone preferentially suppressed a pathophysiological pain hypersensitivity mechanism.

While carbenoxolone blocks gap junctions and hemichannels, it also has other effects among which are modulations of neuronal membrane properties (Chepkova et al., 2008) and synaptic transmission (Tovar et al., 2009). Moreover, carbenoxolone has modulated anion channels on astrocytes (Ye et al., 2009). Although we cannot exclude the possibility that one or more of these other effects of carbenoxolone contributed to its antihypersensitivity effect, we can conclude that i.t. administration of a compound that is a well-established gap junction decoupler reduced REMSD-induced hypersensitivity.

4.2. Attenuation of pain-related behavior by bumetanide

In previous studies, a blocker of NKCC1, bumetanide, has attenuated inflammatory or neuropathic hypersensitivity (Cramer et al., 2008; Granados-Soto et al., 2005; Pitcher et al., 2007; Valencia-de Ita et al., 2006). In the present study, i.t. administration of bumetanide had a strong antihypersensitivity effect in sleep-deprived animals. Importantly, post hoc tests indicated that after REMSD bumetanide attenuated pain behavior at a dose that failed to influence pain behavior in controls. Together, these findings suggest that increased phosphorylation, membrane mobilization or expression of NKCC1 in the spinal cord may play a role in pain hypersensitivity following REMSD as well as following inflammation or nerve injury (Cramer et al., 2008; Galan and Cervero, 2005). In other words, activation of mechanoreceptive nerve fibers may have induced an excessive activation of adjacent nociceptive nerve fibers, through enhanced GABAergic depolarization of their central terminals. Excessive activation of central terminals of nociceptive primary afferent fibers may generate spike activity that facilitates pain-relaying interneurons and pain hypersensitivity rather than suppresses presynaptically the nociceptive discharge. This mechanism presumably contributes to pain hypersensitivity in injured and inflamed conditions (Cervero and Laird, 1996). According to the present results with bumetanide, an increase in intracellular chloride concentration and excessive depolarization of primary afferent terminals might contribute to pain hypersensitivity also following REMSD.

While bumetanide at high doses inhibits also KCC2 (Payne, 1997), it does not seem likely that inhibition of KCC2 could explain the present result with bumetanide. Namely, inhibition of KCC2, through GABAergic disinhibition of spinal interneurons (Price et al., 2009), is expected to cause facilitation of pain, instead of the bumetanide-induced antihypersensitivity effect of the present study.

4.3. Attenuation of pain-related behavior by muscimol

It might be expected that i.t. administration of $GABA_A$ receptor agonist increases pain behavior, if intracellular chloride concentration in primary afferent nociceptors or spinal pain-relay neurons is increased, due to enhanced NKCC1 or decreased KCC2 functions, respectively. In the present study, however, i.t. administration of muscimol, a $GABA_A$ receptor agonist, had a strong antihypersensitivity effect following REMSD. After REMSD, pain behavior was significantly suppressed by muscimol at a dose that failed to influence pain behavior in controls. Since muscimol after i.t. administration acts on both pre- and postsynaptic $GABA_A$ receptors, the results obtained with muscimol reflect the net effect of $GABA_A$ receptors in various locations and cell types that are involved in the complex spinal circuitry involved in pain behavior.

There is lack of neurobiological data on the effect of REMSD on NKCC1, KCC2, or the expression of spinal $GABA_A$ receptors in the spinal cord, while the effect of nerve injury on the GABAergic system and chloride transporters has been studied by a number of investigators. For example, it has been reported that nerve injury may induce down-regulation of KCC2, up-regulation of NKCC1 (Cramer et al., 2008), reduction of GABA and GABAergic neurons (Castro-Lopes et al., 1993; Moore et al., 2002), and up-regulation of $GABA_A$ receptors (Castro-Lopes et al., 1995); some of these changes may reduce and some promote inhibitory action of an exogenous $GABA_A$ receptor agonist in the spinal cord. Without further neurobiological data on REMSD-induced changes in the spinal GABAergic system, the present behavior results only allow concluding that the net effect induced by non-selective activation of spinal $GABA_A$ receptors by an exogenous agonist in REM sleep-deprived animals is inhibition of pain hypersensitivity, an effect that resembles that shown earlier in nerve-injured animals (Hwang and Yaksh, 1997).

4.4. Failure to attenuate pain-related behavior by minocycline

In the present study, minocycline administered systemically once daily failed to prevent development of mechanical hypersensitivity in the REMSD group. In contrast, pretreatment with minocycline (an inhibitor of microglia activation) has been effective in attenuating or delaying development of neuropathic hypersensitivity (Ledeboer et al., 2005; Raghavendra et al., 2003). Moreover, minocycline-induced antihypersensitivity effect in neuropathic animals was associated with decreased microglia activation and decreased levels of proinflammatory cytokines in the cerebrospinal fluid (Ledeboer et al., 2005). Concerning the present negative result with minocycline, it should be noted that we cannot exclude the possibilities that a higher pre-emptive dose of systemically administered minocycline, i.t. treatment, or acute pharmacological effect by minocycline administered shortly before testing had suppressed pain hypersensitivity in the REMSD group. In spite of these limitations, the failure to attenuate REMSD-induced hypersensitivity by pretreatment with the currently used dose of minocycline that has proved effective in nerve-injured animals (Raghavendra et al., 2003) suggests that microglia activation may not play as important role in REMSD-induced hypersensitivity as in neuropathic pain hypersensitivity.

4.5. Conclusions

The present results suggest that hypersensitivities induced by REMSD and nerve injury may share some common underlying spinal mechanisms that can be selectively reversed or suppressed by i.t. treatment with a blocker of NKCC1, a blocker of gap junctions, or $GABA_A$ receptor agonist. Activation of microglia, however, may not be as important for hypersensitivity induced by REMSD as for that induced by nerve injury, since pretreatment with minocycline failed to prevent development of REMSD-induced hypersensitivity at a dose that has been effective in preventing nerve injury-induced hypersensitivity. These results add to previous evidence indicating that REMSD and nerve injury share common spinal mechanisms involving the $mGluR_5$ and nitric oxide (Wei et al., 2007).

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