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Neurophysiological response properties of rostroventromedial medullary pain-control neurons following chronic treatment with morphine or oxycodone: modulation by acute ketamine

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Running head: Chronic opioid-induced changes in RVM cell discharge

Abstract

Descending facilitatory circuitry that involves the rostroventromedial medulla (RVM) exerts a significant role in the development of antinociceptive tolerance and hyperalgesia following chronic morphine treatment. The role of the RVM in the development of antinociceptive tolerance to oxycodone, another clinically used strong opioid, is not yet known. Ketamine, an NMDA receptor antagonist, attenuates opioid antinociceptive tolerance, but its effect on RVM cell discharge in opioid tolerant animals is not known. Here, we compared chronic effects of morphine and oxycodone on the discharge properties of RVM cells and attempted to attenuate chronic treatment-induced changes with ketamine. Parallel recordings of RVM cell discharge and limb withdrawal response were performed under light pentobarbital anesthesia in male rats following sustained systemic treatment with morphine or oxycodone at equianalgesic doses. Ongoing activity and the response to noxious heat and pinch were determined in pronociceptive RVM ON-cells and antinociceptive OFF-cells on the sixth treatment day. Proportions of RVM cell types were not changed. Chronic oxycodone induced antinociceptive tolerance both in limb withdrawal and RVM cell activity. Chronic morphine induced antinociceptive tolerance in limb withdrawal that was accompanied by pronociceptive heat response changes in RVM ON- and OFF-cells. A behaviorally subantinociceptive dose of acute ketamine reversed antinociceptive tolerance both to morphine and oxycodone in limb withdrawal and reversed the chronic morphine-induced pronociceptive discharge changes in RVM cells. The results indicate that an NMDA receptor-dependent descending pronociceptive circuitry involving the RVM has an important role in behavioral antinociceptive tolerance to morphine but not oxycodone.

Keywords: Ketamine; morphine; analgesic opioid tolerance; oxycodone; rostroventromedial medulla

New and noteworthy: Morphine and oxycodone are two clinically used strong opioids. Chronic treatment with oxycodone as well as morphine can lead to analgesic tolerance and paradoxical hyperalgesia. Here we show that an NMDA receptor-dependent pronociceptive change in discharge properties of rostroventromedial medullary neurons controlling spinal nociception has an important role in antinociceptive tolerance to morphine but not oxycodone. Interestingly, chronic oxycodone did not induce pronociceptive changes in the rostroventromedial medulla.

Abbreviations: ANOVA, analysis of variance; intraperitoneal, i.p; MO, morphine; MOR, μ-opioid receptor; NMDA, N-methyl-D-aspartate; OX, oxycodone; PAG, periaqueductal gray; RVM, rostroventromedial medulla; s.c., subcutaneous

Chemical compounds studied in this article: Ketamine hydrochloride (PubChem CID: 15851); Morphine hydrochloride (PubChem CID: 5464110); Oxycodone hydrochloride (PubChem CID: 5462350)

Introduction

Long-term use of strong opioids such as morphine and oxycodone (Caraceni et al. 2012; Fanelli et al. 2016; Tompkins and Campbell 2011) can lead to opioid tolerance and opioid-induced hyperalgesia (OIH). Chronic morphine may enhance descending facilitation from the rostroventromedial medulla (RVM), a major relay for descending pain regulation (Fields et al. 2006). This has been suggested to be one of the mechanisms promoting morphine tolerance and paradoxical pain/hyperalgesia (Meng and Harasawa 2007; Ossipov et al. 2004; Vanderah et al. 2001; Vera-Portocarrero et al. 2007; Xie et al. 2005).

Neurons in the RVM respond to opioids in a unique way (Fields et al. 1983a, 1983b; Heinricher and Fields 1992; Heinricher et al. 1994, 1999, 2001). Pronociceptive RVM ON-cells are activated by noxious stimuli and inhibited by μ-opioid receptor (MOR) agonists, whereas antinociceptive RVM OFF-cells decrease activity during the stimulus just before the nocifensive reflex and increase activity following administration of MOR agonists. In the third type of neurons, NEUTRAL-cells, firing remains unaffected by noxious stimulation or MOR agonists. In morphine tolerance and morphine-induced paradoxical pain/hyperalgesia, MOR agonists fail to produce discharge rate changes in RVM ON- and OFF-cells (Lane et al. 2004; Tortorici et al. 2001), and the proportion of RVM ON-cells has been reported to increase and that of RVM NEUTRAL-cells to decrease (Meng and Harasawa 2007). These changes in functional properties and distribution of RVM neurons could contribute to the predominance of tonic descending facilitation from the RVM that promotes tolerance and that can lead to chronic morphine-induced paradoxical pain/hyperalgesia (Meng and Harasawa 2007; Vanderah et al. 2001).

In the rat, morphine has shown somewhat lower antinociceptive potency compared with oxycodone after systemic administration (Lemberg et al. 2006a) even though oxycodone

has lower efficacy and potency after *ex vivo* G-protein activation assays in the rat brain (Lemberg et al. 2006b; Nakamura et al. 2013; Thompson et al. 2004). The effect of oxycodone, unlike that of morphine, on RVM neuron firing has not yet been investigated. Here we studied chronic effects of morphine and oxycodone on the discharge properties of RVM ON- and OFF cells in male rats. In order to have a behavioral correlate for the neurophysiological findings, heat-evoked limb withdrawal was determined in parallel with recordings of RVM cells. Ketamine (Lilius et al. 2015, 2018) and other N-methyl-D-aspartate (NMDA) receptor antagonists (Tiseo and Inturrisi 1993; Trujillo and Akil 1991, 1994) have been shown to attenuate behavioral antinociceptive tolerance to chronic opioid treatment in experimental studies. Moreover, ketamine has been used in the clinic for prevention and treatment of opioid tolerance (Bell et al. 2017; Clark and Kalan 1995). Therefore, we also studied whether acute subcutaneous ketamine modulates chronic morphine or oxycodone-induced changes in the discharge of RVM cells or pain-related behavior.

Methods

Experimental animals. The provincial government of Southern Finland (Etelä-Suomen aluehallintovirasto, Hämeenlinna, Finland; Permission # ESAVI/10218/04.10.07/2016) had approved the study protocol and the research was performed according to the guidelines of the European Parliament and the Council Directive of 22 September 2010 (010/63/EU), International Association for the Study of Pain (Zimmermann 1983), and the ARRIVE guidelines (Kilkenny et al. 2010; Knopp et al. 2015; McGrath et al. 2010). Male Sprague-Dawley rats (Envigo Laboratories, Horst, the Netherlands) weighing 200–250 g at the beginning of experiments, were used. Animals were housed in standard light- and temperature-controlled rooms (lights on 06:00–18:00 h, temperature 22 ± 2 °C) in groups of

two in individually ventilated plastic cages with free access to tap water and standard laboratory chow. The experiments were performed in accordance to 3R principles and all efforts were made to limit distress to the animals. Chronic vehicle and opioid treatments were randomized and blinded.

Chronic opioid treatments. Morphine and oxycodone tolerance were induced with continuous opioid administration using osmotic mini-pumps (Alzet 2ML1; DURECT, Cupertino, CA, USA). The pumps were filled with morphine 40 mg/ml to deliver morphine 9.6 mg/day, or oxycodone 15 mg/ml to deliver oxycodone 3.6 mg/day. The doses were chosen based on a previous study (Lilius et al. 2018) showing that at these doses, acute administration of morphine and oxycodone induces antinociception of equal magnitude and chronic administration induces similar antinociceptive tolerance. The pumps were not pre-primed and the treatment lasted for 6 days. Administration continued during recording. Morphine and oxycodone were diluted in sterile water, which was also used to fill the control pumps. The pumps were implanted s.c. between the scapulae under brief isoflurane 3.0 % anesthesia on day 0. The adequacy of anesthesia was verified by lack of withdrawal response to tail pinch. The health of the rats was monitored daily after the operation. If any objective signs of pain or discomfort occurred, the animal was euthanized. However, no complications were observed in the present study. Electrophysiological recordings of RVM neurons were performed on day 6 (Fig. 1).

Drugs. Oxycodone hydrochloride was purchased from Sigma-Aldrich (St Louis, MO), morphine hydrochloride and racemic ketamine hydrochloride (Ketaminol vet.; Boxmeer, Netherlands) from The University Pharmacy (Helsinki, Finland). Ketamine was diluted in

physiological saline and administered s.c. in a volume of 2 ml/kg. All drug concentrations were expressed as free base amounts.

Recording of neuronal activity in the RVM. Electrophysiological microelectrode recordings of RVM cells were performed under anesthesia that was induced by administering 50–60 mg/kg of sodium pentobarbital intraperitoneally (i.p.). Following induction of anesthesia, the animal was placed in a standard stereotaxic frame according to the atlas of Paxinos and Watson (1998). Anesthesia was continued by i.p. injections of pentobarbital at intervals of 30 min and at the dose of 15–20 mg/kg. The level of anesthesia was frequently monitored by assessing the size of the pupils, general muscle tone and reflex responses to noxious pinching. Supplemental doses of sodium pentobarbital were administered as required. The rats breathed spontaneously and the body temperature was maintained within a physiological range with a warming blanket. Peripheral perfusion was checked by examining the color of the ears and extremities.

The skull was exposed and a hole drilled for the placement of a recording electrode in the RVM (anteroposterior [AP]: 2.0–2.8 mm from the interaural line; mediolateral [ML]: 0–1 mm; dorsoventral [DV]: 8.9–11 mm from the dura mater; Paxinos and Watson 1998).

Neuronal activity in the RVM was recorded extracellularly with lacquer-coated tungsten electrodes (impedance 5–7 MΩ at 1 kHz; FHC Inc., Bowdoin, ME). The signal was amplified and filtered using standard techniques. Data sampling was performed with a computer connected to a CED Micro 1401 interface and using Spike2 software (Cambridge Electronic Design, Cambridge, UK). Spike2 software classifies waveform shapes based on full wave templating, and in the offline analysis the template matching can be complemented by clustering using principal component analysis, which allows evaluating separately multiple identified units in a single recording session.

During recordings, the microelectrode was first lowered according to the stereotaxic coordinates into the RVM. When neuronal firing was observed, spontaneous ongoing activity was first assessed. Before starting the actual recording of cells, the deep level of anesthesia needed for the surgical procedures was allowed to lighten to a level where the animal did not have any spontaneous limb movements but noxious stimulation caused a brief flexion reflex with no other behavioral responses. The RVM cells were classified based on the concurrently assessed RVM cell and limb flexion response to noxious heat (Fields et al. 1983) in a single trial. The mean ongoing baseline discharge rate a 30 s period just before noxious heat stimulation was subtracted from the discharge rate determined a 3 s period beginning 0.5 s before the paw limb withdrawal, Fig. 2. RVM cells that gave an excitatory discharge >20% just before the heat-evoked limb withdrawal, were classified as pronociceptive ON-cells. RVM cells, in which the ongoing discharge rate was inhibited by >20% just before the heat-evoked limb withdrawal, were classified as antinociceptive OFF-cells. RVM cells that did not respond to noxious heat were considered NEUTRAL-cells. NEUTRAL-cells were not included in this study, except when calculating proportions of RVM cell types. In case the RVM cell responded only to noxious heating of the hind paw but not to noxious mechanical stimulation of the tail, only its heat-evoked response as well as ongoing discharge rate were considered in further analyses of the drug effects.

Somatosensory test stimulation during electrophysiological recordings. Sensitivity to noxious heat stimulus was assessed with a feedback-controlled Peltier device (82.8 mm^2, LTS-3 Stimulator, Thermal Devices Inc., Golden Valley, MN) that was applied to the plantar skin of the left hind paw. The baseline temperature of the thermode was 35 °C. During stimulation, the temperature was increased to 54 °C at a rate of 10 °C/s. The duration of the peak temperature was 10 s, after which the temperature was decreased to the baseline of 35

°C at a rate of 4 °C/s. A miniature piezoelectric movement detector (Siemens Elema Ab, Solna, Sweden) over the gastrocnemius muscle was used to detect the heat-evoked limb withdrawal (Fig. 2).

Mechanical sensitivity in the hind paw was assessed by a brush that produced tactile sensation when applied to the experimenter's hand. Brush stimulation was used only in the first characterization of the neurons. Mechanical nociception was assessed by applying a hemostatic clamp (pinch stimulation) to the tail for 5 s (Gonçalves et al. 2007; Viisanen and Pertovaara 2007). The clamp produced a force of 350 g and it evokes pain-related behavior at a latency of 10 s when applied to the tail of unanesthetized rats (Kauppila et al., 1998) and pain when applied to the finger of the experimenter.

When assessing drug effects on somatic responses, the testing procedure started with the assessment of ongoing discharge rate. Next, the response to noxious heat applied to the hind paw was determined. This was followed by testing the response to tail pinch. The order of testing heat and pinch stimulation was the same in all experimental groups. When determining the neuronal discharge rate during heat stimulation, a 10 s time-period from the start of the stimulus rise was taken into account (Fig. 2). When determining the discharge rate during a noxious tail pinch, a 5 s time period from the start of the pinch was taken into account. In further analysis of the stimulus-evoked discharge responses, the baseline ongoing discharge rate recorded during a 30 s period just before noxious stimulation was taken into account (Figs. 1, 2). When the effect of drug treatments on the stimulus-evoked discharge rates was calculated, the ongoing baseline discharge rate was subtracted from the discharge rate determined during stimulation. Positive values represent excitatory responses evoked by peripheral stimulation whereas negative responses represent inhibitory responses.

Moreover, the latency to the onset of the stimulus-evoked burst discharge and the duration of the stimulus-evoked burst discharge in ON-cells were assessed (Fig. 2). The burst

discharge was defined to start when discharge rate started to increase over the peak ongoing discharge rate. Similarly, the latency to the onset of the stimulus-evoked discharge inhibition and the duration of the stimulus-evoked discharge inhibition in OFF-cells were assessed (Fig. 2). The discharge inhibition was defined to start when discharge rate started to decrease under the lowest ongoing discharge rate. The following parameters in ON-cell discharge were considered to represent a pronociceptive change (and opposite changes an antinociceptive change): increase of ongoing activity, increase of noxious stimulus-evoked discharge rate, decrease of the noxious stimulus-evoked burst discharge latency, and increase of the noxious stimulus-evoked burst discharge duration. The following parameters in OFF-cells were considered to represent an antinociceptive change (and opposite changes a pronociceptive change): increase of ongoing activity, increase of stimulus-evoked activity, increase in the latency of the noxious stimulus-evoked burst discharge inhibition, and decrease in the duration of the noxious stimulus-evoked burst discharge inhibition.

In case the studied RVM ON-cell did not respond to noxious stimulation after acute ketamine injection, the latency to the onset of the stimulus-evoked burst discharge was defined to be 10 s (heat) or 5 s (pinch), and the duration of the stimulus-evoked burst discharge was defined to be 0 s. Similarly, if the RVM OFF-cell did not respond to noxious stimulation after acute ketamine injection, the latency to the onset of the stimulus-evoked discharge inhibition was defined to be 10 s (heat) or 5 s (pinch), and the duration of the stimulus-evoked discharge was defined to be 0 s. In case the ongoing discharge rate was zero after acute ketamine injection, the latency to the onset of the stimulus-evoked discharge inhibition in OFF-cell was defined to be 0 s and the duration of the stimulus-evoked discharge inhibition was defined to be 10 s.

Course of the electrophysiological recordings. First, the RVM cell was characterized as a pronociceptive RVM ON-, an antinociceptive RVM OFF-cell, or a NEUTRAL-cell. NEUTRAL-cells were not studied further. With RVM ON- and OFF-cells, its ongoing discharge rate and the response to noxious thermal and mechanical (pinch) stimulation was assessed. In the same recording session, multiple neurons could be recorded and analyzed separately.

Recordings were performed in animals that were treated during the preceding days (from day 0 to day 6, the recording day) with vehicle, morphine (9.6 mg/day) or oxycodone (3.6 mg/day) (Fig. 1). Recordings of RVM cells were performed on day 6 of chronic drug treatment by determining the ongoing discharge rate of the RVM cells followed by determination of its response to noxious thermal and mechanical (pinch) stimulation. Immediately after this, ketamine was administered at the subanesthetic dose of 10 mg/kg s.c. This was followed by determination of the ongoing discharge rate and the response to noxious thermal and mechanical stimulation 15 min and 30 min after ketamine administration. The latency to the onset of the heat-evoked hind limb withdrawal was determined in parallel with recording of RVM cells. When analyzing the data on chronic opioid treatments, the ongoing discharge rates and peripherally evoked responses in opioid-treated animals were compared with the corresponding values in the vehicle-treated control group. When analyzing the ketamine-induced effect, the ongoing discharge rate and peripherally evoked responses recorded before ketamine administration (= after chronic opioid treatment) were compared with the corresponding values recorded 15 and 30 min after ketamine administration within each chronic treatment group. Discharge properties of RVM neurons following chronic treatments with vehicle, morphine or oxycodone were studied in 23 animals, while heat-evoked limb withdrawal responses were studied in 25 animals that had received chronic opioid or vehicle treatments; i.e., a parallel recording of identified RVM cells and limb

withdrawal was not successful in two animals and therefore, only limb withdrawal responses were determined in these two animals. A total of 41, 29 and 27 RVM neurons were sampled from 9 vehicle-, 7 morphine- and 6 oxycodone-treated animals, respectively (Table 1).

The total duration of the recording sessions varied from 2 to 4 h. At the end of the recording sessions, the animal was given a lethal dose of sodium pentobarbital, an electrolytic lesion was made in the recording site, and the brain was removed, fixed in formalin and sliced for histological verification of the recording sites. Only neurons the location of which in the RVM was confirmed in the histological analysis were included in the study (Fig. 3).

Statistics. Data are presented as mean ± SD. Data analysis was performed using Prism 6.0 software (GraphPad Software, Inc., San Diego, CA). Statistical analyses of neuronal discharge rates, percentage of each RVM cell type and withdrawal responses were performed using mixed-design two-way-analysis of variance (2-w-ANOVA) followed by a t-test with a Bonferroni correction. Statistical analyses of neuronal discharge rates (effect of brush stimulation) were performed using one-way-analysis of variance (1-w-ANOVA) followed by Tukey's test. Changes in the proportion of various RVM neuron types were analyzed using chi-square test. $P < 0.05$ was considered to represent a significant difference.

Results

Proportions of different cell types in the RVM. The numbers of ON-, OFF-, and NEUTRAL-cells sampled in the chronic treatment study did not differ among chronic vehicle, morphine, and oxycodone groups ($\chi^2 = 6.061$, Table 1). In an additional analysis, the percentage of the different types of RVM cells was counted for each individual animal. The

mean percentage of each RVM cell type was then calculated over all animals in each chronic treatment group. The mean percentages of RVM cell types within each chronic treatment group did not differ significantly among the treatment groups ($F_{2,57}$ = 0.00, P = 1; Fig. 4).

Ongoing discharge rates of pronociceptive ON-cells and antinociceptive OFF-cells. Chronic opioid treatment had almost significant main effect on the **ongoing discharge rate** in pronociceptive RVM ON-cells ($F_{2,45}$ = 3.153, P = 0.052; Fig. 5A) and no main effect on ongoing discharge of antinociceptive RVM OFF-cells ($F_{2,27}$= 1.007, P = 0.379; Fig. 5*B*). Acute ketamine treatment had no significant main effect on the ongoing discharge rate in ON-cells ($F_{2,90}$ = 0.734, P = 0.483; Fig. 5*A*) or in antinociceptive RVM OFF-cells ($F_{2,54}$ = 0.115, P = 0.892; Fig. 5*B*).

*Noxious heat-evoked responses of pronociceptive ON-cells***.** Chronic drug treatments had a significant (pronociceptive) main effect on the **heat-evoked discharge rate** of RVM ON-cells ($F_{2,45}$ = 4.694, P < 0.05; Fig. 6*A*). Acute ketamine treatment had a significant (antinociceptive) main effect on the heat-evoked discharge rate in ON-cells ($F_{2,90}$= 5.616, P < 0.01; Fig. 6*A* and Fig. 7), independent of the chronic treatment group (interaction: $F_{4,90}$ = 2.118, P = 0.085). *Post hoc* testing indicated that the noxious heat-evoked discharge rate in ON-cell was significantly increased (pronociceptive effect) by chronic morphine treatment and that the increase was reversed (antinociceptive effect) by acute ketamine (Fig. 6A). In the chronic oxycodone group, the heat-evoked discharge rate in ON-cells was not significantly different from that in the vehicle-treated group, independent of acute ketamine treatment.

Chronic drug treatments had a significant (pronociceptive) main effect on the **latency** of the heat-evoked response (burst discharge) in ON-cells ($F_{2,45}$ = 5.253, P < 0.01; Fig. 6*B*). Acute ketamine treatment had a significant (antinociceptive) main effect on the heat-evoked

ON-cell response latency ($F_{2, 90}$ = 18.16, P < 0.0001; Fig. 6*B*), independent of the chronic treatment group (interaction: $F_{4, 90}$ = 0.947, P = 0.441). *Post hoc* testing indicated that before acute ketamine treatment, the differences in the heat-evoked response latencies among chronic treatment groups were not significant. Acute ketamine significantly increased the response latency (antinociceptive effect) in the chronic vehicle and morphine groups (Fig. 6*B*). Moreover, the ketamine-induced antinociceptive effect (increase of the response latency) was significantly stronger in the chronic vehicle than chronic oxycodone or morphine groups.

Duration of the heat-evoked response (burst discharge) in RVM ON-cells was not significantly influenced by chronic drug treatments ($F_{2, 45}$ = 1.594, P =0.215; Fig. 6*C*). However, acute ketamine treatment had a significant (antinociceptive) main effect on the duration of the heat-evoked ON-cell response ($F_{2, 90}$ = 16.51, P < 0.0001; Fig. 6*C*), independent of the chronic treatment group (interaction: $F_{4, 90}$ = 0.573, P = 0.683). *Post hoc* testing indicated that following acute ketamine administration, the duration of the heat-evoked ON-cell response was significantly decreased (antinociceptive effect) in all chronic treatment groups (Fig. 6*C*).

Noxious mechanical stimulation-evoked responses of pronociceptive ON-cells.

Noxious mechanical stimulation-induced discharge rate of RVM ON-cells was not significantly influenced by chronic drug treatments ($F_{2, 33}$ = 2.469, P = 0.100; Fig. 6*D*). Acute ketamine treatment had a significant (antinociceptive) main effect on the noxious mechanical stimulation-evoked discharge rate in RVM ON-cells ($F_{2, 66}$ = 4.124, P < 0.05; Fig. 6*D*), independent of the chronic treatment group (interaction: $F_{4,66}$ = 1.771, P = 0.145). *Post hoc* testing indicated that following acute ketamine administration, the noxious mechanical stimulation-evoked discharge rate in ON-cells was significantly decreased (antinociceptive effect) in the chronic vehicle-treated group (Fig. 6*D*).

Response latency of ON-cells to noxious mechanical stimulation (latency to the onset of the stimulation-evoked burst discharge) was not significantly influenced by chronic drug treatments ($F_{2, 33} = 0.694$, $P = 0.507$; Fig. 6*E*). However, acute ketamine treatment had a significant (antinociceptive) main effect on the ON-cell response latency to noxious mechanical stimulation ($F_{2, 66} = 10.89$, $P < 0.0001$; Fig. 6*E*) that varied with the chronic treatment group (interaction: $F_{4, 66} = 2.965$, $P < 0.05$). *Post hoc* testing indicated that after acute ketamine treatment the ON-cell response latency was significantly prolonged (antinociceptive effect) in the chronic vehicle and chronic oxycodone treatment groups.

Duration of the noxious mechanical stimulation-evoked response (burst discharge) in ON-cells was not significantly influenced by chronic drug treatments ($F_{2, 33} = 0.3112$, $P = 0.7347$; Fig. 6*F*). However, acute ketamine treatment had a significant (antinociceptive) main effect on the duration of the ON-cell response ($F_{2, 66} = 8.780$, $P < 0.001$; Fig. 6*F*) that varied with the chronic treatment group (interaction: $F_{4, 66} = 2.580$, $P < 0.05$). *Post hoc* testing indicated that following acute ketamine administration, the duration of the mechanical stimulation-induced ON-cell response was significantly decreased (antinociceptive effect) in the chronic vehicle and oxycodone groups.

Innocuous mechanical stimulation (brush, 5 s) of the hind paw was used in the preliminary characterization of the RVM neuron's response properties to assess whether chronic opioid treatments induce an increased response to innocuous mechanical stimulation that might underlie tactile-allodynia-like behavior observed in unanesthetized animals following chronic opioid treatment (Vanderah et al. 2001). Chronic drug treatments had no significant main effect on the innocuous brush-evoked discharge rate in RVM ON-cells ($F_{2, 45} = 1.721$, $P = 0.190$; not shown).

Noxious heat stimulation-evoked responses of antinociceptive OFF-cells. **Heat-induced discharge rate** in RVM OFF-cells was not significantly influenced by chronic drug treatments ($F_{2, 27} = 0.718$, $P = 0.497$; not shown) or by acute ketamine ($F_{2, 54} = 0.564$, $P = 0.572$; an example shown in Fig. 7).

Latency to the onset of the heat-evoked discharge inhibition in OFF-cells was not significantly influenced by chronic drug treatments ($F_{2, 27} = 0.045$, $P = 0.957$; Fig. 8*A*). Acute ketamine treatment had a significant (prolonging / antinociceptive) main effect on the onset latency of the heat-evoked discharge inhibition in OFF-cells ($F_{2, 54} = 3.858$, $P < 0.05$; Fig 8*A*), independent of the chronic treatment group (interaction: $F_{4,54} = 0.498$, $P = 0.737$). *Post hoc* testing, however, failed to reveal a significant difference in the onset latency of the heat-evoked OFF-cell discharge inhibition determined before versus after acute ketamine in any of the chronic treatment groups (Fig. 8*A*).

Duration of the noxious heat-induced discharge inhibition of OFF-cells was not significantly influenced by chronic drug treatments ($F_{2, 27} = 1.458$, $P = 0.251$; Fig. 8*B*). Acute ketamine treatment had a significant (shortening / antinociceptive) main effect on the duration of the heat-evoked discharge inhibition of RVM OFF-cells ($F_{2, 54} = 5.981$, $P < 0.01$; Fig. 8*B*) that varied with the chronic treatment group (interaction: $F_{4,54} = 3.718$, $P < 0.01$). *Post hoc* testing indicated that chronic morphine-induced a prolongation in the duration of the heat-evoked discharge inhibition of OFF-cells (pronociceptive effect) that was reversed (antinociceptive effect) by acute ketamine treatment (Fig. 8*B*).

Noxious mechanical stimulation-evoked responses of antinociceptive OFF-cells. **Noxious mechanical stimulation-induced discharge rate** in RVM OFF-cells was not significantly influenced by chronic drug treatments ($F_{2, 17} = 0.332$, $P = 0.722$; not shown) or acute ketamine treatment ($F_{2, 34} = 1.877$, $P = 0.169$; not shown).

Latency to the onset of the noxious mechanical stimulation-induced discharge inhibition of OFF-cells was not influenced by chronic drug treatments ($F_{2,17} = 0.246$, $P = 0.785$; Fig. 8*C*). Acute ketamine treatment had a significant (prolonging / antinociceptive) main effect on the onset latency of the mechanical stimulation-induced OFF-cell discharge inhibition ($F_{2, 34} = 10.41$, $P < 0.001$; Fig. 8*C*), independent of chronic treatment condition (interaction: $F_{4,34} = 0.537$, $P = 0.710$). *Post hoc* testing indicated that ketamine significantly increased onset latencies (antinociceptive effect) of the mechanically evoked OFF-cell discharge inhibition in the vehicle and chronic morphine groups.

Duration of the noxious mechanical stimulation-induced discharge inhibition of OFF-cells was not significantly influenced by chronic drug treatments ($F_{2, 17} = 0.372$, $P = 0.694$; Fig. 8*D*). Acute ketamine treatment had a significant (shortening / antinociceptive) main effect on the duration of the mechanically induced discharge inhibition of OFF-cells ($F_{2, 34} = 7.064$, $P < 0.01$; Fig. 8*D*), independent of the chronic treatment group (interaction: $F_{4,34} = 2.063$, $P = 0.107$). *Post hoc* testing indicated that the acute ketamine-induced decrease in the duration of the noxious mechanical stimulation-induced OFF-cell discharge inhibition (antinociceptive effect) was significant in the chronic vehicle and morphine groups.

Innocuous mechanical stimulation-evoked discharge rate of RVM OFF-cells was not significantly influenced by chronic drug treatments ($F_{2, 27} = 0.990$, $P = 0.385$; not shown).

Noxious heat-evoked limb withdrawal. Heat-evoked limb withdrawal latencies were assessed on day 6 in the chronic drug treatment groups, before and after acute ketamine treatment, in parallel with recordings of RVM cells.

The main effect of chronic drug treatments on the heat-evoked limb withdrawal latency was not significant ($F_{2, 21} = 2.77$, $P = 0.086$; Fig. 9). Acute administration of ketamine had a significant (antinociceptive) main effect on the limb withdrawal latency ($F_{2,42} = 21.21$,

$P < 0.0001$; Fig. 9) that varied with the chronic treatment group (interaction: $F_{4,42} = 5.50$, $P < 0.01$). *Post hoc* tests indicated that before acute ketamine treatment, there were no significant differences in the withdrawal latencies between the vehicle and opioid treatment groups indicating development of antinociceptive tolerance to morphine and oxycodone. Acute administration of ketamine had no significant effect on the limb withdrawal latency in the chronic vehicle treatment group. However, in the chronic morphine and oxycodone groups acute administration of ketamine significantly increased the heat-evoked limb withdrawal latency (antinociceptive action) (Fig. 9).

Discussion

In the present electrophysiological study, we compared chronic effects induced by systemically administered morphine and oxycodone, two clinically used MOR agonists, on response characteristics of pro- and antinociceptive neurons in the RVM, a major relay for descending pathways controlling spinal nociception (Fields et al. 2006). A parallel behavioral assay (heat-evoked limb withdrawal) showed that the animals had developed antinociceptive tolerance to both morphine and oxycodone by the time point of neuronal recordings (day 6 of chronic opioid administration). In line with this, RVM neurons in neither chronic morphine nor chronic oxycodone treated animals showed discharge rate changes shown to promote antinociception following acute administration of opioids in previous studies (Fields et al., 2006). A marked difference in the chronic actions of the two studied MOR agonists was that RVM neurons in animals treated with chronic morphine, but not oxycodone, showed significant pronociceptive changes in their discharge characteristics. These chronic morphine treatment-induced pronociceptive changes were most prominently shown as an increase in the heat-evoked discharge rate of pronociceptive RVM ON-cells and as a prolonged duration of the heat-evoked discharge inhibition of antinociceptive RVM OFF-cells. To assess contribution of NMDA receptors to the chronic opioid-induced effects, ketamine that is used to prevent opioid tolerance in the clinic (Bell et al. 2017; Clark and Kalan 1995) was administered at a dose that alone did not influence pain behavior in controls. Acute administration of ketamine reversed the chronic morphine-induced pronociceptive changes in response characteristics of RVM cells as well as the behaviorally determined antinociceptive tolerance to morphine and oxycodone. Under the current experimental conditions, the proportion of various RVM cell types was not significantly changed following chronic morphine or chronic oxycodone.

Effect by chronic administration of opioids on the discharge of RVM neurons.

Characteristic antinociceptive actions of acute morphine in the RVM are suppression of pronociceptive ON-cell responses and disinhibition of antinociceptive OFF-cell activity (Fields et al. 2006). These antinociceptive actions induced by acute morphine were not observed following chronic morphine or oxycodone treatment in RVM ON- or OFF-cells, independent of noxious test stimulus modality, suggesting development of antinociceptive tolerance. These findings following a 6-day systemic opioid treatment are in line with earlier results showing that following a 3-day morphine treatment of the ventrolateral PAG, animals developed antinociceptive morphine tolerance as shown by a failure to observe characteristic changes induced by acute morphine on RVM ON- or OFF-cell activity or on pain behavior (Lane et al. 2004; Tortorici et al. 2001). Earlier results on the effect by a 7-day systemic morphine treatment on RVM cell discharge (Meng and Harasawa 2007) are partly in line with the present results. As in the present study, systemically administered chronic morphine did not induce antinociceptive changes in baseline activity of ON- or OFF-cells or in the heat-evoked discharge rate of OFF-cells (Meng and Harasawa 2007). Neither was the heat-evoked ON-cell discharge rate influenced by chronic morphine in the earlier study indicating development of antinociceptive tolerance (Meng and Harasawa 2007), whereas in the present study the heat-evoked discharge rate of ON-cells was increased in the chronic morphine group suggesting development of heat hypersensitivity. The increase in the heat-evoked ON-cell discharge in the chronic morphine group of the present study was accompanied by an increase in the duration of the heat-evoked OFF-cell inhibition, a response parameter that was not assessed in the previous study (Meng and Hararasawa 2007) and that is expected to reflect heat hypersensitivity as well. However, it should be noted that Meng and Harasawa (2007) used morphine pellets that keep plasma morphine levels constantly at around 450 ng/ml,

whereas our earlier measurements indicate that the plasma level of morphine with the currently used Alzet pumps was only about 300 ng/ml (Lilius et al. 2015). This difference in morphine levels may have contributed to differences between the findings. An additional explanation for the difference in the heat-evoked ON-cell response between the present and the previous study is that the radiant heat stimulation used in the previous study (Meng and Harasawa 2007) may not have been as effective in revealing the chronic morphine-induced heat hypersensitivity of RVM ON-cells as the high-intensity contact heat stimulation of the present study (see for further discussion the chapter on *Limitations*).

The present findings are in line with earlier behavioral results showing that pain hypersensitivity induced by prolonged systemic administration of morphine is driven by descending circuitry involving the RVM (Vanderah et al. 2001; Vera-Portocarrero et al. 2007; Xie et al. 2005). Sensitization of RVM ON-cells to noxious thermal stimulation has been described also during opioid withdrawal (Bederson et al. 1990; Kaplan and Fields 1991). In the present study, however, heat hypersensitivity of RVM ON-cells was not due to opioid withdrawal, since opioid exposure was sustained throughout the recordings.

Effect of chronic oxycodone treatment on RVM cell discharge has not been studied earlier. In the present study, chronic oxycodone treatment had no significant effect on baseline activity or the response to noxious mechanical or heat stimulation of RVM ON- or OFF-cells. This finding indicates that the antinociceptive oxycodone tolerance shown in behavioral assays when using the currently used chronic oxycodone treatment procedure (Lilius et al. 2018; the present behavioral results) is accompanied by response characteristics mimicking antinociceptive tolerance in RVM neurons. In contrast to chronic morphine treatment, chronic oxycodone did not induce pronociceptive changes in RVM ON- or OFF-cells.

Effect of acute ketamine on discharge of RVM neurons in chronic opioid-treated animals. Role of NMDA receptors in the chronic opioid-induced effects was studied by acute administration of ketamine at a dose that according to the behavioral assay was subantinociceptive in control animals. In general, acute ketamine promoted antinociception, independent of neuron type, submodality of noxious test stimulation or treatment group. The most prominent effect by acute administration of ketamine in the RVM was the reversal of chronic morphine-induced heat hypersensitivity effect in RVM ON- and OFF-cells. Moreover, acute ketamine at a behaviorally subantinociceptive dose induced behavioral antinociception in animals that had developed antinociceptive tolerance to oxycodone as well as morphine. These findings are in line with earlier results showing that ketamine (Lilius et al. 2015, 2018; Shimoyama et al. 1996) and other NMDA receptor antagonists (Tiseo and Inturrisi 1993; Trujillo and Akil 1991, 1994) attenuate the development of morphine and oxycodone tolerance for antinociception to noxious heat. Interestingly, among mechanisms through which acute ketamine has been shown to potentiate MOR-mediated signaling is the enhancement of the levels of MOR-mediated ERK1/2 phosphorylation in heterologous cells expressing MOR; i.e., a non-NMDA receptor-mediated action (Gupta et al. 2011).

In the chronic morphine group, acute ketamine reversed heat hypersensitivity-promoting changes in RVM neurons in parallel with the reversal of antinociceptive morphine tolerance in a behavioral assay of heat nociception. This supports the hypothesis that the RVM, an important relay between the PAG and the spinal cord, exerts a significant role in antinociceptive morphine tolerance and in its attenuation by acute ketamine. Previous studies have shown that microinjections of NMDA receptor antagonists into the RVM can attenuate pain behavior in various experimental models of pain hypersensitivity (e.g., Da Silva et al. 2010; Wei and Pertovaara 1999). Moreover, local administration of an NMDA receptor antagonist prevented noxious chemical stimulation-induced activation of pronociceptive

RVM ON-cells (Xu et al. 2007). Together these findings suggest that the systemic ketamine-induced reversal of the chronic morphine-induced heat hypersensitivity in RVM ON-cells may be due to a direct action of ketamine on the RVM, although we cannot exclude the possibility that ketamine action was at least partly due to block of NMDA receptors upstream e.g. in the PAG (Rodríguez-Muñoz et al. 2012). Interestingly, ketamine at a behaviorally subantinociceptive dose induced a significant antinociceptive change in the discharge of RVM ON- and OFF-cells in the chronic vehicle group suggesting that the RVM discharge was a more sensitive assay for ketamine-induced antinociception than the currently used behavioral assay.

In lightly anesthetized animals of the present study, 10 mg/kg of acute s.c. ketamine alone had no significant antinociceptive effect on the heat-evoked paw withdrawal response. In contrast, some earlier studies in unanesthetized animals have shown that acute ketamine alone may significantly attenuate pain-related behavior in the tail immersion test following s.c. administration at the dose of 10 mg/kg s.c. (Hoffmann et al. 2003) or in the hot-plate test following a dose as low as 5 mg/kg i.v. (Radford et al. 2017). On the other hand, our earlier behavioral results in unanesthetized animals indicate that 10 mg/kg of acute s.c. ketamine alone has no significant effect on the tail-flick test (Lilius et al. 2015, 2018), although in the hot-plate test the effect of ketamine varied from no (Lilius et al. 2015) to weak antinociceptive effect (Lilius et al. 2018).

Mechanisms potentially explaining the partly different effects of morphine and oxycodone in the RVM. Even though both morphine and oxycodone are MOR agonists, they had partly different effects on the discharge of RVM neurons following chronic systemic administrations. This was the case in spite of the fact that the doses of morphine and oxycodone were equianalgesic according to tail-flick and hot plate tests in a previous study

(Lilius et al. 2018). The fact that oxycodone has a lower potency to activate MOR in the PAG and the spinal cord than morphine (Lemberg et al. 2006b) is one potential mechanism explaining the difference. PAG is an important structure for mediating systemic opioid-induced actions (Yaksh 2006) and it influences spinal nociception through a relay in the RVM (Millan 2002; Pertovaara and Almeida 2006). A weaker activation of the PAG by systemic oxycodone than by systemic morphine is expected to produce a weaker drive of the RVM leading to weaker chronic effects on pain-controlling RVM neurons. A weaker MOR-induced activation of the PAG and thereby a weaker descending drive of the RVM in the chronic oxycodone group might explain why RVM cells did not develop heat hypersensitivity as they did in the chronic morphine group. In line with this, when oxycodone and morphine were microinjected into the PAG, the dose of oxycodone producing equal antinociceptive effect as morphine was more than ten times higher (Morgan et al. 2014). Also, following intrathecal administrations to the lumbar level of the spinal cord, the dose of oxycodone needs to be higher than that of morphine to produce an equianalgesic effect (Lemberg et al. 2006b). In contrast, with systemic administrations an equal behavioral antinociceptive effect was obtained, when the dose of oxycodone was about one third of that of morphine (Lilius et al. 2018) suggesting that mechanisms other than activation of brainstem MORs by oxycodone are likely to have a key role in its systemic antinociceptive action. Systemic morphine and oxycodone may also have influenced RVM neurons directly, but even in that case it might be expected that oxycodone's potency to activate MOR in the RVM as well as in the PAG or the spinal cord is lower than that of morphine. However, we cannot exclude the possibility that various other differences in the receptor actions and metabolism of morphine and oxycodone contributed to the differences in their RVM actions in the present study.

It is noteworthy that acute ketamine effectively reversed antinociceptive tolerance in the behavioral assay but had only a weak effect on RVM cell discharge in the chronic

oxycodone group. This finding further supports the proposal that the role of the RVM in oxycodone tolerance markedly differs from the pronociceptive role the RVM has in morphine tolerance. In line with this, an earlier study showed that 10 mg/kg of ketamine increased the brain and serum concentrations of morphine, but not oxycodone (Lilius et al. 2018).

In the clinic, opioid rotation (i.e.; switching from one opioid drug to another or changing an opioid's administration route) has been used with variable success to reduce adverse side-effects as well as opioid tolerance and hyperalgesia (Treillet et al. 2018). Differences in tolerance mechanisms, such as described in the present study between morphine and oxycodone, might be of clinical relevance when choosing opioids for rotation in the treatment of chronic pain.

Limitations. In the present study, noxious heat stimulation consisted of stimuli of 10 s duration delivered to the hind paw with a contact thermostimulator. The stimulus temperature was 54 ^{o}C, which is about 10 ^{o}C above the threshold for nociception. The high intensity of heat stimulation eliciting a limb withdrawal response within 3 s needs to be taken into account when interpreting the following findings.

First, the present study failed to reveal a significant chronic opioid-induced change in the proportion of different RVM cell types, although results of an earlier study indicated that the proportion of RVM NEUTRAL-cells is decreased and that of RVM ON-cells is increased following a 7-day morphine treatment (Meng and Harasawa 2007). The earlier study describing chronic morphine-induced changes in the proportions of RVM cell types used a radiant heat stimulus that produced about 0.5 s longer limb withdrawal latency than in the present study; i.e., the stimulus was weaker than in the present study (Meng and Harasawa 2007). It may be speculated that in the current control group, the high intensity of heat stimulation unmasked ON-cell-like response properties in a subpopulation of NEUTRAL-

cells that had been classified as ON-cells only after chronic morphine treatment when using a lower test stimulus temperature for classification. In line with this explanation, the proportions of RVM NEUTRAL-cells and ON-cells after chronic morphine treatment in the study of Meng and Harasawa (2007) were in the same range as in the vehicle-treated controls of the present study.

Second, high intensity of the currently used heat stimulus may have contributed to the finding that the chronic morphine-induced pronociceptive changes of RVM neurons were observed only with noxious heat but not noxious mechanical stimulation. Namely, the intensity of nociception induced by the currently used heat stimulus was stronger than that induced by the currently used noxious mechanical stimulus (hemostatic clamp pinching the tail) as indicated by behavioral response latencies of 3 s and 10 s, respectively (Kauppila et al. 1998). Thus, it remains to be seen whether a higher intensity of noxious mechanical stimulation might have revealed a pronociceptive action of chronic morphine on mechanically as well as heat-evoked responses of RVM cells.

Third, high intensity of heat stimulation provides a plausible explanation for the failure to observe chronic opioid-induced heat hypersensitivity in the current behavioral assay, limb withdrawal. Namely, the latency of the heat-evoked limb withdrawal in the present control group was so short (3 s) that it may not have been possible to induce a further decrease in the withdrawal latency following chronic opioid treatments (floor effect). In line with this interpretation, behavioral studies demonstrating chronic morphine-induced heat hypersensitivity in unanesthetized animals have used heat stimuli producing considerably longer baseline latencies of the limb withdrawal response (10–20 s; e.g.: Meng and Harasawa 2007; Vanderah et al. 2001). Importantly, since identical test stimuli were used in all chronic treatment groups of the present study, properties of the currently used heat stimulation cannot

explain the marked difference in the heat-evoked responses of RVM cells between the chronic morphine and oxycodone groups.

The current experiments were performed under light pentobarbital anesthesia. It should be noted that anesthesia provides a potential confounding factor for assessment of neuronal responses and drug actions. For example, anesthesia may significantly influence response properties of RVM neurons (Olivéras et al. 1991) and the effect of opioids in the RVM (McGaraughty et al. 1993). Moreover, an earlier study indicated that light pentobarbitone anesthesia markedly suppresses tactile allodynia-like behavior, with little or no influence on the response to noxious mechanical or thermal stimulation (Pertovaara et al. 2001). This may explain the failure to find RVM neurons responding to innocuous brushing in the chronic opioid groups, although in awake animals the RVM is known to drive tactile allodynia-like behavior following chronic morphine treatment (Vanderah et al. 2001) as well as following peripheral nerve injury (Pertovaara et al. 1996). It should also be pointed out that the current experiments were performed in male rats. This also limits interpretations, since opioid effects at least partly vary with the sex (Fullerton et al. 2018).

Conclusions. At a time point when chronic morphine and chronic oxycodone had developed behavioral antinociceptive tolerance, neither of these chronically administered opioids induced antinociception-promoting discharge changes in pain-control cells of the RVM. In contrast, chronic morphine, but not chronic oxycodone, induced pronociceptive discharge changes in RVM cells. Acute ketamine reversed the behavioral antinociceptive tolerance to morphine and oxycodone and importantly, reversed the chronic morphine-induced pronociceptive discharge properties of RVM cells. The most prominent difference in the chronic effect of morphine and oxycodone is that only chronic morphine induced a significant pronociceptive action through an NMDA receptor-dependent descending circuitry

involving the RVM. This descending pronociceptive circuitry is likely to contribute to antinociceptive morphine tolerance and morphine hyperalgesia, but not to oxycodone tolerance.

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DISCLOSURES

The authors have nothing to disclose.

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LEGENDS FOR FIGURES

Figure 1. *Scheme of the chronic opioid experiments*. The ongoing discharge rates, and responses evoked by noxious heat (H) and mechanical stimulation (M) in rostroventromedial medullary neurons as well as noxious heat-evoked limb withdrawal latencies were assessed on day 6 of a continuous treatment period from day 0 onwards with vehicle, morphine (9.6 mg/day) or oxycodone (3.6 mg/day). The testing was replicated 15 min and 30 min after acute subcutaneous (s.c.) administration of ketamine (10 mg/kg). BL_1: baseline ongoing discharge rate 30 s before noxious stimulations, BL_2: baseline ongoing discharge rate 60 s before, 15 min and 30 min after ketamine injection.

Figure 2. *An example of recordings of neuronal responses in the rostroventromedial medulla (RVM).* Traces I– IV. **I**: withdrawal of the stimulated hind limb as assessed by a piezoelectric device (withdrawal shown as a deflection starting from the dotted vertical line that is to the right from the letter A). **II**: the temperature of the contact thermostimulator (baseline 35°C, peak 54°C) applied to the left hind paw. **III**: time-histogram of the noxious heat-evoked neuronal discharge in a pronociceptive RVM ON-cell. **IV**: time-histogram of the heat-evoked neuronal discharge in an antinociceptive RVM OFF-cell. Intervals A–C. A: heat-evoked limb withdrawal latency. B: latency to the onset of the heat-evoked burst discharge (ON-cell) or discharge inhibition (OFF-cell). C: duration of the heat-evoked burst discharge (ON-cell) or discharge inhibition (OFF-cell). The horizontal bar above trace I represents a 3 s time period used in the classification of RVM neurons (beginning 0.5 s before the paw withdrawal). Mean discharge rate was measured before and during noxious heat stimulation.

Figure 3. *A schematic diagram showing the histologically verified recording sites and a photographic example of a recording site in the rostroventromedial medulla*. The arrow head indicates the recording site. The numbers above the schematic graphs represent antero-posterior distances from the ear bar. Brain slice example (lower right corner) was stained with

hematoxylin and eosin. Gi = gigantocellular reticular nucleus; GiA = gigantocellular reticular nucleus, alpha part; Rmg = raphe magnus nucleus.

Figure 4. *Effect of chronic drug administrations on the proportion of rostroventromedial medullary cell types*. Treatment groups were vehicle, morphine (9.6 mg/day) or oxycodone (3.6 mg/day). Cell types are expressed as the mean (+ SD) percentage of the cells recorded *per* rat in each chronic treatment group. N = 9 (vehicle), n = 7 (morphine), n=6 (oxycodone).

Figure 5. *Ongoing discharge rates of pronociceptive rostroventromedial medullary (RVM) ON-cells (**A**) and antinociceptive RVM OFF-cells (**B**).* Effects of chronic treatment with s.c. vehicle, morphine (9.6 mg/day) and oxycodone (3.6 mg/day) on ongoing discharge rates before, 15 and 30 minutes after s.c. acute ketamine (10 mg/kg) injections. Data represent mean + SD. The numbers of the registered cells were 13–21 (A) and 7–13 (B). * $P < 0.05$ (t-test with Bonferroni correction).

Figure 6. *Heat-evoked (**A–C**) and mechanically evoked (**D–F**) responses of pronociceptive rostroventromedial medullary ON-cells*. The effects of chronic treatment with s.c. vehicle, morphine (9.6 mg/day), and oxycodone (3.6 mg/day) on the stimulus-evoked discharges (**A, D**), on the latency to the onset of the stimulus-evoked burst discharge (**B, E**) and on the duration of the stimulus-evoked burst discharge (**C, F**) before, 15 and 30 minutes after s.c. acute ketamine (10 mg/kg) injections. Figure A and D show the effects of stimulus-evoked discharge rate changes; i.e., stimulus-evoked discharge rate minus ongoing discharge rate. Decreases in mechanically evoked discharge rate changes (D) are considered to reflect antinociception. Increases in heat-evoked discharge rate changes (A) are considered to reflect increased nociception. Increases in the latency to the onset of the stimulus-evoked burst discharge (B, E) and decreases in the duration of the stimulus-evoked burst discharge (D, F) are considered to reflect antinociception. Data represent mean + SD. The numbers of the registered cells were 13–21 (A–C) and 9–17 (D–F). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t-test

with Bonferroni correction), # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ (t-test with Bonferroni correction, reference: the corresponding value without ketamine treatment).

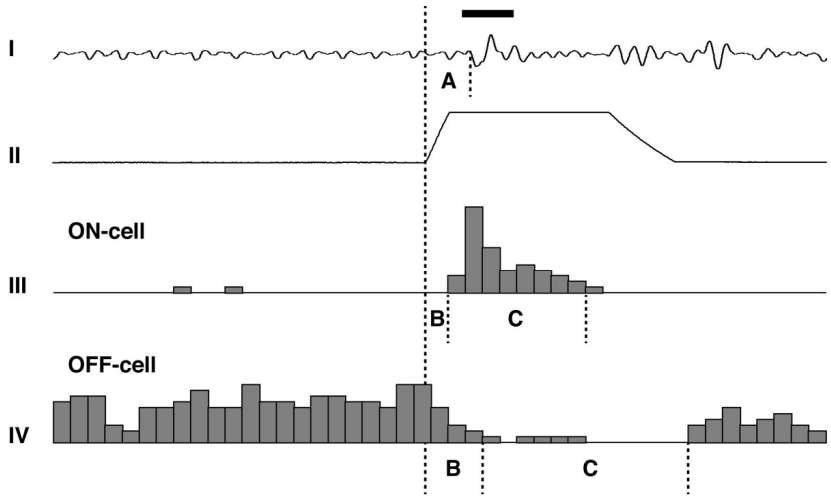
Figure 7. *An example of simultaneous original recordings of one ON-cell and one OFF-cell in the rostroventromedial medulla.* Effect of chronic treatment with s.c. morphine (9.6 mg/day) on the pronociceptive ON-cell and antinociceptive OFF-cell and their modulation by ketamine (10 mg/kg). Traces I– IV. **I**: raw data of neuronal recordings. **II**: time-histogram of an ON-cell and an OFF-cell discharge. The vertical bars represent discharge rate of 40 Hz. **III**: the temperature of the contact thermostimulator (baseline 35°C, peak 54°C) applied to the left hind paw. **IV**: withdrawal of the stimulated hind limb as assessed by a piezoelectric device. The horizontal bar indicates the duration of 10 s. The arrow indicates the start of the limb withdrawal. In I, switching on and off the thermal device caused the long vertical spike artefacts. Insets show templates of the recorded action potentials.

Figure 8. *Heat-evoked (**A–B**) and mechanically evoked (**C–D**) responses of antinociceptive rostroventromedial medullary OFF-cells*. The effects of chronic treatment with s.c. vehicle, morphine (9.6 mg/day) and oxycodone (3.6 mg/day) on the latency to the onset of the stimulus-evoked discharge inhibition (**A, C**) and on the duration of the stimulus-evoked discharge inhibition (**B, D**) before, 15 and 30 minutes after s.c. acute ketamine (10 mg/kg) injections. Increases in the latency to the onset of the noxious stimulus-evoked discharge inhibition (A, C) and decreases in the duration of the noxious stimulus-evoked burst inhibition are considered to reflect antinociception (B, D). Data represent mean + SD. The numbers of the registered cells were 7–13 (A, B) and 4–9 (C, D). * $P < 0.05$ (t-test with Bonferroni correction). # $P < 0.05$, ## $P < 0.01$, #### $P < 0.0001$ (t-test with Bonferroni correction, reference: the corresponding value without ketamine treatment).

Figure 9. *Noxious heat-evoked limb withdrawal latencies before and after acute ketamine following chronic treatments*. Chronic treatment groups were vehicle, morphine (9.6 mg/day) or oxycodone (3.6 mg/day) followed by administration of ketamine (10 mg/kg). Data represent mean + SD. N = 9 (vehicle+ketamine), n = 9 (morphine+ketamine), n = 6 (oxycodone+ketamine).

* P<0.05, ** P<0.01 (t-test with Bonferroni correction). ## $P < 0.01$, #### $P < 0.0001$ (t-test with Bonferroni correction, reference: the corresponding value without ketamine treatment). Increase in withdrawal latency represents antinociception.

Figure 1.



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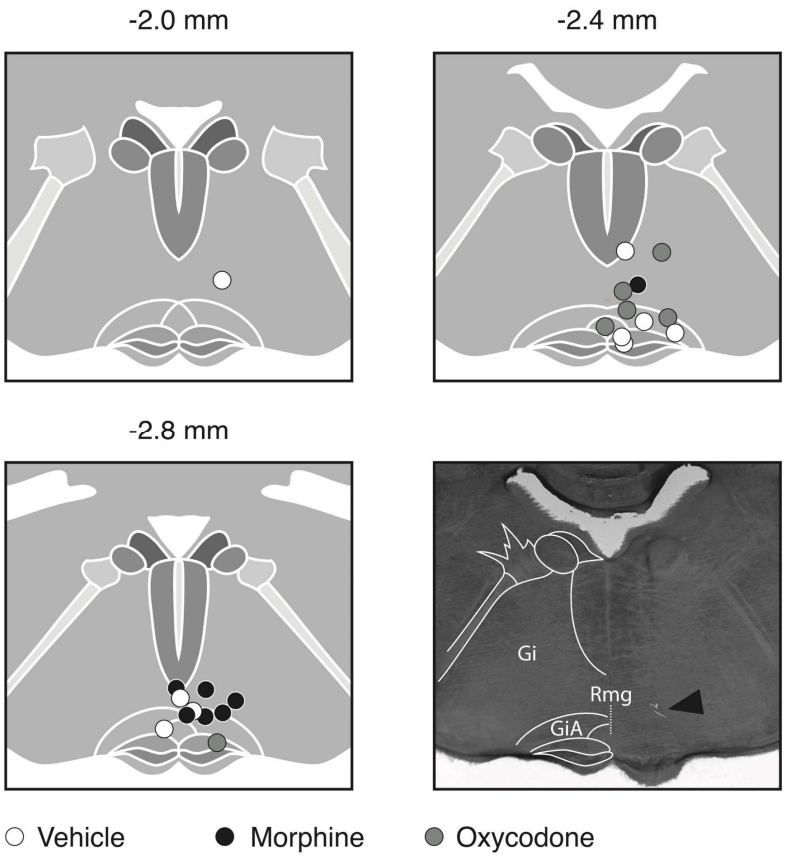

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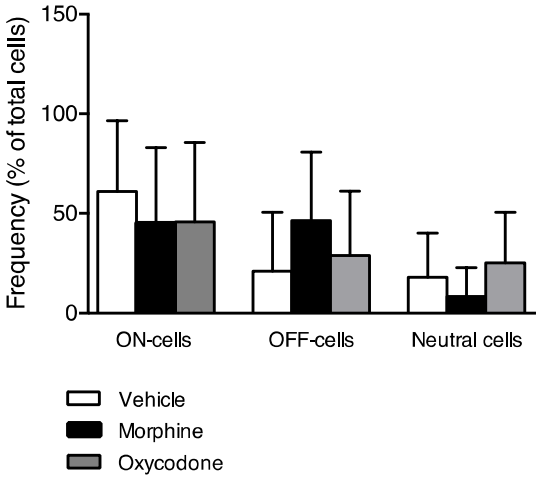

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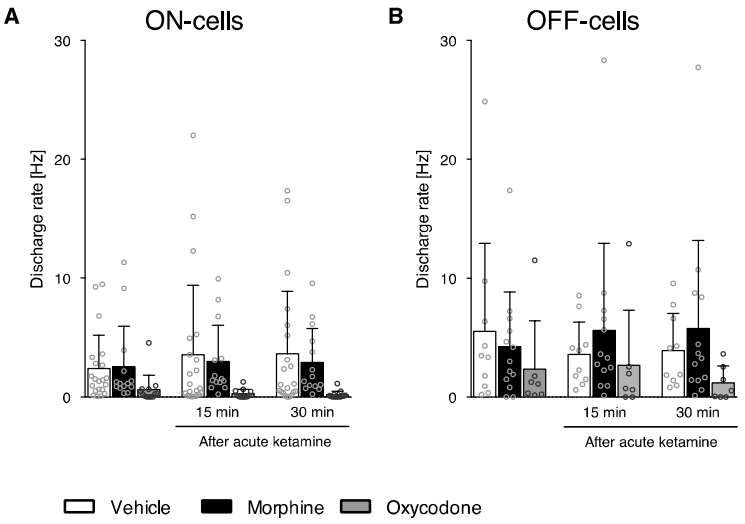

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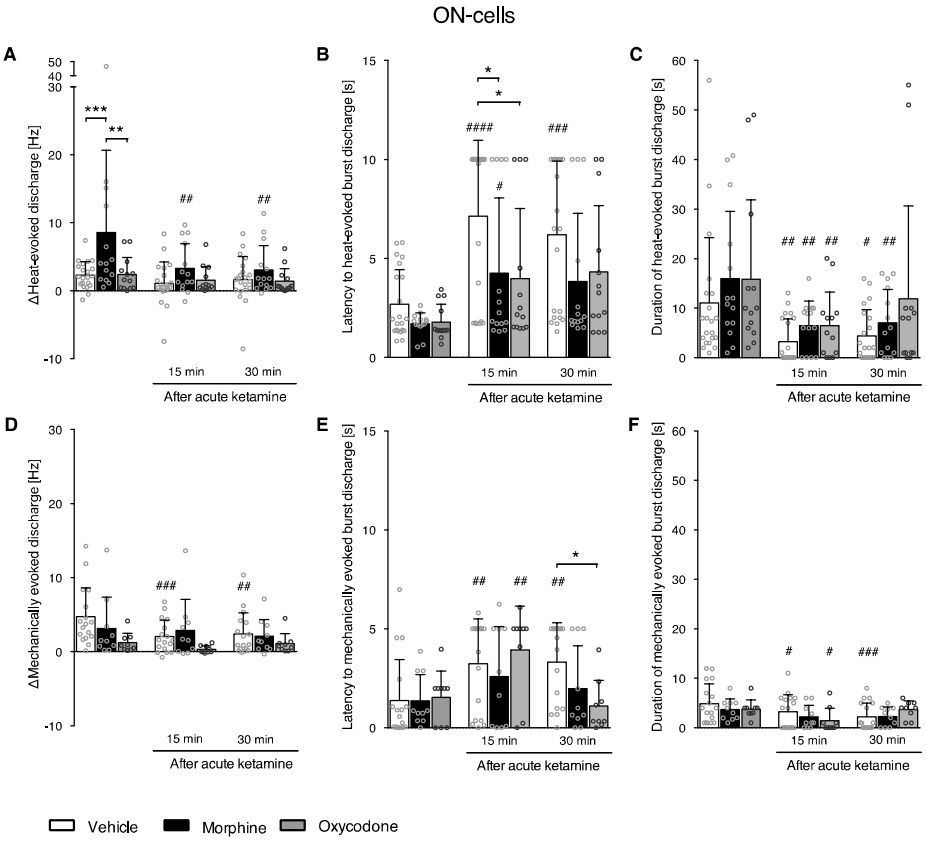

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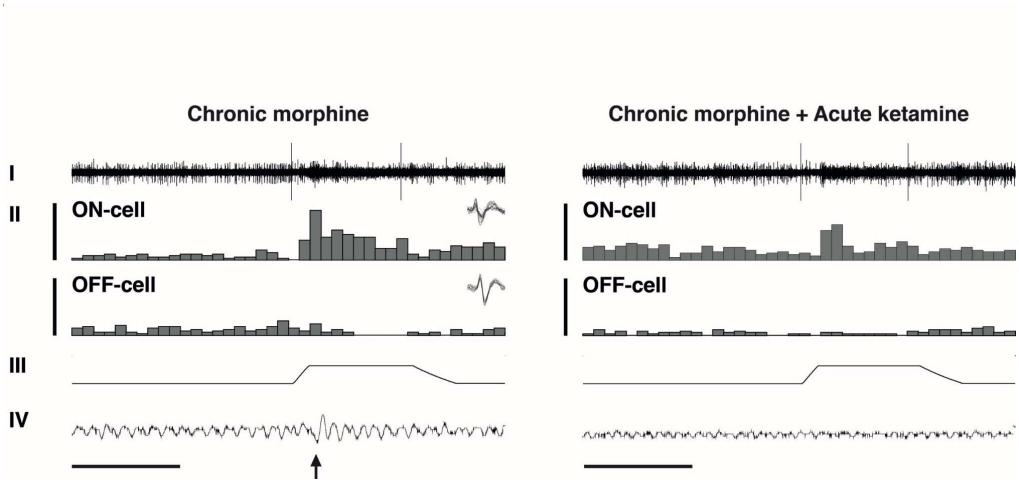

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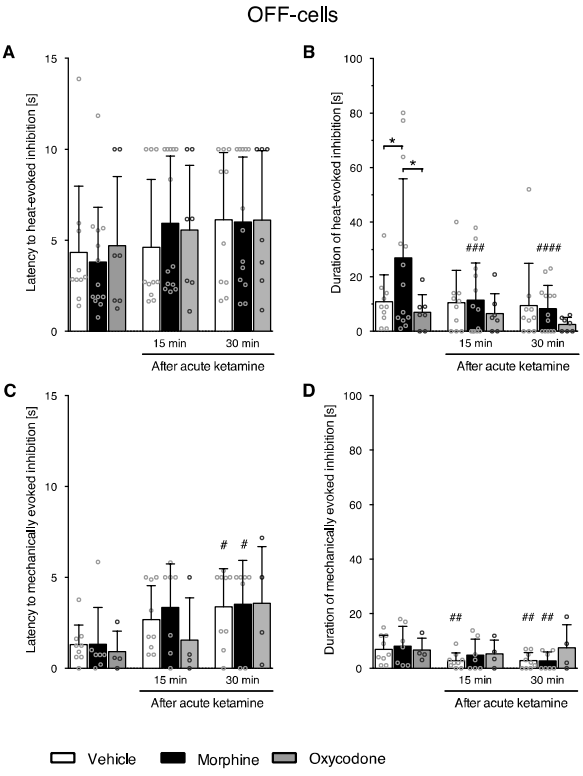

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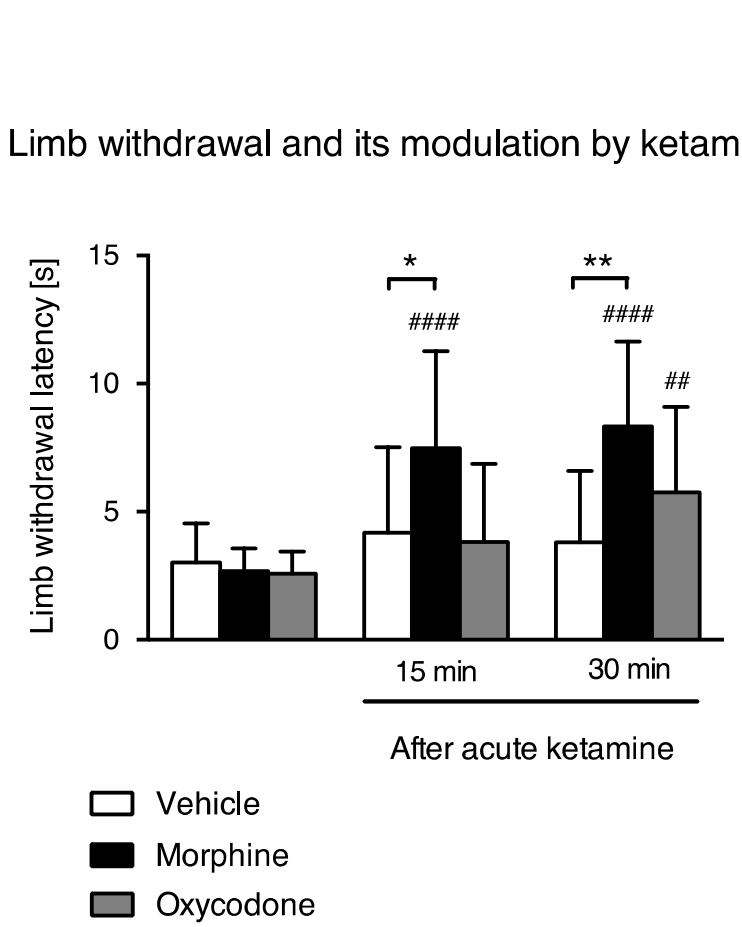
Limb withdrawal and its modulation by ketamine
Limb withdrawal latency [s]
15
10
5
0
*
**
####
####
##
15 min
30 min
After acute ketamine
Vehicle
Morphine
Oxycodone

Figure 9.