Research Report

An anti-Parkinson drug ropinirole depletes orexin from rat hypothalamic slice culture

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ABSTRACT

Non-ergot type dopamine receptor agonists such as ropinirole are used for the treatment of Parkinson disease, but they occasionally show serious side effects including sleep attacks and daytime sleepiness. These symptoms are reminiscent of narcolepsy, a major sleep disorder. Because narcolepsy is thought to result from deficiency of a hypothalamic neuropeptide orexin, we examined whether ropinirole affected the integrity of orexin-containing neurons, using organotypic slice culture of rat hypothalamus. Application of ropinirole induced a significant decrease in the number of orexin-immunoreactive neurons. The same treatment showed no significant effect on the number of melanin-concentrating hormone-immunoreactive neurons. The decrease of orexin-immunoreactive neurons was reversible after washout of ropinirole and was not accompanied by induction of cell death. Antagonism of dopamine D_2 receptors and of serotonin 5-HT_{1A} receptors attenuated the effect of ropinirole, suggesting involvement of these receptors in depletion of orexin. On the other hand, a moderate concentration of *N*-methyl-D-aspartate that excited orexin neurons counteracted the effect of ropinirole on the number of orexin-immunoreactive neurons. These results suggest that ropinirole can cause deficiency of orexin by inhibiting excitatory activities of orexin neurons, which may be relevant to the adverse actions of this drug on sleep and wakefulness.

Key words: hypocretin; melanin-concentrating hormone; sleep attack; ropinirole; talipexole; pramipexole; c-Fos

1. Introduction

Parkinson disease is characterized by a progressive decrease of midbrain dopaminergic neurons and resultant shortage of dopamine in the striatum. Besides levodopa as a dopamine precursor, dopamine receptor agonists are useful drugs for the therapy of Parkinson disease (Katzenschlager and Lees, 2002; Foley et al., 2004). That is, stimulation of dopamine D_2 receptors in the striatum normalizes the balance between the direct and the indirect pathways of the extrapyramidal system to alleviate the symptoms associated with the disease such as akinesia, rigidity and tremor. Historically, ergot-type dopamine receptor agonists including bromocriptine and pergolide have been used either alone or in combination with levodopa. A problem associated with the usage of these drugs is frequent occurrence of adverse actions that mainly appear as gastrointestinal damages and cardiac valvular disease (Horvath et al., 2004).

An alternative choice of dopamine agonists is non-ergot type compounds such as ropinirole, pramipexole and talipexole (Hobson et al., 1999). In contrast to ergot-type drugs that have mixed agonist activity on D_1 and D_2 receptors, non-ergot compounds selectively stimulate D_2 family receptors and have little adverse actions compared to ergot-type drugs (Steiger et al., 2009). However, these compounds still produce several undesirable effects. A characteristic case is the occurrence of sudden sleep attacks and daytime sleepiness that can lead to automobile accidents, although the cellular mechanisms of this action are unknown (Frucht et al., 1999; Paus et al., 2003).

Sudden sleep attacks and excessive daytime sleepiness are characteristic symptoms also observed in narcolepsy patients. Narcolepsy is a neurological disorder associated with disturbance of sleep and wakefulness (Scammell, 2003). Neuropathological studies in the patients as well as genetic and neurobiological studies in animals have shown that deficiency of orexins (also called hypocretins) is a culprit of this neurological disorder (Chemelli et al., 1999; Nishino et al., 2001). Orexin-A and -B, neuropeptides derived from a common

precursor peptide preproorexin, are produced by a small population of neurons located in the lateral hypothalamic area and the perifornical region of the hypothalamus (Sakurai, 2007). Orexin concentrations in the cerebrospinal fluid are decreased to undetectable level in the majority of narcolepsy patients (Nishino et al., 2001), and a substantial decrease in orexin neurons is found in postmortem brain of the patients (Peyron et al., 2000; Thannickal et al., 2000). The decrease is specific to orexin neurons because melanin-concentrating hormone (MCH)-containing neurons in the hypothalamus show no change.

During investigations aimed to elucidate the mechanisms of loss of orexin neurons, we have found that orexin neurons are vulnerable to excitotoxicity compared to MCH neurons (Katsuki and Akaike, 2004). On the other hand, we also found that inhibition of neural activities led to reversible depletion of orexin immunoreactivity from hypothalamic slice culture (Michinaga et al., 2010). These findings suggest that alterations of neuronal excitability in either direction can lead to dysfunction of orexin neurons. Based on these precedent findings, we investigated whether a non-ergot type dopamine receptor agonist, ropinirole, could affect the integrity of hypothalamic orexin neurons.

2. Materials and methods

2.1. Preparation of hypothalamic slice culture

Organotypic slice cultures of rat hypothalamus were prepared as described in our previous studies (Katsuki and Akaike, 2004; Michinaga et al., 2010). Experimental procedures were approved by our institutional ethical committee concerning animal experiments, and animals were treated in accordance with the guidelines of the U.S. National Institutes of Health regarding the care and use of animals for experimental procedures. Briefly, brains were obtained from 7 to 8 day-old Wistar rats (Nihon SLC, Shizuoka, Japan) under deep anesthesia with diethyl ether and cut into two hemispheres under sterile conditions. Then, coronal

brain slices of 300 μm thickness were prepared with a tissue chopper. Five consecutive hypothalamic slices containing the lateral hypothalamic area were placed onto a culture insert with microporous membrane (Millicell-CM; Millipore, Bedford, MA, USA) and cultivated in a six-well culture plate. Culture medium consisted of 50% minimum essential medium with Earle's salts (Gibco, Invitrogen Japan, Tokyo, Japan), 25% Hanks' balanced salt solution (Gibco), and 25% horse serum (Gibco) with 6.5 mg/ml glucose, 2 mM L-glutamine, 100 U/ml penicillin G potassium, and 100 μg/ml streptomycin sulfate (Gibco). The medium was supplied at 0.9 ml/well and replaced by fresh one on the next day of culture preparation, and then, twice a week. Slices were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 34 °C for 18 days.

2.2. Drug treatment

At 18 days in vitro, drug treatment was achieved by transfer of culture inserts into wells containing fresh medium with added drugs. In several sets of experiments, post-incubation for 72 h in drug-free culture medium was performed after 72 h of drug treatment.

Ropinirole was obtained from LKT Laboratories Inc. (St. Paul, MN, USA). Talipexole and pramipexole were provided by Boehringer Ingelheim (Ingelheim, Germany). Sulpiride, picrotoxin, (+)-MK-801 hydrogen maleate and propidium iodide were from Sigma Aldrich Chemicals (St. Louis, MO, USA). *N*-methyl-D-aspartate (NMDA) and pindolol was from Wako Chemicals (Osaka, Japan). Yohimbine was from Nacalai Tescue (Kyoto, Japan). (*S*)-WAY 100135 dihydrochloride was from Tocris Bioscience (Ellisville, MO, USA).

2.3. Histochemical examinations

After drug treatment, double immunofluorecence histochemistry against orexin and MCH was performed, which was followed by cell counting of immunoreactive neurons, as described (Katsuki and Akaike, 2004; Michinaga et al., 2010). Goat polyclonal anti-orexin

A (C-19) antibody (1:150; sc-8070, Santa Cruz Biotech., Santa Cruz, CA, USA) and rabbit anti-MCH antiserum (1:800; H-070-47, Phoenix Pharmaceuticals Inc., Belmont, CA, USA) were used as primary antibodies. Secondary antibodies were Alexa Fluor 488-labeled donkey polyclonal anti-goat IgG (H+L) antibody (1:200; A-11055, Molecular Probes, Eugene, OR, USA) and Alexa Fluor 594-labeled donkey polyclonal anti-rabbit IgG antibody (1:500; A21707, Molecular Probes). An epifluorescence microscope was used to count the number of immunopositive cells in an area of $420 \times 640\ \mu m^2$ in each slice. When c-Fos expression was examined, anti-c-Fos (Ab-5) (4-17) rabbit pAb (1:500, Calbiochem) was used as a primary antibody instead of anti-MCH antiserum. Laser-scanning confocal microscope system (Fluoview FV300; Olympus, Tokyo, Japan) was used to examine co-localization of immunoreactivities against orexin and c-Fos, and also to obtain clear images of immunoreactive cells as presented in figures.

Propidium uptake into dead cells was used to address whether cell death was induced in orexin neurons (Michinaga et al., 2010). Propidium iodide (5 μg/ml) was applied to slice cultures during drug treatment. After treatment, slices were processed for orexin immunofluorescence histochemistry, and fluorescence signals were examined by laser-scanning confocal microscopy.

2.4. Statistical Analysis

Data on the number of immunoreactive cells were expressed as means ± SEM, the mean number of immunoreactive cells in control slices being set at 100%. Statistical significance of difference between groups was evaluated by one-way analysis of variance, followed by Student-Newman-Keuls tests. Probability values less than 0.05 were considered significant.

3. Results

3.1. Ropinirole causes a selective decrease of orexin-immunoreactive neurons

Ropinirole at various concentrations (50 μM – 1 mM) was applied for 72 h to hypothalamic slice cultures. Examinations of immunoreactivities against orexin and MCH revealed that ropinirole caused a concentration-dependent decrease in the number of orexin-immunoreactive neurons. The effect of ropinirole reached statistical significance at 100 μM (Fig. 1A, B). Notably, the number of MCH-immunoreactive neurons within the same slice cultures showed no change after treatment with ropinirole. In the following sets of experiments we used ropinirole at 1 mM, a concentration that exhibited most potent effect on orexin neurons.

Next we examined the time-course of the effect of ropinirole, by varying the period of drug treatment from 6 h to 72 h. A small but significant decrease in the number of orexin-immunoreactive neurons was evident already at 6 h after initiation of ropinirole treatment. The number of orexin-immunoreactive neurons gradually decreased in a time-dependent manner, and reached a plateau level at 24 h (Fig. 1C).

We also tested the effect of talipexole and pramipexole, other non-ergot drugs used for the treatment of Parkinson disease. Application of these drugs at 1 mM for 24 – 72 h caused a significant decrease in the number of orexin-containing neurons, although the degree of the decrease was smaller than that induced by ropinirole (Fig. 1D). Both talipexole and pramipexole showed little effect on the number of MCH-containing neurons.

3.2. Depletion of orexin, but not cell death, is responsible for the decrease of orexin immunoreactivity

Disappearance of orexin immunoreactivity may result either from cell death of orexin neurons or from depletion of the peptide normally present in orexin neurons. To distinguish these possibilities, we examined whether the effect of ropinirole on the number of orexin-immunoreactive neurons was reversible after washout of the drug. In this

experiment, a group of slices received treatment with 1 mM ropinirole for 72 h, then cultured in drug-free medium for additional 72 h. Consistent with the results shown in the previous section, slices examined promptly after 72 h treatment with ropinirole exhibited a significant decrease in the number of orexin-immunoreactive neurons. In contrast, the number of orexin-immunoreactive neurons in slices examined after 72 h of post-incubation did not show any significant changes compared to that in control slices (Fig. 2A, B). This result indicates that the decrease of orexin-immunoreactive neurons was reversible.

To further verify if the decrease of orexin immunoreactivity was irrelevant to cell death events, we examined propodium uptake into ropinirole-treated slice cultures. Propidium iodide is conventionally used to detect dead cells as this reagent passes damaged cell membrane of dead cells, binds to nuclear DNA and exhibits bright fluorescence (Lossi et al., 2009). We confirmed that, as a positive control, NMDA (60 μM) induced a robust increase in the number of propidium-positive nuclei. In sharp contrast, treatment with ropinirole for 24 - 72 h was not accompanied by appearance of propidium-positive cells (Fig. 2C).

3.3. Dopamine D_2 and serotonin 5-HT_{1A} receptors are involved in depletion of orexin

Ropinirole is known as an agonist selective for dopamine D_2 receptors (Ravikumar and Sridhar, 2006). To address whether the effect of ropinirole on orexin neurons was mediated by activation of D_2 receptors, we examined potential counteraction of the effect of ropinirole by sulpiride, a D_2 receptor antagonist. As shown in Fig. 3A, co-appilication of sulpiride (50 – 200 μM) significantly attenuated the effect of ropinirole. Sulpiride (200 μM) alone showed no effect on the number of orexin-immunoreactive neurons.

Although ropinirole is selective for D_2 receptors, it is uncertain whether the drug retains receptor specificity at a concentration as high as 1 mM (Hobson et al., 1999; Gerlach et al., 2003). Therefore, we next examined the effects of several antagonists at neurotransmitter receptors that have been reported to inhibit electrical excitability of orexin neurons (Li et al.,

2002; Muraki et al., 2004; Li and van den Pol, 2005). Antagonists used were picrotoxin (for $GABA_A$ receptors), yohimbine (for α_2 adrenergic receptors) and pindolol (for serotonin $5\text{-}HT_{1A}$ receptors). We found that pindolol (30 μM) partially but significantly attenuated the effect of ropinirole, whereas picrotoxin (30 μM) and yohimbine (5 μM) showed no significant effect (Fig. 3B). Because pindolol blocks β adrenergic receptors as well as $5\text{-}HT_{1A}$ receptors, we also examined the effect of WAY 100135, a selective $5\text{-}HT_{1A}$ receptor antagonist. WAY 100135 (100 nM) significantly attenuated the effect of ropinirole on the number of orexin-immunoreactive neurons (Fig. 3C). These results indicate that $5\text{-}HT_{1A}$ receptor stimulation may contribute to the decrease of orexin immunoreactivity induced by ropinirole.

3.4. Stimulation of NMDA receptors prevents depletion of orexin by ropinirole

A previous study has indicated that D_2 receptor stimulation inhibits neural activities of orexin neurons by presynaptically suppressing excitatory inputs to these neurons (Alberto et al., 2006). The major excitatory inputs to orexin neurons are glutamatergic neurons (Horvath and Gao, 2005; Sakurai, 2007). In addition, we reported that NMDA receptor stimulation supported orexin expression in hypothalamic slice culture (Michinaga et al., 2010). Accordingly, we examined whether activation of NMDA receptors could counteract depletion of orexin by ropinirole. We used a moderate concentration of NMDA (20 μM) that showed little cytotoxicity within a period of 24 h (Katsuki and Akaike, 2004). Immunostaining against c-Fos, a marker of neuronal excitation, confirmed that NMDA induced a robust increase in c-Fos-positive orexin neurons after 24 h. The effect of NMDA on c-Fos expression was observed even in the presence of ropinirole, although the effect was somewhat weak as compared to that in the absence of ropinirole. Ropinirole alone tended to decrease the percentage of c-Fos-positive orexin neurons (Fig. 4A, C). Under these conditions, we observed that co-application of NMDA for 24 h significantly attenuated the

effect of ropinirole on the number of orexin-immunoreactive neurons (Fig. 4B). To further clarify the relationship between glutamatergic system and the effect of ropinirole, we examined the effect of MK-801, a non-competitive NMDA receptor antagonist, in combination with ropinirole. Application of MK-801 (10 μM) alone for 24 h significantly decreased the number of orexin-immunoreactive neurons, which is consistent with our previous report showing the effect of 72-h treatment with MK-801 (Michinaga et al., 2010). Importantly, in the presence of 1 mM ropinirole, MK-801 did not decrease the number of orexin-imunoreactive neurons further (Fig. 4D). These results suggest that inhibition of glutamatergic system underlies the effect of ropinirole with respect to depletion of orexin.

4. Discussion

Ropinirole has been used for treatment of Parkinson disease, and promotion of sleepiness is well recognized as a characteristic adverse action of this drug (Frucht et al., 1999). Reportedly, about 10% of patients treated with combination of ropinirole and levodopa experience sleep attacks (Paus et al., 2003). In the present study, we examined whether ropinirole could affect hypothalamic orexin neurons, because orexin plays an important role in maintenance of the arousal state. We demonstrated here that ropinirole decreased orexin-immunoreactive neurons in organotypic slice culture of rat hypothalamus. The effect was selective for orexin neurons, because MCH neurons in the same culture were not affected by this drug.

The decrease of orexin-immunoreactive neurons was attributable to depletion of orexin peptide stored within the neurons, rather than to induction of cell death. This view was supported by the observations that the effect of ropinirole was reversible after washout, and that ropinirole did not increase propidium-stained cells indicative of the occurrence of cell death. Interestingly, these observations are similar to those of our previous study showing

the effect of inhibition of neural activity (Michinaga et al., 2010). That is, treatment of hypothalamic slice cultures with tetrodotoxin or elevated extracellular Mg^{2+} caused reversible depletion of orexin, without affecting MCH. NMDA receptor antagonists also decreased orexin immunoreactivity, suggesting that spontaneous activity of glutamatergic excitatory inputs plays an important role in the maintenance of orexin expression (Michinaga et al., 2010). Notably, stimulation of D_2 receptors has been shown to reduce the frequency of miniature excitatory postsynaptic currents recorded from orexin neurons in acute hypothalamic slices (Alberto et al., 2006). Therefore, depletion of orexin by ropinirole may be caused by D_2 receptor-mediated presynaptic suppression of excitatory inputs to orexin neurons. Indeed, the effect of ropinirole and that of MK-801 on the number of orexin-immunoreactive neurons were not additive, suggesting that these drugs affect common pathways to deplete orexin. In this context, we have previously demonstrated that ω-conotoxin MVIIC, a blocker of N- and P/Q-type Ca^{2+} channels that mediate presynaptic Ca^{2+} entry, also decreases the number of orexin-immunoreactive neurons (Michinaga et al., 2010). Orexin neurons themselves do not appear to possess dopamine D_2 receptors (Sakurai, 2007): mRNAs encoding D_2 receptors are rarely expressed in the lateral hypothalamus / perifornical area where orexin neurons are located (Bubser et al., 2005).

Similar to ropinirole, other D_2 receptor agonists such as talipexole and pramipexole induced a selective decrease of orexin-immunoreactive neurons, supporting the notion that the effect of ropinirole was mainly mediated by D_2 receptors. Another line of evidence for involvement of D_2 receptors is that the effect of ropinirole was largely attenuated by sulpiride, a D_2 receptor antagonist. On the other hand, we also suggest that 5-HT_{1A} receptors are partly involved in the observed effect of ropinirole, because pindolol and WAY 100135 partially but significantly reversed the decrease of orexin immunoreactivity by ropinirole. Indeed, ropinirole exhibits weak affinity to 5-HT_{1A} receptors (Newman-Tancredi et al., 2002). 5-HT_{1A} receptors are expressed on orexin neurons, and application of 5-HT_{1A} receptor

agonist causes hyperpolarization of orexin neurons (Muraki et al., 2004). Therefore, 5-HT_{1A} receptor stimulation by high concentrations of ropinirole may inhibit the excitatory activity of orexin neurons postsynaptically, accelerating the decrease of orexin in combination with D_2 receptor-mediated presynaptic inhibition of excitatory inputs.

The counteracting effect of NMDA lends further support for the proposal that inhibition of excitatory neural activities of orexin neurons is responsible for ropinirole-induced depletion of orexin. NMDA was previously shown to reverse tetrodotoxin-induced depletion of orexin from hypothalamic slice culture (Michinaga et al., 2010). We showed here that NMDA was also effective in preventing the decrease in orexin immunoreactivity by ropinirole. As revealed by induction of c-Fos expression, direct stimulation of postsynaptic NMDA receptors produced robust excitation of orexin neurons, overcame inhibition by ropinirole and thereby supported orexin expression in the presence of ropinirole.

Detailed mechanisms of activity-dependent regulation of orexin expression remain unsolved. As discussed in our previous report (Michinaga et al., 2010), Ca^{2+}-dependent transcriptional regulation by monocyte enhancer factor 2 (MEF2) is a candidate mechanism, since a consensus site for the binding of MEF2 has been found in the promoter region of preproorexin gene (Kato et al., 2007). Other kinds of Ca^{2+}-dependent pathways such as Ca^{2+}/calmodulin-dependent activation of CREB, might also be involved in regulation of preproorexin gene expression (Greer and Greenberg, 2008). Notably, orexin neurons show frequent spike activity during waking, whereas they are relatively silent during sleep (Lee et al., 2005; Mileykovskiy et al., 2005). Therefore, possible diurnal variations of orexin expression in vivo might be an interesting issue to be determined. As for in vitro preparations, orexin neurons in acute hypothalamic slices exhibit frequent spontaneous activity as if they are in waking state in vivo (Eggermann et al., 2003).

At present, we do not directly relate the present findings to clinical conditions because ropinirole at or over 100 μM, concentrations that were beyond the therapeutic range, was

required to significantly affect the number of orexin-immunoreactive neurons. However, the observed effect of ropinirole on orexin neurons was mainly mediated by the principal action of this drug as anti-Parkinson drug, i.e., stimulation of dopamine D_2 receptors. Provided that suppression of presynaptic glutamate release was responsible for the effect of ropinirole, the effect might have been diminished in slice cultures that have lost several, if not all, neural connections present in vivo. Moreover, the effect of ropinirole was determined within a short period of 72 h in the present study, whereas long-term treatment for months or years is common in the case of treatment of Parkinson disease. Interestingly, several reports have shown that the hypothalamus of Parkinson disease patients exhibits a reduced number of orexin neurons as compared to that of age-matched controls (Fronczek et al., 2007; Thannickal et al., 2007). Altogether, decreased levels of orexin expression induced by long-term treatment with non-ergot D_2 receptor agonists, in conjunction with a decreased number of orexin neurons in Parkinson disease patients, might result in severe deficiency of orexin that leads to failure to maintain the arousal state. Examination of the effect of these drugs on orexin neurons in vivo, either in normal animals or in Parkinson disease model animals, is a next step to substantiate this proposal.

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Figure Legends

Fig. 1. Ropinirole and other non-ergot dopamine receptor agonists induce a selective decrease of orexin neurons in hypothalamic slice culture. (A) Representative photographs of orexin and MCH-immunoreactive neurons in hypothalamus slice culture. Ropinirole at indicated concentrations was applied for 72 h. Scale bars = 50 μm. (B) Concentration-dependency of the effect of ropinirole applied for 72 h on the number of orexin- and MCH-immunoreactive neurons. (C) Time-course of the effect of 1 mM ropinirole on the number of orexin-immunoreactive neurons. (D) Selective decrease of orexin neurons by 1 mM talipexole and pramipexole. Drugs were applied for indicated periods. n = 12-18 for each condition in panels B-D. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

Fig. 2. Decrease of orexin neurons is reversible and does not accompany cell death. (A) Photographs showing orexin-immunoreactive neurons in slices cultured under control conditions (Control), treated with 1 mM ropinirole for 72 h (Ropinirole), or cultured for 72 h after 72-h treatment with 1 mM ropinirole (72 h post). Scale bar = 50 μm. (B) Quantitative results of the effect of post-incubation. n = 12 for each condition. $^{***}p < 0.001$ vs control; $^{\#\#\#}p < 0.001$. (C) NMDA, but not ropinirole, induces cell death. Ropinirole (1 mM) or NMDA (60 μM) was applied for 24 or 72 h as indicated. Green signals correspond to orexin immunoreactivity, and red signals correspond to propidium iodide (PI) fluorescence indicative of cell death. Scale bar = 50 μm.

Fig. 3. D_2 and $5\text{-}HT_{1A}$ receptors are involved in the decrease of orexin. (A) Effect of sulpiride. Sulpiride at indicated concentrations was applied for 72 h concomitantly with 1 mM ropinirole, and the number of orexin-immunoreactive neurons was determined. n = 12-18 for each condition. $^{***}p < 0.001$ vs control (no drug treatment); $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ vs ropinirole alone. (B) Effects of 30 μM picrotoxin (PTX), 5 μM yohimbine (Yoh) and 30

μM pindolol (Pind). Drugs were applied for 72 h concomitantly with 1 mM ropinirole. n = 12-18 for each condition. $^{***}p < 0.001$ vs control (Cont); $^{\#}p < 0.05$. (C) Effect of 100 nM WAY 100135 (WAY). WAY 100135 was applied for 24 h concomitantly with 1 mM ropinirole. n = 15 for each condition. $^{***}p < 0.001$ vs control (Cont); $^{\#\#\#}p < 0.001$ vs ropinirole alone.

Fig. 4. Stimulation of NMDA receptors activates orexin neurons and prevents depletion of orexin. (A) Double immunofluorescence staining against orexin (green) and c-Fos (red). Slices were treated for 24 h with 1 mM ropinirole, either alone or in combination with 20 μM NMDA. Note that NMDA induced a robust increase in c-Fos-positive orexin neurons. Scale bars = 50 μm. (B) Effect of NMDA on ropinirole-induced decrease in the number of orexin-immunoreactive neurons. Slices were treated with 20 μM NMDA and 1 mM NMDA for 24 h. (C) Percentage of c-Fos-positive cells within orexin-immunoreactive neurons, after treatment for 24 h with 1 mM ropinirole and 20 μM NMDA, either alone or in combination. n = 18 for each condition in panels B and C. $^{*}p < 0.05$; $^{***}p < 0.001$ vs. control (Cont); $^{\#\#\#}p < 0.001$. (D) Effect of combined treatment with MK-801 and ropinirole on the number of orexin-immunoreactive neurons. MK-801 (10 μM) was applied for 24 h, either alone or concomitantly with 1 mM ropinirole. n = 15 for each condition. $^{\#\#\#}p < 0.001$ vs. control (Cont); n.s., not significant.