

Clozapine Blocks Sympathetic and Thermogenic Reactions Induced by Orexin A in Rat

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Summary

This experiment tested the effect of clozapine on the sympathetic and thermogenic effects induced by orexin A. The firing rates of the sympathetic nerves to interscapular brown adipose tissue (IBAT), along with IBAT and colonic temperatures were monitored in urethane-anesthetized male Sprague-Dawley rats before and for 5 h after an injection of orexin A (1.5 nmol) into the lateral cerebral ventricle. The same procedure was carried out in rats treated with orexin A plus an intraperitoneal administration of clozapine (8 mg/kg bw), an atypical antipsychotic that is largely used in the therapy of schizophrenia. The same variables were monitored in rats with clozapine alone. A group of rats with saline injection served as control. The results show that orexin A increases the sympathetic firing rate, IBAT and colonic temperatures. Clozapine blocks completely the reactions due to orexin A. These findings suggest that clozapine influences strongly the thermogenic role of orexin A. Furthermore, the remarkable hyperthermic role played by orexin A is confirmed.

Key words

Body temperature • Clozapine • Orexin A • Rat • Sympathetic activity

Introduction

Orexin A is a novel hypothalamic neuropeptide (De Lecea *et al.* 1998), which was named after its influence on eating behavior (Lubkin and Stricker-Krongrad 1998, Wolf 1998, Sweet *et al.* 1999). The orexin A influences various physiological variables. An intracerebroventricular (icv) injection of orexin A induces an increase in heart rate, blood pressure (Shirasaka *et al.* 1999), and metabolic rate (Lubkin and Stricker-Krongrad 1998). It thus indicates that this neuropeptide plays a role in the control of vegetative functions. The expression pattern of mRNA encoding two orexin receptors in the rat

brain has been described by Trivedi *et al.* (1998). This expression of mRNA in the hypothalamus supports the role of orexin A in the regulation of feeding (Sakurai *et al.* 1998), while the presence of orexin receptors in other cerebral areas suggests the additional functions played by orexin A (Marcus *et al.* 2001). A role of orexins in a sleep regulation has been demonstrated (Beuckmann and Yanagisawa 2002). A deficiency in orexin neurotransmission results in a sleep disorder narcolepsy in mice, dogs, and human (Taheri *et al.* 2002). Orexin A also influences body temperature. An icv administration of orexin A definitely increases firing rate of the sympathetic nerves to interscapular brown adipose tissue

(IBAT), accompanied with a rise in IBAT and colonic temperatures (Monda *et al.* 2001). These changes in body temperature are primary and predominant in comparison with those in eating behavior. For this reason, we suggested the name “hyperthermine A” as additional denomination of “orexin A” (Monda *et al.* 2003b).

Clozapine, an atypical antipsychotic, is able to induce modification of body temperature. An undesirable effect of clozapine is the hypothermia in patients treated with this drug (Heh *et al.* 1988). Intraperitoneal administration of clozapine also produces hypothermic effects in the rats (Millan *et al.* 1995, Oerther and Ahlenius 2000). This hypothermia appears to be unrelated to drug effects on movement. In fact, intraperitoneal administration of clozapine lowers core temperature, having minimal motor side effects (Huberman *et al.* 2000).

Since we have recently demonstrated that haloperidol, a typical antipsychotic drug, modifies slightly the thermogenic responses to orexin A (Monda *et al.* 2003a), we have tested the effects of clozapine on the sympathetic and thermogenic changes induced by orexin A to evaluate the influence of this antipsychotic drug on the orexin-induced activity.

Methods

Animals

Male Sprague-Dawley rats (n=24), 3 months old and weighing 270-320 g were used in the experiments. The rats were housed in pairs at controlled temperature (22±1 °C) and humidity (70 %) with a 12:12 h light-dark-light cycle from 07:00 to 19:00 h. The experiments were in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Apparatus

A pair of silver wire electrodes recorded the firing rate of nerves to IBAT. The electrical pulses were amplified by a condenser-coupled amplifier and were filtered by band-pass filters (NeuroLog System, Digitimer). The raw pulses were displayed on an oscilloscope (Tektronix) and sent to a window discriminator. Square waves from the discriminator were sent to an analog-digital converter (DAS system, Keithley) and stored on a computer (PC AT, IBM) every 5 s. A ratemeter with a reset time of 5 s was also used to observe the time course of the nerve activity recorded by pen recorder (Dynograph, Beckman). Because signal-to-

noise ratio depended on the number of nerve filaments and the condition of contact between nerve and electrodes, the basal burst rates were different for each rat. The threshold level of the event detector was fixed during the experiment at 50 % of the peaks of the largest pulses and above background noise. Thermocouples (Ellab) were used to monitor colonic and IBAT temperatures (T_C and T_{IBAT}) and the values were stored on a chart recorder.

Drugs and doses

We used a dose of 1.5 nmol of orexin A (Peninsula, England) after a preliminary experiment with doses of 0.15, 1.5, and 15 nmol in three groups of 3 rats. This test showed a dose-effect curve on T_C . The temperature changes were: a) from 37.15±0.11 to 37.79±0.15 with dose of 0.15 nmol; b) from 37.10±0.09 to 38.40±0.16 with dose of 1.5 nmol; c) from 37.12±0.12 to 38.71±0.13 with dose of 15 nmol. We chose the sub-maximal dose of 1.5 nmol. A similar preliminary test was carried out with doses of 4, 8, 12 mg/kg bw of clozapine (Sigma, Italy) in three groups of 3 rats. The temperature changes were: a) from 37.11±0.10 to 36.75±0.18 with dose of 4 mg/kg bw; from 37.17±0.12 to 36.03±0.18 with dose of 8 mg/kg bw; from 37.15±0.13 to 35.91±0.18 with dose of 12 mg/kg bw. The dose of 8 mg/kg was utilized. These preliminary experiments were merely indicative to determine the optimal dose.

Surgery

All animals were anesthetized with ip pentobarbital (50 mg/kg bw) and a 20-gauge stainless guide cannula was positioned stereotaxically (Pellegrino *et al.* 1979) above a lateral cerebral ventricle at the following coordinates: 1.7 mm lateral to the midline, 0.4 mm posterior to the bregma, 3.0 mm from the cranial theca. The rats were given 7-10 days to recover from surgery judged by recovery of preoperative body weight.

Procedure

After the recovery, six animals (group 1) were anesthetized with ethyl-urethane (1.2 mg/kg bw ip) and mounted in a stereotaxic instrument (Stoelting). The level of anesthesia was kept constant and evaluated by skeletal muscle relaxation, eye and palpebral responses to stimuli. Nerve activity was recorded by small nerve bundles dissected from the intercostal nerves supplying the right side of IBAT. Nerve filaments were isolated from the central cut end of these nerve bundles under a dissecting

microscope; the efferent activity was recorded with a pair of silver wire electrodes. The nerve filaments were covered with a mixture of vaseline and liquid petroleum at 37 °C to avoid dehydration. The firing rate was recorded over 60 min before and 300 min after the injection of orexin A (1.5 nmol dissolved in 5 µl of 0.9 % NaCl sterile solution). The orexin A was delivered into the left cerebral ventricle by gravity flow over 2 min. The cannula for the injection was 0.4 mm longer than the guide cannula. Furthermore, T_C and T_{IBAT} were monitored at the same time as the nerve activity. T_C was measured by inserting the thermocouple into the colon 4 cm from the anus, while T_{IBAT} was monitored by inserting the thermocouple in the left side of IBAT. The same variables were recorded in additional 6 animals (group 2), but clozapine (8 mg/kg bw dissolved in 2 ml of 0.9 % NaCl sterile solution) was injected ip 30 min before the icv injection of orexin A. In the other six rats treated with an ip injection of clozapine (group 3), saline was injected into the cerebral ventricle and the same variables were monitored. In six control rats (group 4), saline was injected both in the peritoneal cavity and in the lateral ventricle. The baseline values of T_C from all animals used were maintained constant by a heating pad. The electrical energy supplied to pad was not altered during the experimental period. At the end of the experiment, the location of the cannula was identified with histological controls.

Statistical analysis

The experimental scheme was 2x2x12 (icv-orexin/saline x ip-clozapine/saline x 12 time intervals). The values were presented as means \pm S.E.M. Statistical analysis was performed using analysis of variance. Multiple comparisons were performed by Newman-Keuls *post hoc* test.

Results

Figure 1 shows the percentage changes in the firing rate of sympathetic nerves to IBAT. The icv injection of orexin increased the firing rate in the rats with ip administration of saline. The rats treated with icv saline plus ip clozapine showed the same decrease in the sympathetic discharge as the rats treated with icv orexin A plus ip clozapine. Injections of saline alone did not induce modifications. The analysis of variance showed significant effects for orexin [$F(1, 20) = 287.1$, $p < 0.01$], for clozapine [$F(1, 20) = 1379.6$, $p < 0.01$], for time [$F(11,$

220) = 27.1, $p < 0.01$], for interaction orexin x clozapine [$F(1, 20) = 286.1$, $p < 0.01$], orexin x time [$F(11, 220) = 17.4$, $p < 0.01$], clozapine x time [$F(11, 220) = 34.1$, $p < 0.01$], and orexin x clozapine x time [$F(11, 220) = 21.9$, $p < 0.01$]. The *post hoc* test showed that saline+orexin group was different from clozapine+orexin group at 0 to 5 h.

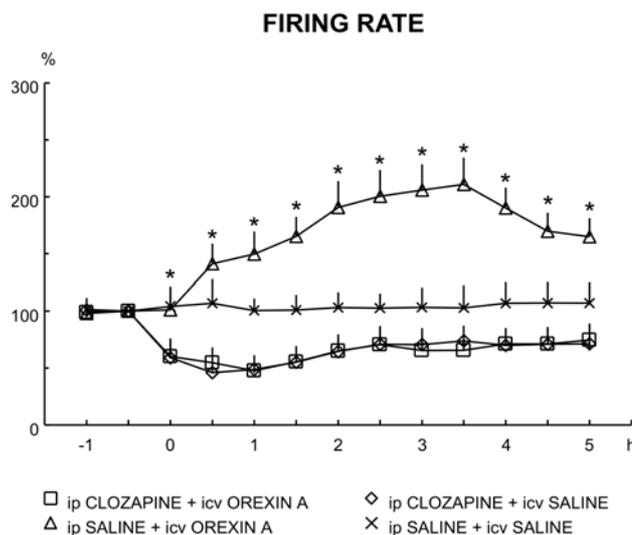


Fig. 1. The changes in firing rate of nerves to interscapular brown adipose tissue. Intraperitoneal (ip) injection of saline or clozapine (8 mg·kg⁻¹ bw) was made at -0.5 h. Intracerebroventricular (icv) injection of saline or orexin A (1.5 nmol) was made at time 0. * Statistical significance ($p < 0.05$) between clozapine+orexin group and saline+orexin group. Data are means \pm S.E.M.

The baseline absolute values were respectively of 40.2 \pm 7.8 spikes/5 s in group 1, 38.4 \pm 9.2 in group 2, 41.7 \pm 6.1 in group 3 and 43.6 \pm 8.4 in group 4. There were no differences in the baseline absolute values of all groups. Examples of the changes in firing rate are shown in Figure 2.

T_{IBAT} changes are shown in Figure 3. The icv injection of orexin caused a rise in the animals ip injected with saline. There was the same decrease in the rats with icv saline plus ip clozapine and in the rats with icv orexin A plus ip clozapine. Double injection of saline did not cause any modifications. The analysis of variance showed significant effects for orexin [$F(1, 20) = 38.6$, $p < 0.01$], for clozapine [$F(1, 20) = 597.8$, $p < 0.01$], for time [$F(11, 220) = 58.0$, $p < 0.01$], for interaction orexin x clozapine [$F(1, 20) = 48.1$, $p < 0.01$], orexin x time [$F(11, 220) = 30.6$, $p < 0.01$], clozapine x time [$F(11, 220) = 171.9$, $p < 0.01$], and orexin x clozapine x time [$F(11, 220) = 31.4$, $p < 0.01$]. Newman-Keuls *post hoc* test showed that the saline+orexin group was different from

clozapine+orexin group at 0 to 5 h.

Figure 4 represents T_C changes. The icv injection of orexin induced a rise in the rats ip injected with saline. Clozapine induced the same decrease in the rats icv injected with saline or orexin A. No changes were seen in the animals with saline administrations. The analysis of variance showed significant effects for orexin [$F(1, 20) = 39.9, p < 0.01$], for clozapine [$F(1, 20) = 631.7, p < 0.01$], for time [$F(11, 220) = 58.1, p < 0.01$], for interaction orexin x clozapine [$F(1, 20) = 52.2, p < 0.01$], orexin x time [$F(11, 220) = 31.9, p < 0.01$], clozapine x time [$F(11, 220) = 171.1, p < 0.01$], and orexin x clozapine x time [$F(11, 220) = 30.4, p < 0.01$]. Newman-Keuls *post hoc* test showed that the saline+orexin group was different from clozapine+orexin group at 0 to 5 h.

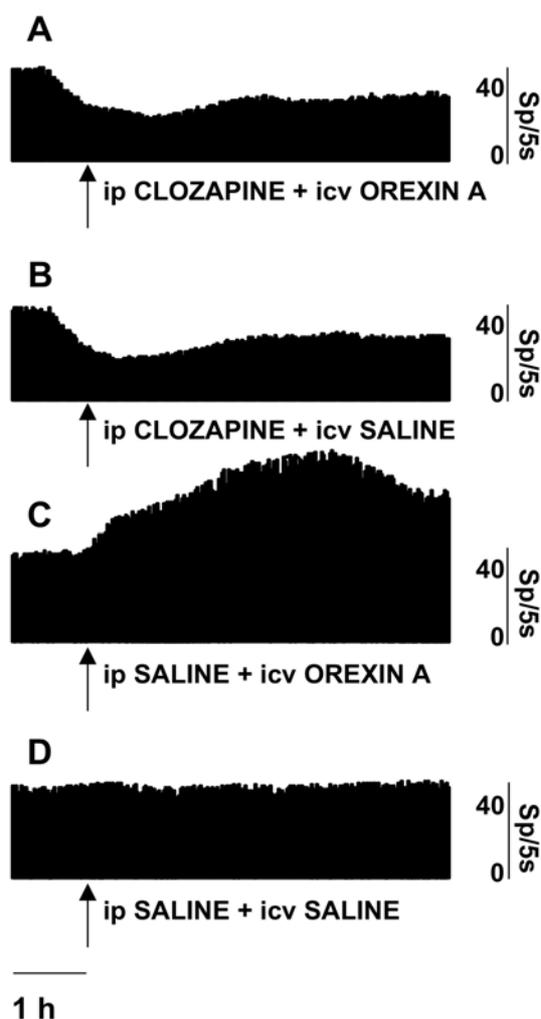


Fig. 2. Actual changes of firing rate in a rat receiving intraperitoneal (ip) injection of clozapine plus intracerebroventricular (icv) injection of orexin A (panel A) or saline (panel B), and in a rat receiving ip injection of saline plus icv injection of orexin A (panel C) or saline (panel D). The arrow indicates the time of icv injection. Ip administrations were made 30 min before the icv injection.

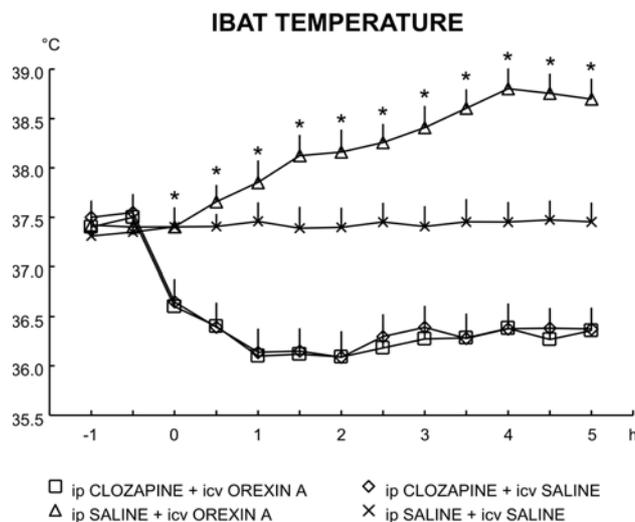


Fig. 3. The changes in temperature interscapular brown adipose tissue (IBAT). Intraperitoneal (ip) injection of saline or clozapine ($8 \text{ mg} \cdot \text{kg}^{-1} \text{ bw}$) was made at -0.5 h . Intracerebroventricular (icv) injection of saline or orexin A (1.5 nmol) was made at time 0. * Statistical significance ($p < 0.05$) between clozapine+orexin group and saline+orexin group. Data are means \pm S.E.M.

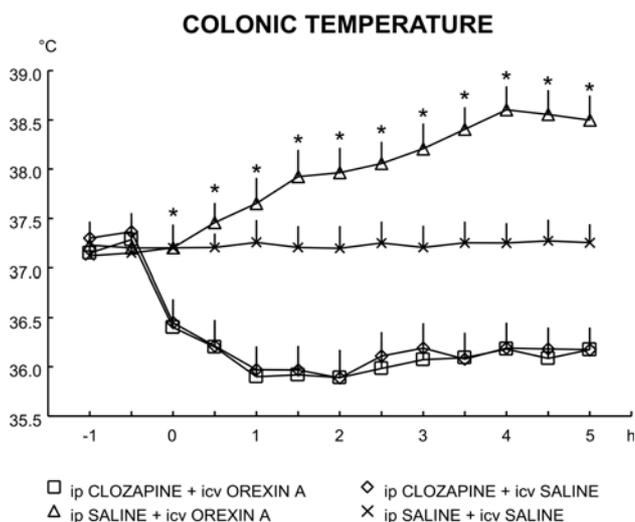


Fig. 4. The changes in colonic temperature. Intraperitoneal (ip) injection of saline or clozapine ($8 \text{ mg} \cdot \text{kg}^{-1} \text{ bw}$) was made at -0.5 h . Intracerebroventricular (icv) injection of saline or orexin A (1.5 nmol) was made at time 0. * Statistical significance ($p < 0.05$) between clozapine+orexin group and saline+orexin group. Data are means \pm S.E.M.

Discussion

These results are the first to demonstrate that an ip administration of clozapine completely blocks the sympathetic and thermogenic reactions induced by orexin A, while an activation of the sympathetic nervous system due to orexin A has been already demonstrated (Monda *et al.* 2001, Shirasaka *et al.* 1999). The present experiment

confirms the role of orexin A in the control of the autonomic nervous system and it indicates the strong effect of clozapine on the orexin-induced activity.

Since the increase in the sympathetic and thermogenic activity induced by orexin A is blocked by clozapine, an involvement of the dopaminergic system can be hypothesized, considering the high affinity of clozapine for dopamine receptors (Kulkarni and Ninan 2000). There is evidence showing that dopamine D₃ receptors participate in clozapine-induced hypothermia (Millan *et al.* 1995). An activation of dopaminergic system could be a possible mechanism of sympathetic and thermogenic stimulation induced by orexin A. In this mechanism, the involvement of D₃ receptors could play a key role.

On the other hand, an involvement of serotonergic pathways in the orexin-induced hyperthermia cannot be excluded. A review of the psychiatric cases revealed that nine of ten hypothermic patients were treated with drugs that are potent antagonists of 5-HT₂ receptors (Schwaninger *et al.* 1998). Since clozapine is an antagonist of 5-HT₂ receptors (Gobbi and Janiri 1999), the block of these receptors could be important in the lack of the hyperthermia induced by orexin A. In other words, orexin A can elevate body temperature by acting on 5-HT₂ receptors.

Furthermore, several laboratories found an even higher affinity for α_1 -adrenergic receptors (Brunello *et al.* 1995, Menon *et al.* 1988). It was shown that central blockade of α_1 -adrenergic receptors produces hypothermia (Stone *et al.* 1999) and that α_1 -agonists block the hypothermic effects of clozapine (Menon *et al.* 1990). Thus, the central blockade of α_1 -adrenergic receptors due to clozapine could be involved in the lack of the hyperthermia induced by orexin A.

We have already demonstrated that lysine acetylsalicylate reduces the sympathetic activation induced by orexin A, suggesting that prostaglandins have an implication in the mediation of this phenomenon (Monda *et al.* 2001). Since the clozapine induces an inhibition of prostaglandin synthase activity in the brain (Sokola 1981), we can hypothesize that, at least in part, clozapine could block orexin-induced hyperthermia through an inhibition of the prostaglandin synthesis.

The icv injection of orexin A increases the temperature of IBAT, which is the most important effector of non-shivering thermogenesis in the rat (Cannon *et al.* 1998), illustrating that the rise in heat production is also due to the activation of thermogenesis unrelated to muscle

activity. The increase in colonic temperature emphasizes the effect of orexin A on “core” temperature suggesting the inclusion of orexin A among the peptides controlling body temperature. Clozapine injection blocks both temperatures, indicating that orexin-induced reactions involve an activation of dopaminergic and/or serotonergic and/or adrenergic systems.

Further investigations should be carried out with more specific antagonist for dopamine, serotonin, and norepinephrine receptors simultaneously with clozapine to determine the role played by each group of receptors. Furthermore, the measurements of dopamine, serotonin and norepinephrine after injection of orexin A plus saline or clozapine should be performed. These further experiments should indicate which type of catecholamine receptors is involved in thermogenic reactions induced by orexin A.

The choice of clozapine, a drug that is notorious because of its “dirty” pharmacology, is due to its large use in the treatment of schizophrenia. In this experiment, clozapine blocks completely the hyperthermic action of orexin A. This indicates that patients treated with clozapine are non-responsive to physiological variations of orexin A. This non-responsiveness to orexin A could induce not only hypothermia, but also body weight gain (McIntyre *et al.* 2001). In fact, orexin A is involved in the activation of thermogenesis related to eating behavior. The thermogenesis due to food intake is an important factor in the regulation of body weight and the lack of this thermogenesis causes the obesity (Bray 2000, Monda *et al.* 1997). In this experiment, the complete lack of hyperthermic effects due to injection of orexin A makes this assumption reasonable.

Although the precise mechanisms of clozapine effect must be examined by using more specific antagonists for dopamine, serotonin, and noradrenaline receptors, our present results indicate that the sympathetic effects of orexin A are completely blocked by clozapine. Haloperidol reduces but does not block these reactions due to orexin A (Monda *et al.* 2003a). Clozapine influences strongly the thermogenic role of orexin A, which is ineffective to modify the clozapine-induced hypothermia. These data are useful to address the choice of antipsychotic drugs for the therapy of mental disorders. Furthermore, the treatment of rare but dangerous hypothermia induced by clozapine should exclude substances, which utilize the orexinergic pathway to elevate body temperature. This pathway is completely blocked by clozapine, as demonstrated by the findings of

this experiment.

The present findings confirm that orexin A induces an elevation of body temperature in anesthetized rats, thus independently on food intake. We can suppose that this peptide elevates the thermal set-point, inducing the reactions to reach a new level of body temperature. For this reason, we insist on suggested term “hyperthermine A”, as an additional denomination of “orexin A”. Alternatively, we can drop our campaign to propose a new nomenclature, but the term “orexin A” must be definitively replaced by “hypocretin-1” in the international literature

In conclusion, this experiment strongly indicates that clozapine blocks the reactions induced by orexin A. The remarkable thermogenic role played by orexin A is confirmed.

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Reprint requests

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