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Dopaminergic regulation of orexin neurons

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Abstract

Orexin/hypocretin neurons in the lateral hypothalamus and adjacent perifornical area (LH/PFA) innervate midbrain dopamine (DA) neurons that project to corticolimbic sites and subserve psychostimulant-induced locomotor activity. However, it is not known whether dopamine neurons in turn regulate the activity of orexin cells. We examined the ability of dopamine agonists to activate orexin neurons in the rat, as reflected by induction of Fos. The mixed dopamine agonist apomorphine increased Fos expression in orexin cells, with a greater effect on orexin neurons located medial to the fornix. Both the selective D1-like agonist, A-77636, and the D2-like agonist, quinpirole, also induced Fos in orexin cells, suggesting that stimulation of either receptor subtype is sufficient to activate orexin neurons. Consistent with this finding, combined SCH 23390 (D1 antagonist)–haloperidol (D2 antagonist) pretreatment blocked apomorphine-induced activation of medial as well as lateral orexin neurons; in contrast, pretreatment with either the D1-like or D2-like antagonists alone did not attenuate apomorphine-induced activation of medial orexin cells. *In situ* hybridization histochemistry revealed that LH/PFA cells rarely express mRNAs encoding dopamine receptors, suggesting that orexin cells are transsynaptically activated by apomorphine. We therefore lesioned the nucleus accumbens, a site known to regulate orexin cells, but this treatment did not alter apomorphine-elicited activation of medial or lateral orexin neurons. Interestingly, apomorphine failed to activate orexin cells in isoflurane-anaesthetized animals. These data suggest that apomorphine-induced arousal but not accumbens-mediated hyperactivity is required for dopamine to transsynaptically activate orexin neurons.

Introduction

The orexins/hypocretins are neuropeptides that are expressed in cells of the lateral hypothalamus and contiguous perifornical area (LH/PFA) (Broberger *et al*., 1998; de Lecea *et al*., 1998; Sakurai *et al*., 1998). Despite the small number of orexin cells, orexin axons are distributed across almost all areas of the brain (Peyron *et al*., 1998). Consistent with the broad distribution of orexin neurons is a correspondingly wide distribution of orexin receptors in the central nervous system (Marcus *et al*., 2001).

Orexin potently activates catecholaminergic neurons in the brainstem, including the dopamine (DA) neurons of the ventral tegmental area (VTA) and noradrenergic neurons of the locus coeruleus (Horvath *et al*., 1999; Uramura *et al*., 2001; Korotkova *et al*., 2003); both types of catecholaminergic cells express orexin receptors (Lu *et al*., 2000; Marcus *et al*., 2001). The interaction between orexin and catecholaminergic neurons is reflected in the close proximity of orexin axons to catecholamine neurons, including VTA dopamine neurons and locus coeruleus noradrenergic cells (Horvath *et al*., 1999; Fadel & Deutch, 2002; Baldo *et al*., 2003). The ability of orexin to promote wakefulness is thought to in part to be mediated by catecholamines, including dopamine (Nakamura *et al*., 2000; Uramura *et al*., 2001; Korotkova *et al*., 2003).

Although it is clear that orexin activates dopamine neurons in the VTA, relatively little is known about the effects of catecholamines on orexin neurons. This is of particular interest because wakefulness-promoting drugs (including amphetamines, modafinil, and caffeine) activate orexin neurons (Scammell *et al*., 2000; Estabrooke *et al*., 2001; Fadel *et al*., 2002; Murphy *et al*., 2003). Moreover, the wake-promoting effects of modafinil and methamphetamine are abolished in dopamine transporter knockout mice (Wisor *et al*., 2001).

In order to determine how dopamine regulates orexin neurons, we examined the effects of dopamine receptor agonists on Fos expression in orexin neurons, and determined whether dopamine agonist-elicited effects could be blocked by pretreatment with dopamine antagonists. In order to understand whether dopamine agonists directly activate orexin neurons, we determined whether cells in the lateral hypothalamus express dopamine receptor transcripts. Because previous studies found that nucleus accumbens projections to the LH/PFA can transsynaptically regulate the activity of orexin and other LH/PFA neurons (Stratford & Kelley, 1999; Satoh *et al*., 2003; Zheng *et al*., 2003; Baldo *et al*., 2004), we examined the effects of lesions of the nucleus accumbens (NAS), the neurons of which express D1-like and D2-like dopamine receptors, on dopamine agonist-induced activation of orexin cells. Finally, because orexin is involved in arousal (Hagan *et al*., 1999; Espana *et al*., 2001; Winsky-Sommerer *et al*., 2003), we determined whether DA agonists could activate orexin cells in anaesthetized rats.

Materials and methods

Subjects

Adult male Sprague–Dawley rats (Harlan, Birmingham, AL) were group-housed under a 12-h light : 12-h dark cycle (lights on at 06:00 h) with food and water available *ad libitum*. Experiments were

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performed during the light phase unless specifically mentioned. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Vanderbilt University Animal Care and Use Committee.

Dopamine agonist and antagonist treatment

We determined the effects of administration of the mixed D1/D2 agonist, apomorphine, on Fos expression in orexin neurons. Because orexin cell activity and the behavioural effects of apomorphine are influenced by the time of day (Nakano *et al*., 1980; Nasello *et al*., 1995; Fujiki *et al*., 2001; Espana *et al*., 2003), we first examined the effects of apomorphine when the drug was administered during the animals' dark (active) or light (sleep) phase. Rats were injected with apomorphine (0.6 and 3.0 mg/kg, s.c.) or vehicle ($N = 6$–12/group), starting either three hours after light onset or dark onset, and were killed with isoflurane 2 h later; all experiments concluded at least three hours before the change in lighting (light or dark onset). Subsequent studies were performed during the light phase.

Other groups of rats were treated with either the D1-like agonist, A-77636 (0.3, 0.6 or 3.0 mg/kg, s.c.; $N = 5$–7/group), which targets both D_1 and $D_{1b(5)}$ receptors, or the D2-like agonist, (–)quinpirole HCl (0.25, 0.5 or 2.5 mg/kg, s.c., $N = 5$–7/group), which has a moderately high affinity for D_2, D_3, and D_4 dopamine receptors (Millan *et al*., 2002); control animals were injected with the appropriate vehicle. Rats were killed two hours after dopamine agonist treatment.

We subsequently determined whether pretreatment with D1-like or D2-like receptor antagonists altered apomorphine-elicited Fos expression in orexin cells. Rats ($N = 4$–10/group) were injected with the D1 antagonist SCH 23390 (0.75 mg/kg) or vehicle, followed 30 min later by injections of apomorphine (3 mg/kg) or its vehicle. Other groups of rats ($N = 7$–11/group) were pretreated with the D_2 antagonist haloperidol (1 mg/kg) or vehicle prior to apomorphine challenge. In order to determine whether concurrent D1-like and D2-like dopamine receptor blockade prevents apomorphine-induced activation of orexin neurons, rats ($N = 7$–8/group) were treated with a combination of SCH 23390 (0.5 mg/kg) and haloperidol (0.75 mg/kg), or vehicle, 30 min before apomorphine (3 mg/kg). Rats were perfused two hours after apomorphine administration.

Drugs were obtained from Sigma Chemical Co. (St. Louis, MO) and drug doses were based on *ex vivo* dopamine receptor occupancy (Meller *et al*., 1985; Saller *et al*., 1989).

Ibotenic acid lesions of the nucleus accumbens

In order to determine whether orexin neurons are transsynaptically activated by nucleus accumbens projections to the LH/PFA, the NAS of rats was lesioned by unilaterally infusing ibotenic acid (18 nmols in 300 nL of phosphate buffered saline, pH 7.0; Tocris Cookson, Ellisville, MO) into two accumbal sites (AP, +1.5; ML, 0.9 and 1.4; DV, −7.4 and −8.0; respectively) at a rate of 50 nL/min ($N = 9$); the cannula was left in place for 4 min after the injection. Two weeks after surgery lesioned and sham-lesioned rats were challenged with vehicle or apomorphine (3 mg/kg, s.c.) and killed two hours later.

Assessment of apomorphine-induced Fos expression in anaesthetized animals

We assessed whether dopamine agonist-induced arousal is required for apomorphine to activate orexin cells by anaesthetizing rats with isoflurane (Henry Schein, Melville, NY) before administering apomorphine (3 mg/kg) or vehicle ($N = 6$–7/group. Anaesthesia was maintained throughout the experiment using a vaporizer (VetEquip, Pleasanton, CA) set at 2.0–2.5% isoflurane delivery, with body temperature maintained at 39 °C by a heating pad. Non-anaesthetized rats that were injected with apomorphine or vehicle ($N = 6$–7/group) in their home cage served as controls. Animals were killed two hours after apomorphine administration.

Immunohistochemistry

Rats were transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde, and the brains removed, postfixed overnight, and cryoprotected in 30% sucrose. Coronal sections were cut through the hypothalamus at 42 μm on a freezing microtome. Fos-like immunoreactive (-li) cells and orexin A-li neurons were visualized by a dual immunoperoxidase procedure using a rabbit anti-orexin A (Calbiochem, San Diego, CA, USA; 1 : 3000) and a goat anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 3000), as we have previously described (Bubser & Deutch, 1998; Fadel *et al*., 2002), with a nickel/cobalt-intensified diaminobenzidine product revealing black Fos-li nuclei and a brown diaminobenzidine product marking orexin-li neurons.

The extent of ibotenic acid-induced cell loss in the nucleus accumbens was visualized by staining accumbal sections for the neuronal marker NeuN, using a mouse anti-NeuN antibody (Chemicon, Temecula, CA; 1 : 2000) (Wolf *et al*., 1996).

Localization of dopamine receptor mRNA in the LH/PFA

We determined whether dopamine receptors were expressed on LH/PFA neurons by *in situ* hybridization histochemistry, using [^{35}S]-labelled cRNA probes for D_1, D_2, D_3, D_4, and D_5 dopamine receptor transcripts, following our previously described methods (Damask *et al*., 1996; Ritter & Meador-Woodruff, 1997). Briefly, coronal sections were fixed, acetylated, and dehydrated, and were then incubated with labelled probes (1.0–1.5×10^6 c.p.m./30 μL of hybridization buffer) overnight at 55 °C in humidified chambers. The next day, the sections were washed to a final stringency of 0.5× SSC at 55 °C. After drying, slides were dipped in Kodak NTB-2 emulsion, and developed for ≈30 days. Sections were then briefly stained with cresyl violet, dehydrated, and coverslipped.

Cell counting, lesion reconstruction, and statistical evaluation

Cell counting was performed using our previously described approach (Fadel *et al*., 2002). Briefly, the distributions of single-labelled (orexin or Fos) and double labelled (orexin + Fos) cells in the LH/PFA were plotted using NeuroLucida software (MicroBrightField Inc; Williston, VT). A vertical line bisecting the fornix was used to separate the medial and lateral sectors of the LH/PFA, with the location of cells plotted in sections through two anteroposterior levels of the LH/PFA (see Fadel *et al*., 2002).

In the experiment examining the effects of accumbal lesions on DA agonist-induced activation of orexin neurons, the extent of the NAS lesions was reconstructed from serial sections (spaced ≈200 μm apart), with the distribution of NeuN cells in the ventral striatum charted using the NeuroLucida system. In the hypothalamus, cells expressing Fos and/or orexin in the LH/PFA were charted both ipsilateral and contralateral to the accumbal lesion, and the densities of Fos-li neurons and percentages of orexin-li cells that also expressed Fos were determined.

Data in most experiments were analysed by means of ANOVA, followed by Dunnett's or Bonferroni's tests when indicated by a significant interaction or main effect. In order to determine whether the medial and lateral orexin cells responded to dopamine agonist challenges to different degrees, data were analysed by a univariate ANOVA using adjusted (difference) scores accounting for baseline values.

Results

Effects of apomorphine on Fos expression in orexin neurons

Systemic administration of apomorphine during the light phase dose-dependently increased the percentages of orexin cells expressing Fos in both the medial ($F_{2,22} = 44.3$, $P < 0.001$) and lateral ($F_{2,22} = 27.3$, $P < 0.001$) LH/PFA (Figs 1 and 2). The high dose of apomorphine (3 mg/kg) also increased Fos expression in orexin cells when administered during the dark period [$F_{2,14} = 33.1$, $P < 0.001$ (medial) and $F_{2,14} = 4.48$, $P < 0.001$ (lateral)] (see Fig. 2). In contrast, the low dose of apomorphine (0.6 mg/kg) increased Fos expression only when administered in the light, but not the dark phase.

In animals injected with the higher dose of apomorphine, the dopamine agonist increased Fos expression in orexin neurons located in the medial LH/PFA to a significantly greater degree than in laterally situated orexin cells ($F_{1,13} = 4.78$, $P < 0.05$); there was no significant difference in the response magnitude of medial and lateral orexin cells to the lower dose of apomorphine, although a trend toward a greater effect in medial cells was noted (Fig. 2). Apomorphine also activated non-orexin Fos-positive cells in the LH/PFA, with a greater effect on cells located medial to the fornix than those in the area lateral to the fornix (see Supplementary material, Table S1).

Effects of selective D1 and D2 receptor agonists on orexin neurons

The D1 agonist A-77636 and the D2 agonist quinpirole both induced Fos in orexin cells located in the medial ($F_{6,46} = 14.3$, $P < 0.001$) and lateral LH/PFA ($F_{6,46} = 16.9$, $P < 0.001$) (Fig. 3). With the exception of the low dose of A-77636, all doses of DA agonists increased the percentage of medial orexin cells expressing Fos. The lowest doses of both the D1-like and D2-like agonists failed to activate orexin cells in the lateral LH/PFA. Quinpirole and A-77637 also sharply increased the density of non-orexin LH/PFA cells expressing Fos (see Supplementary material, Table S1).

Pretreatment with D1 and/or D2 receptor antagonists and apomorphine-induced activation of orexin neurons

Pretreatment with the D1 antagonist, SCH 23390, blocked apomorphine-induced Fos expression in orexin cells in the lateral ($F_{3,25} = 8.0$, $P < 0.001$; $t_{15} = 3.78$, $P < 0.01$), but not in the medial LH/PFA ($F_{3,26} = 3.6$, $P < 0.05$; $t_{15} = 2.11$, $P > 0.05$) (see Fig. 4). SCH 23390 also did not alter basal Fos expression, i.e., the percentage of activated orexin cells in the medial ($t_8 = 0.84$, $P > 0.05$) or lateral LH/PFA ($t_7 = 1.27$, $P > 0.05$) of vehicle-treated rats, and did not affect apomorphine-induced Fos expression in non-orexin cells (see Supplementary material, Table S2).

The D2 antagonist, haloperidol, did not attenuate dopamine agonist-induced Fos expression in orexin cells in either the medial ($F_{3,36} = 18.5$, $P < 0.001$; $t_{18} = 1.88$, $P > 0.05$) or lateral LH/PFA ($F_{3,36} = 7.8$, $P < 0.001$; $t_{18} = 0.26$, $P > 0.05$) (see Fig. 4). Haloperidol pretreatments also had no effect on Fos expression in medial ($t_{15} = 0.58$, $P > 0.05$) or lateral ($t_8 = 0.67$, $P > 0.05$) orexin cells of vehicle-treated rats. Haloperidol also did not alter apomorphine-elicited Fos expression in non-orexin cells (see Supplementary material, Table S2).

In contrast to the effects of selective D1 and D2 antagonists, combined pretreatment with SCH 23390 and haloperidol blocked apomorphine-elicited activation of orexin cells in the medial ($F_{3,27} = 26.0$, $P < 0.001$; $t_{12} = 7.74$, $P < 0.001$) as well as lateral LH/PFA ($F_{3,28} = 11.2$, $P < 0.001$; $t_{13} = 5.04$, $P < 0.001$; see Fig. 4). Combined haloperidol–SCH 23390 treatment significantly reduced

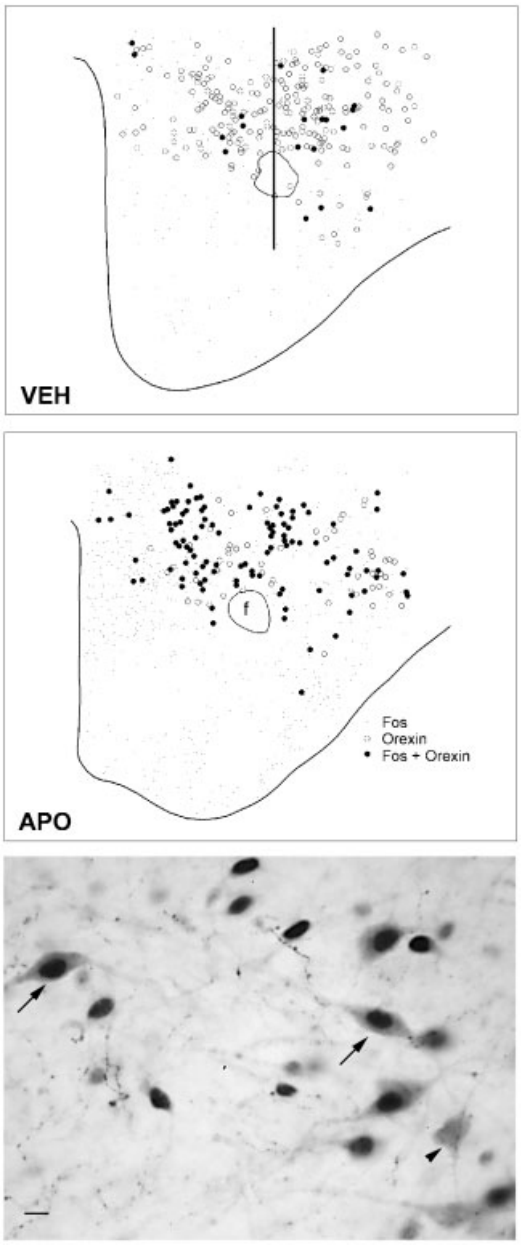

FIG. 1. The distribution of Fos-li cells (black dots), single-labelled orexin cells (open circles), and orexin cells expressing Fos (filled circles) in vehicle-treated (VEH; top panel) and apomorphine (3 mg/kg)-treated rats (APO; middle panel). The photomicrograph (bottom panel) shows Fos-li cells (black), orexin cells (light grey, large arrowheads) and orexin cells expressing Fos (arrows) in an apomorphine-treated rat. The vertical line through the fornix demarcates the medial and lateral LH/PFA. f, fornix. Scale bar, 10 μm.

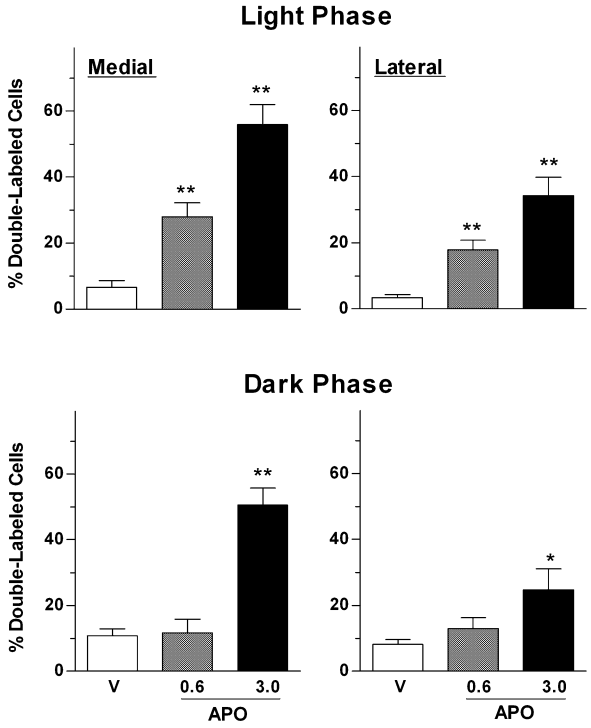

FIG. 2. Effects of the mixed D1–D2 receptor agonist apomorphine on Fos expression in orexin cells at different times of day. When administered during the light phase both low and high doses of apomorphine increase Fos-li expression in orexin cells of the medial and lateral LH/PFA (top panel). During the dark phase only the high dose of apomorphine activates orexin cells (bottom panel). APO, apomorphine; V, vehicle; $*P < 0.05$ $**P < 0.01$ vs. vehicle.

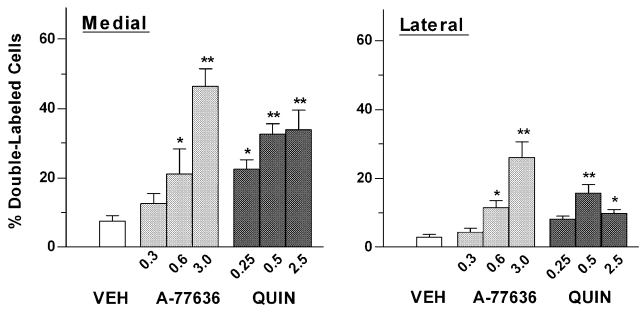

FIG. 3. The D1-like agonist, A-77636, and the D2-like agonist, quinpirole, increase Fos expression in orexin cells in a dose-related fashion in the medial (left panel) and lateral LH/PFA (right panel). QUIN, quinpirole; VEH, vehicle; $*P < 0.05$, $**P < 0.01$ vs. vehicle.

basal Fos expression in medially ($t_{12} = 2.81$, $P < 0.05$), but not in laterally situated orexin cells ($t_{12} = 1.31$, $P > 0.05$). Finally, coadministration of haloperidol and SCH 23390 also reduced the density of non-orexin Fos cells in the lateral but not the medial LH/PFA (see Supplementary material, Table S2).

Dopamine receptor expression in the LH/PFA

We observed a very small number of LH/PFA neurons that expressed detectable amounts of D_2 mRNA (Fig. 5); we did not observed any specific labelling of LH/PFA neurons for D_1, D_3, D_4, and $D_{5(1B)}$ receptor transcripts. The D_2 mRNA-expressing cells in the LH were

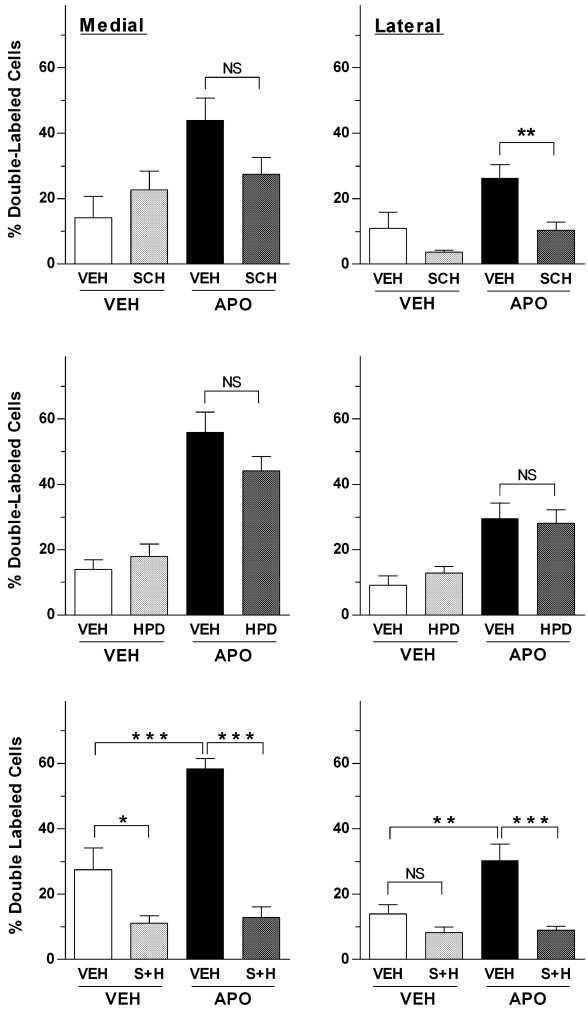

FIG. 4. Effects of single and combined pretreatment with D1-like and D2-like dopamine agonists on apomorphine-induced Fos expression in orexin neurons. (Top panel) SCH 23390 (0.75 mg/kg) significantly attenuated Fos expression in orexin cells in the lateral LH/PFA, but did not reduce Fos expression in medial orexin neurons. (Middle panel) Haloperidol (1 mg/kg) did not significantly attenuate Fos expression in orexin cells of either the medial or lateral LH/PFA. (Bottom panel) Combined pretreatment with SCH 23390 (0.5 mg/kg) and haloperidol (0.75 mg/kg) blocked apomorphine elicited Fos expression in orexin cells of the medial and lateral LH/PFA and also reduced basal Fos expression in orexin cells of the medial LH/PFA. APO, apomorphine; both H and HPD refer to haloperidol; S and SCH refer to SCH 23390; VEH, vehicle; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; NS, not significant.

lightly labelled with silver grains when compared to densely labelled D_2-positive cells observed in the zona incerta or caudal striatum in the same brain section. The rare cells that did express D_2 transcripts were scattered across the LH, but subjectively appeared to be most common in the ventral LH (see Fig. 5).

Effects of nucleus accumbens lesions on apomorphine-induced activation of orexin cells

Ibotenic acid lesions of the nucleus accumbens resulted in a loss of NeuN-positive cells in the nucleus accumbens (see Fig. 6). The lesions involved the medial shell of all animals, with most animals also showing marked loss of neurons in the lateral shell as well as the core (see Fig. 6). Incidental damage to the region medial to the rostral shell was observed in animals with the largest lesions.

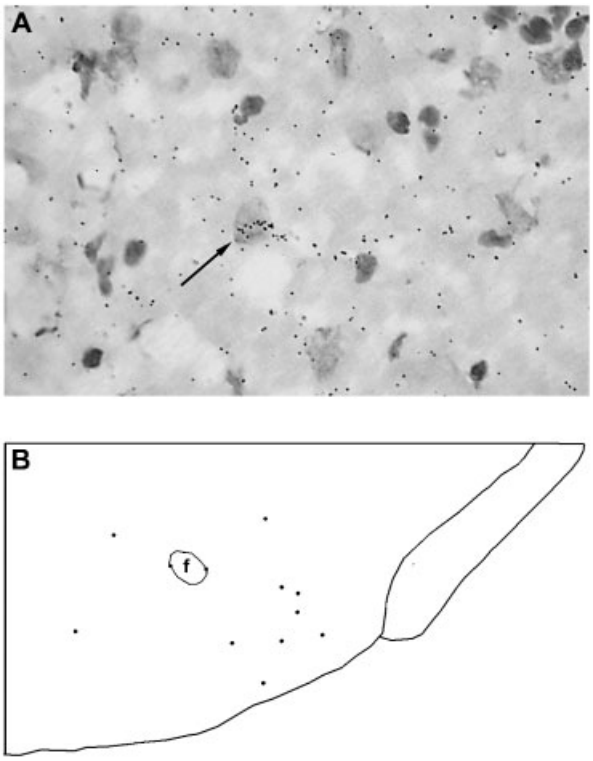

FIG. 5. Dopamine D_2 receptor mRNA is rarely expressed in LH/PFA cells. The photomicrograph (A) shows a Nissl-stained cell in the LH/PFA (arrow) expressing D_2 receptor mRNA (silver grains). The charting in (B) shows the regional distribution of D_2 receptor expressing cells in the LH/PFA. f, fornix.

Analysis of variance did not reveal any significant lesion × drug (apomorphine) treatment × side (ipsilateral vs. contralateral to the accumbal injection) interactions in the medial or lateral LH/PFA. Thus, apomorphine increased Fos expression in orexin cells of the medial and lateral LH/PFA to the same degree in both lesioned and sham-operated control animals (see Fig. 7). The ibotenic acid lesion also failed to alter basal or apomorphine-induced densities of single-labelled Fos-li cells in the LH/PFA (see Supplementary material, Table S3).

Effects of anaesthesia on apomorphine-induced activation of orexin cells

Isoflurane anaesthesia blocked the ability of apomorphine to induce Fos in orexin cells of both the medial ($F_{3,25} = 18.6$, $P < 0.001$; $t_{12} = 5.12$, $P < 0.001$) and lateral LH/PFA ($F_{3,25} = 13.5$, $P < 0.001$; $t_{12} = 5.01$, $P < 0.001$). In vehicle-treated rats the percentage of Fos expressing orexin cells in medial ($t_{10} = 0.11$, $P > 0.05$) and lateral LH/PFA ($t_{10} = 0.10$, $P > 0.05$) was not altered by anaesthesia (see Fig. 8). Similarly, apomorphine did not increase the density of non-orexin Fos-li cells in the LH/PFA of anaesthetized rats (see Supplementary material, Table S4).

Discussion

We found that dopamine agonists activate orexin cells in the LH/PFA, particularly orexin cells medial to the fornix. Apomorphine-elicited Fos-li expression in orexin cells was blocked by concurrent D1-like and D2-like dopamine antagonist pretreatment, but not by pretreatment with either D1 or D2 antagonists alone, consistent with both D1-like and D2-like receptors contributing to the activation of orexin neurons. Given the almost complete lack of dopamine receptor expression by LH/PFA cells, it appears that dopamine agonists indirectly activate orexin neurons, a contention bolstered by the finding that apomorphine did not induce Fos in orexin cells of anaesthetized animals. The transsynaptic activation of orexin cells by dopamine agonists does not appear to involve the NAS, because accumbal lesions do not block apomorphine's effect on orexin cells. However, because NAS lesions do block apomorphine-induced hyperactivity, the increase in locomotor activity elicited by apomorphine cannot be responsible for activation of orexin cells. However, a dopamine agonist-induced change in arousal state may play a role.

Dopamine agonists induce Fos in orexin neurons of the LH/PFA. The ability of dopamine receptor agonists to activate orexin neurons,

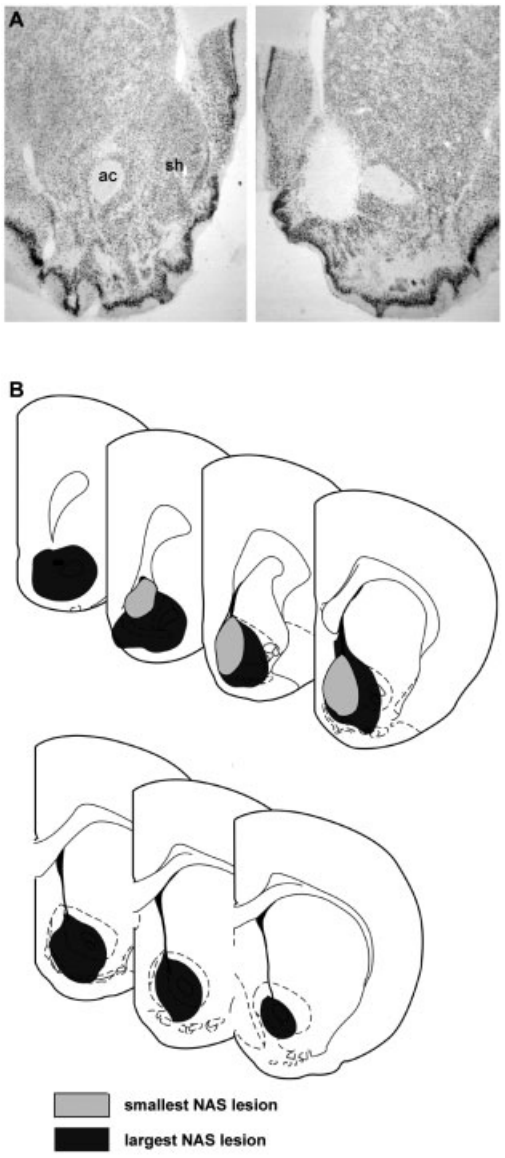

FIG. 6. The loss of NeuN immunoreactive cells reveals the extent of ibotenic acid lesions of the nucleus accumbens. (A) The panels show the nucleus accumbens of control (left panel) and lesioned (right panel) animals. (B) The bottom panel shows the reconstruction of the largest and smallest lesions. The drawings were on templates adapted from Paxinos & Watson (1986). ac, anterior commissure; sh, shell.

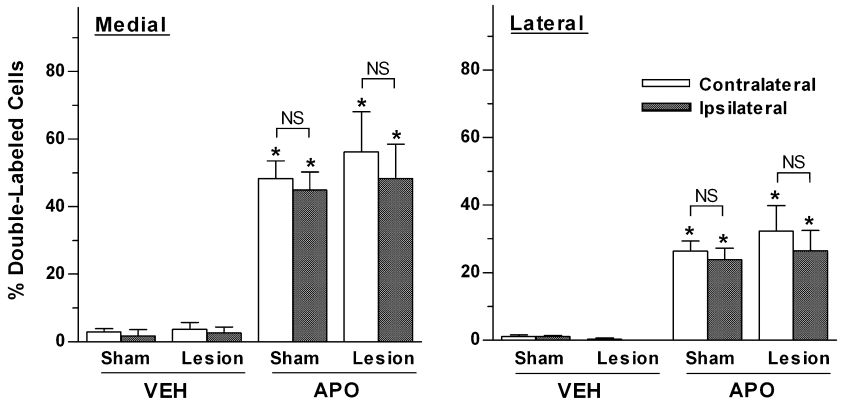

FIG. 7. Ibotenic acid lesions of the medial nucleus accumbens do not alter basal and apomorphine Fos-li expression in the medial (left panel) or lateral LH/PFA (right panel). APO, apomorphine; VEH, vehicle; *$P < 0.01$ vs. corresponding vehicle treatment; NS, not significant.

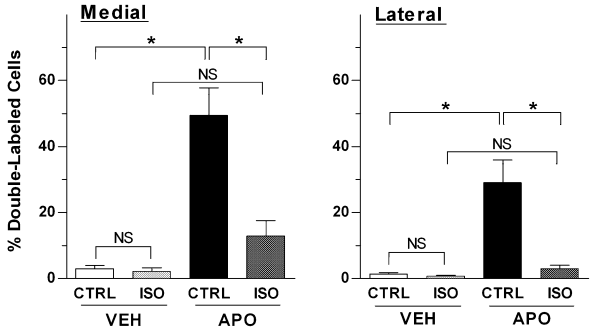

FIG. 8. Effects of isoflurane anaesthesia on apomorphine-induced Fos expression in orexin neurons. In anaesthetized rats apomorphine failed to activate orexin cells in both the medial and lateral LH/PFA. APO, apomorphine; CTRL, control; ISO, isoflurane; VEH, vehicle; *$P < 0.001$; NS, not significant.

as reflected by induction of Fos, extends previous results obtained with amphetamines (Estabrooke *et al.*, 2001; Fadel *et al.*, 2002). Amphetamine-induced activation of orexin neurons may occur secondary to increases in extracellular dopamine or norepinephrine levels; our data suggest that dopamine receptor stimulation is sufficient to activate orexin neurons. Paralleling our previous finding that amphetamine exerted a greater effect in medial than lateral orexin neurons (Fadel *et al.*, 2002), we found that apomorphine activated medial orexin cells (those located medial to the fornix) to a significantly greater degree than laterally situated orexin neurons. Apomorphine-induced activation of orexin cells was more robust when the drug was administered during the light phase, when orexin cell activity is lowest (Espana *et al.*, 2003; Akiyama *et al.*, 2004).

Involvement of D1-like and D2-like dopamine receptors in dopamine agonist-elicited activation of orexin neurons

Administration of the D1-like agonist, A-77636, or the D2-like agonist quinpirole increased the percentage of orexin cells expressing Fos, suggesting that stimulation of D1-like or D2-like receptors alone is sufficient to activate orexin neurons. The D1 agonist, but not quinpirole, elicited a dose-related increase in the percentage of activated orexin cells. As quinpirole and A-77636 do not discriminate well between different D2 (D_2, D_3, and D_4) and D1 (D_1 and D_5) receptor subtypes (Millan *et al.*, 2002), respectively, we cannot ascribe dopaminergic modulation of orexin cells to any specific dopamine receptor subtype.

The mixed D1/D2-like receptor agonist apomorphine also activated orexin cells. However, apomorphine-induced Fos expression in orexin cells was not altered by pretreatment with the D2 antagonist, haloperidol, and attenuated only in the lateral LH/PFA by the D1 antagonist SCH 23390. These findings suggest that stimulation of either D1-like or D2-like dopamine receptors is sufficient to activate orexin cells, but that the combined activation of D1 and D2 receptors by the mixed agonist apomorphine can only be blocked by pretreatment with both haloperidol and SCH 23390. Because we did not conduct experiments in which the effects of apomorphine and selective dopamine agonists were directly compared at the same time, we cannot state unequivocally that apomorphine activates a greater number of orexin cells than D1 or D2 agonists alone. However, our subjective impression after reviewing the data is that the full activation of orexin neurons can be achieved only by concurrent D1–D2 receptor stimulation.

Interestingly, combined haloperidol–SCH 23390 treatment of animals that were subsequently injected with vehicle reduced Fos expression in medial orexin cells, when compared to vehicle-treated animals that were not pretreated with active drug. This finding suggests the presence of a tonic dopaminergic regulation of a subset of orexin neurons.

Dopamine receptors in the LH/PFA

Previous reports have mentioned scattered D_1 and D_2 mRNA cells in the hypothalamus (Mansour *et al.*, 1990, 1991), but there has been no systematic examination of dopamine receptor expression in the LH/PFA. We found very few D_2 mRNA-expressing cells in the LH/PFA; cells in the region did not express detectable levels of any other dopamine receptor transcripts. The low density of dopamine receptors previously reported in autoradiographic and binding studies (Klemm *et al.*, 1979; Leibowitz *et al.*, 1982; Savasta *et al.*, 1986) is probably due to binding sites on axons innervating or passing through the LH/PFA. RT-PCR studies of the hypothalamus have suggested the presence of very low abundance D_2 and D_1 transcripts in the PFC (Fetissov *et al.*, 2002), but it is unclear whether these result from the dissection area encroaching upon cells that express DA receptors in surrounding regions, such as the zona incerta or paraventricular hypothalamus, or alternatively dendritic or even axonal transport of the transcript (Steward & Schuman, 2001; Lee & Hollenbeck, 2003).

Because D_2 mRNA-positive cells are so few in number and no other DA receptor transcript mRNA-expressing cells were present in detectable levels in the LH/PFA, it is unlikely that orexin cells are directly regulated by dopamine.

The nucleus accumbens is not involved in dopamine agonist-elicited activation of orexin neurons

The ability of dopamine agonists to strongly activated orexin neurons despite the lack of dopamine receptors on LH/PFA cells suggests that dopamine may transsynaptically activate orexin neurons. Neurons in a large number of brain regions express D1 or D2 receptors, or both, and project to the LH/PFA, and could therefore be positioned to transsynaptically activate orexin cells. Among such sites is the nucleus accumbens. Previous studies have reported that acute pharmacological inactivation of the NAS elicits feeding and induces Fos in orexin cells of the lateral LH/PFA (Stratford & Kelley, 1999; Satoh *et al*., 2003; Zheng *et al*., 2003; Baldo *et al*., 2004). These observations suggest that accumbal neurons projecting to the LH/PFA may suppress the activity of orexin cells, and conversely that inhibition of NAS projection neurons activates orexin cells. Because NAS neurons project mainly to the region lateral to the fornix but have a very sparse projection to the region that contains medial orexin cells (Usuda *et al*., 1998; our unpublished observations), we anticipated that NAS lesions would attenuate apomorphine-elicited Fos expression in the lateral but not medial LH/PFA. However, ibotenic acid lesions of the medial shell, from which most of the accumbal projections to the LH/PFA originate (Zahm & Heimer, 1993; Usuda *et al*., 1998), failed to attenuate apomorphine-induced activation of either the lateral or medial orexin cells.

Arousal and locomotor activity as mediators of apomorphine-induced activation of orexin cells

Postsynaptically acting doses of mixed and selective D1 or D2 dopamine agonists exert several effects that may contribute to their ability to activate orexin cells. Among these actions are the behavioural effects of dopamine agonists, such as their ability to increase locomotor activity and elicit arousal (Kropf *et al*., 1989; Carr *et al*., 2001; Isaac & Berridge, 2003). We reasoned it was therefore possible that the increase in activity would drive orexin cells. We examined this possibility by treating anaesthetized rats with apomorphine. The dopamine agonist activated orexin cells in awake, freely moving rats, but not in anaesthetized animals.

Several other lines of evidence also argue against locomotor activity driving orexin cells, at least orexin cells in the medial LH/PFA. First, we found that apomorphine-induced activation of orexin neurons is not blocked by pretreatment with either selective D1 or D2 antagonists, yet both D1 and D2 antagonists block the motor stimulant effects of apomorphine (Asper *et al*., 1973; Köhler *et al*., 1981). Second, excitotoxic lesions of the nucleus accumbens suppress apomorphine-induced locomotor activity (Kafetzopoulos, 1986; Weissenborn & Winn, 1992) but do not attenuate apomorphine-induced Fos expression in orexin cells. Finally, studies in sleep-wake consolidated primates suggest that locomotor activity does not increase cerebrospinal fluid orexin-A levels (Zeitzer *et al*., 2004).

In contrast to the hyperactivity produced by dopamine agonists, the wake-promoting or arousing properties of apomorphine may be a prerequisite for activation of medial orexin cells. Intraventricular administration of either D1 or D2 receptors alone awakens animals from sleep and results in an electroencephalographically defined aroused state (Isaac & Berridge, 2003).

Alternative mechanisms subserving dopamine agonist-elicited activation of orexin cells

The paucity of lateral hypothalamic neurons that express detectable levels of DA receptor transcripts suggests that DA agonists activate orexin cells indirectly. However, a direct action of dopamine on orexin cells is possible if dopamine acts on noncognate receptors expressed by orexin neurons. Dopamine has low nanomolar affinity for the α_{2C} receptor, through which dopamine regulates adenylyl cyclase (Zhang *et al*., 1999); this adrenergic receptor is localized to the LH/PFA. In contrast to our data indicating that dopamine activates orexin cells *in vivo*, Li & van den Pol (2005) have recently reported that dopamine directly hyperpolarizes orexin cells in hypothalamic slices. They reported that dopamine hyperpolarized orexin cells through D2 but not D1 receptors. However, the high concentrations (50 μM) of both DA and the D2 antagonist, eticlopride, used in the study suggests that DA may be signalling through some noncognate receptor to hyperpolarize orexin cells. Regardless, it is difficult to reconcile the suggestion of a direct effect of dopamine on orexin neurons with the observation that these cells largely lack DA receptors.

One other mechanism through which dopamine agonists may drive orexin cells is via mobilization of peripherally derived factors that activate orexin cells. Systemic administration of dopamine receptor agonists may effect release of adipocytokines from fat by targeting dopamine receptors on adipocytes or by acting centrally to alter sympathetic outflow (Nisoli *et al*., 1992; Lee *et al*., 1998). Among the adipocytokines that may be involved in the regulation of orexin neurons are leptin and ghrelin. The long form of the leptin receptor is expressed by orexin neurons (Meister, 2000), and peripheral leptin administration induces Fos in LH/PFA neurons (Bouret *et al*., 2004). Ghrelin administration has been reported to induce Fos in orexin neurons (Olszewski *et al*., 2003). The effects of the adipocytokines, resistin and adiponectin, on orexin cells have not been determined, although resistin inhibits depolarization-induced dopamine release in the hypothalamus (Brunetti *et al*., 2004).

Conclusions

Dopamine agonists induce Fos in orexin cells, with both D1-like and D2-like agonists activating orexin cells. Dopamine regulates orexin cells in the medial LH/PFA to a significantly greater degree than laterally placed orexin cells, emphasizing an emerging appreciation of heterogeneity of orexin neurons and suggesting that key afferents to orexin neurons differentially regulate orexin cells, consistent with our findings that dopamine agonists transsynaptically regulate orexin neurons and only rare cells in the LH/PFA express dopamine receptors. The observation that accumbal lesions do not block apomorphine-induced activation of orexin neurons may suggest that dopamine agonist-elicited arousal, but not hyperactivity, plays a critical role in activating orexin cells. This latter finding also suggests that orexin neurons are both central to the arousal state of animals but that arousal may in turn homeostatically regulate orexin.

Supplementary material

The following supplementary material may be found on:
http://www.blackwellpublishing.com/products/journals/suppmat/EJN4121/EJN4121sm.htm

Table S1. Apomorphine and selective D1 and D2 dopamine receptor agonists increase the density of single-labelled Fos-li cells in the LH/PFA.

Table S2. Effects of pretreatment with SCH 23390 and haloperidol, alone or combined on apomorphine-induced increase in the density of single-labelled Fos-li cells in the LH/PFA.

Table S3. Unilateral ibotenic acid lesions of the medial nucleus accumbens fail to alter the density of single-labelled Fos-li cells in the LH/PFA in response to an apomorphine challenge.

Table S4. Isoflurane anaesthesia blocks the ability of apomorphine to increase the density of single-labelled Fos-li cells in the LH/PFA.

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Abbreviations

DA, dopamine; -li, -like immunoreactive, LH/PFA, lateral hypothalamus/perifornical area; NAS, nucleus accumbens; VTA, ventral tegmental area.

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