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ORIGINAL ARTICLE

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Calcium-dependent, D2 receptor-independent induction of c-fos by haloperidol in dopamine neurons

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Abstract Antipsychotic drugs such as haloperidol act as dopamine D2 receptor antagonists to produce a number of cellular effects including the induction of immediate-early genes such as c-fos. It has been hypothesized that blockade of D2 receptors by antipsychotics is responsible for the induction of c-fos, but the mechanism has not been determined. Using cultured ventral tegmental area (VTA) dopaminergic neurons as a model, we report that nanomolar concentrations of haloperidol cause a time-dependent increase in Fos expression in dopaminergic neurons.

Surprisingly, this induction was not mimicked by sulpiride, a selective D2 receptor antagonist, and was not blocked by Rp-cAMPS, an antagonist of protein kinase A (PKA), thus suggesting that D2 receptors and the cAMP cascade are not required. The induction of Fos expression was blocked by tetrodotoxin, BAPTA and KN-93, thus showing that it is activity- and calcium-dependent and requires the activation of a calmodulin-dependent kinase (CaMK). Together, these results suggest that haloperidol induces Fos expression in dopaminergic neurons through a D2 receptor-independent increase in intracellular calcium, leading to CaMK activation.

Keywords Fos · Calcium · Culture · Dopaminergic neurons · Haloperidol · Immunofluorescence

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Introduction

Although the mechanism of action of antipsychotics remains poorly understood, most have the ability to induce the expression of immediate early genes such as c-fos (Robertson and Fibiger 1992; Robertson et al. 1994). C-fos encodes a nuclear phosphoprotein (Fos) that usually acts as a heterodimeric transcription factor. The transient expression of c-fos can be considered as a potential marker of neuronal activation (Dragunow and Robertson 1987; Sagar et al. 1988) and the induction of Fos may participate in the initiation of plastic changes in the nervous system associated with physiological events such as activity-dependent synaptic plasticity and with pathological conditions such as seizures (Dragunow and Robertson 1987; Sagar et al. 1988). Acute administration of the classical antipsychotic haloperidol or the atypical antipsychotic clozapine has been reported to differentially increase the expression of c-fos in a number of nuclei including the nucleus accumbens, the amygdala and the dorsolateral striatum (Deutch et al. 1992; Robertson and Fibiger 1992; Robertson et al. 1994).

Although the different classes of classical and atypical antipsychotic drugs differ in their pharmacological properties, all have in common their ability to act as antagonists or inverse agonists on the dopamine D2 receptor (Hall and Strange 1997; Vanhauwe et al. 2000) (for review see Strange 2001). Therefore, it has been suggested that the induction of Fos by these drugs occurs as a consequence of the activation of signaling pathways previously inhibited by ongoing D2 receptor activation (Dragunow et al. 1990; Robertson and Fibiger 1992). D2 receptors are G protein-coupled receptors and have been demonstrated to couple to a number of signaling pathways including adenylyl cyclase (Onali et al. 1985) and potassium channels (Greif et al. 1995; Kitai and Surmeier 1993; Williams et al. 1989). Under conditions where a basal level of D2 receptor occupation occurs, blockade of D2 receptors with antipsychotics such as haloperidol is thus expected to relieve the inhibition of adenylyl cyclase, leading to activation of PKA and to phosphorylation of the cAMP response

element binding protein (CREB). Phospho-CREB can then interact with the cAMP response element (CRE) site in the promoter region of a number of genes including the c-fos gene (Montminy and Bilezikjian 1987; Sheng et al. 1990). In support of this hypothesis, haloperidol-mediated induction of c-fos in the striatum has been shown to be prevented by intrastriatal injection of specific antisense oligonucleotides directed against CREB mRNA (Konradi and Heckers 1995) and to be blocked in PKA knockout mice (Adams et al. 1997).

Blockade of D2 receptors could also induce Fos expression through cAMP-independent mechanisms. In many preparations, it has been shown that D2 receptors increase outward potassium currents, leading to cell hyperpolarisation (reviewed in Missale et al. 1998). Blockade of dopamine action at D2 receptors by haloperidol is therefore expected to enhance the firing rate of dopaminergic neurons (Bunney et al. 1973), possibly leading to enhanced c-fos transcription. In fact, it has been previously demonstrated that membrane depolarization activates c-fos transcription in PC12 cells (Bartel et al. 1989; Morgan and Curran 1986). Transcriptional activation of c-fos by depolarization in these cells is Ca^{2+}-dependent and is blocked by Ca^{2+} chelation, by specific inhibitors of L-type Ca^{2+} channels and by calmodulin antagonists (Morgan and Curran 1986). This pathway also seems to converge on the CRE element in the c-fos gene promoter region as a result of CREB phosphorylation by CaMK (Sheng et al. 1990).

Considering the above stated hypotheses, the proposed role of the D2 receptor in haloperidol-induced c-fos expression, and the fact that dopaminergic neurons of the substantia nigra (SN) and VTA express considerable levels of D2 receptors (Bouthenet et al. 1991; Meador-Woodruff et al. 1989; Weiner et al. 1991), it may be considered somewhat surprising that the induction of Fos by antipsychotics in dopaminergic cell body regions has not yet been closely studied (but see (Wirtshafter and Asin 1995) for Fos induction by haloperidol in non-identified cells in the VTA). To investigate the mechanism of c-fos induction by haloperidol in dopaminergic neurons, we have used a preparation of VTA neurons in primary culture. We find that haloperidol is indeed able to induce Fos expression in a subset of dopaminergic neurons, but that the D2 receptor and the cAMP cascade are not involved.

Materials and methods

Cell culture. Primary cultures of rat VTA neurons were prepared according to recently described protocols (Michel and Trudeau 2000; Bourque and Trudeau 2000; Congar et al. 2002) derived from Cardozo (1993) and Sulzer et al. (1998). Dissociated VTA neurons were plated on midbrain astrocytes grown in monolayers on pre-coated glass coverslips.

To prepare midbrain astrocytic cultures, Sprague Dawley rats (P0-P2) were cryoanesthetized. Cells from the mesencephalon were enzymatically dissociated using papain (Worthington Biochemical Corp., Lakewood, NJ, USA) and were grown in culture flasks for 5 to 10 days in Basal Medium Eagle with Earl's Salts (Gibco, Burlington, Ontario, Canada) supplemented with penicillin/streptomycin, GlutaMAX-1 (Gibco), Mito+ serum extender (VWR Canlab, Montreal, Canada) and 10% Fetal Calf serum (Gibco). After the first 48 h, astrocyte cultures were vigorously washed with cold medium to remove most neurons, leaving only tightly adherent astrocytes. After reaching confluence, astrocytes were trypsinized, washed, collected and plated at 100,000 living cells per milliliter on collagen/poly-L-lysine-coated coverslips. Three to four days after plating, 5-Fluoro-2-Deoxyuridine (FUDR) was added to halt glial proliferation.

To prepare neurons, a 1 to 2 mm-thick slice was cut at the level of the midbrain flexure. The VTA was isolated using a custom tissue micro-punch. As for preparation of astrocytes, the tissue was digested with papaïn, before being gently triturated. The dissociated cells were then collected by centrifugation and diluted at a density to optimize neuronal viability (240,000 living cells per milliliter) and plated onto a pre-established midbrain astrocyte monolayer. Further astrocytic division was inhibited by a second addition of FUDR 24 h after neurons were plated. Kynurenic acid (0.5 mM) (Sigma Chemicals Co., St. Louis, MO, USA) was added to the culture medium 7 days after neurons were plated in order to prevent excitotoxicity. Cultures were incubated at 37°C in 5% CO_2 atmosphere and maintained in Neurobasal-A/B27 medium (Gibco) supplemented with penicillin/streptomycin, GlutaMAX-1 (Gibco) and 5% Fetal Calf serum (Hyclone Laboratories, Logan, UT, USA). The cultures contained between 15 and 40% dopaminergic neurons and were used between 12 and 16 days after plating. All animal handling protocols were approved by the Université de Montréal animal ethics committee and complied with the European Community guidelines.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde in phosphate-buffered solution (PBS) (pH 7.4) for 30 min. After permeabilization with 0.05% Triton X-100 and blocking with bovine serum albumin and normal goat serum, cells were incubated overnight at 4°C with primary antibodies against tyrosine hydroxylase (Sigma) and Fos (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The rabbit polyclonal Fos antibody that was used is known not to be cross-reactive with Fos B, Fra-1 or Fra-2. The cells were then rinsed several times and incubated at room temperature for 60 min with Alexa-488 and Alexa-546 conjugated secondary antibodies (Molecular Probes Inc., Eugene, OR, USA). Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed by epifluorescence microscopy on a Nikon Eclipse TE-200 inverted microscope. Images of immunofluorescent labeling were acquired using a Hamamatsu Orca-II digital cooled CCD camera and an Inovision workstation using Isee software (Inovision Corporation, Raleigh, NC, USA.).

In order to evaluate the relative proportion of dopaminergic cells expressing Fos, cells which were doubled labeled for tyrosine hydroxylase and Fos were observed by epifluorescence microscopy and counted. The number of doubly positive cells, that is dopaminergic cells expressing Fos, was evaluated for every coverslip and expressed as a percentage of the total number of dopaminergic cells on the coverslip. In each experiment, the proportion of dopaminergic cells expressing Fos was expressed as a percentage of the value measured in the control condition, performed in parallel. Such a normalization was required to control for the variability in the proportion of dopaminergic neurons expressing Fos under control conditions in different cultures (range: 1.8 to 21.5%; mean: 8.6±6.1%, n=40). In each experiment, coverslips from the same culture were used for each different treatment. All experiments include results from at least two different cultures.

In situ hybridization. Cultured neurons were fixed in 4% paraformaldehyde for 30 min. Immunolabeling of dopaminergic neurons was performed prior to hybridization, using a primary antibody against tyrosine hydroxylase (Sigma) followed by a biotin-SP-conjugated anti-mouse antibody and horseradish peroxidase conjugated-streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The labeling was revealed with a 0.05% solution of 3,3'-diaminobenzidine (Sigma) containing 0.02% H_2O_2. Then, a specific ^{35}S-UTP radiolabeled cRNA probe was used. The rat D_2

receptor probe consisted of a fragment of 1,032 bp (1,311–2,343) as previously described (Dearry et al. 1990). The probe recognized both the short and the long isoforms of the receptor. The fragment was subcloned into a pBluscript SK+ vector (Stratagene, La Jolla, CA, USA) and linearized with *EcoRI*. Single-stranded complementary RNA (cRNA) probes were synthesized and labeled using a Riboprobe system (Promega, Madison, WI, USA) with [^{35}S]UTP (Dupont NEN, Boston, MA, USA) using the T_3 RNA polymerase. Cells were permeabilized in 2 × SSC containing 0.1% Triton-X-100 for 10 min at room temperature. In situ hybridization was performed overnight at 55°C in a standard hybridization buffer containing 50% formamide as previously described (Beaudry et al. 2000). The slides were then washed, dehydrated, air-dried and dipped in LM-1 photographic emulsion (Amersham/Pharmacia Biotech, Baie d'Urfé, Québec, Canada) melted at 43°C, air-dried for 3 h and stored in the dark for 6 weeks at 4°C. The emulsion was developed in D-19 developer (Kodak) for 5 min, rinse in deionized water and fixed for 10 min in rapid fixer solution (Kodak). Slides were rinsed under tap water for 30 min, dehydrated and mounted using Permount media (Fisher). Positive cells were identified under bright field illumination. Images were captured with a Nikon Coolpix 990 digital camera.

RT-PCR. To identify the subtype of dopamine D2 family class of receptors present in our VTA primary culture model, we performed a RT-PCR analysis. Total RNAs were extracted from VTA primary cultured cells and total rat brain (used as positive control for the PCR amplification) according to the Chomczynsky and Sacchi method (Chomczynski and Sacchi 1987). One microgram of total RNAs was used for reverse transcription (RT) using 50 U of Expand reverse transcriptase (Roche, Laval, Québec, Canada) following the conditions suggested by the manufacturer for 1h 30 at 42°C with a poly (dT)15 primer. Two microliters of the RT reaction was used for the amplification of both D2 and D3 receptors and 5 µl was used for the amplification of the D4 receptor. PCR conditions included a 5 min at 94°C incubation step to active the Taq platinum (1 U, Invitrogen, Carlsbad, CA, USA) followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C with a final elongation period of 7 min at 72°C. The PCR mixture obtained from VTA primary cultures was re-amplified under the same conditions to avoid false negative results. The following set of primers were used for the PCR reactions: for the D2 receptor: sense: 5'-gcagtcgagctttcagagcc-3'; antisense: 5'-tctgcggctcatcgtcttagg-3', which generate two fragments of 316 bp and 403 bp corresponding to the short (D2S) and the long (D2L) isoforms of the D2 receptor, respectively, for the D3 receptor : sense: 5'-agcatctgctccatctccaaccc-3'; antisense: 5'-aggagttccgagtcctttccacg-3' for a fragment of 459 bp and for the D4 receptor : sense: 5'-ctcatcggcatggtgttggca-3'; antisense: 5'-atcagcgtggacaggtttgtg-3' for a fragment of 256 bp.

Drugs. All chemicals were obtained from Sigma Chemicals Co. (St-Louis, MO, USA) except for forskolin (Calbiochem, San Diego, CA, USA), TTX (Alomone Laboratories, Jerusalem, Israel), BAPTA-AM (Molecular Probes Inc., Eugene, OR, USA) and LR-172 (ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine) (Tocris Cookson, Ballwin, MO, USA). Haloperidol, Forskolin, BAPTA-AM and KN-93 were dissolved as stock solutions in dimethyl sulfoxide (DMSO). The final concentration of DMSO was equal to or lower than 0.001%. Stock solutions of TTX, dibutyryl-cAMP, Rp-cAMPS and LR172 were prepared in water. Sulpiride was prepared in ethanol. All drugs were diluted directly in the culture medium to their final concentration except for BAPTA-AM, which was loaded into cells after dilution in physiological saline.

Statistics. Statistical analysis were performed on raw data. For simple two group comparisons, the data were analyzed using the Student *t*-test. Multi-group data were otherwise compared using either a one-way or two-way analysis of variance (ANOVA), as appropriate, followed by the Fisher LSD post-hoc test, when required.

Results

Haloperidol induces Fos expression in VTA dopaminergic neurons

Experiments were performed on rat VTA neurons in culture. Fos expression was detected by immunocytochemistry. In order to confirm the dopaminergic phenotype of neurons, double labeling was performed using an antibody directed against tyrosine hydroxylase (TH). The cultures contained between 15 and 40% dopaminergic neurons. Treatment of VTA cells for 60 min with the classical antipsychotic haloperidol produced an increase in the proportion of dopaminergic neurons displaying nuclear Fos immunoreactivity (Fig. 1A). The effect of acute haloperidol on Fos expression by dopaminergic neurons was characterized by pharmacological studies. A dose-response relationship was established (Fig. 1B) using concentrations of haloperidol ranging from 0.1 nM to 1 µM. The quantification of neurons doubly positive for TH and Fos was performed by epifluorescence microscopy (see Materials and Methods for details). In untreated, control cells, 8.6±6.1% of dopaminergic neurons expressed Fos. Haloperidol caused a significant increase in Fos expression (one-way ANOVA, $F(5)=2.58$, $p=0.047$). The dose-response relationship displayed an inverted U shape (Fig. 1B). The maximal response was elicited by 1 nM haloperidol, which caused more than a 3-fold increase in the proportion of doubly positive neurons (340±32%, $n=6$, Fisher LSD, $p=0.008$). Although all subsequent experiments were performed on dopaminergic neurons, we found that at 1 nM, haloperidol also caused a 3-fold increase in the proportion of non-dopaminergic neurons immunoreactive for Fos (389±67%, $n=6$ coverslips, Student *t*-test, $p<0.001$, data not shown). In these cultures a majority of the non-dopaminergic neurons are GABAergic (not shown). Astrocytes, also present in the cultures were unaffected (not shown).

A kinetic study of the effect of haloperidol on Fos expression by dopaminergic neurons was performed. Cultured VTA neurons were incubated with 1 nM haloperidol for 15 to 90 min (Fig. 1C). Fos expression was evaluated as described previously. A 60 min long treatment was necessary to stimulate maximally Fos expression (342±18%, $n=4$, one-way ANOVA $F(3)=19{,}422$, Fisher LSD, $p<0.01$). On the basis of these results, all subsequent experiments were conducted using an incubation time of 60 min with 1 nM haloperidol.

Lack of involvement of D2 receptors

Cultured dopaminergic neurons in our model demonstrate presynaptic inhibition in response to D2 receptor agonists (Congar et al. 2002; Sulzer et al. 1998) and express abundant levels of D2 receptor mRNA, as confirmed by in situ hybridization (Fig. 2A) and by RT-PCR analysis (Fig. 2B). In contrast, D3 and D4 mRNA could not be detected

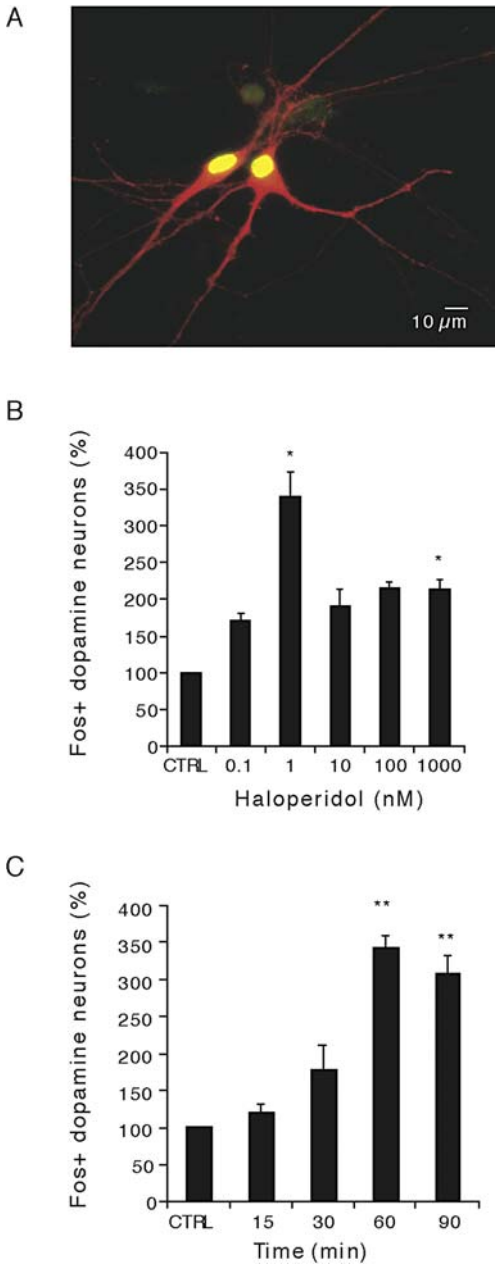

Fig. 1A–C Haloperidol induces Fos expression in dopaminergic neurons in culture. **A** Immunofluorescence image of neurons double-labeled for tyrosine hydroxylase (*red*) and Fos (*green*). The nuclear region expresses the two markers and appears *yellow*. **B**, **C** Histograms illustrating the dose-response relationship and time course of haloperidol's effect on Fos. Haloperidol concentrations are expressed in nM. The percentage of dopaminergic neurons expressing Fos (Fos+ dopamine neurons) was normalized to the untreated control condition of each experiment (n=6). Data are expressed as mean ± SEM. *p≤0.05, **p≤0.01

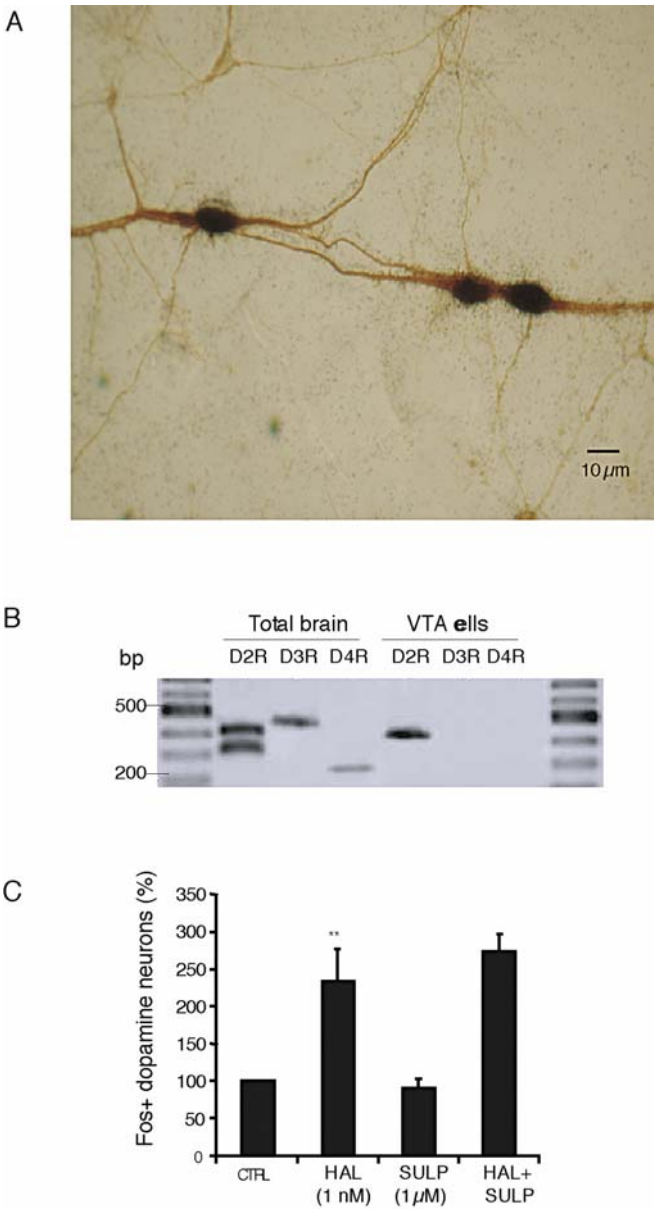

Fig. 2A–C D2 receptor blockade by haloperidol is not necessary to induce c-fos expression in dopaminergic neurons. **A** In situ hybridization illustrating the specific labeling of cultured dopaminergic neurons by radiolabeled D2 receptor mRNA probes (*silver grains*). Dopaminergic neurons were identified using tyrosine hydroxylase immunostaining (brown diaminobenzidine deposit). **B** Expression of the D2 receptor family in rat VTA primary culture determined by RT-PCR analysis. Specific sets of primers were used to amplify each D2-like receptor subtypes. Corresponding length of fragments generated are D2S: 316-bp, D2L: 403-bp, D3: 459-bp and D4: 256-bp. Total brain mRNA was used as a positive control. A 100-bp ladder is shown on the left and right. The position of the 200-bp and 500-bp fragments is identified next to the left lane. **C** Histogram illustrating the lack of effect of the D2 receptor antagonist sulpiride (1 μM) on haloperidol-induced Fos expression in dopaminergic neurons (n=6). Data are expressed as mean ± SEM. **p≤0.01

(Fig. 2B). To assess whether the induction of Fos expression by haloperidol in dopaminergic neurons was dependent on D2 receptor binding, we evaluated whether its effect could be mimicked by (±)-sulpiride, another D2 receptor antagonist. We treated VTA neurons with sulpiride at a concentration which should saturate D2-type receptors (1 μM). Sulpiride produced no significant change in the percentage of dopaminergic neurons expressing Fos (SULP= 90±12% of control, n=6, Two-Factor ANOVA, main ef-

fect, $F(1)=0.616$, $p=0.442$) (Fig. 2C). As an additional test of the implication of D2 receptors in the effect of haloperidol, we applied haloperidol (1 nM) in the presence of sulpiride (1 µM). This manipulation, which should prevent haloperidol from binding to D2 receptors, did not prevent induction of Fos expression by haloperidol (HAL= 243±42%, HAL+SULP=275±23% Two-Factor ANOVA, interaction, $F(1)=0.727$, $p=0.404$). These results suggest that binding of haloperidol to the dopamine D2 receptor is not necessary and sufficient to stimulate c-fos transcription in dopaminergic neurons.

Lack of involvement of sigma receptors

Besides its high affinity to D2 receptors, haloperidol is also recognized as a high affinity ligand of sigma receptors (Seth et al. 1998), a family of receptors whose endogenous ligand and physiological role is unclear but that are located in areas rich in dopaminergic innervation (Ceci et al. 1988; Graybiel et al. 1989). Using LR172, a selective sigma receptor ligand (de Costa et al. 1992), we assessed the implication of sigma receptors in the induction of c-fos caused by haloperidol in dopaminergic neurons. LR172 is usually considered an agonist at sigma receptors (Vilner and Bowen 2000; Vilner et al. 1995), although it has been suggested that it can also act as an antagonist on some responses (McCracken et al. 1999). Cells were exposed for 60 min to a concentration of LR172 high enough to saturate most sigma receptors (100 nM). By itself LR172 produced no significant change in the proportion of dopaminergic neurons expressing Fos (LR172= 263±70% of control, $n=6$, Two-Factor ANOVA, main effect, $F(1)=1.414$, $p=0.250$) (not shown). Furthermore, coapplication of LR172 with haloperidol (1 nM) did not prevent the effect of haloperidol on Fos expression (HAL= 467±80%, HAL+LR172=489±111%, Two-Factor ANOVA, interaction, $F(1)=2.032$, $p=0.171$). These results suggest that the binding of haloperidol to sigma receptors is not required to stimulate c-fos transcription in dopaminergic neurons.

Implication of a PKA independent mechanism

Several second-messenger pathways have been implicated in c-fos transcriptional activation. One of the better characterized is the PKA pathway, which can activate c-fos transcription through phosphorylation of CREB and the activation of a calcium/cyclic-adenosine monophosphate response element (CaRE/CRE) in the c-fos promoter region (Konradi et al. 1994; Montminy and Bilezikjian 1987). We found that activation of the PKA signaling pathway with the adenylyl cyclase activator Forskolin mimicked the effect of haloperidol on Fos expression (Fig. 3A). Forskolin (10 µM) produced a fourfold increase in the percentage of dopaminergic neurons showing Fos labeling (Forsk=422±51%, One-way ANOVA, $p<0.001$). Direct activation of PKA with the cAMP analog Bt_2-cAMP (100 µM) produced a similar effect (Bt_2-cAMP=248±25%, t-test, $p<0.01$) (not shown). To determine whether the activation of PKA was necessary for haloperidol to induce c-fos transcription in cultured dopaminergic neurons, cells

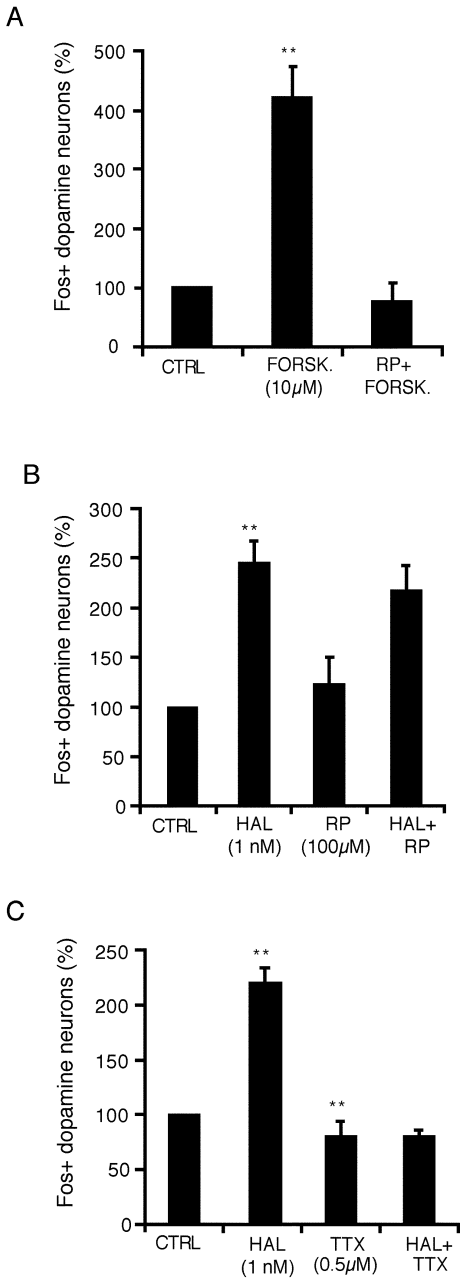

Fig. 3A–C Haloperidol induces Fos expression by an activity-dependent mechanism that is independent of PKA activation. **A** Histogram illustrating that elevations of intracellular cAMP levels using the adenylyl cyclase activator forskolin (*FORSK*) (n=4) increases the proportion of dopaminergic neurons expressing Fos. This increase is prevented by the membrane-permeant PKA inhibitor Rp-cAMPS (*RP*). **B** Histogram illustrating that Rp-cAMPS fails to prevent haloperidol-induced Fos expression in dopaminergic neurons ($n=6$). **C** Histogram illustrating that a pre-treatment with the sodium channel blocker tetrodotoxin (*TTX*) prevents haloperidol-induced Fos expression ($n=5$). Data are expressed as mean ± SEM. $^{**}p\leq 0.01$

were exposed to the PKA inhibitor Rp-cAMPS (RP). Rp-cAMPS was applied in the culture medium at a final concentration of 100 µM for 120 min, alone or in the presence of haloperidol for the last 60 min. In the presence of Rp-cAMPS, haloperidol was still able to stimulate Fos expression in dopaminergic neurons (Fig. 3B). This effect was not significantly different from that produced by haloperidol alone (HAL+RP=217±25%, HAL=244±23%, Two-Factor ANOVA, interaction, $F(1)=1.266$, $p=0.274$). However, incubation of cells with Rp-cAMPS was effective in preventing Forskolin-induced Fos expression (RP+FORSK=78±27%, FORSK=422±51%, One-way ANOVA, $p<0.001$) (Fig. 3A). These results show that activation of PKA is not necessary for the effect of haloperidol on Fos expression.

Haloperidol induces Fos expression by an activity-dependent mechanism

To determine whether Fos induction by haloperidol was activity-dependent, we exposed cultured dopaminergic neurons to the sodium channel blocker tetrodotoxin (TTX). Cells were treated with haloperidol alone for 60 min or with 0.5 µM TTX for 70 min in the absence or in the presence of haloperidol for the last 60 min. TTX treatment completely prevented the induction of Fos expression caused by haloperidol (HAL=221±13%, HAL+TTX=80±5%, $n=5$, Two-Factor ANOVA, interaction, $F(1)=37.380$, $p\leq0.001$) (Fig. 3C). The effect of haloperidol on Fos expression by dopaminergic neurons is thus dependent on cell firing.

Fos induction by haloperidol requires calcium

Studies performed on PC12 cells have suggested that Ca^{2+} is a major second messenger regulating immediate early gene expression in excitable cells (Greenberg et al. 1986). We used the membrane-permeable Ca^{2+} chelator BAPTA-AM to determine whether a rise in intracellular Ca^{2+} is required for haloperidol to enhance Fos expression in dopaminergic neurons. Cultured neurons were exposed to BAPTA-AM (10 µM) for 90 min alone or in the presence of haloperidol (1 nM) for the last 60 min. Changes in the percentage of double-labeled neurons were evaluated as described previously. Incubation of cells with BAPTA-AM alone caused a small but significant reduction in the percentage of double-labeled neurons in comparison to the untreated condition (BAPTA=80±33%, $n=6$, Two-Factor ANOVA, $F(1)=17.189$, $p=0.001$) (Fig. 4A). Furthermore, chelation of intracellular Ca^{2+} by BAPTA-AM prevented Fos induction by haloperidol in dopaminergic neurons (HAL=206±84%, HAL+BAPTA=104±42%, $n=6$, Two-Factor ANOVA, interaction, $F(1)=5.565$, $p=0.029$). These results suggest that an increase in intracellular Ca^{2+} is necessary for haloperidol to enhance Fos expression in cultured dopaminergic neurons.

KN-93 blocks the induction of c-fos caused by haloperidol in dopaminergic neurons

Two major pathways have been identified as regulating immediate early gene expression through CREB phosphorylation: PKA and Ca^{2+}/CaMK signaling pathways. Considering the block of c-fos induction by BAPTA, a reasonable hypothesis was that the induction of c-fos expression by dopaminergic neurons requires Ca^{2+} and CaMK activation. Therefore, using KN-93, a CaMK-II and IV inhibitor, we assessed the role of the CaMK signaling pathway in the induction of c-fos by haloperidol in dopaminergic neurons. Cultured cells were exposed to 10 µM KN-93 alone or in the presence of haloperidol. By itself, KN-93 significantly reduced the percentage of dopaminergic neurons expressing Fos (KN-93=32±10% Two-Factor ANOVA $F(1)=26.713$, $p<0.001$) (Fig. 4B). Moreover, KN-93 treat-

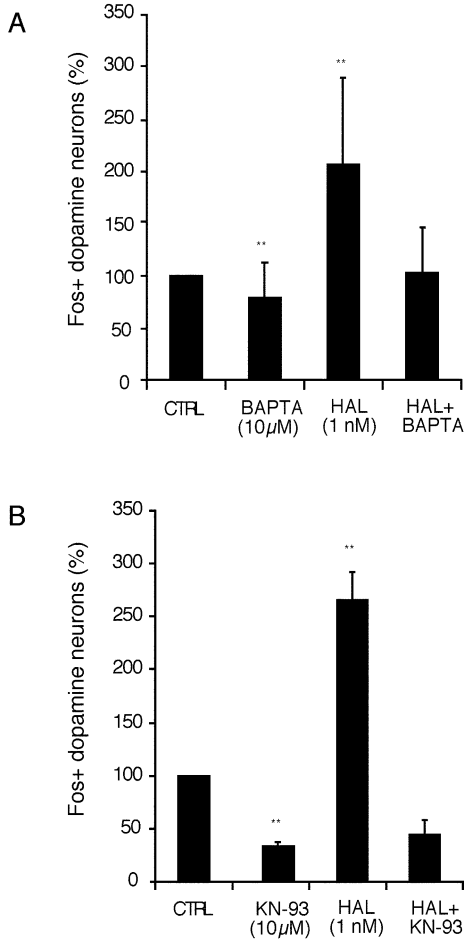

Fig. 4A, B Effect of calcium chelation or calmodulin-dependent kinase inhibition on Fos expression induced by haloperidol. **A** Histogram showing that chelation of intracellular calcium using the membrane-permeant intracellular calcium chelator BAPTA-AM (*BAPTA*) prevents haloperidol-induced Fos expression in dopaminergic neurons (*HAL+BAPTA*) ($n=6$). **B** Histogram showing that the calmodulin-dependent kinase inhibitor KN-93 prevents haloperidol-induced Fos expression (*HAL+KN-93*) ($n=6$). Data are expressed as mean ± SEM. $^{**}p\leq0.01$

ment prior to haloperidol application completely prevented the induction of Fos expression caused by haloperidol (HAL=266±30%, HAL+KN-93=44±13%, n=6, Two-Factor ANOVA, interaction, F(1)=13.097, p=0.002). These results suggest that the Ca^{2+}/CaMK signaling pathway is involved in the activating effect of haloperidol on c-fos expression in cultured dopaminergic neurons.

Discussion

Several studies have demonstrated that the antipsychotic agent haloperidol causes an increase in the expression of the transcription factor Fos in the central nervous system (Dragunow et al. 1990; Robertson and Fibiger 1992). However, the mechanism by which haloperidol induces Fos expression remains to be clearly elucidated. The present work provides novel information on the mechanism of c-fos induction by haloperidol in neurons. We have made two major observations. First, we have shown for the first time that at least in culture, haloperidol can induce Fos expression in dopaminergic neurons through a D2-independent mechanism. Second, we have shown that haloperidol induces Fos expression in neurons through an activity-dependent, Ca^{2+}- and CaMK-dependent mechanism.

Expression of Fos in dopaminergic neurons

Our finding that haloperidol can induce Fos expression in dopaminergic neurons is a novel finding that extends previous work showing abundant Fos expression in response to haloperidol in the dorsal striatum, the nucleus accumbens and the amygdala (Dragunow et al. 1990; Robertson and Fibiger 1992; Robertson et al. 1994). The reason why previous studies have not focused much attention or reported a response in the VTA is unclear. However, it is likely that modest changes in the proportion of Fos-positive dopaminergic neurons may have escaped detection in vivo, especially considering the heterogeneity of the cell population in the VTA and the fact that previous studies have not performed systematic double-labeling for tyrosine hydroxylase, as was done in the current study. Alternately, the receptor(s) involved in initiating the signaling cascade leading to Fos expression in response to haloperidol may be expressed at higher levels in culture than in vivo.

Identity of the receptor mediating the c-fos response in dopaminergic neurons

Antipsychotic drugs share the ability to bind with high affinity to the dopamine D2 receptor and to induce c-fos expression. Considering these facts, the simplest hypothesis is that in all brain areas, c-fos induction in response to haloperidol results directly from the blockade of D2 receptors. In support of this possibility, it has been shown that in the striatum the induction of c-fos by haloperidol is reduced by selective D2 receptor agonists (Miller 1990) and mimicked by the selective D2 receptor antagonists raclopride and sulpiride (Ozaki et al. 1997; Robertson and Fibiger 1992).

In the present report, blockade of the D2 receptor by haloperidol cannot explain the induction of Fos expression in rat VTA dopaminergic neurons in culture. Indeed, the selective D2 receptor antagonist sulpiride did not mimic the effect of haloperidol. In addition, exposure of neurons to haloperidol in the presence of a concentration of sulpiride which should saturate D2 receptors, failed to block the induction of Fos. These results cannot be explained by the lack of D2 receptor expression in our model. Indeed, our in situ hybridization results confirmed that much like in vivo (Bouthenet et al. 1991), cultured VTA dopaminergic neurons under our experimental conditions express D2 receptor mRNA (Fig. 2A). Our recent demonstration that D2 receptor agonists produce presynaptic inhibition in cultured dopaminergic neurons provides additional evidence for the expression of functional D2 receptors in our model (Congar et al. 2002). It should be noted that in our RT-PCR experiments, the long isoform of the D2 receptor was the most abundant. Although in one RT-PCR experiment low levels of the short isoform could also be detected (results not shown), only the long isoform was detected in another (Fig. 2B).

Although haloperidol can bind to D3 and D4 dopamine receptors with relatively high affinity, our finding that the mRNA for these receptors cannot be detected by RT-PCR in our cultures strongly argues against their implication. In addition, even though haloperidol displays some significant affinity for D1 dopamine receptors, it is unlikely that these receptors participate in the effect of haloperidol on dopaminergic neurons since there are no D1-like binding sites on VTA dopaminergic neurons (Boyson et al. 1986) and no significant levels of D1 mRNA can be detected in this structure (Mengod et al. 1991). Thus, dopaminergic neurons in our model of cultured VTA neurons probably do not express dopamine D1 receptors. It is thus reasonable to hypothesize that another class of receptor must be involved. The finding of a D2-independent induction of c-fos by haloperidol has been reported previously in PC-12 cells. Although haloperidol potently induces c-fos expression in these neuron-like cells, this effect is not mimicked by the selective D2 receptor antagonist sulpiride (Esteve et al. 1995).

In the present report, we found that haloperidol maximally induced Fos expression at 1 nM. Increasing the concentration of haloperidol did not further increase the response. In fact the proportion of dopaminergic neurons expressing Fos was even lower at higher concentrations. As haloperidol can bind to a number of receptors with affinity in the high nanomolar range (Hartman and Civelli 1996), a possible explanation is that at higher concentrations the effect on dopaminergic neurons is heterogenous as a consequence of the blockade of different receptors. Nonetheless, our observation of robust effects of haloperidol at low nanomolar concentrations is compatible with the involvement of a receptor to which haloperidol binds

with high affinity. We have not performed a broad screening of all receptors possibly involved. However, considering the high affinity of haloperidol for sigma receptors (K_D of 3 nM at α-1 receptor (Seth et al. 1998), and the observation that selective sigma receptors ligands induce Fos expression in rat brain (Dahmen et al. 1996; Sharp 1997), we investigated the possible implication of this class of receptors. Our finding that the selective sigma receptor ligand LR-172 failed to enhance Fos expression and to block the effect of haloperidol argues against the involvement of sigma receptors.

In addition to binding to the D2 family receptors and sigma receptors, haloperidol has the ability to bind to 5-HT_{2A} (Egan et al. 1998; Leveque et al. 2000) and α1-adrenergic receptors (Hartman and Civelli 1996; Rosen et al. 1995) with an affinity in the nanomolar range. These receptors may be possible targets and may explain the induction of c-fos by haloperidol in our model. However, in the dorsolateral striatum and the nucleus accumbens, ritanserin and prasozin, selective antagonists of these receptors, do not prevent the induction of Fos immunoreactive nuclei by haloperidol (Fink-Jensen et al. 1995). Further experiments will be required to test this possibility in VTA neurons and to identify more precisely the receptor(s) specifically involved.

Lack of implication of the PKA pathway

The signaling cascade mediating the induction of c-fos by haloperidol in vivo is not completely characterized. Several signaling pathways could mediate c-fos transcriptional activation (Bartel et al. 1989; Sheng et al. 1990). The most completely described pathway involves PKA-mediated phosphorylation of CREB (Gonzalez and Montminy 1989), followed by binding of phospho-CREB to the CRE element in the c-fos gene promoter region (Montminy and Bilezikjian 1987; Sheng et al. 1990). Compatible with the involvement of this pathway, haloperidol-induced Fos expression in vivo has been shown to be absent in PKA knockout mice (Adams et al. 1997). However, in a recent report, Adams and Keefe (2001) have cast some doubt on this hypothesis by showing that c-fos activation by the D2 receptor antagonist eticlopride in the striatum is not blocked by a PKA antagonist. In the present report, our results also argue against a critical role of PKA. Although we were able to confirm that under our experimental conditions activation of the PKA pathway was effective at producing c-fos transcriptional activation, we found that the PKA inhibitor Rp-cAMPS was unable to block c-fos activation by haloperidol.

Implication of an activity- and calcium-dependent mechanism

We found that preventing action potential generation using the sodium channel blocker TTX completely blocked the induction of c-fos caused by haloperidol. Based on these results, we can conclude that haloperidol acts through an activity-dependent mechanism on dopaminergic neurons. However, an indirect effect implicating nondopaminergic neurons cannot be excluded. These results are compatible with work on PC12 cells showing that extracellular stimuli leading to membrane depolarization induce several immediate early genes including c-fos (Bartel et al. 1989; Morgan and Curran 1986).

An enhancement in the firing rate of dopaminergic neurons could cause the opening of voltage-sensitive Ca^{2+} channels and an increase in intracellular Ca^{2+}. Considering several studies describing the essential role of Ca^{2+} in c-fos transcriptional activation (Ghosh et al. 1994; Morgan and Curran 1986; Sheng et al. 1990), we examined the role of Ca^{2+} in haloperidol-induced Fos expression. We found that Ca^{2+} chelation by BAPTA blocked the ability of haloperidol to induce Fos expression. These results are in agreement with the work of Esteve et al. (1995) who showed that incubation of PC12 cells in Ca^{2+}-free media prevents haloperidol-induced Fos expression.

In our studies, only a subset of dopaminergic neurons expressed Fos after haloperidol exposure. Although a number of interpretations are possible, it may be that the population of dopaminergic neurons in our cultures is heterogeneous and that only a subset of these neurons express the receptor(s) targeted by haloperidol at a sufficiently high level.

Role of Ca^{2+}/calmodulin-dependent protein kinase

Expression of the c-fos gene is under the control of two regulatory element named SRE and CaRE/CRE. The SRE element has been described as necessary for c-fos induction by serum, growth factors and PKC-activators (Treisman 1985). Binding of phospho-CREB to the CaRE/CRE element leads to c-fos transcription. Both PKA, activated by cAMP (Gonzalez and Montminy 1989), and CaMK, activated by the Ca^{2+}-dependent activation of calmodulin (Sheng et al. 1991; Wu et al. 2001), have the ability to phosphorylate CREB. In support of the implication of the later pathway, we report here that the CaMK inhibitor KN-93 blocks Fos induction by haloperidol in dopaminergic neurons. Although we have not directly measured phospho-CREB in the present set of experiments, taken together our results support the hypothesis that haloperidol induces Fos expression in dopaminergic neurons by increasing intracellular Ca^{2+}, leading to CaMK activation and CREB phosphorylation. Considering that c-fos induction by haloperidol was completely blocked by a CaMK inhibitor, a major role for other pathways that could regulate c-fos such as those involving PKC or MAP kinases cannot be excluded, but is unlikely.

In summary, the present study represents the first report describing the effect of haloperidol on Fos expression specifically in dopaminergic neurons. In our model of cultured VTA neurons, we have identified a mechanism of c-fos induction by the typical antipsychotic haloperidol that is independent of D2 receptor binding. This induction

of c-fos occurs through an activity-dependent increase in intracellular Ca^{2+} leading to an activation of CaMK. Although the physiological relevance of our observations remain to be specified, the fact that our main effect is obtained at low nanomolar concentrations of haloperidol suggests that it has the potential to contribute to the therapeutic action or side effects of this drug. Considering the obvious limitations of primary culture models in terms of level of receptor expression and lack of normal neuronal circuitry, it would be important to attempt to replicate our results in a more intact preparation. Systematic and quantitative double-labeling experiments would be required.

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