

Chronic Antipsychotic Drug Treatment Induces Long-lasting Expression of *fos* and *jun* Family Genes and Activator Protein 1 Complex in the Rat Prefrontal Cortex

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We have characterized the effects of chronic clozapine and haloperidol treatments on the expression of fos (c-fos, fosB, fra-2) and jun (c-jun, junB, junD) family genes in the rat forebrain. The effects of chronic (17d) clozapine and haloperidol on mRNA expression were determined two hours, 24 hours, and six days after the last drug injection, and the DNA-binding activity of the activator protein-1 (AP-1) complex was studied after washout periods of 24 hours and six days. Chronic clozapine treatment with a 6 d washout period induced the expression of several fos and jun family genes in cortical regions, including the prefrontal cortex (PFC), and in the caudate putamen and nucleus accumbens. Moreover, the DNA-binding activity of the AP-1 complex was greatly increased in the anterior cingulate cortex-PFC in mobility shift assays already after 24 h, and remained increased after a 6d washout

period. Chronic administration of haloperidol upregulated fos and jun family mRNA expression that was detectable 24 h and 6 d after cessation of the treatment mainly in the cortex. However, the DNA-binding activity of the AP-1 complex was not altered in the anterior cingulate cortex-PFC by chronic haloperidol administration at any of the time points studied. Thus, chronic treatments with clozapine and haloperidol induce a long-lasting enhancement of fos and jun family transcription factors that continues for several days after the cessation of the treatments in the cortex. These lasting effects might represent events that are potentially involved in the mechanisms of antipsychotic drug action.

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Antipsychotic drugs increase the expression of immediate early gene protein c-Fos (Dragunow et al. 1990; Miller 1990; Nguyen et al. 1992), which is considered a marker of neuronal activation (Sagar et al. 1988; Dragunow and Faull 1989; Morgan and Curran 1991). Both acute haloperidol and clozapine treatments induce c-Fos in the shell compartment of the nucleus accumbens (AcbSh) and in the lateral septal nucleus, whereas haloperidol induces c-Fos also in the core of the nucleus accumbens (AcbC) and in the dorsal striatum (Dragunow et al. 1990; Miller 1990; Nguyen et al. 1992; Robertson and Fibiger 1992; Merchant and Dorsa 1993). Activation of striatal c-Fos by typical (including haloperidol), but

not by atypical antipsychotics (including clozapine), has been hypothesized to result from the blockade of D₂ receptors by typical antipsychotic drugs and thus to reflect the manifestation of extrapyramidal side effects (Robertson et al. 1994). Unlike haloperidol, clozapine characteristically induces c-Fos in the prefrontal cortex (PFC), and this region has been proposed as a site underlying the unique effects of clozapine treatment (Robertson and Fibiger 1992; Fink-Jensen and Kristensen 1994; Robertson et al. 1994; Sebens et al. 1995; Deutch and Duman 1996; Kinon and Lieberman 1996).

Although the blockade of dopamine receptors by most antipsychotics takes place immediately after drug administration, the clinical antipsychotic effect develops slowly over the first days and weeks of treatment. The mechanisms behind the delayed drug effectiveness are unknown, but receptor-mediated transcriptional changes ultimately leading to alleviation of psychotic symptoms may occur. Alterations in neuronal activation following chronic, rather than acute antipsychotic drug treatment, may model the long-term drug effects in clinical use. However, only a few studies characterizing the outcome of chronic antipsychotic drug treatment on c-Fos expression have been undertaken. Chronic administration of haloperidol produces a c-Fos expression pattern essentially similar to that induced by acute treatment, but the induction is diminished especially in the striatum and nucleus accumbens (Sebens et al. 1995; Sun et al. 1998). Long-term haloperidol treatment also increases c-Fos in the limbic cortex such as in the anterior cingulate and entorhinal cortices, but not in the PFC (Merchant et al. 1996; Sun et al. 1998). Likewise, chronic treatment with clozapine induces a c-Fos expression pattern that is similar to acute clozapine treatment, but the induction is attenuated in many areas, except the nucleus accumbens (Sebens et al. 1995, 2000; Merchant et al. 1996).

Activator protein 1 (AP-1) complexes are formed by heterodimers between members of the Fos family (c-Fos, FosB, Fra-1 and Fra-2) and the Jun family (c-Jun, JunB and JunD) proteins, or by Jun protein homodimers. AP-1 complexes bind with high affinity to AP-1 DNA consensus sequence sites in regulatory regions of Fos-inducible genes. Binding of the AP-1 complex usually activates the transcriptional activity of the target gene but, depending on the types of Fos and Jun proteins in the AP-1 complex, may also repress it (Herdegen and Leah 1998). Dopamine receptor antagonists are able to modulate the protein combination in the AP-1 complex. Acute administration of D₂ antagonists haloperidol and (–)-sulpiride induces the expression of c-Fos, FosB, Fra-1, c-Jun and JunD proteins, whereas treatment with acute SCH23390, a D₁ selective antagonist, results in up-regulation of FosB, Fra-1 and JunD proteins (Ozaki et al. 1997, 1998). Acute and chronic haloperidol treatments increase the DNA-binding activity of the AP-1 complex

in the rodent caudate putamen and whole brain extracts (Nguyen et al. 1992; Ozaki et al. 1997, 1998; Atkins et al. 1999), while the effects of clozapine on AP-1 complex binding activity remain more obscure.

A characteristic property of the *fos* and *jun* family genes is their rapid induction and downregulation (Herdegen and Leah 1998). Following activation, the mRNA levels of the immediate early genes usually decay within one hour, and the amounts of the corresponding proteins peak after one hour and decay within three to four hours (Herdegen and Leah 1998). These fast kinetics can also occur in the presence of continuous stimulation. Some stimuli are able to induce secondary waves of immediate early gene expression; however, even the secondary expression waves are typically gone by 12–24 h (Herdegen and Leah 1998).

Recently, *fos* family transcription factors have been classified into three categories: c-Fos, 'acute' Fra's, and 'chronic' Fra's (Nestler et al. 1999). As compared with rapid induction of c-Fos, 'acute' Fra's (e.g., FosB, ΔFosB, Fra-1, Fra-2) are induced somewhat later and their expression lasts longer than that of c-Fos. The 'chronic' Fra's, expressed after repeated inducing stimuli, are biochemically modified forms of ΔFosB protein, which have a very long life-span (Nestler et al. 1999). They are induced after chronic perturbations such as seizure, axotomy or transplantation, and following repeated exposure to neuroactive agents, such as cocaine, kainic acid, and antipsychotic drugs (Hope et al. 1994; Nye et al. 1995; Bing et al. 1996; Doucet et al. 1996; Herdegen and Leah 1998; Atkins et al. 1999; Kelz et al. 1999; Nestler et al. 1999; Morris et al. 2000).

We have here carried out an *in situ* hybridization examination of the expression of *fos* and *jun* family genes in several cortical and subcortical regions following chronic treatment by clozapine and haloperidol. We have also studied the functional activity of the AP-1 complex after the drug treatments in the anterior cingulate cortex-PFC. The differential and long-lasting regulation of Fos and Jun family mRNAs in the limbic system by clozapine and haloperidol suggests a wider range of antipsychotic drug effects on brain than previously reported.

MATERIALS AND METHODS

Animal Care and Drug Administration

All animal experiments were performed in accordance to the guidelines of the Society for Neuroscience and were accepted by the experimental animal ethics committee of the University of Kuopio. Male Wistar rats (~200 g, National Laboratory Animal Center, University of Kuopio, Kuopio, Finland) were kept under standardized temperature, humidity and lighting conditions with free access to food and water. Animals were handled briefly during two days prior to the experi-

ments. Each treatment group consisted of five to six animals. Rats were injected intraperitoneally either with saline (1 ml/kg, control), haloperidol (1 mg/kg, Sere-nase, Orion, Finland, diluted in saline) or clozapine (25 mg/kg, Leponex, Wander Pharma, Novartis Pharma GmbH, Nürnberg, Germany), and sacrificed after the treatment period by decapitation under CO₂ anesthesia. Chronic treatments were performed by daily injections for 17 days. The last injection of each treatment or saline was given two hours (17d 2hw), 24 h (17d 24hw), or six days (17d 6dw) before sacrifice. For *in situ* hybridiza-tion, saline treated control animals were injected and matched separately for each drug treatment group to ensure identical handling. All control animals for elec-tromobility shift assay experiments were injected with saline for 17d and were allowed a 24-h washout period.

In situ Hybridization Procedure and Quantitation

For *in situ* hybridization, the brains were collected after appropriate treatment periods, frozen on dry ice, and stored at -75°C prior to cutting into 14 μm thick sections in coronal orientation with a cryostat. Sections were mounted onto Superfrost slides (Fisher Scientific, Toronto, ON, Canada), postfixed in 4% paraformalde-hyde, dehydrated, and stored under ethanol at $+4^{\circ}\text{C}$ until used. The sections were hybridized with oligonu-cleotide probes that had been labeled with [$\alpha^{32}\text{P}$]dATP by terminal deoxynucleotidyl transferase (MBI Fermen-tas, Vilnius, Lithuania). The antisense oligonucleotides recognizing rat c-Fos, FosB, c-Jun, JunB (Koistinaho et al. 1993), Fra-2 (nt 421-456, GenBank accession number U18913), and JunD (nt 890-924, GenBank accession number 450471) mRNAs were designed to minimize nonspecific hybridization. Hybridization was performed with $1\text{--}3 \times 10^3$ cpm/ μl of labeled probe in buffer con-taining 50% formamide, 10% dextran sulfate and $4 \times \text{SSC}$ at $+42^{\circ}\text{C}$ overnight. After incubation the sections were dipped into $1 \times \text{SSC}$ at room temperature, washed for 30 min at $+55^{\circ}\text{C}$ in $1 \times \text{SSC}$, washed sequentially for 3 min each at room temperature in $1 \times \text{SSC}$, $0.1 \times \text{SSC}$, 70% ethanol, and finally for 5 min in 94% ethanol. Sec-tions were opposed onto Hyperfilm- βmax films (Amer-sham, Buckinghamshire, England) for 1-3 weeks and developed for 5 min in D-19 (Kodak, Rochester, NY).

Hybridized sections were quantified with MCIDTM image analysis software (Imaging Research Inc., St. Ca-tharines, Ontario, Canada) and optical densities were converted to specific binding using ^{14}C scaling. As the mRNA expression levels were quantified, it was not consistently possible to distinguish layer-specific ex-pression patterns due to homogeneous expression of some of the *fos* and *jun* family genes. Therefore, the cor-tical layers were divided in superficial and deep layers. Quantitated brain regions were as follows: superficial

and deep layers of the prefrontal cortex (PFC outer and inner, respectively) consisting of the cingulate cortex re-gions 1 and 3 (Cg1 and Cg3), and medial orbital cortex/ infralimbic cortex (IL) were all quantitated from sec-tions cut within the distance of $+3.20 - +4.20$ mm from the bregma (see Figures 6-8 in Paxinos and Watson 1986). Superficial and deep layers of the anterior cingu-late cortex (Cg outer and inner, respectively) consisting of the cingulate cortex regions 1 and 2 (Cg1 and Cg2), the superficial layers of the frontal cortex (Fr1 and Fr2), the caudate putamen (CPu), and the core (AcbC) and shell (AcbSh) compartments of the nucleus accumbens were all quantified from sections cut within the dis-tance of $+0.70 - +1.60$ mm from the bregma (see Fig-ures 12-15 in Paxinos and Watson 1986). The analyzed brain regions are schematically shown in Figure 1, pan-els A and B. Hybridization intensities were quantified from matching brain regions of drug treated and identi-cally saline treated control sections. The data are mean values of the specific binding of all sections from the treat-ment group, and are presented as percentage of the mean specific binding of the corresponding control sections.

Electromobility Shift Assay Procedure

For electromobility shift assays, the forebrain region comprising the prefrontal and anterior cingulate cortex was dissected after washout periods of 24 hours and six days following the chronic drug treatments, rapidly frozen on dry ice, and stored at -75°C . Nuclear pro-teins were extracted from the tissues by homogeniza-tion into hypotonic buffer containing 10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM (*p*-amidino-phenyl)methanesulfonyl fluoride (PMSF), 1 mM dithiotre-itol (DTT), 1 $\mu\text{g}/\text{ml}$ leupeptin and 1 $\mu\text{g}/\text{ml}$ aprotinin. The homogenate was centrifuged for 15 min at ~ 1500 g (4000 rpm) at $+4^{\circ}\text{C}$ and resuspended in a mixture of low-salt buffer (20 mM Hepes (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 1.2 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 1 mM DTT, 1 $\mu\text{g}/\text{ml}$ leupeptin and 1 $\mu\text{g}/\text{ml}$ aprotinin) and high salt buffer (same as low-salt buffer but with 20 mM KCl) in the ratio of 2:1. After 30 min incubation on ice the homogenate was centrifuged at ~ 15000 g (13000 rpm) for 30 min at $+4^{\circ}\text{C}$ and the nu-clear proteins were collected and stored in -75°C .

Activator protein-1 (AP-1) specific oligonucleotide (Promega, Madison, WI) was labeled with [$\gamma^{32}\text{P}$]ATP using T4 polynucleotide kinase (Promega). Five micro-grams of nuclear protein extract isolated from the ante-rior cingulate cortex-PFC was combined with labeled AP-1 oligonucleotide in binding buffer containing 10 mM Tris/HCl (pH 7.5), 5% glycerol, 1 mM MgCl₂, 50 mM NaCl and 0.5 mM EDTA. Poly dI/dC (2 μg) was added to inhibit non-specific DNA binding (Amersham Phar-macia Biotech, Buckinghamshire, UK). For super-shift experiments, 2 μg of antibodies raised against Fos fam-

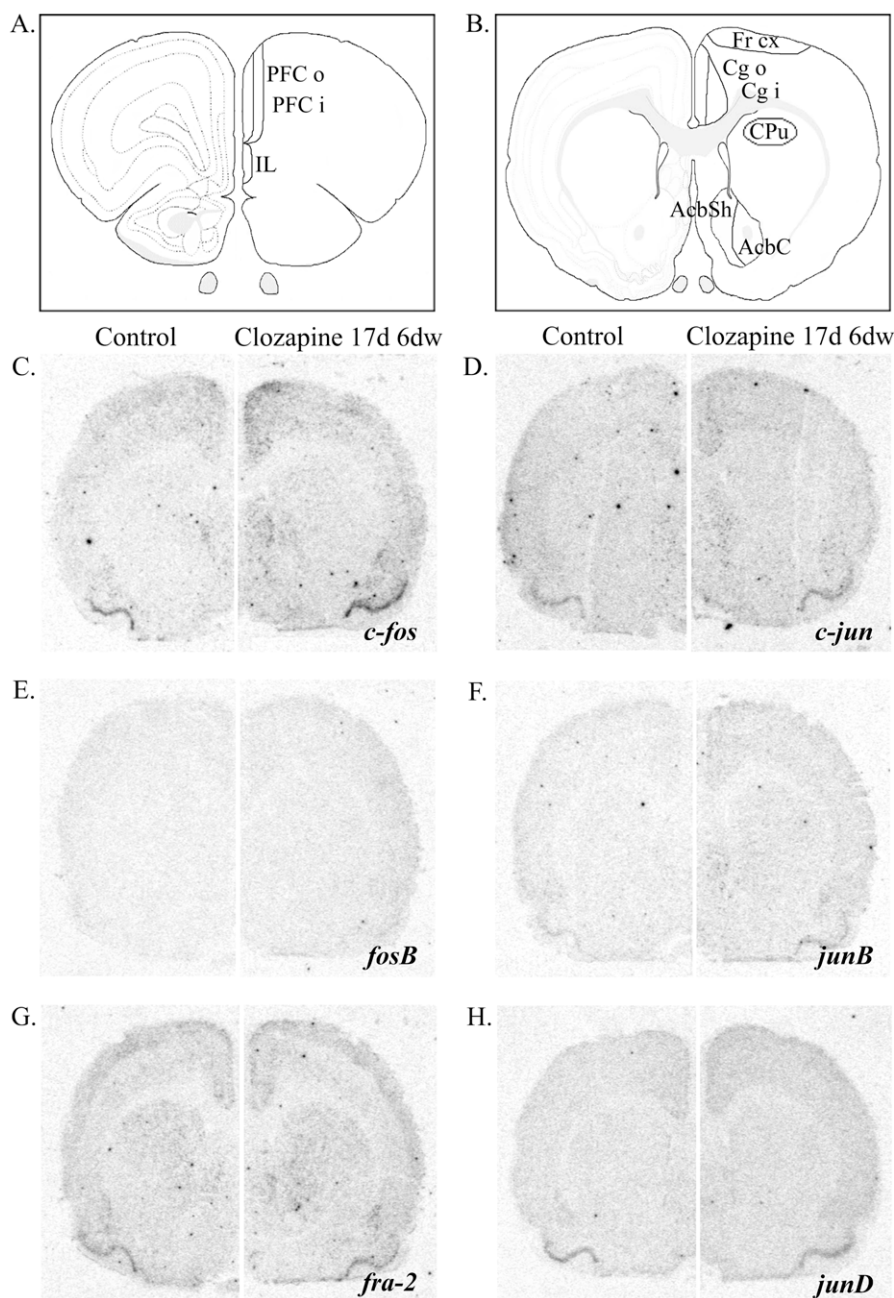


Figure 1. Expression patterns of the studied *fos* and *jun* family genes after chronic clozapine treatment followed by a 6-d washout period (17d 6dw). Schematic representation of the quantitated brain regions in *in situ* hybridization studies showing the regions of the (panel A) prefrontal (PFC) and infralimbic cortices (IL), and (panel B) frontal cortex (Fr cx), anterior cingulate cortex (Cg cx), caudate putamen (CPu), and nucleus accumbens core (AcbC) and shell (AcbSh). Brain regions were quantitated at indicated levels or at their proximity (see Materials and Methods). Representative autoradiograms show mRNA expression of (panel C) *c-fos*, (panel E) *fosB*, (panel G) *fra-2*, (panel D) *c-jun*, (panel F) *junB*, and (panel H) *junD* both in control (left panel) and clozapine (right panel) treated sections.

ily proteins (recognizing c-Fos, FosB, Fra-1 and Fra-2, catalog number sc-253 X, Santa Cruz Biotechnology Inc., Santa Cruz, CA) or Jun family proteins (recognizing c-Jun, JunB and JunD, catalog number sc-44 X, Santa Cruz) was added to the nuclear protein extract prior to addition of labeled AP-1 oligonucleotide, and incubated for 2h at +4°C in a rotator. DNA-protein complexes were allowed to form for 20 min on ice and were resolved by 4% polyacrylamide gel electrophoresis at 100 V for 1.5 h in 1x Tris/glycine buffer, pH 8.5. The gels were then dried and exposed to X-ray films. DNA binding activity was quantitated with MCID™ image analysis software. Activities were converted to percents of AP-1 binding in comparison to protein extracts from saline treated animals.

Data Analysis

The results of *in situ* hybridization experiments were analyzed by ANOVA followed by post-hoc tests (two-tailed Student's *t*-test). Electromobility shift assay results were analyzed by ANOVA followed by Dunnett's multiple comparison test.

RESULTS

We compared the effects of chronic treatment with clozapine and haloperidol on the expression levels of the *fos* (*c-fos*, *fosB* and *fra-2*) and *jun* (*c-jun*, *junB* and *junD*) family

immediate early gene mRNAs in the rat forebrain using *in situ* hybridization. The drugs were administered for 17 days and washout periods of two hours, 24 h, and six days were selected to further characterize the temporal expression patterns of *fos* and *jun* family genes. The functional activity of the AP-1 complex was measured in the anterior cingulate cortex-PFC after chronic drug treatment by electromobility shift assay. The effects on mRNA expression were dependent upon the drug administered and the duration following the last drug injection.

Effects of Chronic Drug Treatment on *fos* and *jun* Family Gene Expression

Quantified intensity values from *in situ* hybridized sections of all drug treatments, time points, studied genes, and brain regions were analyzed with ANOVA. A significant correlation was observed between the treatment groups (control and drug treated) and time points (17d 2hw, 17d 24hw, and 17d 6dw). The analysis indicated highly significant differences both for clozapine treated samples ($F > 8.9$, $df = 2$, $p < .0002$), and for haloperidol treated samples ($F > 4.4$, $df = 2$, $p < .02$). The data was further analyzed to identify the time points, genes and brain regions altered by the treatments.

Chronic clozapine treatment followed by a 2-h washout period downregulated the mRNA expression levels of *fosB* in the infralimbic cortex (Table 1), whereas the expression of *c-jun* was induced in the anterior cingulate cortex and in AcbSh (Table 2). No changes were detected in *c-fos*, *fra-2*, *junB* and *junD* mRNA expression at this time point.

Chronic haloperidol treatment followed by a 2-h withdrawal period downregulated the expression levels of *c-fos* (Table 1) and *junB* (Table 2) in the PFC. Moreover, *junB* expression was decreased in the anterior cingulate and infralimbic cortices, and in the AcbSh (Table 2). Levels of *fosB*, *fra-2*, *c-jun* and *junD* mRNA expression were not altered by chronic haloperidol treatment after a 2-h washout period.

After a 24-h washout period following chronic clozapine treatment, the expression of most *fos* and *jun* gene family mRNAs was unchanged. Only *junB* mRNA was upregulated in the PFC, especially in the deep cortical layers (Figure 2, panel D), and *junD* was downregulated in the anterior cingulate cortex (Table 2).

In contrast, chronic haloperidol treatment followed by a 24-h washout period induced the expression of *c-fos* in the frontal cortex, PFC, and in the anterior cingulate cortex, where the increase was most prominent

Table 1. Changes in the *fos* Family Gene mRNA Levels after Chronic Treatments

Gene	Region	Clozapine			Haloperidol		
		17d 2hw	17d 24hw	17d 6dw	17d 2hw	17d 24hw	17d 6dw
<i>c-fos</i>	Frontal cx	n.s.	n.s.	n.s.	n.s.	+++	++
	PFC	n.s.	n.s.	++	—	+	n.s.
	Cg cx	n.s.	n.s.	+	n.s.	++	n.s.
	IL cx	n.s.	n.s.	+	n.s.	n.s.	n.s.
	CPu	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	AcbC	n.s.	n.s.	++	n.s.	n.s.	n.s.
	AcbSh	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>fosB</i>	Frontal cx	n.s.	n.s.	++	n.s.	n.s.	n.s.
	PFC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Cg cx	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	IL cx	—	n.s.	n.s.	n.s.	n.s.	n.s.
	CPu	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	AcbC	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	AcbSh	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
<i>fra-2</i>	Frontal cx	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	PFC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Cg cx	n.s.	n.s.	+	n.s.	n.s.	n.s.
	IL cx	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	CPu	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	AcbC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	AcbSh	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Rats were injected with clozapine (25 mg/kg) or haloperidol (1 mg/kg) for 17d, and were sacrificed either 2h (17d 2hw), 24h (17d 24hw) or 6d (17d 6dw) after the last injection. mRNA expression levels were determined by *in situ* hybridization. Symbols: n.s., not significant; *n.d.*, not determinable; + or —, increase or decrease, respectively, $p < .05$; ++ or — —, increase or decrease, respectively, $p < .01$; + + +, increase $p < .001$ (ANOVA followed by two-tailed Student's *t*-test). Abbreviations: Frontal cx = frontal cortex, PFC = prefrontal cortex, Cg cx = cingulate cortex, IL cx = infralimbic cortex, AcbC = nucleus accumbens core, and AcbSh = nucleus accumbens shell.

Table 2. Changes in the *jun* Family Gene mRNA Levels after Chronic Treatments

Gene	Region	Clozapine			Haloperidol		
		17d 2hw	17d 24hw	17d 6dw	17d 2hw	17d 24hw	17d 6dw
<i>c-jun</i>	Frontal cx	n.s.	n.s.	+	n.s.	n.s.	++
	PFC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Cg cx	+	n.s.	n.s.	n.s.	n.s.	++
	IL cx	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	CPu	n.s.	n.s.	n.s.	n.s.	n.s.	++
	AcbC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	AcbSh	+	n.s.	++	n.s.	n.s.	n.s.
<i>junB</i>	Frontal cx	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	PFC	n.s.	+	+	—	+	n.s.
	Cg cx	n.s.	n.s.	++	—	n.s.	n.s.
	IL cx	n.s.	n.s.	+	—	n.s.	n.s.
	CPu	n.s.	n.s.	++	n.s.	n.s.	n.s.
	AcbC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	AcbSh	n.s.	n.s.	+	—	n.s.	n.s.
<i>junD</i>	Frontal cx	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	PFC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Cg cx	n.s.	—	n.s.	n.s.	n.s.	n.s.
	IL cx	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	CPu	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	AcbC	n.s.	n.s.	n.s.	n.s.	<i>n.d.</i>	n.s.
	AcbSh	n.s.	n.s.	n.s.	n.s.	<i>n.d.</i>	n.s.

Rats were injected with clozapine (25 mg/kg) or haloperidol (1 mg/kg) for 17d, and were sacrificed either 2h (17d 2hw), 24h (17d 24hw) or 6d (17d 6dw) after the last injection. mRNA expression levels were determined by *in situ* hybridization. Symbols: n.s., not significant; *n.d.*, not determinable; + or —, increase or decrease, respectively, $p < .05$; ++ or — —, increase or decrease, respectively, $p < .01$; +++ or — — —, increase $p < .001$ (ANOVA followed by two-tailed Student's *t*-test). Abbreviations: Frontal cx = frontal cortex, PFC = prefrontal cortex, Cg cx = cingulate cortex, IL cx = infralimbic cortex, AcbC = nucleus accumbens core, and AcbSh = nucleus accumbens shell.

in the deep cortical layers (Figure 3, panel A). In addition, the expression of *junB* was increased in the PFC (Figure 3, panel D). Expression levels of *fosB*, *c-jun* and *junD* mRNAs were not significantly altered at 24 h after chronic haloperidol treatment.

At six days after the last clozapine injection the expression of *c-fos*, *fosB*, *c-jun* and *junB* mRNA levels were significantly upregulated. The induction of *c-fos* was the most pronounced in the PFC, especially in the superficial cortical layers (Figure 2, panel A), in the anterior cingulate cortex (Figure 1, panel C, and Figure 2, panel A), and in the infralimbic cortex (Table 1). Occasionally, an induction of *c-fos* mRNA was detected in the frontal cortex, but the induction did not reach statistical significance. In addition, *c-fos* was induced in the AcbC (Figure 1, panel C, and Table 1). The expression level of *fosB* mRNA was very low throughout the brain. Yet, a modest but consistent increase of *fosB* transcript was observed in the frontal cortex after a 6-d washout period (Figure 1, panel E, and Figure 2, panel B). The mRNA expression of *c-jun* was also increased in the frontal cortex six days following the cessation of clozapine treatment (Figure 1, panel D, and Figure 2, panel C). Moreover, *c-jun* expression was upregulated in the AcbSh

(Figure 1, panel D, and Table 2). The mRNA levels of *junB* were generally induced and the upregulation was the most pronounced in the PFC (especially in the superficial layers) (Figure 2, panel D), and in the anterior cingulate cortex (Figure 1, panel F, and Figure 2, panel D). In addition, *junB* was induced in the infralimbic cortex (Table 2), in the caudate putamen and AcbSh (Figure 1, panel F, and Table 2). *fra-2* (Figure 1, panel G) and *junD* (Figure 1, panel H) mRNAs remained essentially at their basal expression levels in the quantitated regions.

Chronic haloperidol treatment followed by a 6-d washout period led to activated transcription of *c-fos* and *c-jun* mRNAs predominantly in the cortical regions. In the frontal cortex, expression of both *c-fos* and *c-jun* mRNAs was significantly increased (Figure 3, panels A and C, respectively). Upregulation of *c-jun* expression was also detected in the anterior cingulate cortex (Figure 3, panel C), and in the caudate putamen (Table 2). At this time point, other genes studied were not significantly regulated from their basal levels.

In general, the magnitude of gene induction in the cortex by chronic antipsychotic drug treatments with variable washout periods was around the same level or less than that produced by acute drug treatments (un-

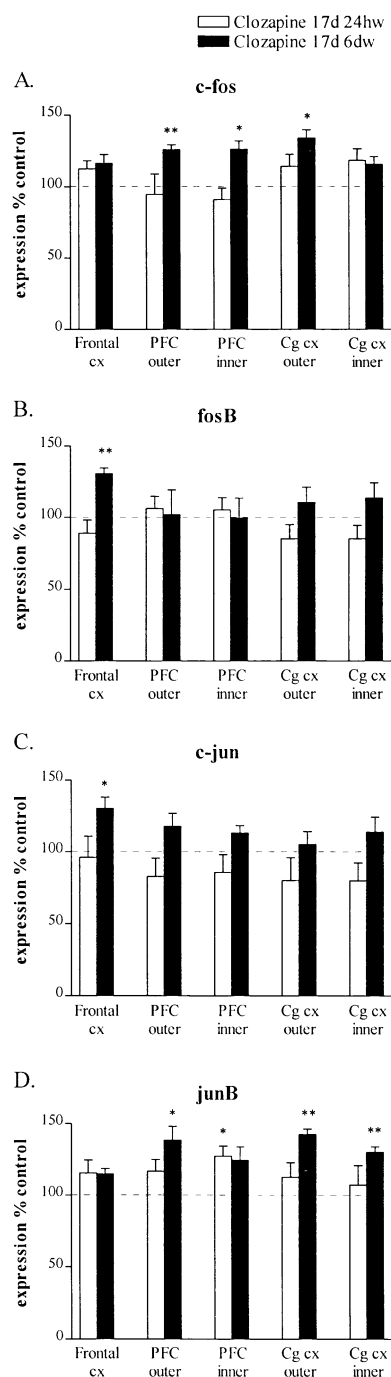


Figure 2. Effects of chronic (17 d) administration of clozapine (25 mg/kg) with different washout periods on the expression of (panel A) *c-fos*, (panel B) *fosB*, (panel C) *c-jun*, and (panel D) *junB* mRNA levels as studied with *in situ* hybridization. The expression levels were quantitated following 24-h (17d 24hw) and 6-d (17d 6dw) washout periods after the cessation of repeated clozapine treatment. *, $p < .05$; **, $p < .01$ as compared with respective control sections. Data are mean \pm SEM values ($n = 5-6$, two-tailed Student's *t*-test). PFC: prefrontal cortex; Cg cx: anterior cingulate cortex; outer: superficial layers; inner: deep layers (see Materials and Methods).

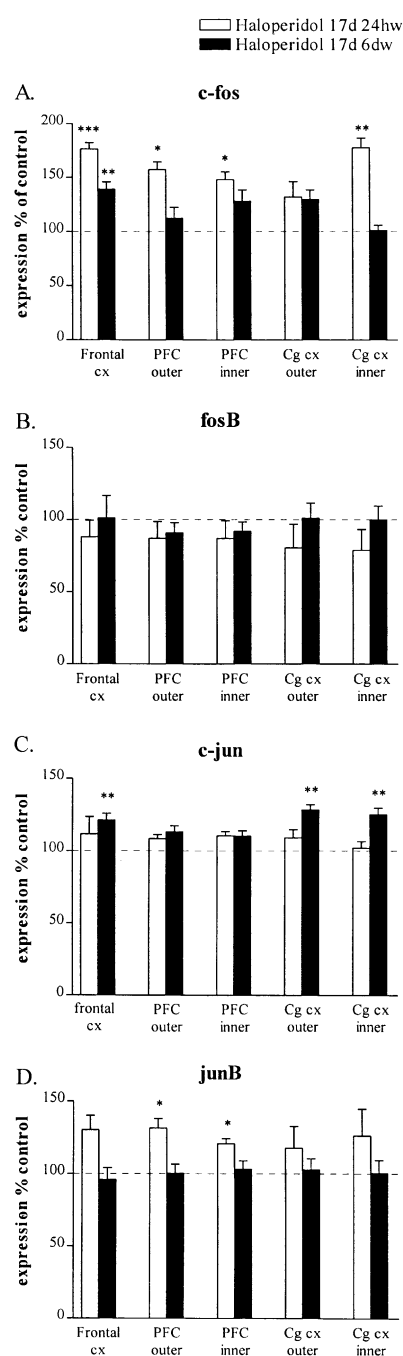


Figure 3. Effects of chronic (17 d) administration of haloperidol (1 mg/kg) with different washout periods on the expression of (panel A) *c-fos*, (panel B) *fosB*, (panel C) *c-jun*, and (panel D) *junB* mRNA levels as studied with *in situ* hybridization. The expression levels were quantitated following 24-h (17d 24hw) and 6-d (17d 6dw) washout periods after the cessation of haloperidol treatment. *, $p < .05$; **, $p < .01$; ***, $p < .001$ as compared with respective control sections. Data are mean \pm SEM values ($n = 5-6$, Student's *t*-test). PFC: prefrontal cortex; Cg cx: anterior cingulate cortex; outer: superficial layers; inner: deep layers.

published data, O.K. and E.C.). With haloperidol treatment, the induction of *c-fos* in the cortex with chronic treatment was, however, always less than the induction of *c-fos* in the striatum after acute treatment (unpublished data, O.K. and E.C.).

Regulation of the DNA-binding Activity of the AP-1 Complex after Chronic Drug Treatment

We next studied whether the induction of mRNAs for *fos* and *jun* gene family members by chronic drug treatments was accompanied with changes in the DNA binding activity of the AP-1 complex in the anterior cingulate cortex-PFC. Chronic clozapine treatment followed by withdrawal periods of 24 h and six days greatly increased the DNA binding activity of the AP-1 complex as shown by electromobility shift assay (Figure 4, panel A). Quantitation revealed that the DNA binding activity of the AP-1 complex was increased about twofold at both time points studied as compared with saline treated (17d 24hw) control samples (Figure 4, panel B). The increased DNA binding activity of the AP-1 complex after chronic clozapine treatments was inhibited by the addition of Fos and Jun family protein antibodies in super-shift assays (Figure 4, panel C). Both after 24-h and 6-d washout periods, the DNA-protein complexes were shifted by Fos family antibody, whereas the addition of Jun family antibody decreased the DNA binding activity of the AP-1 complex without producing a shift in the mobility of the complexes.

Next, we investigated the effect of chronic haloperidol treatment followed by 24-h and 6-d washout periods on the DNA binding activity of the AP-1 complex. In contrast to clozapine treatment, chronic administration of haloperidol did not result in altered DNA-binding activity of the AP-1 complex at any of the time points studied in the anterior cingulate cortex-PFC (data not shown).

DISCUSSION

Several studies have reported alterations in c-Fos expression as a marker of changes in transcriptional activity induced by acute antipsychotic drug treatment (Dragunow et al. 1990; Miller 1990; Nguyen et al. 1992; Robertson and Fibiger 1992; Merchant and Dorsa 1993; Fink-Jensen and Kristensen 1994; Sebens et al. 1995; Deutch and Duman 1996). However, only a few studies have characterized the outcome of chronic antipsychotic drug treatments on c-Fos expression (Sebens et al. 1995, 2000; Merchant et al. 1996; Sun et al. 1998). In addition to c-Fos, there are various other gene members of *fos* and *jun* families, which act as powerful regulators of gene expression. Most of the previous studies on c-Fos expression by antipsychotic drug treatments have em-

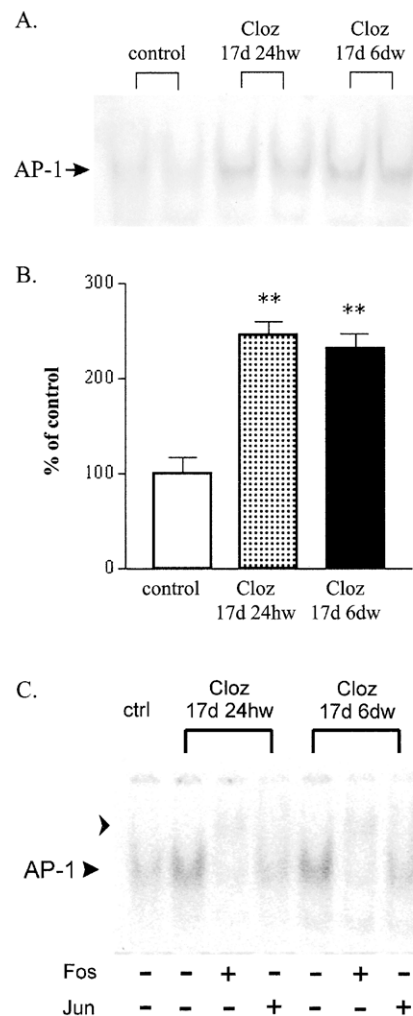


Figure 4. Induction of the DNA-binding activity of the AP-1 complex in the anterior cingulate cortex-PFC of rat brain in response to chronic (17 d) clozapine (25 mg/kg) treatment. A. Duplicate representatives of the gel shift assays show increased AP-1 binding after 24 hours (17d 24hw) and six days (17d 6dw) of withdrawal following repeated clozapine administration. Control tissues were treated with saline for the period of 17d 24hw. B. Quantified results of the AP-1 binding activity as compared with saline treated control tissues ($n = 5-6$). C. Addition of Fos and Jun protein antibodies inhibits the induced DNA-binding activity of the AP-1 complex in chronic clozapine treated samples. Fos antibody-shifted band is indicated with an arrowhead. **, $p < .01$. Data are mean \pm SEM values (Dunnett's multiple comparison test).

ployed immuno-labeling techniques, while we have here characterized the changes in several *fos* (*c-fos*, *fos-B*, *fra-2*) and *jun* (*c-jun*, *junB*, *junD*) family gene members' mRNA expression levels using radioactive *in situ* hybridization. We have investigated alterations in mRNA levels in several potential target regions of chronic antipsychotic drug treatments. We also measured the DNA binding activity of the AP-1 complex formed by Fos and Jun family proteins in order to correlate the mRNA

expression with the presence and function of the proteins. We describe here an unexpectedly long-lasting induction of *fos* and *jun* family genes in the rat forebrain following chronic treatment with clozapine and haloperidol.

Effects of Chronic Clozapine Treatment

The effect of chronic clozapine treatment on *fos* and *jun* family gene transcript expression was dependent on the washout period, since only minor regulation in limited brain areas was observed at two and 24 h after the last injection. This is in accordance with previous studies that have reported tolerance in the induction of Fos protein expression immediately after chronic clozapine treatment (Sebens et al. 1995, 2000; Merchant et al. 1996; Sun et al. 1998).

In contrast to the short washout periods, a marked induction of the *fos* and *jun* family gene mRNA levels was detected in several regions of the rat forebrain following a 6-d washout period after the cessation of chronic clozapine treatment. Induction of *c-fos* and *junB* was observed in cortical areas, as well as in the caudate putamen and nucleus accumbens. Moreover, the expression of *fosB*, *fra-2* and *c-jun* was induced in more restricted regions. Increased expression of *fosB* mRNA in the frontal cortex after chronic clozapine treatment is in line with recently reported increase in the FosB immunoreactivity after long chronic (six months) clozapine treatment (Rodriguez et al. 2001). Although we were able to detect limited changes in the expression of *fosB* mRNA after chronic drug treatment, the possible role of Δ FosB isoforms in long-term clozapine effects cannot be excluded.

Interestingly, although only minor regulation of the transcripts was observed after a 24-h washout period, the DNA-binding activity of the AP-1 complex was strongly enhanced in the anterior cingulate cortex-PFC at this time point. The substantial induction in the AP-1 binding persisted following six days of drug-free period after chronic clozapine administration. Super-shift experiments revealed that the majority of the DNA-protein complexes after both washout periods involve Fos family proteins, as the DNA binding of the AP-1 complex was shifted by the addition of Fos family antibody. It appears that Jun family proteins are less abundant in the AP-1 complexes after chronic clozapine treatments since the AP-1 binding was reduced, but not shifted, with the addition of Jun family antibody. These data suggest that chronic clozapine treatment induces persistent transcription of a subset of *fos* and *jun* family genes, and concomitantly increases the functional activity of the Fos and Jun family proteins.

Effects of Chronic Haloperidol Treatment

Cessation of chronic haloperidol administration either had no effect on or reduced the mRNAs of *fos* and *jun*

family genes at two hours after the last injection, but thereafter led to long-lasting induction that was observed after 24 hours and six days of drug-free period. The lack of *fos* and *jun* family mRNA upregulation after a 2-h washout period is consistent with previous reports that have showed tolerance in the inducibility of Fos protein expression immediately after chronic haloperidol treatment (Sebens et al. 1995, 2000; Merchant et al. 1996; Sun et al. 1998).

After 24 h washout period, the expression of *c-fos* was the most markedly induced in the frontal cortex, but we detected a minor increase in *c-fos* expression also in the PFC. Induced *c-fos* mRNA level in the PFC is not consistent with previously published expression pattern of c-Fos by chronic haloperidol treatment (Merchant et al. 1996; Sun et al. 1998). This discrepancy may be produced by differences in the studied time point and in the methodology of detection. Increase in *c-fos* expression was the most marked in the frontal cortex where the induction was still observed after a 6-d drug-free period. At this time point the transcript levels of *c-jun* were also induced in cortical regions and in the caudate putamen.

Despite of the induction in the *fos* and *jun* family transcript expression, the DNA-binding activity of the AP-1 complex was not altered by chronic haloperidol treatment in the anterior cingulate cortex-PFC at any of the time points investigated. Fos and Jun proteins are known to be capable of heterodimerizing with other transcription factors, and these heterodimers tend to shift the binding affinity from AP-1 sequence site to other DNA motifs (Herdegen and Leah 1998). This may explain our inability to detect changes in AP-1 binding affinity following chronic administration of haloperidol.

Conclusions

Repeated administration of clozapine and haloperidol may lead to drug accumulation in brain, and thus to result in altered gene expression several days after cessation of the treatment. Clozapine administered with i.p. injection has been reported to have a terminal half-life of 1.5–1.6 h in the rat brain, and it does not significantly accumulate in brain after a week of daily dosing (Baldessarini et al. 1993). Therefore, it is unlikely that significant amounts of clozapine are present in brain 24 h or later after the cessation of chronic treatment. Similarly, haloperidol has been described to have a terminal half-life of 1.5 h in rat (Cheng and Paalzow 1992), but longer half-lives have also been reported (Cohen et al. 1992). Consequently, residual amounts of haloperidol may still be present in brain after several days of drug withdrawal. However, because the most widespread and functionally significant changes were observed after chronic treatment with clozapine, it is unlikely that accumulation of drugs in brain can explain the extended induction and function of *fos* and *jun* family genes.

Finally, long-term drug treatments, such as with cocaine, antidepressants and ethanol, are known to cause tolerance and dependence, and as the treatment is reversed, are accompanied with symptoms of drug withdrawal. Others, and we here, have also detected tolerance in the inducibility of immediate early gene expression immediately after chronic administration of antipsychotic drugs (Sebens et al. 1995, 2000). However, the initial tolerance in *fos* and *jun* family gene expression after the cessation of chronic antipsychotic drug treatments was followed here by a marked induction in the gene expression as the drug-free period progressed. Clinical studies have indicated certain cases of relapse of schizophrenic patients after long-term antipsychotic drug medication is withdrawn (Ekblom et al. 1984; Gilbert et al. 1995; Meltzer et al. 1996; Shiovit et al. 1996). Moreover, the occurrence of relapse in patients withdrawn from clozapine treatment appears to be higher than that of neuroleptic-treated patients (Meltzer et al. 1996). It is possible, that the induction of *fos* and *jun* family genes one to six days after the cessation of the long-term clozapine and haloperidol treatments may be associated with drug withdrawal effect at the level of gene expression.

Taken together, our results indicate that chronic antipsychotic drug treatments may induce changes in target gene expression through actions of Fos and Jun transcription factors several days after the cessation of chronic antipsychotic drug administration. Experiments using DNA microarray technology might provide further information about the genes regulated by *fos* and *jun* family transcription factors in response to antipsychotic drug medication. This could advance the understanding of the molecular mechanisms of the action of antipsychotic drugs and help to design more selective drugs.

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