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# Comparison of Alterations in *c-fos* and Egr-1 (*zif*268) Expression Throughout the Rat Brain Following Acute Administration of Different Classes of Antidepressant Compounds

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The majority of immediate-early gene (IEG) studies focus on a few key brain regions associated with the class of psychoactive compound being studied. Recently, using a meta-analysis of the *c-fos* literature, we demonstrated the utility of *c-fos* profiling to classify such compounds. The present study examined acute delivery of a range of antidepressant classes; fluoxetine, imipramine, LiCl, and mirtazapine. The dual aims were to study the IEG profiles of these varying classes of antidepressants throughout the rat brain and to compare the utility of *c-fos* or Egr-1 as IEGs to classify clinically efficacious antidepressants. All antidepressants increased *c-fos* mRNA in the central amygdala, as previously shown, while *c-fos* was also increased in the anterior insular cortex and significantly decreased within the septum. Although acute antidepressant administration altered *c-fos* expression in a number of brain regions, Egr-1 expression was only significantly altered in the central amygdala, suggesting that Egr-1 may not be as useful a marker to investigate acute antidepressant treatment. The fact that these drugs, including the previously unclassified antidepressant mirtazapine, share a number of common loci of activation, which are implicated by human and animal studies in depression, adds further support to the use of IEG mapping to classify psychoactive compounds.
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## INTRODUCTION

Since their discovery in the late 1980s, cellular proto-oncogenes (Nishina *et al*, 1990; Sagar *et al*, 1988; Zerial *et al*, 1989) have become widely utilized tools to examine the effect of an acute stimulus; pharmacological or sensory (Beckmann and Wilce, 1997; Herdegen and Leah, 1998; Ziolkowska and Przewlocki, 2002). These transcription factors are also referred to as immediate-early genes (IEG) (Herrera and Robertson, 1996) and can be detected using immunohistochemistry (Dragunow and Faull, 1989) or *in situ* hybridization (Ziolkowska and Przewlocki, 2002) with members of the Fos- and Early growth response (Egr)-families being among the most extensively studied. Egr-1 (also known as *zif*268, *Krox*-24, NGFI-A) has been extensively used to study neuronal responses to sensory stimuli and, unlike *c-fos,* exhibits a high basal expression (Beckmann and Wilce, 1997). Both IEGs are considered to reflect neuronal activity due to their function of controlling gene transcription via their protein forms binding to regulatory sites on DNA (Beckmann and Wilce, 1997; Chaudhuri, 1997; Herdegen and Leah, 1998; Hoffman and Lyo, 2002).

Owing to their utility as neuronal markers, IEGs have been used to examine numerous psychoactive compounds in an effort to understand the mechanism of action of these drugs. Despite the availability of a number of successful antidepressants, the pathology of depression remains unclear. All current drugs have a delayed onset of action and a primary mechanism of action to increase monoamine levels in the brain (see Slattery *et al* (2004) for a review). Although chronic administration of antidepressants is required for clinical benefit, acute IEG induction can give insight into their mechanisms as it reflects longer-term

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alterations in cellular phenotype and provides insight into the brain regions affected by antidepressants (for reviews see Beckmann and Wilce, 1997; Dragunow and Faull, 1989; Herdegen and Leah, 1998; Herrera and Robertson, 1996). The use of IEG profiling can help to elucidate those brain regions that are activated by acute antidepressant administration.

There are a number of considerations to take into account when using IEGs as neuronal activity markers, such as their transient response. Time course studies have demonstrated that *c-fos* mRNA is rapidly induced, within 30–60 min of an acute challenge and c-Fos protein after 90–120 min (Torres *et al*, 1998; Zangenehpour and Chaudhuri, 2002). Both mRNA and protein expression return to baseline after 6 h. Egr-1 expression is less transient, showing similar induction responses to that of *c-fos* but levels remain elevated for up to 6 h following a stimulus (Zangenehpour and Chaudhuri, 2002). A major drawback of IEGs is that certain brain regions do not basally express a particular IEG, while other studies have shown that a wide variety of stimuli do not induce *c-fos* in certain brain regions such as the substantia nigra (Chaudhuri, 1997; Labiner *et al*, 1993); although recently caffeine has been demonstrated to increase Fos in this region (Singewald *et al*, 2003). This concern demonstrates the benefit of employing a number of IEGs to study the response to an acute stimulus, as a lack of induction of one gene may not necessarily mean a lack of neuronal activity. Further, despite Egr-1 and *c-fos* sharing a number of common activation pathways there are a number of differences that influence inducibility (see Herdegen and Leah (1998) for details). Egr-1 belongs to the class of zinc-finger protein IEGs while *c-fos* is a member of the activator protein-1 (AP-1) family of IEGs (see Herdegen and Leah (1998) for a review). Egr-1 profiling has mainly been performed following sensory stimuli while that of *c-fos* has been used extensively following acute drug administration, although a number of drugs have been shown to alter Egr-1 expression (see Beckmann and Wilce, 1997).

The majority of IEG studies only focus on a small number of brain regions that are predicted to be affected by the drug, for example the nucleus accumbens for antipsychotics (see Sumner *et al*, (2004) for an overview). We recently performed a meta-analysis, which suggests that different therapeutic classes of psycho-active compounds can be differentiated based on their Fos profiles (Sumner *et al*, 2004). For example, all antidepressants increased *c-fos* expression in the central nucleus of the amygdala while the same was true of the nucleus accumbens for all antipsychotics analyzed (Sumner *et al*, 2004). Also, psychostimulant compounds displayed the most widespread *c-fos* activation, in numerous cortical and subcortical regions while anxiolytic compounds were shown to activate the fewest number of brain regions of the four classes of psychoactive drugs analyzed (Sumner *et al*, 2004).

Therefore, we determined to examine the effect of acute antidepressant treatment on Egr-1 and *c-fos* mRNA expression to determine whether different classes of antidepressants displayed similar or differential induction of the IEGs. The rationale for using both Egr-1 and *c-fos* in the present study was due to the different induction pathways of these genes, as well as the fact that basal Egr-1 expression has been demonstrated to be under noradrenergic control (O'Donovan *et al*, 1999). Therefore, given the study of serotonergic and noradrenergic antidepressants, we determined to study if there would be similar or differential induction of *c-fos* and Egr-1 mRNA. Additionally, a number of studies have been performed, which demonstrate that there is differential regulation of Egr-1 and *c-fos* following acute antipsychotic administration (Cochran *et al*, 2002), therefore we determined to analyze this in relation to antidepressant administration. A range of different classes of antidepressants were examined, as well as the mood stabilizer LiCl (which also augments antidepressant administration and is used as a prophylactic treatment for depression; Fawcett, 2003), to determine whether there was a correlation in their IEG profiles and a wide number of brain regions, many of which were implicated in depression from both animal and human studies were analyzed, as well as a number of regions not previously implicated.

## MATERIALS AND METHODS

### Animals and Experimental Procedure

Male Sprague–Dawley rats (230–250 g, Harlan Olac, UK) were housed in groups of six on a 12 h light/dark cycle at $22 \pm 2^{\circ}C$ and given free access to tap water and food. All animals received a single intraperitoneal (i.p.) injection of vehicle or psychoactive drug ($n = 6$ per group). Drugs and doses included LiCl (a mood stabilizer; 75 mg/kg), mirtazapine (a noradrenaline and serotonin-selective antidepressant; 2 mg/kg), fluoxetine (a selective serotonin (5-HT) reuptake inhibitor (SSRI), 5 mg/kg), or imipramine (a tricyclic antidepressant (TCA) inhibits NA and 5-HT reuptake; 15 mg/kg) based on previous *c-fos* experiments (Lamprecht and Dudai, 1995; Morinobu *et al*, 1997; Torres *et al*, 1998). The exception was mirtazapine, for which there were no previous IEG studies and this dose was selected on behaviorally active doses in male rats (de Boer, 1996). All drugs were dissolved in 5% mulgofen EL-719 (a detergent which improves solubility; GAF, Ltd, Manchester, UK), in a 0.9% saline solution and administered at 1 ml/kg. Vehicle controls were injected with a 5% mulgofen EL-719 in 0.9% saline solution. At 45 min postinjection, rats were killed in a $CO_2$-chamber and the brains rapidly removed and then snap-frozen in isopentane, cooled on dry-ice to $-32^{\circ}C$. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986.

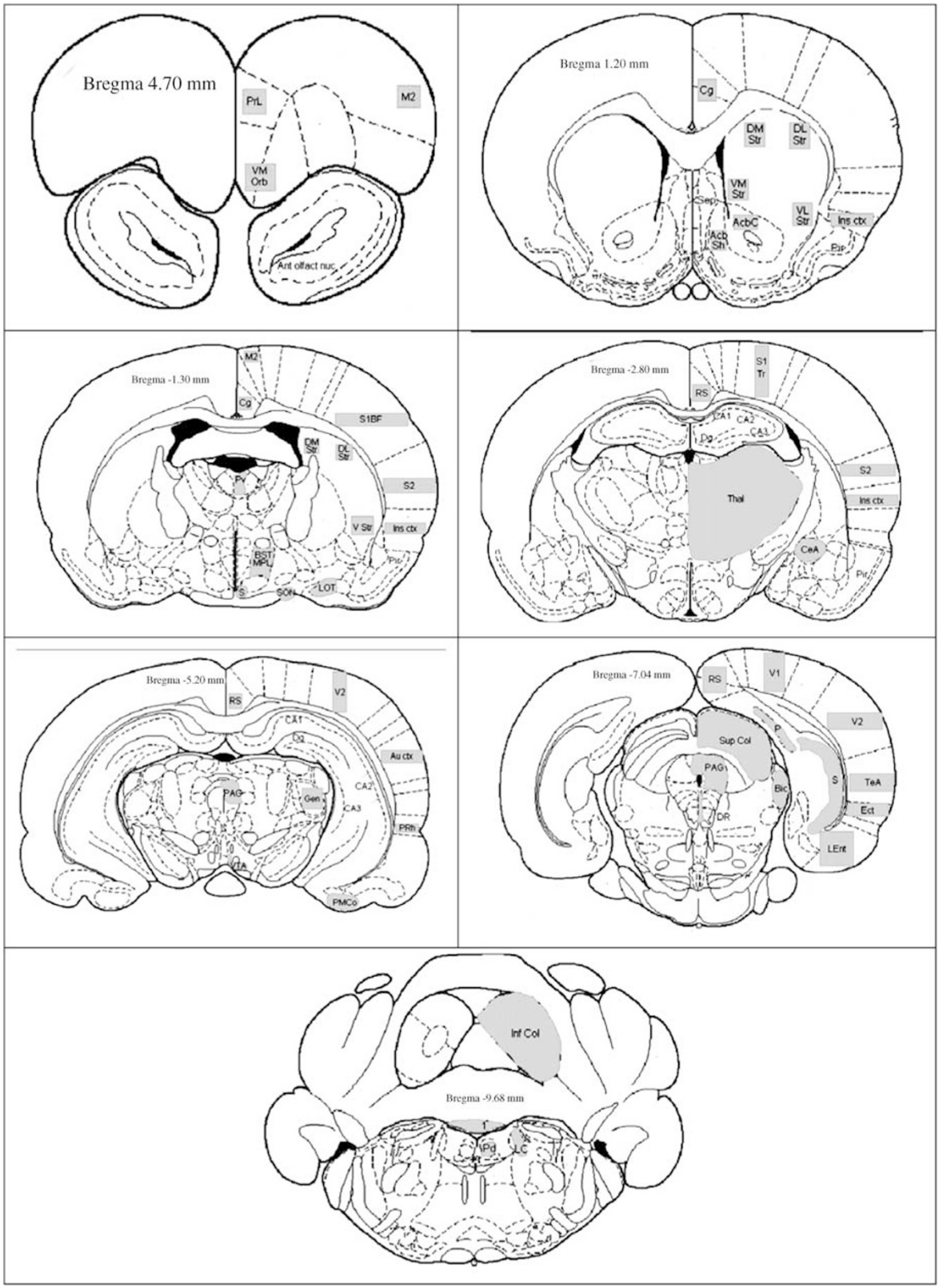
### Tissue Processing

Serial brain coronal sections (20 μm) from 4.7 to −10.3 mm, in seven series of slides comprising 13 slides in each series were cut on a cryostat at $-18^{\circ}C$ and thaw-mounted on to Superfrost Plus electro-statically charged nuclease-free slides (VWR). See Figure 1 for schematic diagram of brain levels sectioned and brain regions analyzed and the manner in which they were quantified (Paxinos and Watson, 1986). Sections were stored at $-80^{\circ}C$ until required for processing. The sections were thawed at room temperature for 30 min then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), rinsed and acetylated in acetic anhydride in PBS (pH 8). After rinsing, the sections were dehydrated

in an ascending series of ethanol (70, 80, 95, and 100%) and stored under ethanol (95%) at 4°C until required for *in situ* hybridization (Henry *et al*, 1999). All solutions were prepared in diethylpyrocarbonate (DEPC)-treated water.

## Probe Labelling

Two 48-base antisense oligonucleotide probes were designed to target different, nonoverlapping sequences of rat *c-fos* mRNA (NCBI Genbank Accession Number: X06769).



Probe 1 was complementary to bases 1001–1048 (probe sequence: 5′-GGC TCC CAG TCT GCT GCA TAG AAG GAA CCA GAC AGG TCC ACA TCT GGC-3′). Probe 2 was complementary to bases 141–188 (probe sequence: 5′-ACT GCA GCG GGA TGA CGC CTC GTA GTC CGC GTT GAA ACC CGA GAA-3′). One 45-base antisense oligonucleotide probe was designed to target rat Egr-1 mRNA (NCBI Genbank Accession Number M18416; probe sequence: 5′-TGG GAG CCC GAC TGA GTG GCG AAG GCT TTG ATA GTG GAT AGT GGA-3′). Using the NCBI Basic Local Alignment Search Tool (BLAST; Altschul *et al*, 1990), the sequences were assessed for homology with other sequences and found to be specific for their respective transcripts. The synthetic oligonucleotide probes were synthesized by Life Technologies (Gibco, BRL, UK). Each oligonucleotide probe, was 3′-endlabelled with [$^{33}$P]dATP as previously described (Henry *et al*, 1999; Sumner *et al*, 2004). The labelled probe was then added to 'maximalist' hybridization buffer to give a final concentration of 0.5 pmol/ml hybridization solution (Henry *et al*, 1999). The final hybridization buffer was used the day it was prepared.

### *In Situ* Hybridization

Labelled hybridization solution was pipetted on to each slide and placed in a hybridization box (150 μl per slide), which were then covered with Hybrislips (Sigma) and incubated in a hybridization oven at 42°C overnight. Following the incubation, the Hybrislips were floated off using 2 × SSC solution and the slides were placed in racks for further processing as previously described (Henry *et al*, 1999; Sumner *et al*, 2004). Once dry the sections were exposed to X-ray film (BioMax MR-1, Kodak) under 'safe' red light conditions for 7 (Egr-1) or 14 (*c-fos*) days. An autoradiographic [$^{14}$C] micro-scale (Amersham) of known radioactivity (range 31–833 nCi/g) was also placed in each cassette to allow conversion of the optical density measurements to nCi/g. The films were then developed under 'safe' light conditions.

### Acetylcholinesterase Activity

In order to assist histological verification of the brain regions analyzed, sites of acetylcholinesterase activity were examined. Untreated cryostat sections were removed from the −80°C freezer and thawed to room temperature for 1 h before use. Acetylcholinesterase incubating medium (13 ml 0.1 M acetate buffer, 2 ml 0.03 M cupric sulfate, 4 ml $dH_20$ and 12 mg ethopropazine and 0.01 g acetylthiocholine iodide) was prepared immediately before use and slides were incubated for 2 h at room temperature in the dark and then washed in $dH_20$ for 1 min. The sections were then subjected to a 1% ammonium sulfide solution (prepared in $dH_20$) for 1 min, to visualize the sites of acetylcholinesterase activity. The slides were then rinsed in several $dH_20$ incubations and allowed to air-dry, then dehydrated and mounted in DPX. This method was adapted from Bancroft and Stevens (1996).

### Data Analysis and Statistics

Densitometric analysis of autoradiographs was performed using a Microcomputer Imaging Device (MCID, InterFocus, Ltd, Haverhill, Suffolk, UK) system. Bilateral relative optical density (ROD) measurements were taken from triplicate sections from each animal ($n = 6$) and converted to nCi/g by creating a standard curve from the [$^{14}$C]-standards. The brain regions analyzed are shown in Figure 1. The majority of brain regions were chosen based on those that have been implicated in major depression from human neuroimaging studies and from preclinical studies involving lesions and prior IEG studies (Drevets, 1999, 2000, 2001; Kirby and Lucki, 1997; Ongur and Price, 2000; Ronan *et al*, 2000; Slattery *et al*, 2004; Sumner *et al*, 2004). These studies have revealed that numerous brain regions that have previously not been implicated in depression seem to play roles; therefore, we also determined to examine a number of other brain regions, at the same bregma coordinates as those implicated in depression. In total, over 50 brain regions were analyzed (not all regions are shown) for alterations in both *c-fos* and Egr-1. In a small number of brain regions, the data shown for imipramine, mirtazapine and fluoxetine have been previously represented in Sumner *et al* (2004) in a format only detailing global alteration in *c-fos* mRNA compared with vehicle as opposed to the actual raw values presented here. A one-way ANOVA with Dunnett's *posthoc* test was performed for each brain region analyzed to determine statistical significance of any drug-induced changes in mRNA using GraphPad Prism v3.02.

## RESULTS

*In situ* hybridization utilizing two probes complementary to the rat *c-fos* or one probe complementary to the rat Egr-1 transcript following acute antidepressant administration demonstrated differing expression profiles for each treatment group. Each of the different drug-treatment groups

**Figure 1** Schematic representation of the seven brain levels sectioned from 4.7 to −10 mm relative to bregma. In all, 13 slides were collected at each level with each slide containing three 20 μm serial brain sections. In each schematic, the brain regions analyzed are shown; where those in grey shade represent the area quantified and not in grey are regions that were quantified using the whole region. Schematics adapted from Paxinos and Watson (1986). Abb: M2 ctx, secondary motor cortex; PrL ctx, prelimbic cortex; VM Orb, ventromedial orbital cortex; AON, anterior olfactory nucleus; Pir, piriform cortex; Cg, cingulate cortex; Ins, insular cortex; DM Str, dorsomedial striatum; DL Str, dorsolateral striatum; VL Str, ventrolateral striatum; VM Str, ventromedial striatum; AcbC, nucleus accumbens core region; AcbSh, nucleus accumbens shell region; Sep, septum; S1BF, primary somatosensory cortex, barrel field,;S2, secondary somatosensory cortex; Pv, anterior paraventricular thalamic nucleus; V Str, caudal ventral striatum; LOT, nucleus of the lateral olfactory tract; SON, supraoptic nucleus; BSTMPL, nucleus of the stria terminalis, medial division, posterolateral part; S, suprachiasmatic nucleus; RS, retrosplenial cortex; S1Tr, primary somatosensory cortex, trunk region; CA1, field CA1 of hippocampus; CA2, field CA2 of hippocampus; CA3, field CA3 of hippocampus; Dg, dentate gyrus; CeA, central amygdaloid nucleus; Thal, thalamus; V2, secondary visual cortex, lateral area; Au ctx, primary auditory cortex; PRh ctx, perirhinal cortex; PAG, periaqueductal gray; VTA, ventral tegmental area; Gen, geniculate nucleus; PMCo, posteromedial cortical amygdaloid nucleus; V1, primary visual cortex; TeA, temporal association cortex; Ect, ectorhinal cortex; LEnt, lateral entorhinal cortex; DR, caudal dorsal raphé; Sup Col, superior colliculus; BIC, nucleus of the brachium of the inferior colliculus; S, subiculum; P, post-subiculum; Inf col, inferior colliculus; LC, locus coeruleus; Pd, posterodorsal tegmental nucleus.

displayed different regulation of *c-fos* and Egr-1 mRNA expression, with both increased and decreased levels compared with vehicle observed. In the present study, *c-fos* expression in the vehicle control groups appeared to be elevated compared with a number of previous studies (Cochran *et al*, 2002; Singewald and Sharp, 2000; Torres *et al*, 1998). However, this may be due to employing the detergent mulogfen in the solutions and would therefore be present in all treatment groups.

### Brain Regions with Common Profile

There were only three brain regions out of more than 50 examined for alterations in *c-fos* and Egr-1 expression (not all data are shown), where acute administration of all antidepressants exhibited a common *c-fos* profile; anterior insular cortex, septum, and central amygdala (see Figure 2). All antidepressants significantly increased *c-fos* expression in the anterior insular cortex (imipramine 38%, mirtazapine 50%, fluoxetine 61%, and LiCl 41% increase compared with vehicle) and central amygdala (imipramine 187%, mirtazapine 131%, fluoxetine 252%, and LiCl 540% increase compared with vehicle), whereas all significantly decreased levels in the septum (imipramine 29%, mirtazapine 30%, fluoxetine 23%, and LiCl 29% decrease compared with vehicle). Furthermore, all antidepressants also increased Egr-1 expression in the central amygdala (imipramine 227%, mirtazapine 209%, fluoxetine 296%, and LiCl 664% increase compared with vehicle; $P<0.001$; Figure 2), which was the only brain region analyzed where acute antidepressant administration altered Egr-1 expression (see Figure 2 and Table 1). Acetylcholinesterase activity was performed to histologically verify the amygdaloid site and demonstrated that the central amygdala was the locus of IEG activation (see Figure 3).

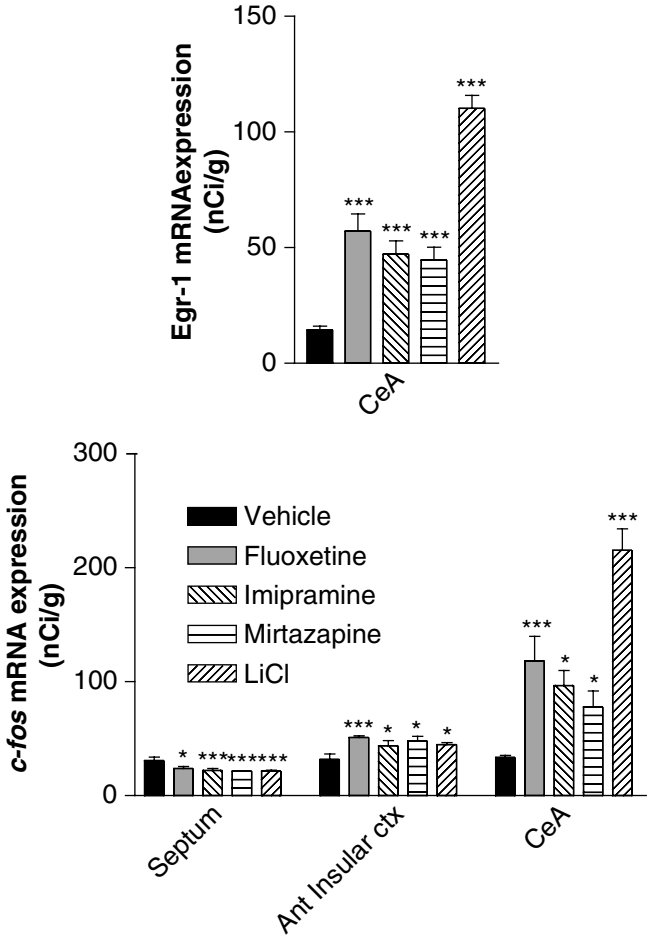



**Figure 2** IEG expression 45-min following acute i.p. vehicle, fluoxetine (5 mg/kg), imipramine (15 mg/kg), mirtazapine (2 mg/kg), or LiCl (75 mg/kg) administration in the central amgydala (CeA), septum, and anterior insular cortex (Ant Insular ctx). Data represent mean ± SEM ($n=6$). One-way ANOVA with Dunnett's *post hoc* test was performed $^{*}P<0.05$ and $^{***}P<0.001$ compared with vehicle.

### Further Alterations of *c-fos* Following Acute Antidepressant Administration

At 45 min following acute fluoxetine administration *c-fos* mRNA expression was significantly increased in the ventromedial orbital cortex ($P<0.05$), ventromedial striatum ($P<0.05$), nucleus accumbens core region ($P<0.05$), nucleus accumbens shell region ($P<0.001$), and in the anterior cingulate cortex ($P<0.001$; see Figure 4). In the striatal and cortical regions, fluoxetine was the only one of the drugs tested which altered *c-fos* mRNA expression, whereas in the nucleus accumbens core and shell regions mirtazapine and LiCl also increased *c-fos* levels ($P<0.001$).

The locus coeruleus and the dorsal raphé are the main sites of noradrenergic and serotonergic origin in the central nervous system (CNS), respectively. In the locus coeruleus, despite a trend towards an increase of *c-fos* expression following mirtazapine or LiCl compared with vehicle, only fluoxetine administration reached significance ($P<0.05$; Table 2). Similarly, no effect of treatment was observed in the caudal dorsal raphé (Table 2). Administration of fluoxetine, imipramine ($P<0.001$) or LiCl ($P<0.05$), but not mirtazapine, increased *c-fos* expression in the perirhinal cortex (Table 2). No alterations in *c-fos* expression were observed in the CA1 field of the hippocampus (Table 2). However, imipramine, mirtazapine, and LiCl administration downregulated mRNA levels in the subiculum (Table 2). The most robust increase in *c-fos* expression was in the supraoptic nucleus following LiCl treatment; a 10-fold increase was observed (Table 2). Despite a trend towards an increase in this brain region following fluoxetine administration, this did not reach significance ($P>0.05$).

## DISCUSSION

The aims of the present study were two-fold: firstly to provide comprehensive analyses of brain regions where acute antidepressant administration alters *c-fos* and Egr-1 expression and secondly to compare the induction profiles of the two IEGs and identify regions of commonality with regard to regional activation.

Overall a number of brain regions showed induction of *c-fos* following acute antidepressant administration, while Egr-1 mRNA was only altered in one brain region analyzed; the central amygdala (see Figure 2 and Table 1). A similar low induction of Egr-1 mRNA following acute antipsychotic treatment has previously been reported in a study examin-

**Table 1** Levels of Egr-1 mRNA Expression in Identified Brain Regions 45-min Following Acute Administration of Vehicle, Fluoxetine (5 mg/kg), Imipramine (15 mg/kg), Mirtazapine (2 mg/kg), or LiCl (75 mg/kg; $n = 6$)

| Brain region | Vehicle (nCi/g) | Fluoxetine (nCi/g) | Imipramine (nCi/g) | Mirtazapine (nCi/g) | LiCl (nCi/g) |
|---|---|---|---|---|---|
| VM Orb ctx | 319.52±11.32 | 317.2±14.91 | 353.42±11.17 | 299.41±13.51 | 274.59±12.25 |
| Septum | 33.26±2.75 | 31.13±1.70 | 33.33±0.33 | 30.38±1.34 | 32.97±0.74 |
| Ant Cg Ctx | 97.75±9.01 | 104.61±6.43 | 112.79±4.76 | 91.61±4.00 | 94.49±5.03 |
| Ant Ins ctx | 41.55±3.02 | 45.69±3.72 | 49.26±3.12 | 44.91±3.01 | 45.34±2.10 |
| VM str | 37.01±4.35 | 36.91±1.48 | 42.31±2.26 | 35.65±2.52 | 33.24±2.71 |
| NA core | 25.11±3.78 | 27.09±2.82 | 26.66±2.77 | 26.22±2.71 | 27.38±1.23 |
| NA shell | 19.92±2.65 | 17.72±1.32 | 17.70±2.56 | 19.86±3.58 | 26.17±1.92 |
| CA1 | 85.29±6.41 | 76.49±4.36 | 80.75±5.14 | 73.53±3.44 | 82.36±8.09 |
| Subiculum | 74.09±9,09 | 67.26±2.66 | 72.92±4.58 | 64.98±3.29 | 59.62±3.62 |
| PRh ctx | 50.97±1.38 | 66.02±2.29 | 64.96±3.56 | 49.04±1.66 | 54.82±6.86 |
| PAG | 3.10±1.44 | 6.99±1.45 | 5.76±1.55 | 3.64±1.38 | 3.96±1.87 |
| VTA | 0.89±0.47 | 3.16±1.07 | 3.62±0.97 | 1.05±0.39 | 1.75±0.89 |
| Dorsal raphe | 17.18±3.01 | 14.65±1.01 | 20.15±1.07 | 13.67±1.09 | 12.75±1.10 |
| Locus coerulus | 12.01±1.13 | 14.62±1.91 | 13.53±2.08 | 17.43±3.00 | 15.33±1.56 |
| Piriform cortex | 118.60±7.94 | 103.63±5.26 | 124.74±7.31 | 108.78±6.30 | 113.80±3.04 |
| S1Tr | 76.21±5.49 | 92.12±5.75 | 93.62±4.89 | 77.76±3.96 | 78.77±3.10 |
| V2 cortex | 85.53±6.48 | 106.79±12.22 | 102.45±6.91 | 72.24±0.87 | 74.10±6.22 |
| Au cortex | 104.81±4.15 | 117.08±4.73 | 110.72±2.31 | 100.54±4.84 | 103.77±6.10 |

Data represent mean±SEM, one-way ANOVA with Dunnett's *post hoc* test was performed. VM Orb ctx, ventromedial orbital cortex; Ant Cg Ctx, anterior cingulate cortex; Ant Ins ctx, anterior insular cortex; VM Str, ventromedial striatum; NA, nucleus accumbens; PRh ctx, perirhinal cortex; PAG, periaqueductal gray; VTA, ventral tegmental area; S1Tr, primary somatosensory cortex trunk region; V2 cortex, secondary visual cortex; Au cortex, auditory cortex.

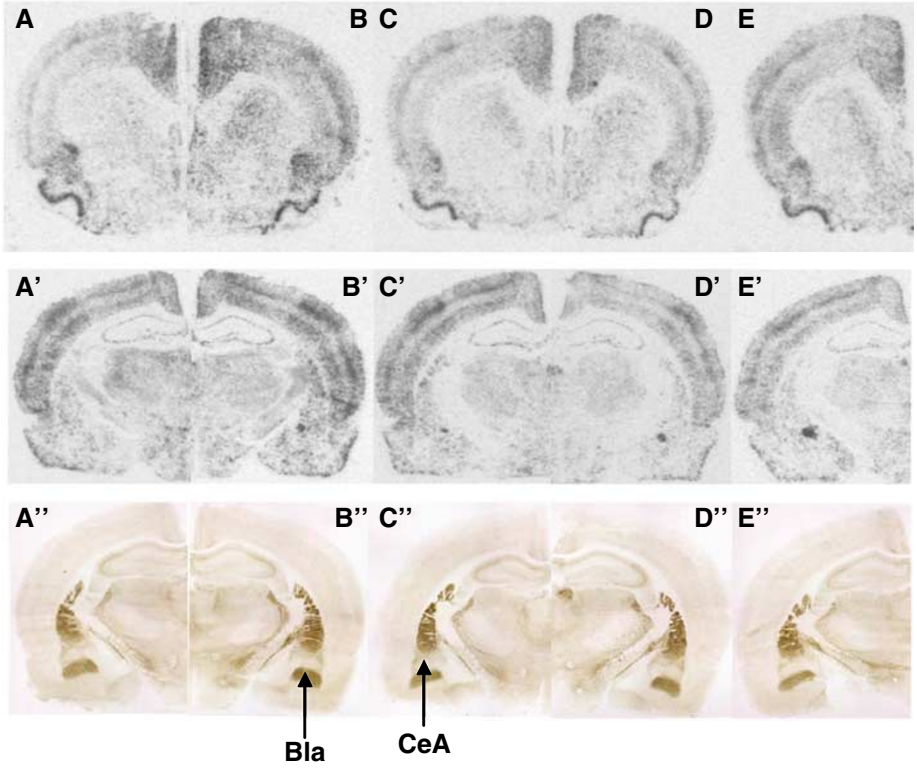



**Figure 3** Representative left or right hemisphere coronal *c-fos* autoradiographs 45-min following acute administration of vehicle (A and A′), fluoxetine (5 mg/kg; B and B′), imipramine (15 mg/kg; C and C′), mirtazapine (2 mg/kg; D and D′), or LiCl (75 mg/kg; E and E′) displaying alterations in the anterior insular cortex and septum or central amygdala, respectively. Corresponding acetylcholinesterase images (A″–E″) enabled delineation between the central amygaloid nucleus (CeA) and basolateral amygaloid nucleus (BlA), which when overlaid with the autoradiograms shows the CeA as the site of *c-fos* induction.

ing both *c-fos* and Egr-1 (Cochran *et al*, 2002). A possible explanation for this is the different intracellular pathways by which, *c-fos* and Egr-1 are induced (see Introduction). Egr-1 tends to be used as a marker following sensory stimuli, although a number of drugs have been demonstrated to activate Egr-1 (see Beckmann and Wilce (1997) for a review), whereas *c-fos* is used more extensively in both sensory and drug administration paradigms. Therefore, the comparison of *c-fos* and Egr-1 profiles demonstrated that Egr-1 is not as versatile as *c-fos* as an IEG to study neuronal induction following acute antidepressant treatment.

### Activation of Common Brain Regions by all Antidepressants

All antidepressants tested increased *c-fos* levels in the anterior insular cortex and central amygdala compared with vehicle, and decreased levels in the septum (Figure 2).

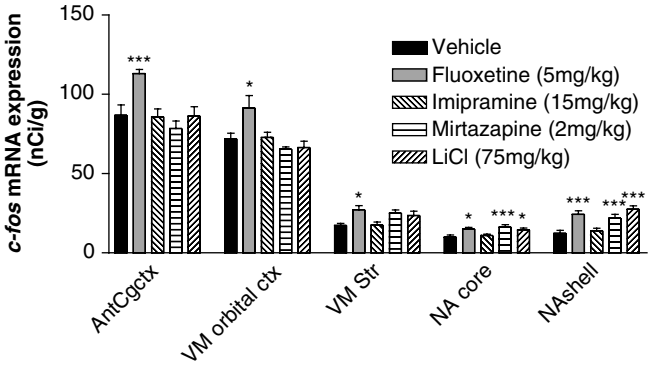



**Figure 4** Alterations in *c-fos* expression in the anterior cingulate cortex (Ant Cg ctx), ventromedial orbital cortex (VM orbital ctx), VM striatum (VM Str), and nucleus accumbens (NA) core and shell regions. Data represent mean ± SEM 45 min following acute administration of vehicle, fluoxetine (5 mg/kg), imipramine (15 mg/kg), mirtazapine (2 mg/kg), or LiCl (75 mg/kg; $n = 6$). One-way ANOVA with Dunnett's *post hoc* test was performed $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with vehicle.

Additionally, the only brain region where Egr-1 mRNA expression was altered was in the central nucleus of the amygdala (CeA), and it was induced by all classes of antidepressants examined (see Figure 2). Intriguingly, these three brain regions have been linked with depression in human neuroimaging studies. These studies have demonstrated an increase in cerebral blood flow and glucose metabolism in the anterior insular cortex and amygdala in depressed patients (the resolution was insufficient to determine the subdivision within the amygdala; Drevets, 2000, 2001). Furthermore, the severity of depression appeared to correlate with cerebral blood flow (CBF) and glucose metabolism in the amygdala (Drevets *et al*, 1992). Rats exposed to fear-conditioned stimuli demonstrate a 53% increase in local CBF, as measured by [$^{14}$C]-iodoantipyrine autoradiography (LeDoux *et al*, 1983), and chronic antidepressant treatment normalizes the hyperactivity of the amygdala in both rats and humans (see Drevets (1999) for a review). Additionally, the central amygdala has previously been demonstrated to be the only common site of *c-fos* activation by antidepressants (Beck, 1995; Duncan *et al*, 1996). However, a number of other psychoactive compounds also elevate *c-fos* in this brain region (see Sumner *et al*, 2004). A previous study has similarly demonstrated that LiCl (Lamprecht and Dudai, 1995) induces Egr-1 expression in the central amygdala. This evidence, coupled with the present study, is suggestive that the central amygdala may be a locus of antidepressant activity as mirtazapine, whose effect on IEGs has not previously been documented, also elevates *c-fos* and Egr-1 in this brain region. However, methamphetamine (40 mg/kg; Thiriet *et al*, 2001), diazepam (2.5 and 5 mg/kg; Malkani and Rosen, 2000), and kainic acid-induced seizures (Gass *et al*, 1993) also induce Egr-1 expression in the central amygdala indicating that other stimuli can also lead to an increase in IEG expression in this area.

The Egr-1 mRNA induction evoked by the LiCl administration in the present study was significantly greater than all other treatments, as was also observed for *c-fos*. The

**Table 2** Levels of *c-fos* mRNA Expression in Identified Brain Regions 45-min Following Injection of Vehicle, Fluoxetine (5 mg/kg), Imipramine (15 mg/kg), Mirtazapine (2 mg/kg), or LiCl (75 mg/kg; $n = 6$)

| Brain region | Vehicle (nCi/g) | Fluoxetine (nCi/g) | Imipramine (nCi/g) | Mirtazapine (nCi/g) | LiCl (nCi/g) |
|---|---|---|---|---|---|
| SON | 26.83 ± 3.18 | 56.42 ± 5.74 | 33.60 ± 2.76 | 44.56 ± 5.47 | 285.89 ± 37.92*** |
| VTA | 19.00 ± 1.27 | 28.64 ± 2.15*** | 22.51 ± 2.44 | 18.83 ± 0.79 | 19.66 ± 1.43 |
| PRh ctx | 54.12 ± 1.05 | 89.01 ± 2.71*** | 67.99 ± 2.62*** | 55.49 ± 1.45 | 61.82 ± 2.02* |
| PAG | 25.57 ± 1.57 | 34.12 ± 1.78*** | 29.05 ± 1.66 | 23.33 ± 1.50 | 24.79 ± 2.34 |
| Dorsal raphe | 22.55 ± 2.11 | 27.31 ± 1.58 | 26.53 ± 1.39 | 24.58 ± 0.90 | 22.7 ± 2.89 |
| Locus coerulus | 35.43 ± 1.98 | 52.79 ± 3.56* | 38.6 ± 3.08 | 47.47 ± 3.38 | 42.61 ± 4.93 |
| CA1 | 21.59 ± 2.55 | 20.41 ± 1.13 | 21.97 ± 1.47 | 19.05 ± 1.22 | 17.20 ± 1.88 |
| Subiculum | 27.32 ± 0.77 | 25.41 ± 0.47 | 22.47 ± 0.67* | 22.19 ± 2.00* | 20.78 ± 1.34** |
| Piriform cortex | 89.85 ± 2.02 | 77.75 ± 3.36* | 76.79 ± 1.62*** | 80.35 ± 3.51 | 77.95 ± 2.84* |
| S1Tr | 39.74 ± 2.76 | 50.26 ± 2.26* | 44.33 ± 2.92 | 34.66 ± 1.74 | 32.73 ± 2.01 |
| V2 cortex | 71.73 ± 3.59 | 94.75 ± 5.03*** | 72.91 ± 3.09 | 56.41 ± 2.03* | 57.74 ± 2.62* |
| Au cortex | 92.54 ± 1.84 | 107.00 ± 5.28* | 90.56 ± 3.32 | 81.97 ± 2.84 | 84.85 ± 3.39 |

Data represent mean ± SEM, one-way ANOVA with Dunnett's *post-hoc* test was performed $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with vehicle. SON, supraoptic nucleus; VTA, ventral tegmental area; PRh ctx, perirhinal cortex; PAG, periaqueductal gray; S1Tr, primary somatosensory cortex trunk region; V2 cortex, secondary visual cortex; Au cortex, auditory cortex.

reason for this is unclear. Spencer and Houpt (2001) demonstrated that LiCl induced *c-fos* as well as another transcription factor, inducible cAMP early repressor (ICER) mRNA, in the central amygdala, which is suggestive of a cAMP-dependent pathway. LiCl injection has been shown to phosphorylate MAP kinase in murine insular cortex and central amygdala 30 min after injection, which would lead to activation of the SRE in the promoter region of *c-fos* and Egr-1 genes. This may explain the high induction of the IEGs by LiCl in the central amygdala, as other antidepressants would be expected to only activate the cAMP-dependent elements in the IEG promoters. Despite a trend of an increase in Egr-1 expression in the insular cortex following LiCl treatment this did not reach significance.

All antidepressants tested in the present trial significantly increased *c-fos* expression in the anterior insular cortex (Figure 2). Similar to the amygdala, human neuroimaging studies in familial major depressive disorder have demonstrated an elevated CBF and glucose metabolism in the anterior insula (Drevets, 2000, 2001). Furthermore, a role for this brain region in sleep control has been implied by studies in healthy volunteers, where an increase in metabolism in the insula from waking to REM sleep was demonstrated (Nofzinger *et al*, 2001). Additionally, the antidepressant bupropion treatment normalized this relationship in depressed patients (Nofzinger *et al*, 2001).

The septum is also believed to be an important brain region in animal models of depression (Kirby and Lucki, 1997). The helpless behavior in learned helplessness is thought to be related to 5-HT levels in the septum, as microinjection of 5-HT into the lateral septal nucleus reverses learned helplessness (Sherman and Petty, 1980). Indeed, Ronan *et al* (2000) demonstrated that rats that do not develop learned helplessness behavior had increased levels of 5-hydroxyindoleacetic acid (5-HIAA) in the lateral septum as measured by microdialysis. This also suggests that 5-HT levels are involved, as higher concentrations of 5-HIAA possibly indicates increased 5-HT metabolism. A recent study reported decreased c-Fos in the lateral septal nucleus of learned helpless rats, and it was hypothesized that this may represent the deficient 5-HT transmission of these rats (Steciuk *et al*, 1999). Another animal model of depression, the forced swim test, has been demonstrated to increase c-Fos in the lateral septum, which is reversed by pretreatment with antidepressants (Duncan *et al*, 1996). Similarly, Salchner and Singewald (2002) have demonstrated that acute fluoxetine administration (5 or 10 mg/kg) induced c-Fos expression in the ventral lateral septal nucleus and exposure to airjet stress potentiated the Fos expression following the higher dose only. Therefore, the fact that all drugs in the present study decreased stress-induced *c-fos* mRNA expression in the septum would appear to contradict the findings from the learned-helplessness study and airjet stressor study but corroborate those of the forced-swim test, although both of these studies examined protein expression rather than mRNA levels of the IEG.

## Most Robust Increase in *c-fos* mRNA

The most robust induction of *c-fos* mRNA observed by any drug treatment was in the supraoptic nucleus following LiCl administration (Table 2). A 10-fold increase compared with vehicle, and five-fold compared with all other drug-treatments was seen. A likely explanation for this comes from the knowledge that the supraoptic nucleus is sensitive to osmotic balance (see Hussy *et al* (2000) for a review) and the injection of a large number of $Li^+$ ions may disrupt this balance. Despite a trend towards an increase following fluoxetine treatment, as previously reported by immunohistochemical studies (Torres *et al*, 1998), this did not reach significance compared with vehicle in the present study (Table 2).

## Brain Regions Activated Primarily by Fluoxetine

Human neuroimaging studies have shown that in familial major depressive disorder there is abnormally elevated CBF and glucose metabolism in the orbital, ventral anterior cingulate cortex, anterior insular cortex, ventral striatum, amygdala, and medial thalamus (see Drevets (2000) for a review); all components of the orbitofrontal circuit. Strong *c-fos* mRNA induction was particularly noted throughout the dorsal and ventral striatum, including the nucleus accumbens core and shell regions (Figure 4). The ventral striatum also projects to the VTA and PAG, in which *c-fos* induction following fluoxetine administration was observed (Table 2). These regions have extensive reciprocal connections with the amygdala and are involved in social withdrawal, fear, and pain; behaviors associated with depression (Drevets, 2000, 2001). The current findings are suggestive that fluoxetine, one of the most commonly prescribed antidepressants, targets a number of brain regions after acute administration, which show abnormal metabolism in depressed patients. It is possible that fluoxetine administration only targets a few of the brain regions in this circuitry but due to reciprocal connections, the increase in neuronal activity in one region affects others. This possibility has been demonstrated for *c-fos*, which can be activated in neurons removed from the site of direct neuronal stimulus (Ziolkowska and Przewlocki, 2002). Other studies analyzing the anterior cingulate cortex have mainly focused on antipsychotics (clozapine 20 mg/kg) and psychostimulant (PCP, amphetamine, caffeine, MK-801, and ketamine) compounds, which have been shown to increase *c-fos* expression in this region (see Sumner *et al*, 2004). However, psychostimulants show a very pronounced cortical, and indeed overall, induction of *c-fos,* which may reflect the nature of these compounds. Nevertheless, it is striking that acute fluoxetine targets so many brain regions known to be associated with depression, especially as very few structures other than those mentioned were observed to be altered by fluoxetine compared with vehicle (piriform cortex, suprachiasmatic nucleus, primary somatosensory cortex, secondary visual cortex, auditory cortex, data not shown).

## Locus Coeruleus and Caudal Dorsal Raphé Nucleus

Another important brain region in depression is the locus coeruleus, which is the major origin of noradrenergic projections in the CNS (Holets, 1990). Although the fluoxetine-evoked increase within the locus coeruleus was

significant, all other antidepressant groups except imipramine demonstrated a nonsignificant trend towards elevated *c-fos* mRNA expression in this region (Table 2). Previous studies that have analyzed this brain region have shown that the antidepressants desipramine (10 mg/kg), tranylcypromine (7.5 mg/kg), and bupropion (20 mg/kg) significantly elevate *c-fos* (Beck, 1995). That all antidepressants show a trend towards an increase in neuronal activity in this region is not surprising given that most increase noradrenergic neurotransmission (Frazer, 2000). Similar findings were observed in this study with the caudal dorsal raphé, as all drugs except LiCl displayed a trend towards an increase in *c-fos* expression. However, only one previous report concerning the caudal dorsal raphé has come to light, which also demonstrated that fluoxetine (10 mg/kg) did not alter *c-fos* expression in the caudal dorsal raphé (Lino-de-Oliveira *et al*, 2001).

Interestingly, given that acute antidepressant treatment is known to induce anxiogenic-like responses acutely in animals, a number of the regions where *c-fos* was elevated by fluoxetine are coincident with areas that are commonly activated by different anxiogenic compounds. For example, Singewald *et al* (2003) demonstrated that acute administration of the anxiogenics caffeine, *m*-chlorophenylpiperazine (mCPP), yohimbine, and FG-7142 (benzodiazepine inverse agoinst) increased c-Fos expression in the septum and central nucleus of the amygdala, which are the two areas that shared a common profile in the present study. This suggests that the similarity between the *c-fos* activation profiles may, in part, be due to the anxiogenic-like effect of antidepressant. However, the posterior agranular insular cortex was activated by all but one compound (mCPP; Singewald *et al*, 2003) but not by any of the antidepressants (data not shown), although the anterior insular cortex was activated by all antidepressants in this study (Figure 2). Additionally, it should be noted that although the anxiogenic compounds have been shown to increase c-Fos expression in the prelimbic cortex, secondary somatosensory cortex, and bed nucleus of the stria terminalis (Singewald *et al*, 2003), we did not find alteration in these regions following antidepressant administration (data not shown).

In conclusion, this study provides further evidence supporting the use of IEG mapping for the classification of novel compounds and identification of brain regions affected by acute drug administration (see Sumner *et al*, 2004). Comparison of the activation profiles of *c-fos* and Egr-1 mRNA expression following acute antidepressant administration demonstrated that Egr-1 expression is only altered within the central amygdala, suggesting that Egr-1 does not provide the level of information afforded by *c-fos* as an IEG in the study of acute antidepressant action. In agreement with previous studies, we have shown that all antidepressants induce *c-fos* in the central amygdala and have demonstrated, for the first time, a similar response following acute mirtazapine administration. Additionally, all compounds in the present study induced *c-fos* expression in the anterior insular cortex and decreased *c-fos* expression in the septum, two regions strongly linked with depression in human or animal models. These findings suggest that, while there are different brain regions activated by different antidepressant compounds, it is possible to employ *c-fos* neuronal activity to profile novel antidepressants based on the three common brain regions activated by a number of different classes of antidepressant compounds.

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