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Effect of 5-HT_{1A} receptor-mediated serotonin augmentation on Fos immunoreactivity in rat brain

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Abstract

The consequences of pharmacologically evoked augmented serotonin (5-hydroxytryptamine, 5-HT) release on neuronal activity in the brain, as reflected by the cellular expression of the immediate early gene c-fos, were studied. Wistar rats were treated with saline, the 5-HT reuptake inhibitor citalopram (10 μmol/kg s.c.), the 5-HT_{1A} receptor antagonist *N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)-*N*-(2-pyridyl)cyclohexane carboxamine trihydrochloride (WAY 100635, 1 μmol/kg s.c.), or the combination of both drugs. At the given dosages, the combination of the drugs has previously been shown to enhance the cerebral release of 5-HT. Two hours and thirty minutes after administration, the brains were fixated, and Fos protein was histologically stained and quantified. The paraventricular nucleus of the hypothalamus, the central nucleus amygdala, the ventromedial hypothalamic nucleus, the dorsolateral striatum, and the nucleus accumbens shell were particularly responsive to increased 5-HT release. The results, illustrating the synergistic consequence of the combined drug treatments, are discussed in terms of activity of the limbic–hypothalamic–pituitary–adrenocortical system.

Keywords: c-fos; Antidepressant; Citalopram; 5-HT_{1A} receptor; WAY 100635; Hypothalamic–pituitary–adrenal axis

1. Introduction

The therapeutic response to selective serotonin reuptake inhibitors is generally attributed to enhanced extracellular levels of 5-hydroxytryptamine (5-HT) and is usually not seen until after 2–4 weeks of medication. A possible explanation for the delayed onset of action is the gradual desensitisation of inhibitory 5-HT autoreceptors, as proposed by Blier et al. (1987a, 1987b). Such consequences of desensitisation can be mimicked by blocking the autoreceptors with antagonists, which instantaneously augments the selective serotonin reuptake inhibitor-induced increase in extracellular 5-HT and may thus accelerate the clinical response to the selective serotonin reuptake inhibitor. Indeed, in several animal studies, augmentation of 5-HT release was observed following acute co-administration of selective serotonin reuptake inhibitors with a 5-HT_{1A} receptor antagonist (Hjorth, 1993; Romero et al., 1996; Sharp et al., 1997; Cremers et al., 2000b) or with a 5-$HT_{1B/1D}$ receptor antagonist (Rollema et al., 1996; Sharp et al., 1997; Gundlah et al., 1997; Cremers et al., 2000b).

Compounds such as the anorectic drug fenfluramine and several antidepressants including the selective serotonin reuptake inhibitors, which increase the release of 5-HT, also induce an enhanced expression of c-fos, a marker of neuronal activity, throughout the brain (Richard et al., 1992; Li and Rowland, 1996; Javed et al., 1997, 1998; Veening et al., 1998). Following challenge with a 5-HT_{1A} or 5-$HT_{2A/C}$ receptor agonist, Fos expression was demonstrated in several brain areas, including the prefrontal cortex, central nucleus of the amygdala, striatum, nucleus accumbens, and paraventricular nucleus of the hypothalamus (Compaan et al., 1996; Moorman et al., 1996; Leslie et al., 1993). The consequences of pharmacologically evoked augmented 5-HT release for neuronal activity in the brain, as reflected by the cellular expression of the immediate early gene c-fos, have not yet been reported.

The effect of augmentation of extracellular 5-HT depends on the brain area investigated and the dose of selective

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serotonin reuptake inhibitor. To show their effect on 5-HT release, autoreceptors need to be sufficiently activated by endogenous 5-HT. Using an optimal dose of both citalopram, a selective serotonin reuptake inhibitor, and the 5-HT_{1A} receptor antagonist *N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)-*N*-(2-pyridyl)cyclohexane carboxamine trihydrochloride (WAY 100635) (Cremers et al., 2000a,b), we studied the effect of combined treatment with citalopram and WAY 100635 on c-fos expression in several brain areas.

2. Materials and methods

2.1. Animals

Male Wistar rats (Harlan, Zeist, the Netherlands) weighing 200–220 g were housed individually under a 12-h light/dark cycle with free access to food and water. The experiments were performed during the light period.

2.2. Design of the study

To minimize the effects of stress, all rats received saline (1 ml/kg/day i.p. and 0.3 ml/kg/day s.c.) for 7 days prior to drug challenge (Sebens et al., 2000). The following day, a single dose of saline (1 ml/kg, $n=6$), citalopram (10 μmol/kg, $n=6$), WAY 100635 (1 μmol/kg, $n=6$), or a combination of citalopram (10 μmol/kg, $n=6$) and WAY 100635 (1 μmol/kg, $n=6$) was administered s.c. The animals were perfused transcardially under pentobarbital anaesthesia 2.5 h after the final drug injection. The study was approved by the Committee on Animal Bio-ethics of the University of Groningen.

2.3. Drugs

Citalopram hydrobromide (generously supplied by Lundbeck, Denmark, courtesy of Dr. Sanchez) and WAY 100635 (synthesized in our own laboratory, courtesy of Dr. Y. Liao) were dissolved in saline. Substances were injected s.c. in a volume of 1 ml/kg; injection of the solutions did not produce any apparent discomfort.

2.4. Immunohistochemistry

Animals were perfused under deep anaesthesia (pentobarbital 100 mg/kg) with saline for 1 min followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 15 min. Brains were removed and postfixed overnight at 4 °C in 4% paraformaldehyde solution before being stored in 50 mM Tris-buffered saline, pH 7.4, containing 0.1% Na-azide. After cryoprotection by overnight immersion in a buffered (50 mM Tris/HCl buffer pH 7.4) 30% sucrose solution at room temperature, the brains were sliced into 30-μm coronal sections using a cryostat microtome. Immunostaining was performed on free-floating sections, according to the previously described procedure (Sebens et al., 1995). Briefly, sections were pretreated with 0.3% H_2O_2 and preincubated in 4% normal goat serum before the Fos primary antiserum (1:10.000, Oncogene Science, Ab-5, Cambridge, MA, USA) was added. A biotinylated anti-rabbit secondary antibody (1:800, Vector Laboratories, Burlingame, CA, USA) was used, followed by an avidin-biotinylated horseradish peroxidase complex (1:125, Vector Laboratories). Intermittent washing was done with Tris-buffered saline. The peroxidase reaction was developed with DAB (3,3′ diaminobenzidine)–Ni (ammoniumnickel-sulphate)/H_2O_2 in Tris buffer. To control for the specificity of immunoreactivity, some of the sections were incubated with omission of the primary or the secondary antibody.

2.5. Quantification and statistical analysis

Schematic drawings of the representative sections used for counting c-Fos-positive cells are shown in Fig. 1.

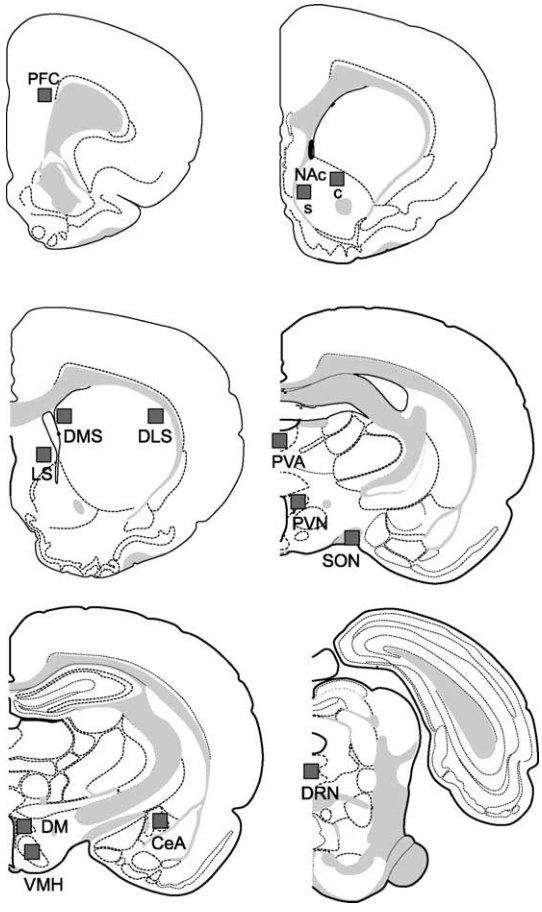

Fig. 1. Schematic representation of the areas used for quantification of Fos protein-positive cells. Grey-filled squares indicate the regions counted. PFC, prefrontal cortex; NAc shell, nucleus accumbens shell; NAc core, nucleus accumbens core; LS, lateral septum; DMStr, dorsomedial striatum; DLStr, dorsolateral striatum; PVN, paraventricular nucleus; SON, supraoptic nucleus; CeA, central nucleus of the amygdala; PVA, paraventricular nucleus; DRN, dorsal raphe nucleus; VMH ventromedial hypothalamic nucleus; DM, dorsomedial hypothalamic nucleus.

The areas counted are indicated by grey-filled squares. Fos-positive cells were counted using a computerized image analysis system. The selected area from structures of interest was digitized using a Sony (SONY, Tokyo, Japan) charge-coupled device digital camera mounted on a LEICA Leitz DMBR microscope (LEICA, Wetzlar, Germany) at $\times 10$ magnification. The numbers of Fos-positive nuclei were counted using a computer-based image analysis system LEICA (LEICA Imaging System, Cambridge, England). The resulting data are reported as the number of positive cells/0.13 mm^2 (Sebens et al., 1995, 2000).

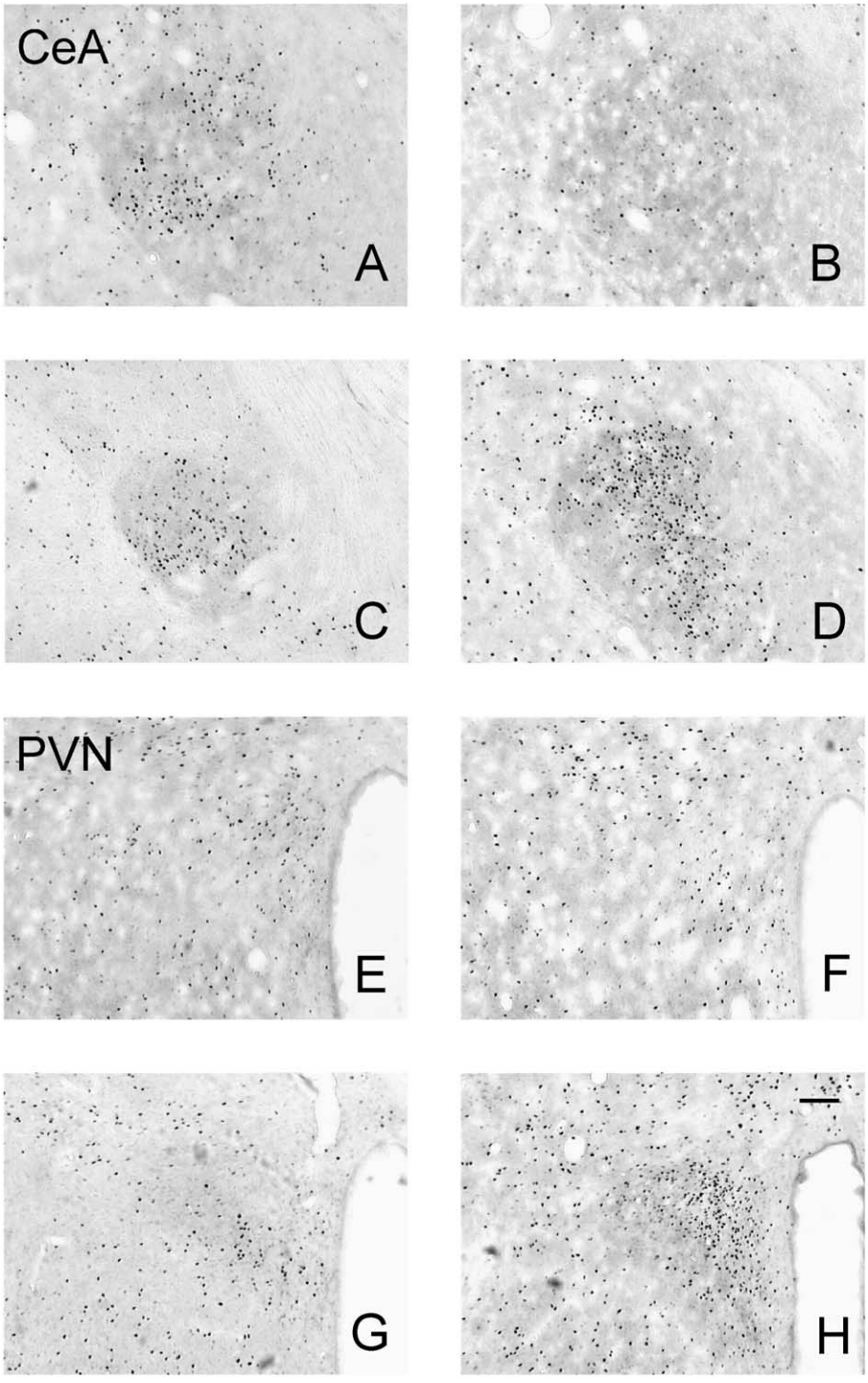

Fig. 2. Representative samples of the distribution of Fos immunoreactivity following saline, WAY 100635, and the combination of WAY 100635 and citalopram, respectively, in the central nucleus of the amygdala (A, B, C, and D) and in the paraventricular nucleus of the hypothalamus (E, F, G, and H). Scale bar = 100 μm. CeA, central nucleus of the amygdala; PVN, paraventricular nucleus of the hypothalamus.

Fos-positive cells were counted bilaterally and averaged per animal. Per experimental group, the mean number (± S.E.M.) of Fos-positive cells was determined. The data of the various groups were compared using a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls or the Dunn's test for multiple comparison procedures. The differences were considered significant if $P < 0.05$.

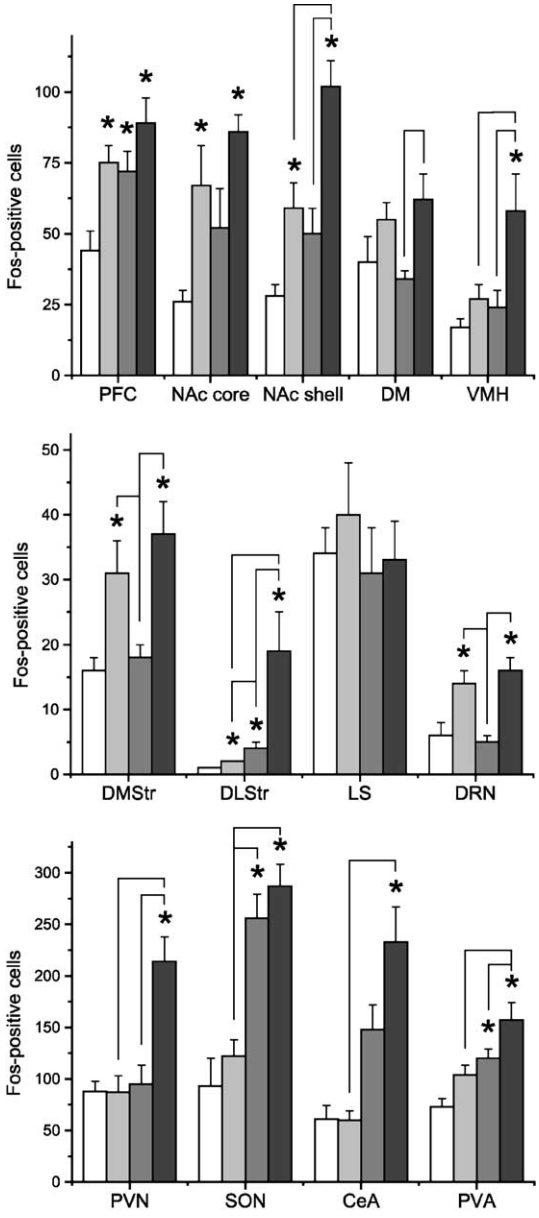

Fig. 3. Distribution patterns of Fos protein-positive cells (mean number ± S.E.M.) induced by a challenge dose of saline (open bars), WAY 100635 (light grey-filled bars), citalopram (grey-filled bars), or a combination of citalopram and WAY 100635 (dark grey-filled bars). Brain regions investigated were as follows: PFC, prefrontal cortex; NAc shell, nucleus accumbens shell; NAc core, nucleus accumbens core; LS, lateral septum; DMStr, dorsomedial striatum; DLStr, dorsolateral striatum; PVN, paraventricular nucleus; SON, supraoptic nucleus; CeA, central nucleus of the amygdala; PVA, paraventricular nucleus; DRN, dorsal raphe nucleus; VMH, ventromedial hypothalamic nucleus; DM, dorsomedial hypothalamic nucleus. *Significantly different ($P < 0.05$, Student's *t*-test) from saline. Connecting lines indicate a significant difference between treatment groups as depicted in graph ($P < 0.05$, Student's *t*-test).

3. Results

Two hours and thirty minutes after administration of either saline, citalopram, or WAY 100635, Fos immunoreactivity was seen throughout the whole brain. The selective serotonin reuptake inhibitor citalopram caused a specific regional pattern of Fos immunoreactivity. Representative sections are shown in Fig. 2. The distribution of Fos-positive cells is shown in Fig. 3. Compared to the c-fos response following saline administration, the most prominent effects of citalopram challenge were seen in the prefrontal cortex, central nucleus of the amygdala, supraoptic nucleus, and paraventricular nucleus of the thalamus, while no effect was seen in the lateral septum, dorsomedial striatum, paraventricular nucleus of the hypothalamus, ventromedial hypothalamic nucleus, and dorsal raphe nucleus (Fig. 1).

Compared to saline, the 5-HT_{1A} receptor antagonist WAY 100635 increased the number of c-fos-positive cells in the prefrontal cortex, nucleus accumbens shell and core, dorsomedial striatum, and dorsal raphe nucleus. In no other areas were significant net effects of WAY 100635 seen.

Co-administration of citalopram and WAY 100635 resulted in a clear increase of Fos-positive cells in all regions of interest, apart from the lateral septum. Four different patterns of response to combined treatment were distinguished: firstly, no difference compared to either one of the treatments (lateral septum); secondly, an increase equivalent to the effect seen following acute citalopram injection (prefrontal cortex, supraoptic nucleus, and paraventricular nucleus of the thalamus); thirdly, an increase comparable to the effect of WAY 100635 (nucleus accumbens core, dorsomedial striatum, and dorsal raphe nucleus and dorsomedial hypothalamic nucleus); and fourthly, an augmentation of the effect of one of the drugs (nucleus accumbens shell, dorsolateral striatum, paraventricular nucleus of the hypothalamus, ventromedial hypothalamic nucleus, and central nucleus of the amygdala).

Brain areas in which no Fos immunoreactivity was found in both control and any of the treated animals included the median raphe nucleus and the hippocampus.

4. Discussion

Compared to saline treatment, co-administration of the selective serotonin reuptake inhibitor citalopram and 5-HT_{1A} receptor antagonist WAY 100635 resulted in a significant increase in c-fos-positive cells in all the investigated brain regions, except the lateral septum. In the prefrontal cortex, supraoptic nucleus, and paraventricular nucleus of the thalamus, this increase can be explained as the exclusive action of

citalopram, whereas in the nucleus accumbens core, dorsomedial striatum, dorsomedial hypothalamic nucleus, and dorsal raphe nucleus, the induction of c-fos appeared to be mainly, if not exclusively, mediated by WAY 100635. Using a dose of citalopram previously shown to activate 5-HT_{1A} receptors (Cremers et al., 2000b), we observed an additive or more than additive effect (augmentation) of the compounds in the central nucleus of the amygdala, paraventricular nucleus of the hypothalamus, ventromedial hypothalamic nucleus, nucleus accumbens shell, and dorsolateral striatum.

Fenfluramine (Richard et al., 1992; Li and Rowland, 1996; Javed et al., 1997, 1998) and other compounds affecting the release of 5-HT (e.g. methylenedioxymethamphetamine, chloroamphetamine, and the monoamine oxidase inhibitor tranylcypromine; Moorman et al., 1995; Moorman and Leslie, 1996, Stephenson et al., 1999) induce c-fos expression in the forebrain (prefrontal cortex, nucleus accumbens, lateral septum, caudate putamen, bed nucleus of the stria terminalis), the midbrain (paraventricular nucleus of the hypothalamus, paraventricular nucleus of the thalamus, central nucleus of the amygdala), and brainstem (lateral parabrachial nucleus, nucleus of the solitary tract).

Selective serotonin reuptake inhibitors induce c-fos activation in the central nucleus of the amygdala, bed nucleus of the stria terminalis, and lateral parabrachial nucleus (Veening et al., 1998). Taken together with the present observations concerning the prefrontal cortex, nucleus accumbens, and paraventricular nucleus of the thalamus, the pattern is similar to that seen following activation with fenfluramine, indicating that 5-HT plays a specific role in the regulation of c-fos expression.

Receptors that are thought to induce c-fos expression include those that stimulate the inositol phosphate pathway and those that increase intracellular cAMP or Ca^{2+} concentration (Sheng et al., 1990, 1991; for review, Sheng and Greenberg, 1990 see Hughes and Dragunow, 1995; Chen et al., 1999). Most serotonergic receptors are positively coupled to their second messenger systems via G_s protein (5-HT_4, 5-HT_6, and 5-HT_7), G_q/G_{11} (5-HT_2), or via ion channels (5-HT_3) (for review, see Uphouse, 1997). Stimulation of the G_i/G_o protein-coupled 5-HT_1 receptor family (5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D}), however, induces inhibition of cAMP and may hence inhibit c-Fos expression. If the effect of selective serotonin reuptake inhibitors on c-fos is indeed directly mediated by an increase in serotonin, the pattern of Fos immunoreactivity is the result of an interplay of all positively coupled serotonergic receptors. Furthermore, blockade of the inhibitory 5-HT_{1A} receptor should increase serotonergic activity, especially in brain areas heavily controlled by 5-HT_{1A} receptors. Although we observed an augmentation of the effect of selective serotonin reuptake inhibitors in the nucleus accumbens shell, dorsolateral striatum, paraventricular nucleus of the hypothalamus, ventromedial hypothalamic nucleus, and central nucleus of the amygdala, none of these regions have a high density of 5-HT_{1A} receptors or are heavily controlled by raphe 5-HT_{1A} receptors (Pazos and Palacios, 1985; Steinbush, 1981). Apart from the low or absent 5-HT_{1A} receptor density, autoreceptor inhibition does not explain the observed results because, in contrast with microdialysis results, no augmented c-fos response could be observed in serotonergic brain areas such as the dorsal raphe nucleus, median raphe nucleus, prefrontal cortex, or hippocampus. These seeming discrepancies are in agreement with results reported in previous studies and have puzzled their authors (Compaan et al., 1996, Hajos-Korcsok and Sharp, 1999).

The pattern of c-Fos expression in the rat brain following the administration of citalopram corresponds neither with the distribution of a particular 5-HT receptor type (Pazos et al., 1985; Pazos and Palacios, 1985; Morilak et al., 1993; Ward et al., 1995; Kilpatrick et al., 1987) nor with the density of 5-HT containing nerve terminals (Steinbush, 1981). This mismatch between receptors, 5-HT innervation and Fos expression does not point to direct coupling between any of the 5-HT receptors and c-fos, but rather indicates that c-fos expression is mediated indirectly through other pathways and/or receptors.

This contention is supported by the notion that stimulation of the 5-HT_{1A} receptor induces Fos immunoreactivity despite its negative coupling to its transducing proteins. So, if c-fos expression is mediated directly via the 5-HT_{1A} receptor, this would more likely lead to an inhibition of its expression (Compaan et al., 1996; Hajós-Korcsok, 1999).

Blocking the 5-HT_{1A} receptor induced an increase in Fos expression in the cortex, nucleus accumbens and striatum. Javed et al. (1998) found the same trend in the cortex. Such differential patterns of Fos expression following administration of the 5-HT_{1A} receptor antagonist WAY 100635 may not only be attributed to brain region specificity but also to the physiological state of the animals (e.g. stress exposure).

We observed augmentation of citalopram-induced Fos expression by WAY 100635, which is in contrast with Javed et al. (1998), who used a combination of WAY 100635 with fenfluramine. Fenfluramine, however, in contrast with citalopram, releases 5-HT via a non-exocytotic mechanism which is independent of the firing rate and hence of 5-HT_{1A} autoreceptor regulation.

The most consistent increases in Fos expression in the augmentation paradigm were observed in the paraventricular nucleus of the hypothalamus, ventromedial nucleus of the hypothalamus, central nucleus of the amygdala, nucleus accumbens shell, and dorsolateral striatum: the first four areas belong to the limbic system. Although no 5-HT_{1A} receptors are present in the paraventricular nucleus of the hypothalamus, co-administration of citalopram with an antagonist seems to be essential to induce elevation of Fos expression in this area. Most likely, corticotropin-releasing hormone cells of the paraventricular nucleus of the hypothalamus are indirectly activated by innervations from other 5-HT_{1A} receptor-containing regions. Indeed, both the bed nucleus of the stria terminalis and the central nucleus of the amygdala innervate the paraventricular nucleus of the hypo-

thalamus (Gray et al., 1989), and involvement in the regulation of the hypothalamic–pituitary–adrenocortical axis has been demonstrated (Feldman et al., 1990, 1994). In addition, the observation that lesion of the ventromedial hypothalamic nucleus disrupts hypothalamic–pituitary–adrenocortical axis feedback control indicates its (in)direct influence on other brain areas of the hypothalamic–pituitary–adrenocortical system (Suemaru et al., 1995). It is remarkable that, when using c-fos as a marker, especially those areas belonging to the limbic–hypothalamic–pituitary–adrenocortical axis responded to serotonergic augmentation.

Dysregulation of the limbic–hypothalamic–pituitary–adrenocortical axis has been considered as part of the pathophysiology of both depression and anxiety. Activity of the paraventricular nucleus of the hypothalamus induces an increase in plasma levels of the stress hormone cortisol (corticosterone in rats) through the release of adrenocorticotropin from the pituitary, which is controlled by the release of corticotropin-releasing hormone from the paraventricular nucleus of the hypothalamus. It has been hypothesized by Barden et al. (1995) (for review, see Holsboer and Barden, 1996) that the mood-stabilizing effect of antidepressants is achieved by their action on the limbic–hypothalamic–pituitary–adrenocortical system. This proposal is not only in line with the present findings, but it also emphasizes the clinical relevance of our observations. Measuring plasma cortisol in depressed or anxious patients gives an indication of the functioning of the paraventricular nucleus of the hypothalamus and the limbic–hypothalamic–pituitary–adrenocortical system. Depressed patients exhibit a blunted response to activation of the hypothalamic–pituitary–adrenocortical axis, which is restored by therapy with antidepressants. Probably an altered functionality of the 5-HT_{1A} receptor contributes to this effect. It would therefore be interesting to follow patients chronically treated with antidepressants, simply by measuring the cortisol release after challenge with a 5-HT_{1A} agonist.

In conclusion, with c-fos expression as a marker of neuronal activity, the greatest effects were seen in areas belonging to the limbic–hypothalamic–pituitary–adrenocortical system, indicating that the hypothalamic–pituitary–adrenocortical axis is a potential target for selective serotonin reuptake inhibitors. These augmentation effects do not necessarily correspond to the effects on the release of 5-HT. Whereas measuring extracellular 5-HT concentration provides insight into processes directly controlling the release of 5-HT, c-fos expression may provide information about which brain regions are activated as an indirect consequence of the manipulation of the release and activity of 5-HT neurons.

In some of our previous studies, it was shown that repeated activation of the c-fos system leads to a rapid attenuation (tolerance) of drug-evoked responses (Sebens et al., 1995, 1996). Whether such c-fos desensitisation can be found following chronic treatment with selective serotonin reuptake inhibitors in the limbic–hypothalamic–pituitary–adrenocortical system has as yet to be assessed.

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