Serotonergic augmentation strategies; possibilities and limitations

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CHAPTER 3

The effect of chronic selective serotonin reuptake inhibitor treatment on serotonin_{1B} receptor sensitivity and HPA-axis activity


Abstract

The authors have investigated 5-HT$_{1B}$ receptor function in prefrontal cortex and dorsal hippocampus as well as the HPA-axis response after subchronic (24 hr) and chronic (15 day) treatment with the SSRI citalopram. All experiments were carried out in presence of citalopram to prevent rapid resensitisation of the 5-HT$_{1B}$ receptors. Moreover, this more closely resembles the clinical situation. The concentration of citalopram was measured in both brain areas to ensure comparable levels in the different treatment groups. Using microdialysis, the authors found that under those conditions the effect of the 5-HT$_{1B}$ receptor antagonists SB 224289 and the mixed 5-HT$_{1B/1D}$ receptor antagonist GR 127935 on extracellular levels of 5-HT was unaltered by duration of treatment. Basal levels of 5-HT however, were increased in the dorsal hippocampus following chronic treatment. In addition, plasma levels of the catecholamines adrenaline and noradrenaline and the HPA-axis hormones ACTH and corticosterone were all decreased after chronic treatment.
1. Introduction

Antidepressants are clinically effective only after prolonged treatment, indicating that adaptive mechanisms are involved in the therapeutic effect. This delayed response may be linked to a gradual desensitization of firing rate and release controlling 5-HT autoreceptors (Blier et al., 1987). This idea was based on electrophysiological experiments in rats, wherein acute administration of antidepressants decreased the firing rate of 5-HT neurons, which normalized after prolonged administration (Blier et al., 1987; Chaput et al., 1986).

Using 5-HT$_{1A}$ or 5-HT$_{1B}$ receptor antagonists, microdialysis studies in rodents have demonstrated that acute administration of an SSRI activates both types of autoreceptors (Cremers et al., 2000a; Hjorth, 1993; Invernizzi et al., 1997; Rollema et al., 1996). Both electrophysiology and microdialysis have revealed a reduction of 5-HT$_{1A}$ receptor functionality following chronic administration (Cremers et al., 2000b; Invernizzi et al., 1994; Kreiss and Lucki, 1995; Le Poul et al., 1995). In contrast with single unit recordings, changes in 5-HT$_{1B}$ receptor functionality following chronic antidepressant treatment could not be demonstrated using microdialysis (Auerbach and Hjorth, 1995; Bosker et al., 1995; Chaput et al., 1986; Cremers et al., 2000b; Davidson and Stamford, 1997; Moret and Briley, 1996).

Levels of 5-HT$_{1B}$ mRNA on the other hand, are decreased following chronic treatment with antidepressants, but rapidly return to normal after discontinuation of the antidepressant (Anthony et al., 2000; Neumaier et al., 1996). Arguably, a loss of presynaptic 5-HT$_{1B}$ receptor function may be retrieved within a few days after discontinuation of the drug. Hence, the possibility that presynaptic 5-HT$_{1B}$ receptors rapidly resensitize is worth investigating. This is further emphasized by the common practice in microdialysis studies to use a washout period (2-7 days) to minimize pharmacological interference by residual antidepressant.

Next to these central serotonergic adaptations, peripheral alterations of stress hormone release might also play a role in the therapeutic effect of long term treatment with SSRIs. Stress activates many physiological systems, such as the sympathetic system, resulting in rapid release of the catecholamines adrenaline and noradrenaline from the adrenal medulla. This is followed by activation of the hypothalamic-pituitary-adrenal (HPA)-axis, resulting in release of ACTH from the pituitary which induces the release of cortisol (corticosterone in rats) from the adrenal cortex. Prolonged elevation of catecholamines and cortisol by chronic stress could be a factor in stress related pathology, including depression. Pharmacological intervention in stress related processes might therefore help to slow down exacerbation of depressive symptoms. This is supported by the observation that the HPA-axis hyperactivity in depressed patients is normalized after clinical
remission due to chronic antidepressant treatment (Barden et al., 1995; Holsboer and Barden, 1996).

In this study, the authors have investigated whether presynaptic 5-HT$_{1B}$ receptors become less responsive during chronic treatment with the SSRI citalopram. Citalopram was administered via osmotic minipumps to obtain steady state levels within the clinically effective range (Cremers et al., 2000b). To circumvent resensitization of presynaptic 5-HT$_{1B}$ receptors, all microdialysis experiments were performed in the presence of the drug. Extracellular levels of 5-HT and citalopram were measured in prefrontal cortex and dorsal hippocampus, brain areas that have been implicated in depression and anxiety, respectively. Responsiveness of presynaptic 5-HT$_{1B}$ receptors was measured by the ability of the 5-HT$_{1B}$ antagonists GR 127935 and SB 224289 to augment the effect of citalopram. In addition, the authors have measured plasma levels of catecholamines, ACTH and corticosterone to investigate the effect of treatment duration on peripheral levels of stress hormones.
2. Materials and methods

2.1 Animals

Male Harlan rats (Zeil, Netherlands) weighing 285-320 g were housed eight per cage under standard conditions (22-24 °C, 12/12 light/dark cycle, food and water ad libitum). Following implantation of the minipump, rats were housed in pairs. After stereotaxic surgery and during the microdialysis experiments the rats were housed separately. All animal experiments were according to the governmental guidelines for care and use of laboratory animals and were approved by the Committee for Animal Research of the Medical Faculty of the Groningen University.

2.2 Drugs

The following drugs were used: Citalopram hydrobromide (kindly donated by Lundbeck (Denmark) courtesy Dr. Sanchez), GR 127935 (N-[4-methoxy-3-(4-methyl-1-piperizinyllphenyl]-2’-methyl-4’-(5-methyl-1,2,4-oxadiazone-3-yl)[1,1’-biphenyl]-carboxamide, synthesised in our own laboratory, courtesy Dr. Y Liao and Dr. M Mensonides) and SB 224289 (2,3,6,7-tetrahydro-1’-methyl-5-(2’-methyl-4’-[5-methyl-1,2,4-oxqadiazole-3-yl]-biphenyl-4-yl]carbon-yl)furo[2,3-f]indole-3-spiro-4’-piperidine oxalate, purchased from Sigma-Alldrich). GR 127935 was dissolved in saline with a drop of acetic acid and administered at a dose of 1 µmol/kg, SB 224289 was dissolved in a 10% dimethylsulfoxide (DMSO)-saline solution and administered at a dose of 4 mg/kg. Both substances were injected subcutaneously in a volume of 1 ml per kg. Both dosages were chosen for their ability to augment SSRI induced increase of 5-HT to the same extent (Cremers et al., 2000a; Roberts et al., 1999).

2.3 Surgery

Minipumps:

Osmotic minipumps (2ML2 Alzet, USA, 5 µl/h, 2 weeks) were either filled with saline or 50 mg/ml citalopram hydrobromide dissolved in saline under aseptic conditions. During isoflurane anaesthesia (2.5%, 400ml/min N₂O, 600 ml/min O₂), minipumps were implanted subcutaneously on the left side of the back of the rat. After 14 days, all minipumps were replaced with citalopram filled minipumps. Hereafter the microdialysis probes were implanted.
Chapter 3

Probes:
During isoflurane anaesthesia (2.5 %, 400 ml/min N₂O, 600 ml/min O₂), a home made concentric microdialysis probe (i.d. 220 µm, o.d. 310 µm, AN 69, Hospal, Italy), made of polyacrylonitrile /
sodium methyl sulphonate copolymer dialysis fiber was stereotaxically implanted in the prefrontal cortex (PFC) or the dorsal hippocampus (dHC) using the following coordinates: PFC;
inscisorbar at -3.3 mm (posterior: +3.5 mm, lateral: +0.9 mm, ventral from dura: –6.0 mm),
exposed tiplength was 4 mm. DHC; incisorbar at +5.0 mm (posterior: -4.0 mm, lateral: -1.2 mm,
ventral from skull: -5.5 mm), exposed tiplength was 2 mm. (Paxinos and Watson, 1982). The
probes were secured in place with dental cement.

2.4 Microdialysis experiments
Microdialysis experiments were performed 24 hrs and 48 hrs after stereotaxic surgery. The saline
pretreated animals had hence received a 24 hr citalopram treatment at the start of the
microdialysis experiments, which is referred to as subchronic treatment. The citalopram
pretreated animals received a 15 day treatment which is referred to as chronic treatment. All
experiments were carried out in presence of citalopram delivered systemically via minipumps.
To avoid carry over effects, if any, pharmacological challenges with SB 224289 or GR 127935
were randomly allocated for each experiment to the first or second day. Thus, all animals
received two different challenges in a randomized fashion.

The probes were perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 1.2 mM CaCl₂,
pH6-7), using a CMA /102 microdialysis pump at a constant flow rate of 1.5 µl/min.

After a stabilization period of two hours, 15 min samples were collected into vials containing 7.5
µl of 0.02 M acetic acid. All experiments were performed in conscious and freely moving
animals.

2.5 Analytical procedures
2.5.1. Serotonin
Analysis of 5-HT was performed by high-performance liquid chromatography (HPLC) with
electrochemical detection. Briefly, 20 µl samples were injected into a HPLC (Shimadzu, LC-
10AD liquid chromatograph) equipped with a reversed-phase column (phenomex hypersil 3 : 3
µm, 100 x 2.0 mm, C18, Bester, Amstelveen, the Netherlands) and an electrochemical detector
(ANTEC Leyden, Leiden, the Netherlands) at a potential setting of 500 mV vs. Ag/AgCl
reference electrode. Chromatography was performed at 30 °C using the integrated column oven of the Antec potentiostat.

The mobile phase consisted of 5 g/l (NH₄)₂SO₄, 50 mg/l heptane sulphonic acid sodium salt, 500 mg/l EDTA, 4.5% methanol, 30 µl/l triethylamine, adjusted to pH 4.65 with diluted acetic acid. The flow rate was 0.4 ml/min. The detection limit for 5-HT was 0.2 fmol/sample (signal to noise ratio 2)

2.5.2 Citalopram
Citalopram was measured according to Oyehaug et al. (1982) with minor modifications. Dialysate samples were injected into an HPLC (1084B Liquid Chromatograph, Hewlett Packard) which was connected with a fluorescence detector (470 Scanning Fluorescence detector, Waters, England) operating at an absorption wavelength of 240 nm, an emission wavelength of 296 nm, and a slitwidth of 12 nm. Separation was performed using a Supelcosil HPLC column (5 µm, C18, 250 x 46 mm, Supelco, the Netherlands), at ambient temperature. The mobile phase consisted of 46% v/v acetonitrile, 54% v/v potassium dihydrogen phosphate buffer (4.3 g/l) and 1 mM tetramethylammonium, at pH 3.0. The flow rate was set at 0.75 ml/min. The detection limit was 5 nM (signal to noise ratio = 2)

2.5.3. Catecholamines
Norepinephrine and epinephrine were extracted according to Smedes et al. (1982). Analysis was performed by high-performance liquid chromatography (HPLC) with electrochemical detection. Briefly, 20 µl samples were injected into an HPLC (Shimadzu, LC-10AD liquid chromatograph) equipped with a reversed-phase column (phenomex hypersil 3 : 3 µm, 100 x 2.0 mm, C18, Bester, Amstelveen, the Netherlands) and an electrochemical detector (ANTEC Leyden, Leiden, the Netherlands) at a potential setting of 500 mV vs. Ag/AgCl reference electrode. Chromatography was performed at 30° C using the integrated column oven of the Antec potentiostate.

The mobile phase consisted of 0.05 g/l EDTA, 4.1 g/l sodium acetate, 0.14 g/l octane sulphonic acid and 1.8% v/v methanol, at pH 4.10. Flow rate was set at 0.25 ml/min and the detection limit was 0.5 fmol/sample (signal to noise ratio = 3).

2.5.4. Corticosterone
Plasma was extracted twice with diethyl ether, the combined ether layers were evaporated under a stream of nitrogen. The residue was dissolved in mobile phase, vortexed and centrifuged. 50 µl of the supernatant was injected into the HPLC system, consisting of a 1084B Liquid Chromatograph, Hewlett Packard HPLC, connected with a Jasco FP 1520 UV detector. Separation was performed using a Supelcosil HPLC column (5 µm, C18, 250 x 46 mm, Supelco, the Netherlands). Wavelength was set at 254 nm. The mobile phase consisted of 30 % v/v
acetonitrile and the flow rate was set at 1 ml/min. The detection limit was 0.7 pmol/sample (signal to noise ratio = 3).

2.5.5 ACTH
ACTH levels in plasma were determined using a commercially available RIA kit from Nichols Institute Diagnostics, San Clemente, CA.

2.6 Histology
Following the termination of each experiment, rats were brought under isoflorane anaesthesia in a small plexiglass box. Blood was collected by cardiac puncture during isoflurane anaesthesia (2.5%, 400 ml/min N₂O, 600 ml/min O₂). The animals were killed by decapitation, and the brains were removed and fixed in a 5% formaldehyde solution. Correct placement of the implanted cannulas was histologically verified.

2.7 Statistics
The data are presented as percentages of basal values calculated as individuals means of the first four consecutive microdialysis samples. Statistical analysis was performed using Statistica for windows. Treatment effects were compared using a student’s T-test or one way ANOVA for repeated measurements. Level of significance was set at \( P < 0.05 \).
3. Results

3.1. Citalopram brain levels
The extent of augmentation by 5-HT1B receptor antagonists will likely depend on the concentration of the SSRI. To compare the effect of different treatment conditions, the concentrations of the SSRI in the region of interest should at least be comparable. To ensure this, citalopram was measured in dialysates from prefrontal cortex and hippocampus. Citalopram levels in dialysates from prefrontal cortex obtained from subchronically treated animals $17.6 \pm 1.5$ nM ($n = 13$) were not significantly different from those obtained from chronically treated animals $13.6 \pm 1.2$ nM ($n = 12$).

The same outcome was obtained for dialysates from dorsal hippocampus, showing citalopram levels of $14.2 \pm 1.6$ nM ($n = 13$) and $11.7 \pm 1.5$ nM ($n = 12$) for sub-chronically and chronically treated animals, respectively. These values are not corrected for in-vitro recovery ($\sim 25\%$), suggesting that the actual brain concentrations are considerably higher.

3.2. Basal 5-HT levels in prefrontal cortex and dorsal hippocampus
Cortical 5-HT level in the subchronic treatment group was $22.9 \pm 3.6$ fmol/sample ($n = 13$), which was not significantly different from the basal level of $29.6 \pm 3.1$ fmol/sample ($n = 12$) as measured in the chronic treatment group.

In dorsal hippocampus basal 5-HT levels in the chronic treatment group ($37.0 \pm 5.9$ fmol/sample ($n = 12$)), were significantly larger ($P = 0.013$, Fig. 1) than those measured in the subchronic treatment group ($20.5 \pm 4.7$ fmol/sample ($n = 13$)).

3.3. GR challenge
Subcutaneous administration of GR 127935 (1 µmol/kg) significantly elevated baseline levels of 5-HT in the prefrontal cortex ($F(10,120) = 2.566$), which did not differ between treatment groups ($F(1,12) = 1.925$ (Fig. 2a)).

Levels in the dorsal hippocampus significantly increased to about 120% of baseline value following GR challenge ($F(10,110) = 2.703$), this effect was unaltered by pretreatment ($F(1,11) = 0.155$ n.s.) (Fig. 2b).

3.4. SB challenge
Administration of SB 224289 (8.45 µmol/kg s.c.) significantly augmented 5-HT to about 150% and 180% of baseline value in cortex ($F(10,90) = 8.092$) and dorsal hippocampus ($F(10,80) = 8.092$).
6.973), respectively (Figs. 3a and b). Statistical analysis did not reveal significant differences between the two treatment groups for both the PFC (F(1,9) = 2.353) and dorsal hippocampus (F(1,8) = 1.386).

3.5. Corticosterone and ACTH plasma levels
Plasma levels of corticosterone were 0.66 ± 0.05 μM and 0.46 ± 0.07 μM for the subchronic and chronic treatment group, respectively. ACTH levels were 0.13 ± 0.02 nM in the subchronically treated animals and 0.07 ± 0.01 nM in the chronically treated animals. Compared to subchronic treatment, chronic treatment significantly decreased both the plasma levels of corticosterone ($P = 0.048$) and ACTH ($P = 0.031$)(Fig. 4a).

3.6. Noradrenaline and adrenaline plasma levels
Plasma levels of noradrenaline and adrenaline were 11.8 ± 1.4 nM and 14.3 ± 1.8 nM, respectively, for the subchronic treatment group, and 5.5 ± 1.0 nM and 5.7 ± 2.0 nM, respectively, for the chronic treatment group. Compared to subchronic treatment, chronic treatment significantly decreased both the plasma levels of noradrenaline ($P = 0.003$) and adrenaline ($P = 0.008$)(Fig. 4b).

![Fig. 1. Effect of duration of treatment on basal levels of 5-HT in the PFC and dHC. Subchronic (24h) treated animals black bars (n = 13); Chronic treated animals light gray bars (n = 12). * P < 0.02. All experiments were carried out in presence of subcutaneous implanted minipumps filled with citalopram.](image-url)
Fig. 2a and b. Effect of GR 127935 1 µmol/kg s.c. on 5-HT release in PFC (a) and dHC (b). Subchronic treated (n = 8; filled squares); chronic treated (n = 6; filled circles). All experiments were carried out in presence of subcutaneous implanted minipumps filled with citalopram.
Fig. 3a and b. Effect of SB 224289 8.45 µmol/kg s.c. on 5-HT release in PFC (a) and dHC (b). Subchronic treated (n = 8; filled squares); chronic treated (n = 6; filled circles). All experiments were carried out in presence of subcutaneous implanted minipumps filled with citalopram.
Fig. 4a and b. Effect of treatment on plasma levels of corticosterone and ACTH (a) and noradrenaline and adrenaline (b). Subchronic (24h) treated animals black bars (n = 9); Chronic treated animals light gray bars (n = 6). * P < 0.05, ** P < 0.01. All experiments were carried out in presence of subcutaneous implanted minipumps filled with citalopram.
4. Discussion

5-HT$_{1B}$ receptor sensitivity

Early electrophysiological data suggest that both 5-HT$_{1A}$ and 5-HT$_{1B}$ autoreceptors desensitize following chronic treatment with antidepressants (Blier et al., 1987; Chaput et al., 1986). Microdialysis studies were able to reproduce these findings for the 5-HT$_{1A}$ autoreceptor, but not for the 5-HT$_{1B}$ autoreceptor (Auerbach and Hjorth, 1995; Bosker et al., 1995; Cremers et al., 2000b).

This may partly be explained in terms of experimental conditions, however differences in pharmacokinetics should also be taken into account. In a previous study, it was emphasized that the plasma levels of citalopram during chronic treatment should be in the same range as those measured in patients (Cremers et al., 2000b). Another relevant factor could be the length of the washout period following chronic treatment. To exclude any possibility of interference, previous microdialysis experiments were performed in absence of the SSRI. However, receptor responsivity is a dynamic process and hence theoretically, resensitization could occur during washout. This is supported by an in-situ hybridization study showing that after 24 hours upon drug discontinuation 5-HT$_{1B}$ receptor mRNA was rapidly recovered (Anthony et al., 2000; Neumaier et al., 1996). To circumvent this problem, the present experiments were performed in the presence of citalopram, mimicking the clinical situation. In a previous study, we measured plasma levels throughout a similar treatment using minipumps delivering 0.25 mg/h citalopram, resulting in plasma levels of 0.3 µM citalopram (Cremers et al., 2000b), which is within the clinically effective range in humans (0.12-0.84 µM, (Baumann, 1992)). In the present study, brain citalopram concentrations were determined in both brain areas. Concentrations of citalopram in the brain were within the same range for chronically and sub-chronically treated animals, which enables direct comparison between the two conditions.

In presence of citalopram, augmentation of the 5-HT release with 5-HT$_{1B}$ receptor antagonist SB 224289 or mixed 5-HT$_{1B/1D}$ receptor antagonist GR 127935 was comparable in both chronic and subchronic treatment group, suggesting that the sensitivity of 5-HT$_{1B}$ receptors had not changed with duration of treatment.

These results do not differ from microdialysis studies performed in absence of the SSRI following a washout period (Auerbach and Hjorth, 1995; Bosker et al., 1995; Cremers et al., 2000b), indicating that 5-HT$_{1B}$ receptor resensitization does not occur. This is in contrast with the findings of in situ hybridization studies (Anthony et al., 2000; Neumaier et al., 1996). It must be realized, however, that mRNA and protein levels do not always correlate. Moreover, plasticity of
pre- and postsynaptic 5-HT$_{1B}$ receptors may be different, which is hard or impossible to discriminate at the level of mRNA.

Although in the present study no alteration was found of 5-HT$_{1B}$ autoreceptor responsivity, chronic treatment with citalopram significantly increased basal 5-HT levels in the dorsal hippocampus. This could be related to a diminished functionality of the 5-HT$_{1A}$ receptor mediated inhibitory control of the local 5-HT release, as demonstrated previously (Bosker et al., 2001; Cremers et al., 2000b). So despite the lack of effect on the 5-HT$_{1B}$ receptor, long-term treatment does induce several other pharmacological alterations which might be involved in the therapeutic effect of SSRIs.

**HPA-axis activity**

Besides changes within the central serotonergic system, chronic treatment with citalopram also changed several peripheral markers. The chronically treated animals had significantly lower plasma levels of adrenaline and noradrenaline, ACTH and corticosterone, suggesting a decreased activity of the HPA-axis. This is in agreement with the observation that chronic treatment with antidepressants restores HPA-axis hyperactivity in depressive patients (Barden et al., 1995; Inder et al., 2001) and that it reduces basal levels of corticosteroids and ACTH in rodents (Reul et al., 1993). Cortisol release in humans can be regulated by activation of 5-HT$_{1A}$ receptors. This response is blunted following chronic antidepressant therapy (Berlin et al., 1998; Lerer et al., 1999), which indicates a strong interaction between the serotonergic system and HPA axis activity and thus further supports our results.

Levels of corticosterone and catecholamines found in the subchronic treatment group are 10-fold higher than baseline levels reported in literature (Gomez et al., 1998; Reul et al., 1993), and comparable with a restraint stress induced response (Ginsberg et al., 2003). The termination procedure used in the present study induced acute stress in all animals, which likely explains the high plasma levels of stress hormones. Interestingly, stress hormone levels were significantly reduced in the chronic treatment group. Our results are in line with the observation that rats chronically treated with an SSRI have a blunted corticosterone response to citalopram (Jensen et al., 1999), as well as a reduced HPA-axis activation in response to acute stress (Reul et al., 1993). In short, the present study confirms that control of stress hormone release is affected by chronic SSRI treatment.
Conclusion

It can be concluded that as 5-HT_{1B} receptor function remains unaltered following chronic citalopram treatment, this receptor is not involved in the observed reduction of the stress hormone release. The notion that 5-HT_{1B} receptors do not desensitize in presence of citalopram suggests that the therapeutic effect of long-term antidepressant treatment could be improved by co-administration of a 5-HT_{1B} receptor antagonist. Further research should reveal how HPA-axis attenuation might play a role in achieving the therapeutic effect of SSRIs.


