# Serotonergic augmentation strategies; possibilities and limitations

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# CHAPTER 6

*Effect of chronic and acute administration of citalopram on serotonin synthesis, storage and metabolism in the rat brain*

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Submitted

## Abstract

While extracellular serotonin is commonly used to assess the effects of long-term treatment with selective serotonin reuptake inhibitors, it is still not clear how this affects serotonergic markers like synthesis, storage and metabolism. It is conceivable that in order to maintain intracellular stores of serotonin, synthesis needs to adjust to the conditions of prolonged reuptake inhibition. In the present study, we investigated the effect of chronic SSRI treatment on serotonin, its metabolite HIAA and the precursor 5-hydroxy tryptophan in tissue of rat brain. It was found that chronic treatment resulted in a dramatic depletion of the serotonin content in the brain, which most likely is the result of insufficient serotonin synthesis caused by prolonged autoreceptor activation.

In addition, we have demonstrated that a washout period rapidly reverses the effects of chronic treatment in terminal areas only, indicating a role for the 5-$HT_{1B}$ receptor. This might parallel the clinical phenomenon of rebound depression, which occurs when suddenly discontinuing treatment with antidepressants. These findings further support the clinical practice to slowly phase out SSRI treatment.

## 1. Introduction

The serotonergic system and consequently also its response to serotonergic drugs like antidepressants are known to be under tight control of a number of feedback mechanisms. Increased levels of serotonin (5-HT) caused by serotonergic reuptake inhibitors (SSRIs) activate several inhibitory autoreceptors controlling serotonergic synthesis (Moret and Briley, 1997;Barton and Hutson, 1999;Hjorth et al., 1995), release (Invernizzi et al., 1997;Rollema et al., 1996;Hjorth, 1993;Cremers et al., 2000) and turnover (Stenfors and Ross, 2002;Fuller, Perry et al., 1974). Starting chronic SSRI treatment, all processes immediately lessened, but steadily normalized or even increased during treatment (Kreiss and Lucki, 1995;Le Poul et al., 1995;Esteban et al., 1999;Stenfors and Ross, 2002), suggesting a gradually reduced functionality of the serotonergic autoreceptors. It is generally believed that the increase of extracellular serotonin as a result of diminished autoreceptor control might underlie the therapeutic response to antidepressants (Blier, de Montigny et al., 1987;Briley and Moret, 1993). Whereas the effect of long term antidepressant treatment on serotonergic release, turnover and synthesis has been investigated extensively, it still remains unknown if intracellular serotonin stores are affected. Serotonergic cells partly rely on synthesis and partly on reuptake of extracellular serotonin to maintain their intracellular concentration. So theoretically, intracellular serotonin stores could get depleted if synthesis rate remains unadjusted while reuptake is continuously blocked by chronic treatment.

In the present study, we investigated the effect of chronic citalopram treatment on brain serotonin, synthesis and metabolism in tissue of several brain areas.

The conversion of tryptophan into 5-HTP is considered to be the rate-limiting step in the synthesis of serotonin, as under normal conditions, 5-HTP is immediately converted into 5-HT by the non-specific enzyme aminoacid decarboxylase. The rate of serotonin synthesis was measured as the amount of 5-HTP accumulated when blocking this final step. The ratio of 5-HIAA/5-HT was used as an index of serotonergic metabolism or turnover.

As a marker of the serotonergic system, extracellullar serotonin measured by microdialysis is generally used to assess the effect of prolonged antidepressant treatment. However, this represents only a fraction of the total serotonin content in the brain as the amount stored intracellular is a 1000 fold higher. In the present study, tissue destruction was used to investigate effects on total levels of serotonin, HIAA and 5-HTP, which includes both intra- and extracellular levels, but merely represents the intracellular situation. Compared to extracellular measurements, this might give a better view of general changes in serotonergic homeostasis throughout the whole brain.

The effect of chronic treatment is commonly assessed by a challenge following a washout period in order to prevent pharmacological interference with the treatment. However, it has been shown that adaptive processes seen after chronic treatment can revert whithin the time span of the washout period (Neumaier, Root et al., 1996). This implies a rapid adaptation of the serotonergic system and also questions the effects observed after a washout. In the present study, citalopram was delivered by osmotic minipumps to ensure stable plasma levels and analyzed to evaluate kinetics. The effect of chronic treatment on a challenge with citalopram was studied both after a washout and in presence of the minipump. The latter could give a better insight in the effects of chronic treatment on the serotonergic homeostatis because it more closely resembles the clinical situation.

## 2. Materials and methods

### *2.1 Animals*

Male Harlan rats (Zeist, Netherlands) weighing 285-320 g were housed eight per cage under standard conditions (22-24 $^{o}$C, 12/12 light/dark cycle, food and water ad libitum). Following implantation of the minipump, rats were housed in pairs of two. All animal experiments were performed 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 Medical Faculty of the Groningen University.

### *2.2 Treatment*

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%, 400 ml/min $N_2O$, 600 ml/min $O_2$), minipumps were implanted subcutaneously on the left side of the back of the rat.

In the treatment group including a washout period, the osmotic minipumps were removed after 14 days, the remaining subcutaneous cavity was flushed twice with 5 ml of sterile saline and animals were sacrified 48 hours after removal of their minipump. The other treatment groups include a 14 day saline treatment and a 14 day citalopram treatment, these animals were sacrified with their minipump still in place.

At the day of the termination, animals were challenged with either citalopram 10 μmol/kg sc or saline. After 45 min. NSD 1015 was injected intraperitoneally at a dose of 100 mg/kg. Another 45 min later, animals were anaesthetized with isoflurane anaesthesia (2,5 %, 400ml/min $N_2O$, 600 ml/min $O_2$), blood was taken by cardiac puncture, brains were removed, rapidly frozen at dry ice and stored at –80 $^{o}$C.

### *2.3 Tissue dissection*

Brains were sliced on a cryostat and punches were taken from nine brain areas; anterior cingulate cortex (ACAD), nucleus accumbens (NAc), caudate putamen (CP), paraventricular nucleus of the hypothalamus (PVN), dorsal hippocampus (dHC), ventral hippocampus (vHC), central amygdala (Amy). Brain samples were homogenized with 100 μL of 0.1 M perchloric acid and centrifuged at 14000 rpm for 10 min at 4 $^{o}$C. The supernatant was removed and assayed for 5-HT, 5-HIAA and 5-HTP.

## 2.4 Drugs

The following drugs were used: Citalopram hydrobromide (kindly donated by Lundbeck (Denmark) courtesy Dr. Sanchez) and NSD 1015 (purchased from Sigma).

## 2.5 Analytical procedures

### 2.5.1. 5-HT, 5-HIAA and 5-HTP

Analysis of 5-HT and 5-HIAA 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 (ESA, Chelmsford, MA, USA) at a potential setting of 600 mV vs. Ag/AgCl reference electrode. The mobile phase consisted of 4.1 g/l Na acetate , 150 mg/l octane sulphonic acid sodium salt, 10 % methanol, adjusted to pH 4.1 with acetic acid. 5-HTP was analyzed by adjusting the methanol content to 5%. The flow rate was 1.0 ml/min.

### 2.5.2. Citalopram

Citalopram was measured in plasma 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 wavelenght 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)

## 2.6 Data processing and statistics

Levels are depicted as percentage of the control group, the saline treated animals receiving a saline challenge. All data are depicted in table 1, results which are discussed are presented in grahps and have been statistically analyzed. Statistical analysis was performed using Sigmastat for windows (Jandel Software, SPPS Inc., Chicago, IL, USA). Treatment or challenge effects were evaluated using one way ANOVA.

## 3. Results

### *Effect treatment on intracellular serotonin stores*

Compared to saline treated animals, chronic treatment with a subsequent washout period of 48 hours reduced intracellular serotonin throughout the brain, showing a statistical difference in the NAc ($P = 0.0017$), CP ($P = 0.0063$), dHC ($P = 0.00816$) and PVN ($P = 0.0095$). Following chronic treatment without washout, this effect was even stronger, now being statistically different in Acad ($P = 0.0077$), NAc ($P = 0.0179$), CP ($P = 0.0402$), dHC ($P = 0.0044$) and PVN ($P = 0.0007$) (Fig. 1).

### *Effect washout on treatment*

After a washout period of 48 hours, both turnover and synthesis were increased following chronic treatment. In absence of a washout period, these processes were decreased. Although a clear trend in all brainareas, this difference was significant in the Nac ($P = 0.0016$), vHC ($P = 0.0093$), Amy ($P = 0.0004$) and PVN ($P = 0.0117$) for the turnover, synthesis statistically differed in Acad ($P = 0.0257$), CP ($P = 0.0344$), dHC ($P = 0.0378$), vHC ($P = 0.0303$), PVN ($P = 0.0329$) and DRN ($P = 0.0417$) (Figs. 2 and 3).

### *Effect acute and chronic treatment with citalopram on plasma levels*

Obviously, following chronic saline treatment, a challenge with saline did not have any effect plasma levels of citalopram. An acute challenge of citalopram increased levels to $2.26 \pm 0.28$ μM ($P<0.0001$). A 48 hour washout period following a 14 day treatment with citalopram was sufficient to reduce the amount of citalopram below a functional level, as plasma levels were below detection limit. Challenging the animal subsequently with citalopram raised levels to $1.91 \pm 0.15$ μM ($P<0.0001$). Chronic treatment without a washout resulted in plasma levels of $1.44 \pm 0.05$ μM, a challenge with citalopram increased plasma levels further to $3.38 \pm 0.63$ μM ($P = 0.0401$). Plasma levels of citalopram following either acute administration or prolonged treatment without washout are comparable. As pharmacokinetics do not differ, group effects should be attributed to treatment duration.

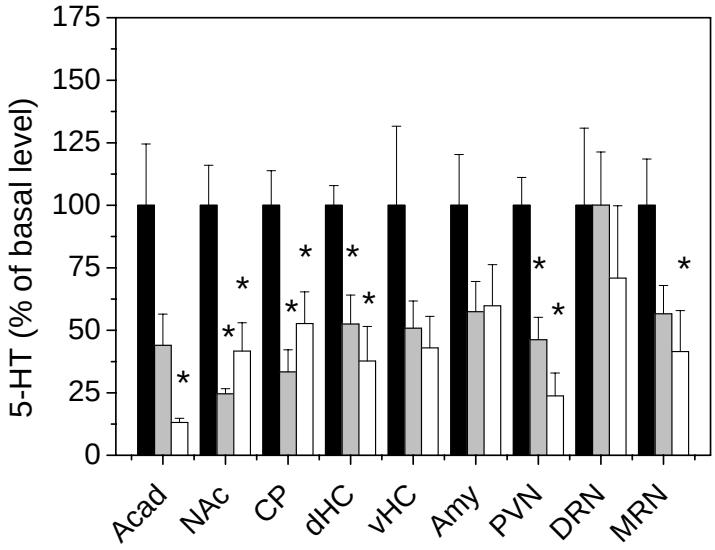


***Fig. 1.*** *Effect of chronic treatment on intracellular serotonin stores*
*Black bars; saline treatment, saline challenge. Grey bars; citalopram treatment, 48 hour washout, saline challenge. White bars; citalopram treatment, no washout, saline challenge. * P < 0.05 versus saline treatment.*

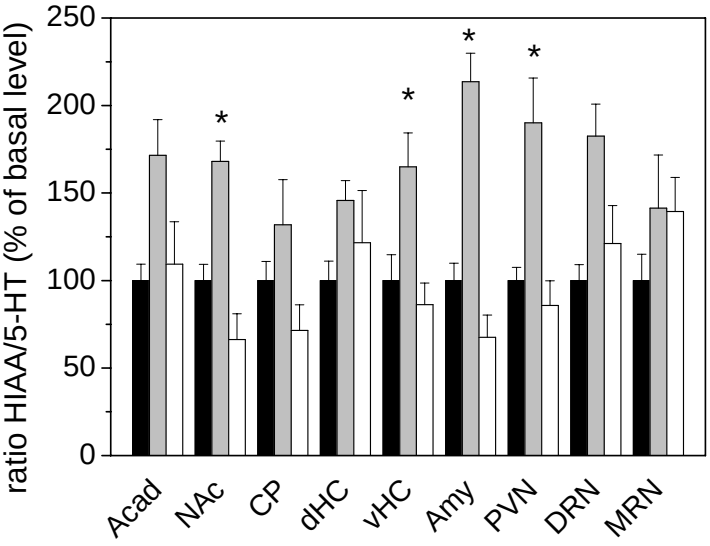


***Fig. 2.*** *Effect of washout on serotonin turnover*
*Black bars; saline treatment, saline challenge. Grey bars; citalopram treatment, 48 hour washout, saline challenge. White bars, citalopram treatment, no washout, saline challenge. * P < 0.05 washout versus no washout.*

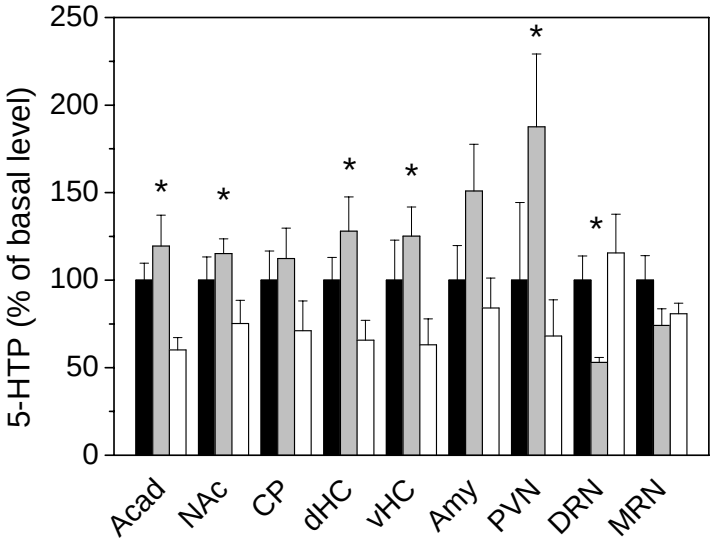


***Fig. 3.*** *Effect of washout on serotonin synthesis*
*Black bars; saline treatment, saline challenge. Grey bars; citalopram treatment, 48 hour washout, saline challenge. White bars, citalopram treatment, no washout, saline challenge. * P < 0.05 washout versus no washout.*

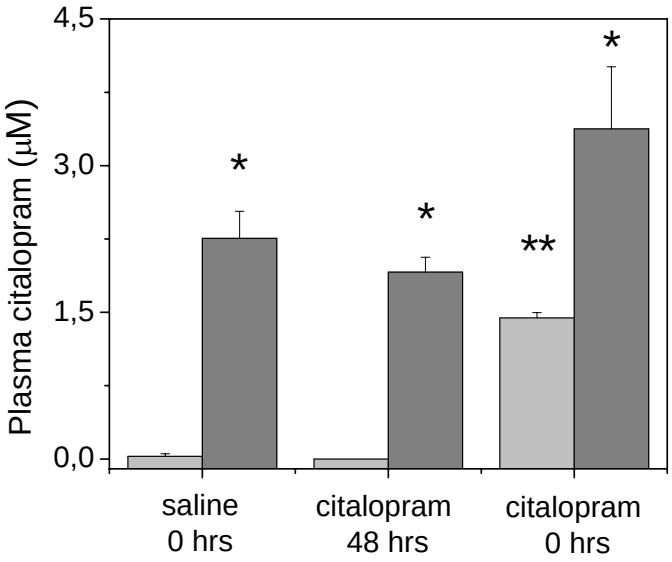


***Fig. 4.*** *Effect acute and chronic treatment on plasma levels of citalopram*
*Light grey bars; saline challenge. Dark grey bars; citalopram challenge.*
** P < 0.05 versus saline challenge, ** P < 0.05 versus other saline challenged groups. Saline 0 hrs = 14 day saline treatment; citalopram 48 hrs = 14 day citalopram treatment with 48 washout; citalopram 0 hrs = 14 day citalopram treatment with no washout.*

| ***treatment*** | *saline* | | *saline* | | *saline* | | *citalopram* | | *citalopram* | |
|---|---|---|---|---|---|---|---|---|---|---|
| ***washout*** | *0 hrs* | | *0 hrs* | | *0 hrs* | | *48 hrs* | | *0 hrs* | |
| ***challenge*** | *saline* | | *saline* | | *citalopram* | | *saline* | | *saline* | |
| **5-HT** | ***fmol*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** |
| Acad | 780,1 ± | 191,4 | 100,0 ± | 24,5 | 55,7 ± | 6,5 | 51,2 ± | 12,5 | 13,2 ± | 1,7 |
| Nac | 663,8 ± | 106,5 | 100,0 ± | 16,0 | 90,1 ± | 22,7 | 25,1 ± | 2,5 | 41,7 ± | 11,3 |
| CP | 370,6 ± | 51,2 | 100,0 ± | 13,8 | 51,9 ± | 12,9 | 37,5 ± | 9,5 | 52,7 ± | 12,7 |
| Amy | 2059,8 ± | 417,3 | 100,0 ± | 20,3 | 67,5 ± | 15,1 | 68,1 ± | 7,3 | 59,8 ± | 16,5 |
| dHC | 546,0 ± | 43,1 | 100,0 ± | 7,9 | 57,7 ± | 4,2 | 63,4 ± | 5,1 | 37,7 ± | 13,8 |
| PVN | 1084,1 ± | 121,0 | 100,0 ± | 11,2 | 61,7 ± | 3,9 | 55,1 ± | 1,1 | 23,8 ± | 9,1 |
| vHC | 1407,8 ± | 445,2 | 100,0 ± | 31,6 | 37,7 ± | 9,7 | 61,5 ± | 2,9 | 43,0 ± | 12,6 |
| DR | 1676,5 ± | 517,2 | 100,0 ± | 30,8 | 95,9 ± | 10,5 | 120,6 ± | 6,9 | 70,9 ± | 28,9 |
| MRN | 1328,5 ± | 245,2 | 100,0 ± | 18,5 | 92,2 ± | 0,6 | 66,2 ± | 7,9 | 41,5 ± | 16,4 |
| **HIAA** | ***fmol*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** |
| Acad | 483,7 ± | 93,1 | 100,0 ± | 19,3 | 61,4 ± | 12,3 | 80,5 ± | 17,9 | 18,3 ± | 5,2 |
| Nac | 666,6 ± | 145,7 | 100,0 ± | 21,9 | 49,5 ± | 8,0 | 48,6 ± | 6,0 | 31,8 ± | 4,1 |
| CP | 543,0 ± | 98,0 | 100,0 ± | 18,0 | 36,5 ± | 3,8 | 44,7 ± | 5,9 | 38,1 ± | 1,8 |
| Amy | 557,1 ± | 158,5 | 100,0 ± | 28,5 | 79,8 ± | 21,4 | 137,1 ± | 32,0 | 42,0 ± | 4,1 |
| dHC | 546,3 ± | 91,9 | 100,0 ± | 16,8 | 47,1 ± | 5,0 | 87,1 ± | 19,7 | 47,0 ± | 7,2 |
| PVN | 310,6 ± | 67,7 | 100,0 ± | 21,8 | 71,6 ± | 23,1 | 103,6 ± | 21,4 | 25,8 ± | 7,2 |
| vHC | 542,0 ± | 141,3 | 100,0 ± | 26,1 | 41,6 ± | 6,2 | 130,6 ± | 13,4 | 40,9 ± | 5,0 |
| DR | 1019,0 ± | 241,2 | 100,0 ± | 23,7 | 50,7 ± | 13,1 | 196,6 ± | 13,8 | 58,9 ± | 20,5 |
| MRN | 1072,0 ± | 245,4 | 100,0 ± | 22,9 | 45,9 ± | 13,9 | 118,3 ± | 31,7 | 55,2 ± | 11,6 |
| **Turnover** | ***ratio*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** |
| Acad | 0,765 ± | 0,07 | 100,0 ± | 9,34 | 74,7 ± | 10,6 | 171,6 ± | 20,4 | 109,4 ± | 24,2 |
| Nac | 1,163 ± | 0,11 | 100,0 ± | 9,24 | 52,6 ± | 6,8 | 168,1 ± | 17,6 | 66,3 ± | 14,7 |
| CP | 1,639 ± | 0,18 | 100,0 ± | 10,91 | 67,4 ± | 8,2 | 131,9 ± | 8,4 | 71,5 ± | 14,7 |
| Amy | 0,321 ± | 0,03 | 100,0 ± | 9,91 | 98,5 ± | 19,6 | 213,6 ± | 16,5 | 67,6 ± | 12,8 |
| dHC | 1,121 ± | 0,12 | 100,0 ± | 11,12 | 74,2 ± | 8,4 | 145,8 ± | 12,7 | 121,7 ± | 29,7 |
| PVN | 0,330 ± | 0,02 | 100,0 ± | 7,55 | 70,6 ± | 14,8 | 190,1 ± | 6,8 | 85,7 ± | 14,2 |
| vHC | 0,499 ± | 0,07 | 100,0 ± | 14,76 | 102,1 ± | 24,0 | 164,9 ± | 11,0 | 86,2 ± | 12,4 |
| DR | 0,550 ± | 0,05 | 100,0 ± | 9,09 | 83,2 ± | 9,9 | 182,5 ± | 13,2 | 121,2 ± | 21,7 |
| MRN | 0,949 ± | 0,14 | 100,0 ± | 15,02 | 63,2 ± | 1,8 | 141,4 ± | 37,7 | 139,4 ± | 19,6 |
| **5-HTP** | ***fmol*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** | ***%*** | ***SEM*** |
| Acad | 102,8 ± | 9,96 | 100,0 ± | 9,69 | 66,9 ± | 12,9 | 119,4 ± | 17,7 | 60,1 ± | 7,1 |
| Nac | 97,9 ± | 43,48 | 100,0 ± | 44,41 | 82,7 ± | 18,2 | 187,6 ± | 41,6 | 68,1 ± | 20,6 |
| CP | 176,4 ± | 29,35 | 100,0 ± | 16,64 | 110,1 ± | 24,5 | 112,4 ± | 17,3 | 71,1 ± | 17,1 |
| Amy | 83,8 ± | 10,90 | 100,0 ± | 13,01 | 64,2 ± | 16,0 | 128,0 ± | 19,6 | 65,7 ± | 11,4 |
| dHC | 195,7 ± | 44,74 | 100,0 ± | 22,86 | 62,6 ± | 10,8 | 125,2 ± | 16,7 | 63,1 ± | 14,8 |
| PVN | 218,9 ± | 43,22 | 100,0 ± | 19,75 | 93,5 ± | 6,0 | 150,9 ± | 26,6 | 84,1 ± | 17,0 |
| vHC | 111,7 ± | 14,82 | 100,0 ± | 13,27 | 85,7 ± | 12,7 | 115,2 ± | 8,4 | 75,2 ± | 13,3 |
| DR | 2178,4 ± | 301,20 | 100,0 ± | 13,83 | 83,8 ± | 10,2 | 53,1 ± | 2,7 | 115,6 ± | 22,1 |
| MRN | 1328,9 ± | 185,41 | 100,0 ± | 13,95 | 70,2 ± | 8,1 | 74,2 ± | 9,4 | 80,8 ± | 6,0 |

***Table 1.*** *Effect of acute and chronic treatment with citalopram in absence and presence of a washout period. Values of the control group (saline treated, saline challenge) are presented both in amount and % of basal level, values of all other groups are presented as % of control group (basal level).*

## 4. Discussion

The present study confirms the general observation that synthesis, release and metabolism of serotonin are all diminished in response to acute antidepressant treatment, but steadily revert to normal levels following chronic treatment. This reduced functionality of inhibitory feedback mechanisms is commonly explained by a gradual desensitization of the serotonergic autoreceptors. However, in contrast with these observations, our data indicate that intracellular serotonin remains decreased even upon chronic treatment, both after washout or in presence of citalopram. The amount of serotonin stored intracellular depends on both synthesis and reuptake of previously released serotonin. Theoretically, if synthesis cannot keep up with the conditions of chronic reuptake inhibition as induced during prolonged treatment with SSRIs, depletion of these stores could occur. Previous studies report restored (Esteban et al., 1999;Stenfors and Ross, 2002) or even increased levels of 5-HTP following chronic treatment (Moret and Briley, 1992), suggesting adaptation. However, in all cases a washout period was included which might have interfered. Like treatment itself, a certain period of drug absence after treatment could also induce pharmacological changes on the cellular level. During a washout period, resensitization or adaptation can take place, altering or even reversing the effects of chronic treatment (Neumaier et al., 1996). This is indeed confirmed by our own results, as both turnover and 5-HTP are increased after washout but showed opposite effects if no washout was included. In addition, storage was only further decreased upon treatment, which can be better explained by a simultaneous reduction in synthesis too. So arguably, the situation without washout, in presence of citalopram, more accurately depicts the neurochemical effects of the chronic treatment itself. Clinically, this is interesting too, as the pharmacological situation in presence of citalopram more closely resembles the clinical situation.

Introducing a washout out period could bear some similarity with the clinical effect known as rebound depression. When suddenly discontinuing antidepressant therapy, patients have been reported to relapse into a depressive state, suffering from an immediate reversal of all therapeutic effects. In the present study, this is resembled by the situation after a washout, which shows the effects of a sudden discontinuation of treatment rather than the effect of the treatment itself. This is most obviously seen in the amygdala, thalamus and forebrain regions. Interestingly, these areas are reported to have a high density of 5-$HT_{1B}$ receptors, which control serotonin release and synthesis. In contrast to 5-$HT_{1A}$ receptors, these receptors do not desensitize, so under contitions of increased extracellular serotonin, both synthesis and release are continuously inhibited as a result of 5-$HT_{1B}$ receptor activation. A fall in extracellular levels due to sudden treatment discontinuation will reverse this process. The enhanced levels of 5-HIAA/5-HT ratio and 5-HTP

accumulation seen after washout indeed point at an increase in release and synthesis, respectively. This process might very well explain the clinical effect known as rebound. Consequently, the present study provides the neurochemical evidence to gradually phase out antidepressant treatment in order to prevent rebound effects.

But almost as important as this finding, our observations also indicate that intracellular serotonin stores in the brain are slowly depleted during chronic treatment. Although it sounds rather alarming, the clinical interpretation of these results remains unclear. If the therapeutic effect of antidepressants should be assigned to increased levels of extracellular 5-HT, it seems to be a paradox that the total amount of brain serotonin gets depleted, which is a rather unwanted side-effect in this case. On the other hand, it might be that the neurochemical basis of the therapeutic effect is not restricted to the extracellular level. By adjusting both metabolism and synthesis, the resetting of the serotonergic system as a whole could also attribute to therapeutic success.

From the present study it can be concluded that, although a washout period after chronic treatment is generally thought to be essential in order to prevent pharmacological interference, it is the washout period itself that interferes with the effect of the chronic treatment. The reversal of effects observed after a washout might refer to the rebound effect seen in the clinic and supports a gradual discontinuation to prevent a relapse.

It should also be noted that as a result of continuous reuptake inhibition and a decreased synthesis rate, intracellular serotonin stores are steadily depleted upon treatment. Further research should reveal how this affects the therapeutic effect of SSRIs as it is still unknown how to interprete this on a clinical level.

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