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# Searching for neural and behavioral parameters that predict anti-aggressive effects of chronic SSRI treatment in rats

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## HIGHLIGHTS

- In an outbred strain of Long Evans rats aggressive behavior varied from 0 to 37%.
- Aggressive behavior after chronic citalopram treatment showed increased variance.
- Anxiety and cue responsivity parameters did not predict the effect of citalopram.
- Regional brain 5-$HT_{1A}$ receptor densities did not predict the treatment effect.

## ARTICLE INFO

*Keywords:*
SSRI
Aggression
Resident-intruder test
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## ABSTRACT

*Rationale:* Only a subset of impulsive aggressive patients benefits from selective serotonin reuptake inhibitor (SSRI) treatment, confirming contradictory results about the association between serotonin (5-hydroxytryptamine, 5-HT) and aggression. This shows the need to define behavioral characteristics within this subgroup to move towards individualized pharmacological treatment of impulsive aggression.
*Methods:* Here we submitted an outbred strain of Long Evans rats to a crossover design treatment regimen with the SSRI citalopram, to test its anti-aggressive effect. Behavioral characteristics were baseline aggression, anxiety parameters as measured in the elevated plus maze and open field and cue responsivity as indicated by sign vs. goal tracking behavior. 5-$HT_{1A}$ receptor densities as measured by *ex vivo* [$^{18}$F]MPPF binding were determined in the dorsal raphe nucleus, dentate gyrus, orbitofrontal cortex, infralimbic cortex and prelimbic cortex, because of the receptors' involvement in the therapeutic delay of SSRIs and aggression.
*Results:* We found statistically significant increased variance in aggressive behavior after citalopram treatment. However, none of the selected parameters predicted the citalopram treatment effect.
*Conclusion:* Since aggression after citalopram treatment decreased in a subgroup of animals and increased in the other, future research should focus on other possible predictors to support treatment strategies in aggressive patients.

## 1. Introduction

Treatment of impulsive aggression is dependent on a thorough understanding about the neurobiological mechanisms underlying aggressive behavior and the environmental triggers that cause it. Currently, a wealth of clinical studies implies that there is an inverse relationship between aggression and brain serotonin (5-hydroxytryptamine, 5-HT) (Montoya et al., 2012; Olivier, 2004; Rosell and Siever, 2015). Indirect support comes from decreased levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in

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cerebrospinal fluid of aggressive individuals (Brown et al., 1979; Linnoila et al., 1983; see (Duke et al., 2013) for review) and manipulation of the serotonergic system affecting aggressive behavior (Coccaro, 1989) (Bjork et al., 1999; Chamberlain et al., 1987). In addition, decreased serotonin levels were measured directly in brain tissue of aggressive mice (Giacalone et al., 1968; Haney et al., 1990; Welch and Welch, 1968). Hence, these findings have pushed pharmacotherapeutic research strategies towards enhancing serotonergic neurotransmission in order to compensate for this deficiency and decrease aggressive behavior with selective serotonin reuptake inhibitors (SSRIs).

The anti-aggressive effect of SSRIs has been shown in numerous studies in subjects suffering from schizophrenia, bipolar disorder and major depression (Butler et al., 2010; Coccaro and Kavoussi, 1997; Fava et al., 2000; Rinne et al., 2002; Salzman et al., 1995; Vartiainen et al., 1995). Furthermore, in intermittent explosive disorder (IED), a specifically characterized disorder of impulsive aggression, SSRI treatment has been shown to decrease aggression, although only 29% reaches full and 46% partial remission as reflected by the IED criteria (Coccaro, 2012; Coccaro et al., 2009). There even are incidental reported findings of increased aggression and violent suicidal thoughts after SSRI treatment (Fergusson et al., 2005; Moore et al., 2010; Spigset, 1999; Troisi et al., 1995). These findings challenge the inverse relationship between serotonin and aggression and imply that aggression is not necessarily attributed to low serotonin levels.

Animal research strengthens the contradicting results of SSRIs on aggressiveness (see (Carrillo et al., 2009) for review). In line with literature on the inverse relationship between serotonin and aggression, genetically modified serotonin reuptake transporter (SERT) knockout mice and rats show decreased aggression as a result of increased extracellular serotonin levels (Holmes et al., 2002; Homberg et al., 2007). Furthermore, blocking the SERT chronically with SSRIs decreased aggression in several animal models (Caldwell and Miczek, 2008; Perreault et al., 2003; Pinna et al., 2003; Sànchez, 1997). However, some of these effects are not behaviorally-specific as they result in a more general sedation, leading to decreased social interest and inactivity (Olivier et al., 1995; Veening et al., 2005). Some studies even showed an increase in aggressive behavior after chronic SSRI treatment (Carlini and Lindsey, 1982; Johns et al., 2005; Mitchell and Redfern, 2005; Ossowska et al., 2004; Taravosh-Lahn et al., 2006). These contradicting results indicate again that the association between serotonin and aggression is not as straight-forward as generally thought.

The functional complexity and homeostatic feedback control of the serotonergic system is further reflected by the delayed therapeutic effect of SSRIs, which is suggested to be mediated by adaptive neuronal changes. In particular, the 5-$HT_{1A}$ receptor is thought to be implicated in this process as transgenic mice with high levels of the 5-$HT_{1A}$ autoreceptors, do not show a behavioral response after fluoxetine treatment in the novelty suppressed feeding test (Richardson-jones et al., 2010). This could indicate that high 5-$HT_{1A}$ receptor densities in the dorsal raphe nucleus (DRN) predict a low or absent anti-aggressive SSRI effect. Furthermore, a causal role has been suggested for these 5-$HT_{1A}$ autoreceptors in tonically suppressing brain 5-HT neurotransmission activity and predisposing to high and excessive aggressive responding (Caramaschi et al., 2007; de Boer et al., 2001; van der Vegt et al., 2001). And adult transgenic mice with conditional overexpression of somatodendritic autoreceptors chronically suppress 5-HT neural firing and phenotypically exhibit heightened aggressiveness (Audero et al., 2013).

Another requirement for therapeutic SSRI action seems to be hippocampal neurogenesis, as it has been shown that chronic SSRI treatment increases neurogenesis in the dentate gyrus dependent on 5-$HT_{1A}$ receptor activation (Santarelli, 2003) (Banasr et al., 2004). Low 5-$HT_{1A}$ receptor levels in the dentate gyrus would then impede anti-aggressive SSRI effects. Furthermore, reduced impulsive aggression after SSRI treatment has been associated with an increased relative metabolic rate in the orbitofrontal cortex (New et al., 2004). Furthermore, aggressive behavior is associated with decreased postsynaptic 5-$HT_{1A}$ receptor mRNA levels (Popova et al., 2005) and decreased 5-$HT_{1A}$ binding potential as measured using PET in cortical areas (Witte et al., 2009). Altogether, these data propose the idea that decreased expression of the 5-$HT_{1A}$ receptor in (sub)cortical areas can negatively affect the anti-aggressive SSRI response.

In addition to 5-$HT_{1A}$ receptor density, behavioral parameters may predict anti-aggressive SSRI responsivity. In naked mole-rats, differential effects of chronic fluoxetine on aggressive behavior were found to be dependent upon the social status (dominant-subordinate) of the animals (Mongillo et al., 2014). Furthermore, Phan et al. showed that high neuroticism and harm avoidance scores predicted a larger decrease in aggression level after SSRI treatment (Phan et al., 2011). The disposition to react anxious is considered as one of the facets contained in the personality trait of neuroticism, and shows an inverse relation with aggression (Veenema et al., 2007). Furthermore, increased aggressive behavior mediated by decreased serotonergic activation was associated with, increased risk-taking behavior in the elevated plus maze and open field, in a line of rats selectively bred for high locomotor response in a novel environment (bHRs) (Clinton et al., 2012; Kerman et al., 2011; Stead et al., 2006). These animals also displayed deficits in withholding impulsive action and attribute an excessive incentive salience to rewarding cues in a sign vs goal task (Flagel et al., 2010). It seems that anxiety and cue responsivity are closely associated with serotonergic deficits and increased aggression, and may be predictive of the anti-aggressive effect of SSRIs.

In this study we aimed to identify distinctive neural and behavioral parameters that predict anti-aggressive responsiveness to SSRI treatment. Aggressive behavior was measured in male Long Evans rats in a resident-intruder test after chronic citalopram treatment by osmotic pumps using a within-subject crossover design. We investigated if anti-aggressive SSRI responsivity was linked to 5-$HT_{1A}$ receptor density (measured by autoradiography), and the behavioral parameters baseline aggression, anxiety (open field (Simon et al., 1994) and elevated plus maze tests (Pellow et al., 1985) and cue responsivity (sign vs goal task (Flagel et al., 2008)). Considering the available literature on the link between aggression and the serotonergic system and these behavioral parameters, it is expected that these parameters might have predictive value in the anti-aggressive potential of SSRIs. This would potentially contribute to a more effective personalized treatment strategy for impulsive aggressive patients.

## 2. Materials and methods

### 2.1. Animals

Adult Long Evans male rats (n = 35) were used as residents and obtained from Charles River (n = 18) (Den Bosch, The Netherlands) and Envigo (n = 17) (Horst, The Netherlands). Testing started at the age of postnatal day 95–105, with animals weighing 445 ± 11 g. Adult Wistar male rats (n = 36) were used as intruders and obtained from Charles River (n = 18) (Den Bosch, The Netherlands) and Envigo (n = 18) (Horst, The Netherlands). At the start of the experiment these animals were at the age of postnatal day 90, weighing 368 ± 5 g. Adult Long Evans female rats were used as companions for the intruders and obtained from Envigo (n = 18) (Horst, The Netherlands). All animals were housed in temperature- (21 ± 1 °C) and humidity-controlled (45–60% relative humidity) rooms, with a 12 h reversed light-dark cycle (lights on at 8:00 p.m.). Standard housing conditions were social, with two Long Evans females per type III open cage and four Wistar males per type IV open cage. Long Evans males were housed individually in type III open cages due to the risk of excessive aggression in the home cage. For all cages, sawdust bedding and cage enrichment were provided, and the animals had *ad libitum* access to water and rodent chow (V1534, Sniff, long-cut pellet, Bio Services, Uden, The Netherlands).

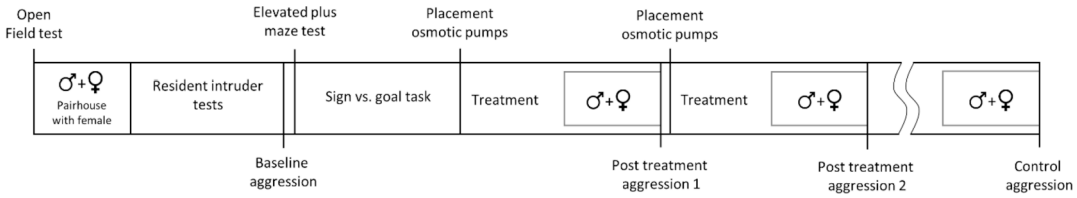


**Fig. 1.** Timeline of all experiments in eighteen weeks. All 35 Long Evans males performed all experiments, but only twenty were selected for the cross over treatment design. In total four aggression measurements were performed: baseline aggression, post treatment aggression 1, post treatment aggression 2 and finally a control aggression measurement 40 days after post treatment 2. All aggressive interactions were preceded by 7 days of housing together with a female rat. Both treatment periods lasted 14 days, with the aggressive interaction on the 15th day.

All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, and all efforts were made to minimize animal suffering and to reduce the number of animals used according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

### 2.2. Timeline

Experiments were performed in all 35 Long Evans males over a time span of eighteen weeks (Fig. 1). First, an open field test was performed, followed by the assessment of baseline aggression in the resident-intruder test. To further characterize the animals, an elevated plus maze and sign vs. goal tracking test were performed. After the baseline characterization, ten high aggressive animals per batch were selected for selective serotonin reuptake inhibitor (SSRI) treatment, ending up with a total of twenty animals being treated. These animals were treated for two weeks by continuous subcutaneous administration via osmotic pumps and received both SSRI and saline treatment in a cross-over design. Aggression levels were measured after both treatment periods using the resident-intruder test. After 40 days, all animals performed a final neutral resident-intruder test and were sacrificed two days later. So aggression levels were measured four times in total for all 35 animals, independent of selection for treatment. All behavioral set ups are described below.

### 2.3. Behavioral assessments

#### 2.3.1. Open field test

The experimental arena was made of polyvinylchloride (50 × 50 × 50 cm; 7 lux center). A square of 10 × 10 cm in the middle of the box was designated as the center. Rats were put in the corner of the arena facing the wall, and allowed to freely move throughout the arena for the next 5 min. Behavior was registered automatically by Ethovision 9.0 (Noldus, Wageningen, the Netherlands). Behaviors registered included total distance moved, frequency entering and time spent in the center and border areas near the walls. Data are expressed as a percentage of time spent in center (center time/(center time + border time) x 100) and percentage of time spent in border (border time/(center time + border time) x 100).

#### 2.3.2. Elevated plus maze test

The maze was made of polyvinylchloride and elevated to a height of 50 cm with two open (50 × 10 cm; 8 lux) and two enclosed (50 × 10 × 40 cm; 2 lux) arms. Rats were put in the center facing one of the open arms, and allowed to freely explore the maze for the next 5 min. Behavior was registered automatically by Ethovision 9.0 (Noldus, Wageningen, the Netherlands). Behaviors registered included total distance moved, frequency entering and time spent in the center, closed and open arms. Data are expressed as a percentage of total time spent in the open arms vs. the closed arms ((open arm time)/(open arm time + closed arm time) x 100) and percentage of total open arm vs. closed arm entries ((open arm entries/(open arm entries + closed arm entries) x 100).

#### 2.3.3. Sign vs. goal task

The tests were performed in operant conditioning chambers ((24 × 21 × 29 cm); Med Associates, St. Albans, VT, USA). The chambers were equipped with two illuminated retractable levers on each side of a food magazine for 45 mg sucrose pellet delivery. Here, an adapted version of the sign-versus-goal-tracking paradigm was used as described by (Flagel et al., 2008). In brief, during three training sessions animals received a total of 50 sucrose pellets per session on a random interval schedule (30 s mean inter trial interval). The purpose of this training was to familiarize the rats with pellet retrieval. Subsequently, 15 acquisition sessions were performed during which sign-versus-goal-tracking behavior was examined. The same interval schedule as described above was used, but now prior to each pellet delivery, one lever was extended for 8 s (CS + lever). The CS + lever location was counterbalanced within the group, and the remaining lever served as a control as it was explicitly unpaired with sucrose pellet delivery (CS− lever) and also presented for 8 s on a random 30s interval schedule. In each session, the rats were given 30 CS+ and 30 CS− trials in randomized order. All lever presses were recorded, but did not have any consequences, as sucrose pellet delivery was independent of the rats' behavior. Rats were tested in daily sessions for eighteen days. Total number of lever presses and magazine entries during CS+ and CS− trials and ITIs were scored automatically by MedState Notation using MED-PC for Windows.

#### 2.3.4. Resident-intruder test

Here an adapted version of the resident-intruder paradigm was used as described by (Koolhaas et al., 2013). Long Evans male residents were housed together with companion Long Evans females in a PhenoTyper 4500 cage (45 × 45 cm) (Noldus, Wageningen, the Netherlands) to allow sufficient space for the full range of aggressive behaviors during the aggressive interactions. Females were sterilized by ligation of the oviducts, to keep them hormonally intact and receptive for the males. Bedding material in PhenoTyper cages was not cleaned during the experiment to ensure an undisturbed territory. One week before the start of the experiment, residents and females were housed together in the PhenoTyper 4500 cages to increase territorial behavior and prevent social isolation. These pairs stayed the same over all experiments, to reduce stress of an unfamiliar partner.

On every testing day, 1 h before the interaction females were removed, and directly before testing, cage enrichment, food and water were removed. Next, an unfamiliar Wistar male was introduced as intruder in the cage. For the first eight test days, the resident-intruder interactions were stopped after the first resident attack or after 10 min. In that way, a stable baseline aggression level was ensured. After every interaction, females and cage accessories were returned and animals were left undisturbed until the next test day. The full range of aggressive behaviors was measured at the ninth test day (baseline aggression), after both treatment periods (effect of SSRI and vehicle on

aggression; post treatment aggression 1 and post treatment aggression 2) and 40 days after the final treatment period (control). All these test days started with the same protocol, but lasted for a maximum of 20 min. After the first resident attack or after the first 10 min, the animals were allowed 10 min of aggressive interaction, during which a range of offensive behaviors was scored offline using Observer XT12 (Noldus, Wageningen, the Netherlands): chase, clinch attack, dragging, keep down, lateral threat, move towards aggressive and upright posture aggressive. The sum of these parameters resulted in a percentage of time spent on total offensive behavior per 10 min of interaction per animal. After these full interactions, males were again housed individually and females socially in pairs in type III open cages.

#### 2.3.5. SSRI treatment

From both batches the ten most aggressive animals were selected for SSRI treatment after a first evaluation of total offensive behavior. Videos were evaluated in more detail after finishing all experiments, so comparison between time points was feasible. These twenty selected animals received two weeks of SSRI treatment and two weeks of vehicle in a randomized order. The SSRI citalopram hydrobromide (Sigma-Aldrich GmbH, Sternheim, Germany) was selected for its high selectivity for the serotonin reuptake site (Sánchez and Hyttel, 1994) and because it minimally affects the uptake of noradrenaline and dopamine and binding to dopamine, noradrenaline, serotonin, histamine, gamma aminobutyric acid (GABA), acetylcholine, and morphine receptors (Hyttel, 1982) (Richelson and Pfenning, 1984). Despite the fact that citalopram is a racemic mixture of an S (+)- and R (−)-enantiomer, in which R-citalopram seems to counteract the S-citalopram induced increase in extracellular serotonin levels, citalopram does increase extracellular serotonin levels (Ceglia et al., 2004; Mørk et al., 2003; Sánchez et al., 2003). Chronic administration for 14 days was realized by the use of osmotic pumps (2ML2 Alzet, Durect Corporation, USA; 5 µl/h for 14 days). Pumps (2 ml) were filled with either saline or citalopram hydro bromide dissolved in saline (20 mg/kg bodyweight/day), and placed subcutaneously under isoflurane anesthesia (2.5%, 400 ml/min $N_2O$, 600 ml/min $O_2$). Two days post-surgery animals were weighed and given 0.1 ml/100 g body weight subcutaneous injections of carprofen (Rimadyl; 50 mg/ml) and enrofloxacin (Baytril; 25 mg/ml). On the 15th day after treatment, a resident-intruder test was performed and the next day osmotic pumps were replaced under isoflurane anesthesia by new pumps, or removed after the second treatment period. No residual volumes of citalopram were found in the pumps after removal.

### 2.4. Autoradiography

#### 2.4.1. Tissue preparation

Animals were sacrificed by decapitation, brains were removed, rapidly frozen at dry ice and stored at −80 °C until further use. Coronal slices of 20 µm were taken at a cryostat at −20 °C from the following brain areas: ventral orbital, infralimbic and prelimbic cortex (bregma 3.72 to 3.00 mm), dentate gyrus (bregma −2.40 to −3.00 mm) and dorsal raphe nucleus (bregma −7.32 to −7.56 mm). Localization of these brain regions was based on a rat brain atlas (Paxinos and Watson, 2004). Slices were thaw-mounted on superfrost microscope slides (Menzel Glaser, Thermo Scientific, Braunschweig, Germany) and stored in boxes at −80 °C until further use.

#### 2.4.2. [$^{18}$F]MPPF binding

4-(2′-methoxyphenyl)-1-[2'-(N-2″-pyridinyl)-p-[$^{18}$F]fluorobenzamido]ethylpiperazine, ([$^{18}$F]MPPF) was obtained from RTM (Radboud Translational Medicine, Nijmegen, The Netherlands). For the autoradiography, slides were taken from storage and washed twice with 0.1 M phosphate buffer containing 1% bovine serum albumin (Sigma-Aldrich). Sections were incubated with 1 ml of this buffer containing 400 kBq/ml [$^{18}$F]MPPF (molar activity 9.1 GBq/µmol) for 1 h at room temperature. After this incubation period slides were washed three times with phosphate buffer and three times with demi-water. Sections were allowed to dry in the fumehood and put in cassettes afterwards, which were exposed to phosphor imaging plates overnight. These plates were developed using the Typhoon FLA 7000 Phosphor Imager. ImageJ software was used to measure mean gray values per selected region of interest, which are proportional for binding of [$^{18}$F]MPPF.

#### 2.4.3. Statistical analysis

All data were checked for normality using the Shapiro–Wilkinson test and homogeneity of variance with Levene's test. Paired t-tests were applied to compare aggression levels over different time points within animals, compare anxiety parameters within elevated plus maze and open field, and compare aggression levels after citalopram compared to saline treatment. For the sign vs. goal task, a repeated measures analysis of variance (ANOVA) was performed using the 15 sessions as dependent variable and response type (positive responders, negative responders) as factor. A one-way ANOVA was used to compare positive and negative responders on selected parameters. When sphericity assumption was violated, results were corrected by the Greenhouse Geisser procedure. Pearson correlation analysis was performed to correlate baseline aggression with aggression at the final resident-intruder interaction, and treatment effect with selected parameters. All results are expressed as average values ± SEM (standard error of the mean), unless stated differently. Level of significance was set at $p < 0.05$ two sided. All statistical analyses were performed using SPSS version 23 (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Baseline aggression

Before treatment, aggression levels of rats (n = 35) were determined in a resident-intruder test. Percentage of time spent on aggressive behavior in the 10 min confrontation with the intruder varied from 0 to 41% at first evaluation (Fig. 2), allowing to classify these Long Evans rats in the low and medium aggression scale according to (Koolhaas et al., 2013). The final resident-intruder test was performed 40 days after the final treatment period to check stability over time in all 35 rats. Here aggressive behavior varied from 0 to 33% and was decreased compared to final baseline evaluation (0–37%) ($t(34) = 5.439$, $p < 0.001$). However, aggressive behavior at baseline and at the final interaction correlated positively with each other ($r = 0.573$, $p < 0.001$), indicating an overall decrease in aggressive behavior. Over all four time points (i.e. baseline, post treatment 1, post treatment 2 and control) aggressive behavior reduced significantly ($F(2.399, 38) = 6.533$, $p = 0.001$).

### 3.2. Behavioral parameters

All 35 animals were subjected to the open field test, elevated plus maze and sign vs. goal task. Animals spent more time in the border area of the open field (88 ± 1%) compared to the center area (12 ± 1%) ($t(34) = -30.990$, $p < 0.001$). Comparable results were found for the elevated plus maze data, where animals spent more time in the closed arms (63 ± 3%) compared to the open arms (37 ± 3%) ($t(34) = -4.205$, $p < 0.001$). In the sign vs. goal task lever presses and magazine entries increased over time ($F(3.307) = 8.038$, $p < 0.001$; $F(3.566) = 2.940$, $p = 0.03$), but remained stable over the final five sessions ($F(4) = 0.123$, $p = 0.98$; $F(3.213) = 1.669$, $p = 0.17$). None of these parameters showed a correlation with baseline aggression (data not shown).

### 3.3. Citalopram treatment and aggression

The ten most aggressive animals per batch (twenty animals in total) were selected for treatment (Fig. 2) in a crossover design, during which

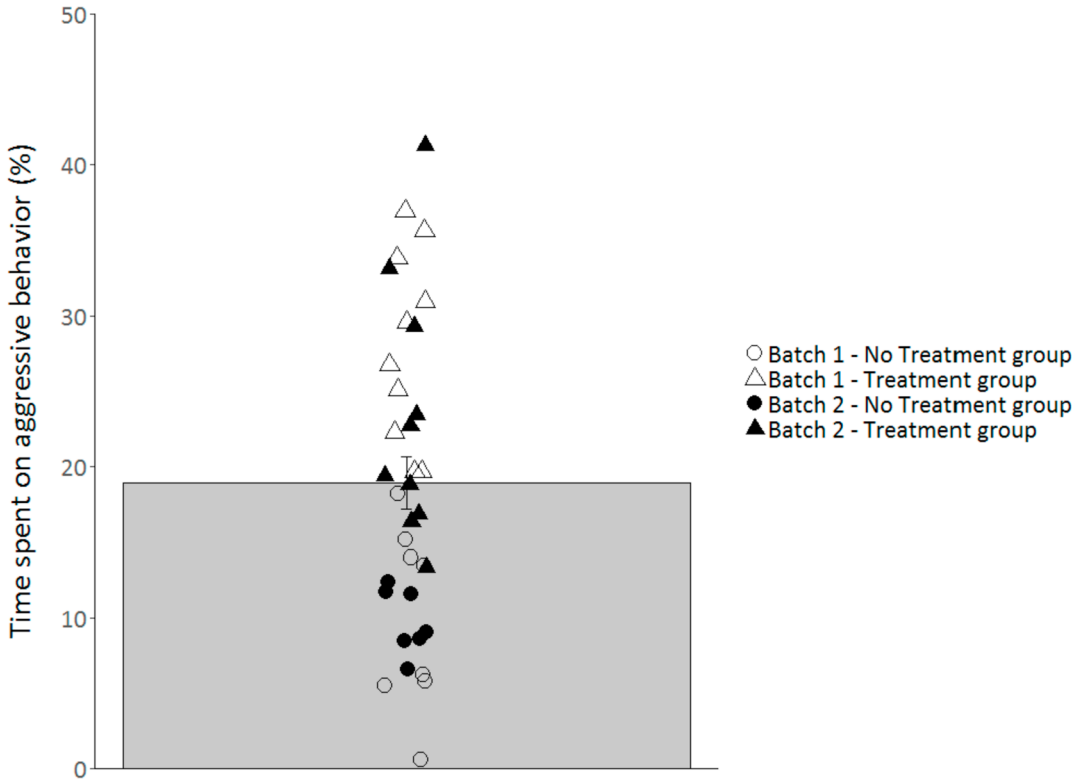


**Fig. 2.** Baseline individual levels of time spent on aggression in a 10-min interaction after first evaluation (n = 35) used to select animals for treatment presented in a scatter plot summarized as mean of the whole group ( ± SEM). Animals represented by a triangle were selected for treatment, whereas animals represented by a circle were not. Open shapes indicate animals from the first batch, whereas black filled shapes indicate animals from the second batch.

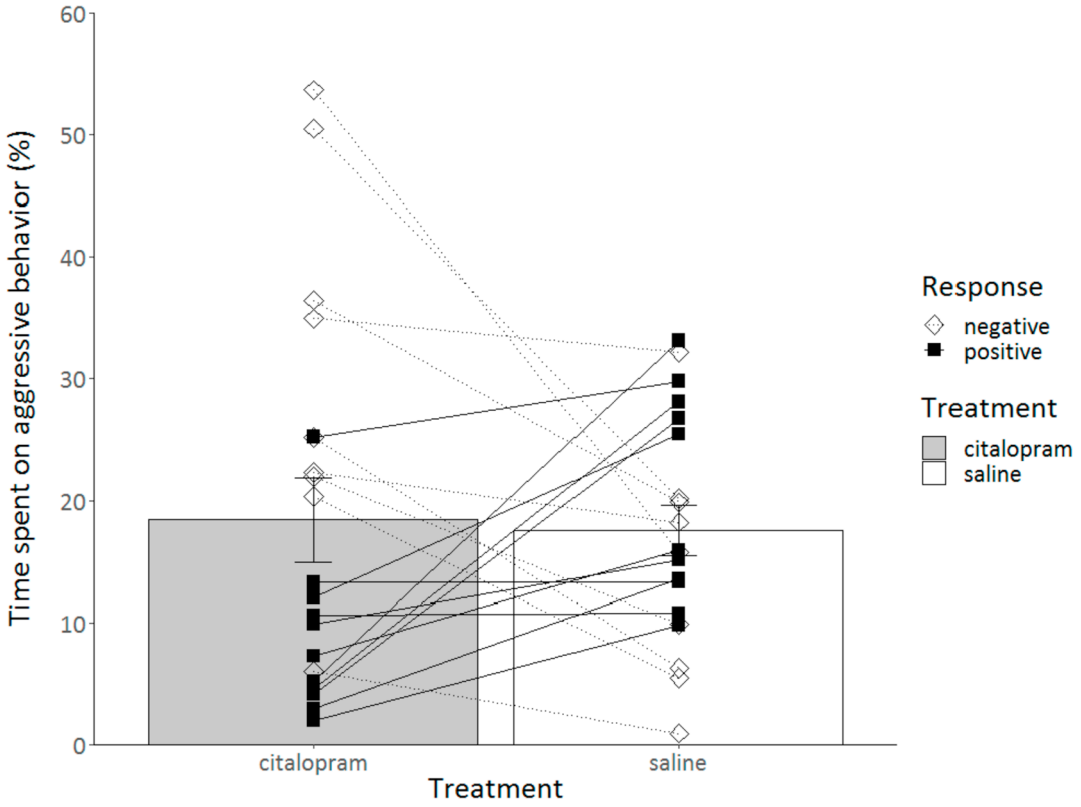


**Fig. 3.** Individual levels of time spent on aggression in a 10-min interaction after saline and citalopram treatment represented as mean ( ± SEM) and individual data points (n = 20). Animals showing a decrease in aggressive behavior after citalopram treatment are presented as positive responders (black square, continuous line; n = 11), whereas animals showing an increase in aggressive behavior after citalopram treatment are presented as negative responders (open diamond, dotted line; n = 9).

**Table 1**
Statistical results for correlation analysis with behavioral parameters and treatment effect (=aggression after citalopram – aggression after saline).

| Baseline aggression | |
|---|---|
| Total offensive behavior | r = 0.065, p = 0.79 |
| **Open Field test** | |
| Percentage time spent in center | r = 0.097, p = 0.69 |
| **Elevated plus maze test** | |
| Percentage time spent in open arm | r = 0.082, p = 0.73 |
| **Sign versus goal task (over last five sessions)** | |
| Mean number of lever presses | r = 0.065, p = 0.78 |
| Mean number of magazine entries | r = −0.039, p = 0.87 |

they received both citalopram and saline treatment in a random order. Aggression was determined after both treatment periods, resulting in two post-treatment aggression measurements. After grouping results per treatment, it was shown that overall levels of aggression did not significantly change after citalopram treatment compared to saline (t (19) = 0.232, p = 0.82), but Levene's test indicated unequal variances (F (1,38) = 4.704, p = 0.04). The variance in the citalopram treated animals was found to be increased compared to the saline treated group (Fig. 3). While nine animals showed an increase in aggressive behavior after citalopram treatment (negative responders; open diamond, dotted line), eleven animals showed a decrease in aggression after citalopram treatment (positive responders; black square, solid line).

No changes in aggression over time were observed from baseline measurement to post-treatment session one and two without taking treatment into account (F (1.478,38) = 2.351, p = 0.13). Aggression between subjects after citalopram and saline treatment did not differ for batch one (t (9) = 0.398, p = 0.70) and batch two (t (9) = -0.310, p = 0.76). When considering aggression for both batches together but per treatment period, behavior did also not differ between citalopram and saline treatment after treatment period one (F (1,18) = 0.333, p = 0.57) and treatment period two (F (1,18) = 0.268, p = 0.61). These parameters indicate that there was no effect of time, batch or treatment order on aggressive behavior.

### 3.4. Association between behavioral parameters and treatment effect

To determine whether behavioral parameters predict citalopram responsivity, we calculated the treatment effect by subtracting the level of aggression after saline treatment from the level of aggression after citalopram treatment. Behavioral parameters in the baseline resident intruder test, open field test, elevated plus maze test and sign vs. goal task did not correlate with the treatment effect (Table 1). Based on aggressive behavior after citalopram compared to saline treatment, we identified two response types: a group of negative responders (increased aggression) and a group of positive responders (decreased aggression) (Fig. 3). Total offensive behavior in the baseline resident intruder test did not differ between positive and negative responders (data not shown). Furthermore, these groups did not show any differences in total distance moved or percentage of time spent in the open arms of the elevated plus maze and the center of the open field (data not shown). In the sign versus goal tracking experiment lever presses during CS + increased over 15 sessions for both groups, indicating a sign tracking preference for all animals (Table 2). No other session, response type (e.g. positive or negative response) or session x response type interactions were observed here (see Table 2).

### 3.5. Association between 5-HT$_{1A}$ receptor density and treatment effect

In line with earlier publications, increased [$^{18}$F]MPPF binding compared to background could be observed in expected regions such as dentate gyrus, dorsal and medial raphe nucleus (Fig. 4).

Percentage of time spent in the open arm of the elevated plus maze and the center of the open field were correlated with 5-HT$_{1A}$ receptor density in the regions of interest. Prelimbic, infralimbic and orbitofrontal cortical 5-HT$_{1A}$ receptor densities correlated negatively with percentage of time spent in the center of the open field (Table 3). Furthermore, a positive correlation between 5-HT$_{1A}$ receptor density in the dorsal raphe nucleus with percentage of time spent in the center of the open field was found (Table 3). However, 5-HT$_{1A}$ receptor densities in dorsal raphe nucleus, orbitofrontal cortex, infralimbic cortex, prelimbic cortex and dentate gyrus did not correlate with the citalopram treatment effect (Table 4).

## 4. Discussion

Here we show that chronic treatment with the SSRI citalopram has a dual effect on aggressiveness as it both increased and decreased aggressive behavior in a group of male Long Evans rats. Hence, these qualitatively opposite treatment effects in different individuals resulted in no significant citalopram effect on aggressiveness at group level.

**Table 2**
Statistical results for sign vs goal task, showing effects of response type (e.g. positive or negative responders), session and the interaction between these two variables. Significant results are indicated by *.

| Dependent variable | Response type | Session | Response type x Session |
|---|---|---|---|
| Lever press during CS+ | F (1,18) = 0.147, p = 0.71 | F (3.354,60.365) = 4.341, p = 0.006 * | F (3.354,60.365) = 0.562, p = 0.66 |
| Magazine entry during CS+ | F (1,18) = 0.005, p = 0.95 | F (2.904,52.277) = 1.379, p = 0.26 | F (2.904,52.277) = 1.080, p = 0.36 |

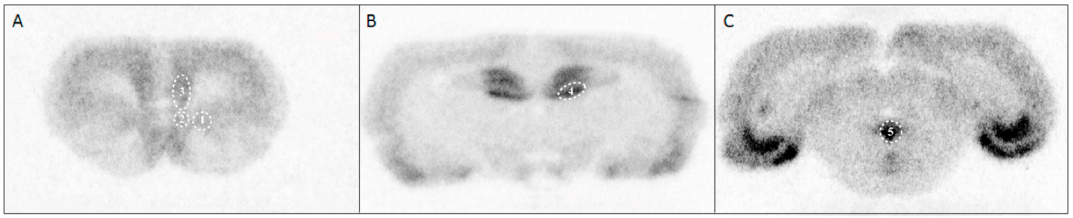


**Fig. 4.** Autoradiograms of [$^{18}$F]MPPF binding to coronal sections of A) ventral orbital[1], infralimbic[2] and prelimbic cortex[3], B) dentate gyrus[4] and C) dorsal raphe nucleus[5].

**Table 3**
Statistical results for correlation analysis with 5-$HT_{1A}$ receptor densities as measured by [$^{18}$F]MPPF autoradiography in regions of interest with duration in the open arms of the elevated plus maze and center of the open field. Significant results are indicated by *.

| Region of interest | Percentage open arm time (elevated plus maze) | Duration center (open field) |
|---|---|---|
| Dorsal raphe nucleus | r = 0.133, p = 0.58 | r = 0.491, p = 0.03 * |
| Orbitofrontal cortex | r = 0.146, p = 0.60 | r = −0.523, p = 0.05 * |
| Infralimbic cortex | r = 0.178, p = 0.50 | r = −0.505, p = 0.04 * |
| Prelimbic cortex | r = 0.129, p = 0.62 | r = −0.691, p = 0.002 * |
| Dentate gyrus | r = 0.009, p = 0.97 | r = 0.150, p = 0.53 |

**Table 4**
Statistical results for correlation analysis with 5-$HT_{1A}$ receptor densities as measured by [$^{18}$F]MPPF autoradiography in regions of interest with treatment effect (=aggression after citalopram – aggression after saline).

| Region of interest | Treatment effect |
|---|---|
| Dorsal raphe nucleus | r = −0.121, p = 0.61 |
| Orbitofrontal cortex | r = −0.291, p = 0.29 |
| Infralimbic cortex | r = −0.323, p = 0.21 |
| Prelimbic cortex | r = −0.122, p = 0.64 |
| Dentate gyrus | r = −0.026, p = 0.91 |

Eleven out of twenty animals showed a decrease in aggressive behavior after citalopram treatment (i.e. positive responders), whereas nine animals showed an increase in aggression after treatment (i.e. negative responders). Variability in aggressive behavior after citalopram was significantly larger compared to that after saline treatment, which indicates a relevant effect of the SSRI. This matches the contradicting literature on the anti-aggressive effects of SSRIs, in which facilitatory, null and inhibitory effects are found (Carrillo et al., 2009). In the current study, behavioral parameters reflecting baseline aggression, anxiety and cue responsivity did not predict whether citalopram had an aggressive or anti-aggressive effect. Cue responsivity did not differ between the groups of positive and negative responders as all animals showed a comparable increase in lever responses over sessions in the sign vs. goal task. Furthermore, 5-$HT_{1A}$ receptor binding in relevant brain regions did not correlate with the outcome of SSRI treatment. However, the increased variability in aggression after citalopram treatment suggests a characteristic distinction between positive and negative responders. Our findings do not preclude that other factors may be predictive of the anti-aggressive effect of citalopram.

An outbred strain of Long Evans rats was used to observe a more natural variation in offensive aggressiveness in the resident-intruder test. No surgical or pharmacological manipulations to induce aggression were needed, which increases the validity of the model and makes it more straightforward to interpret results (Miczek et al., 2013). A disadvantage of this approach is that this laboratory strain of animals only spent a maximum of 37% of their time on offensive behavior, which is considered as a medium aggression level when compared with high aggressive animals from a feral strain showing offensive behavior for more than 55% of the time in a resident-intruder paradigm (Koolhaas et al., 2013). However, the variability of 9–37% in the treated group of animals still gives room for pro- or anti-aggressive effects after SSRI treatment. By repetition of successful resident-intruder interactions at baseline, a winner effect was introduced which established a stable plateau of offensive aggressive behavior. This baseline level of aggression has been associated before with the treatment effect of several serotonergic compounds. Chronic administration of 5-$HT_{1A}$ receptor agonists (S-15535 and alnespirone), the SSRI fluoxetine or the serotonin releasing agent MDMA, decreased aggressive behavior in high-aggressive animals, whereas it increased aggressiveness in low aggressive animals (de Boer et al., 2017; Wallinga et al., 2009). However, here we did not find any correlation between baseline aggressiveness and SSRI treatment effect. Mixed results in this study could be explained by classification of the treated Long Evans rats in the medium aggression class.

No effect of time, batch or treatment order on aggression was found over baseline and post-treatment measurements. However, aggression did not remain stable over the complete experimental timeline as aggression did decrease during the final resident intruder test (after completing both treatment regimens) compared to baseline. There are several possible explanations for this result. The animals were housed individually from the finish of the final treatment period until they were housed together with a female again one week before the final resident-intruder interaction took place. This social isolation could have caused stress, but was necessary to prevent aggressive animals from wounding each other. Furthermore, all animals received citalopram treatment for two weeks, which could have caused long-term effects, resulting in a decreased aggression level. Since aggression at baseline correlated with aggression at the final test, we can conclude that there was an overall decrease in aggression level, which does not interfere with interpretation of the results.

Decreased aggressive behavior has been shown before in males after a chronic citalopram treatment period with a dosage of 20 mg/kg bodyweight (Kugelberg et al., 2001; Manhaes De Castro et al., 2001). However, we couldn't replicate a decrease in aggressive behavior, but did show an increased variability in aggression after citalopram treatment. This suggests different underlying mechanisms in aggression regulation for animals showing increased as opposed to decreased aggressive behavior after SSRI treatment. Individual differences in 5-HT transporter binding and functioning may affect SSRI sensitivity (Jin et al., 2017). Furthermore, serotonin availability in the hypothalamus induces differences in the anti-aggressive effect of SSRI treatment as has been shown with opposite effects in juvenile and female hamsters compared to adult males (Taravosh-Lahn et al., 2006; Terranova et al., 2016).

With about half of the animals showing a decrease in aggression after citalopram treatment compared to saline, correlation analyses were performed with anxiety parameters as measured in the elevated plus maze and open field and cue responsivity as indicated by sign vs. goal tracking behavior. However, we found no predictive value of these behavioral parameters for the anti-aggressive SSRI response. This is not in line with the results of Phan et al., showing that in patients high neuroticism scores predicted a larger decrease in aggression level after SSRI treatment (Phan et al., 2011). This should translate to our model as human neuroticism shows congruency with anxiety in animal models (Flint, 2004). However, anxiety as measured with the elevated plus maze and open field is considered as state anxiety (Belzung, C.; Griebel, 2001), and no consistent pattern in anxious behavior was found, which complicates translation to the results of Phan et al. Furthermore, here all animals that were selected for treatment showed an increased number of lever responses over sessions, indicating more sign tracking behavior in the conditional approach task. This corresponds to a line of rats selectively bred for high locomotor response in a novel environment (bHRs), which show increased aggressive behavior and increased sign tracking behavior (Flagel et al., 2010). So, the aggressive animals in this study may share a similar set of characteristics as is shown with increased sign tracking behavior, which may explain the absence of predictive value of this and other behavioral parameters.

We also analyzed the correlation between regional brain 5-$HT_{1A}$ receptor density, behavioral parameters and the treatment effect. We found an inverse correlation in cortical 5-$HT_{1A}$ receptor densities and anxiety in the open field test. This is in line with the association between decreased 5-$HT_{1A}$ receptor binding potential in cortical regions and anxiety (Solati et al., 2011; Tauscher et al., 2001). However, 5-$HT_{1A}$ receptor densities in the dorsal raphe nucleus, dentate gyrus,

orbitofrontal cortex, infralimbic cortex and prelimbic cortex were not predictive of anti- or pro-aggressive SSRI treatment effect in this study. Chronic SSRI treatment does not change the number of 5-$HT_{1A}$ receptor binding sites, but rather reduces G protein activity in the DRN and 5-$HT_{1A}$ mRNA levels in the anterior raphe in animals sacrificed within 48 h after a 14-day treatment period (Castro et al., 2003; Hensler, 2002; Pejchal et al., 2002). So although treatment does not interfere with our results here, it may be that not the number of receptors, but the capacity to activate G proteins, is indicative of anti-aggressive SSRI efficacy; i.e., 5-$HT_{1A}$ receptor functionality. This study has several other limitations. First, citalopram and serotonin serum levels were not measured during treatment. This was done to avert stress during the housing period before the resident-intruder test, which is a critical period in establishing territorial behavior (Koolhaas et al., 2013). As other studies showed that chronic treatment using osmotic pumps delivers stable plasma levels of 5-HT and citalopram (Ceglia et al., 2004; Cremers et al., 2000; Rossi et al., 2008; Wegener et al., 2003), and we did not observe residual volumes in the pumps after their removal, we carefully conclude that the citalopram treatment in this study was effectively delivered. Although the most aggressive animals were selected for treatment, none of the animals showed escalated, high levels of aggression as reported in the WTG rat strain (Koolhaas et al., 2013). Hence, the aggression we measured may not represent excessive pathological aggressive behavior. However, the individual variation within this group has a good face validity to human aggressive behavior, and the resident intruder test has been shown to be a translatable model for impulsive aggression (Miczek et al., 2013). Finally, this study only looked into male aggression. However, it has become evident that it is important to include both sexes, as it has become clear that serotonin has opposite effects on aggression in males and females (Terranova et al., 2016).

So, within this model there still may be other parameters that could predict SSRI efficacy. Due to the close association between impulsivity, serotonin and aggression, impulsivity may be an interesting parameter to look into for future research (Lesch and Merschdorf, 2000). In conclusion, the SSRI citalopram induced qualitatively different effects on aggression in individuals of an outbred strain of Long Evans rats. The direction of the effect was neither predicted by behavioral measures reflecting baseline aggression, anxiety or cue sensitivity, nor by 5-$HT_{1A}$ receptor availability. This does not preclude that other characteristics may exist to identify positive responders. Future studies should extent this study by looking into other possible predictors for an anti-aggressive SSRI response.

## Conflicts of interest

Authors have no conflicts of interest to disclose.

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