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# Neurochemical impact of the 5-$HT_{2C}$ receptor agonist WAY-163909 on monoamine tissue content in the rat brain☆

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A B S T R A C T

Serotonin2C receptor (5-$HT_{2C}$) agonists are promising drugs for the treatment of neuropsychiatric diseases. However, their effect is not completely understood in part because they possibly affect several neurobiological networks simultaneously. We studied the effect of the 5-$HT_{2C}$ receptor agonist WAY-163909 (0.3 and 3 mg/kg; i.p.) on the tissue concentration of dopamine (DA), 5-HT and noradrenaline (NA) in 29 rat brain regions related to motor, cognitive, mood and vegetative networks. We found that WAY-163909, without altering the tissue concentration of NA, increased 5-HT concentrations in the medial orbitofrontal cortex and the motor cortex M2 at 3 mg/kg and decreased it in the dorsolateral orbitofrontal cortex at 0.3 mg/kg. WAY-163909 enhanced DA concentrations in the central nucleus of the amygdala at 0.3 mg/kg and reduced it in the dorsal hypothalamus at 3 mg/kg. Using correlative analysis of the tissue content of monoamines, WAY-163909 dramatically changed the profile and the pattern of the correlations within and between monoaminergic systems without drastically changing the total number of these correlations. The profile of these changes in correlations was dose-dependent as it was very different between the two doses within and among monoaminergic systems. In conclusion, the data indicated that the 5-$HT_{2C}$ receptor agonist WAY-163909 quantitatively alters monoamine content in very few regions but promotes multiple changes of monoaminergic connectivity in the brain.

## 1. Introduction

The serotonin2C (5-hydroxytryptamin$_{2C}$, 5-$HT_{2C}$) receptor agonists have potential interest in the treatment of various neuropsychiatric diseases (Chagraoui et al., 2016; Di Giovanni and De Deurwaerdere, 2016; Higgins and Fletcher, 2015; Howell and Cunningham, 2015; Kostrzewa et al., 2007). The 5-$HT_{2C}$ receptor agonists have been tested in humans for schizophrenia and drug addiction, although the only drug that has received the FDA approval so far is the 5-$HT_{2A/2C}$ receptor agonist lorcaserin for the treatment of obesity (Di Giovanni and De Deurwaerdère, 2016). However, the mechanism by which 5-$HT_{2C}$ receptor agonists are effective is still unclear.

The 5-$HT_{2C}$ receptor subtype is diffusely expressed in the CNS (Abramowski et al., 1995; Clemett et al., 2000; Pasqualetti et al., 1999; Pazos and Palacios, 1985). According to this large pattern of expression, the administration of 5-$HT_{2C}$ receptor agonists in rodents is associated with a vast array of behavioral effects. They decrease locomotor activity, feeding behavior, and the reinforcing properties of drug abuse or impulsive responses (Anastasio et al., 2015; Dalton et al., 2004; Di Giovanni and De Deurwaerdere, 2016; Fletcher et al., 2011; Fletcher et al., 2006; Fletcher et al., 2013; Fletcher et al., 2009; Higgins et al., 2016; Howell and Cunningham, 2015). On the other hand, they increase purposeless oral movements, penile erection and grooming

*Abbreviations:* aCg, anterior cingulate cortex; ains, anterior insular cortex; BLA, basolateral nucleus of the amygdala); CE, central nucleus of the amygdala; CNS, central nervous system; core, core of the nucleus accumbens; DA, dopamine; DLO, dorsolateral orbitofrontal cortex; dHP, dorsal hippocampus; DLS, dorsolateral striatum; DMS, dorsomedial striatum; DR, dorsal raphe nucleus; dHY, dorsal hypothalamus; EPN, entodepuncular nucleus; HPLC, high pressure liquid chromatography; IL, infralimbic cortex; LO, lateral orbitofrontal cortex; M2, motor cortex M2; MO, medial orbitofrontal cortex; MR, median raphe nucleus; NA, noradrenalin; OFC, orbitofrontal cortices; pCg, posterior cingulate cortex; pIns, posterior insular cortex; PL, prelimbic cortex; 5-HT, 5-hydroxytryptamine;serotonin; 5-$HT_{2C}$ receptors, Serotonin2C receptors; shell, shell of the nucleus accumbens; SN, substantia nigra; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; vHP, ventral hippocampus; vHY, ventral hypothalamus; VCS, ventrocaudal striatum; VLS, ventrolateral striatum; VMS, ventromedial striatum; VTA, ventral tegmental area; WAY-163909, (7bR,10aR)-1,2, 3,4,8,9,10,10a-octahydro-7bH-cyclopenta-[b][1,4]diazepino[6,7, 1hi]indole]

☆ WAY-163909 and brain monoamines.

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**Table 1**
**Experimental details**. It reports the name of the brain structures (and the corresponding abbreviation) that have been chosen for their involvement in neurobiological networks controlling cognition, mood, motor behavior, food intake. The mean size of punched brain region is reported (in mg ± sem) (see also Fig. 1). No significant differences were observed between the weight of tissue between the groups saline-, WAY-163909 0.3- and WAY-163909 3 mg/kg-treated rats (data not shown). The 3 numbers reported in the columns DA, 5-HT and NA correspond to the number of values considered for the statistical analysis for each brain regions. Starting from 8 observations/group, lower numbers are due to loss of tissue, accidental manipulations, loss of chromatographic signal and/or outliers. The asterisk highlights two groups of data that were not considered for correlations due to the low number of data.

| Brain regions | Abbreviation | weight(mg ± sem) | values (n)/group (saline, 0.3 and 3) | | |
|---|---|---|---|---|---|
| | | | DA | 5-HT | NA |
| ***Orbital Cortex*** | OFC | | | | |
| medial orbital | MO | 1.36 ± 0.06 | 8,8,7 | 8,8,8 | 8,8,8 |
| Lateral orbital | LO | 1.2 ± 0.08 | 4*,7,6 | 7,6,8 | 8,8,8 |
| Dorsolateral orbital | DLO | 1.4 ± 0.08 | 8,8,8 | 8,8,8 | 8,8,8 |
| Motor M2 | M2 | 6.6 ± 0.44 | 8,8,8 | 8,7,7 | 8,7,7 |
| ***Frontal Cortex*** | | | | | |
| prelimbic | PL | 1.8 ± 0.1 | 8,8,8 | 8,8,8 | 8,8,8 |
| infralimbic | IL | 2 ± 0.14 | 8,8,8 | 8,8,8 | 8,8,8 |
| Anterior cingulate | aCg | 2.1 ± 0.08 | 8,7,8 | 8,7,8 | 8,8,8 |
| Posterior cingulate | pCg | 2.1 ± 0.09 | 8,8,8 | 8,8,8 | 8,8,8 |
| Anterior insular | ains | 5.8 ± 0.4 | 6,7,6 | 6,7,6 | 6,7,6 |
| Posterior insular | pins | 5.9 ± 0.16 | 6,8,8 | 6,8,8 | 6,8,8 |
| ***Nucleus accumbens Striatum*** | NAc | | | | |
| Shell | Shell | 1.9 ± 0.07 | 8,8,8 | 8,8,8 | 8,8,8 |
| core | core | 2.1 ± 0.1 | 8,7,8 | 8,7,8 | 8,7,8 |
| Dorsomedial striatum | DMS | 2 ± 0.1 | 8,8,8 | 8,8,8 | 8,8,8 |
| Ventromedial striatum | VMS | 2.1 ± 0.14 | 8,8,8 | 8,8,8 | 8,8,8 |
| Dorsolateral striatum | DLS | 2 ± 0.07 | 8,8,8 | 8,8,8 | 8,8,8 |
| Ventrolateral striatum | VLS | 1.7 ± 0.08 | 8,8,8 | 8,8,8 | 8,8,8 |
| Ventrocaudal striatum | VCS | 3.1 ± 0.23 | 8,8,8 | 7,8,8 | 8,8,8 |
| ***Basal ganglia mesencephalon*** | | | | | |
| Entopuncular nucleus | EPN | 4.5 ± 0.23 | 8,8,8 | 8,7,8 | 8,8,8 |
| Subthalamic nucleus | STN | 2.23 ± 0.09 | 8,8,8 | 8,8,8 | 8,8,8 |
| Substantia nigra | SN | 2.08 ± 0.09 | 8,8,8 | 8,8,8 | 8,8,8 |
| Ventral tegmental area | VTA | 2.48 ± 0.1 | 8,8,8 | 8,8,8 | 8,8,8 |
| Dorsal raphe nucleus | DR | 3.6 ± 0.18 | 7,8,8 | 7,8,8 | 7,8,8 |
| Median raphe nucleus | MR | 2.3 ± 0.11 | 7,7,6 | 8,8,8 | 8,8,8 |
| ***Amygdala*** | | | | | |
| Basolateral nucleus | BLA | 1.38 ± 0.33 | 8,8,8 | 8,8,8 | 8,8,8 |
| Central nucleus | CE | 0.97 ± 0.08 | 8,8,8 | 8,8,7 | 8,7,8 |
| ***Hippocampus*** | | | | | |
| Dorsal, anterior parts | dHP | 3.48 ± 0.26 | 8,6,3* | 7,7,7 | 8,8,8 |
| Ventral, posterior parts | vHP | 3.07 ± 0.18 | 6,6,5 | 6,8,8 | 7,8,8 |
| ***Hypothalamus*** | | | | | |
| dorsal parts | dHY | 2.58 ± 0.13 | 7,6,8 | 7,8,8 | 5,6,6 |
| ventral parts | vHY | 2.63 ± 0.16 | 7,8,7 | 8,8,8 | 8,8,8 |

(Graf, 2006; Kreiss and De Deurwaerdere, 2017; Millan et al., 1997; Navailles et al., 2013b) and have opposite effects on anxiety or epilepsy depending on the agonist and the nature of the trouble (Di Giovanni and De Deurwaerdere, 2016). Specific mechanisms of action and circuits have been proposed to account for the above-mentioned behavioral effects of 5-$HT_{2C}$ receptor agonists but not conclusive proofs are available yet. For instance, it has been largely documented that 5-$HT_{2C}$ receptors inhibit dopamine (DA) neuron activity and DA release (Di Giovanni et al., 2001) in the control of motor behaviors. Yet, this action does not appear essential in the ability of 5-$HT_{2C}$ receptor agonists to reduce behaviors associated with enhanced DA (Cathala et al., 2015; De Deurwaerdere and Di Giovanni, 2017). Alternatively, we have proposed that the effects resulting from the brain 5-$HT_{2C}$ receptor stimulation, such as narrowing DA-dependent behavioral effects or food intake, might emerge from a simultaneous action on the activity of several neurobiological networks and from a diffuse interaction and competition between these neurobiological networks (Di Giovanni and De Deurwaerdere, 2016).

The DA, 5-HT and noradrenaline (NA) systems innervate the brain from the cell bodies located in the substantia nigra (SN) and ventral tegmental area (VTA), dorsal and median raphe nuclei (DR and MR), and locus coeruleus (LC), respectively. Monoamines tissue measurement in the brain gives rough information on the biochemical status of their terminals in one brain region, or in multiple brain regions. The content of monoamines is regulated by local neurons connected to distinct networks. Therefore, the tissue content measurement ultimately permits to address the connectivity of neurochemical, monoaminergic markers within one monoaminergic system and between them through correlational approaches (Fitoussi et al., 2013; Klouche et al., 2015). We hypothesized that pharmacological activation of the 5-$HT_{2C}$ receptors may disrupt the monoaminergic connectivity in the brain.

In the present study, we studied the effect of the intraperitoneal (i.p.) injection of the preferential 5-$HT_{2C}$ receptor agonist WAY-163909 (0.3 and 3 mg/kg, i.p.) on the tissue concentration of DA, 5-HT, and NA in 29 brain regions in rats. WAY-163909 elicited various behaviors (Dunlop et al., 2006; Navailles et al., 2013b) that have no clear association with DA release, c-Fos expression in basal ganglia or changes in electrophysiological activities in the basal ganglia (Lagière et al., 2017). The chosen brain regions included various frontal cortices, some basal ganglia regions, the hippocampus, the hypothalamus, and the amygdala.

## 2. Materials and methods

### 2.1. Animals

Male Sprague Dawley rats weighing 300–400 g were used. They were kept in the animal facility (University of Bordeaux, France) with free access to food and water, in a constant temperature (21 ± 2 °C)

and humidity (60%) levels, under a 12-h day/night cycle. All the animals' procedures were in accordance with the European Council Directive 2010/63/EU and the French National Committee (décret 2001-464), and local committee for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### 2.2. Tissue sampling and conditioning

The rats were sacrificed 45 min after drug injection (see pharmacological treatment) and the brain was quickly removed. The brains were immersed in isopentane (2-methyl butane) (−35 ± 5 °C) and stored in a freezer at −80 °C. The brains were cut using a cryostat at −24 °C, and bilateral "punches" were made of various brain structures of interest using steel cannulae of 500 or 800 µm in diameter except for the subthalamic nucleus (STN) (Fitoussi et al., 2013). The STN was gently taken with the smaller cannula used as a spoon to collect the surface (usually around 200 µm thickness) of the tissue from the medial to the lateral extension of the STN. The operation was performed three to four times to account for the rostro-caudal length of the STN.

We selected brain regions belonging to different neurobiological networks (Table 1). Of note, in the striatum, in addition to the classical ventromedial (VMS), dorsomedial (DMS), ventrolateral (VLS) and dorsolateral (DLS) striatum, we selected the ventro-caudal striatum (VCS), an area thought to participate in abnormal oral movements induced by 5-$HT_{2C}$ agonists (Plech et al., 1995). The location of the punches of the structures has been reported on coronal sections diagrams from the Paxinos and Watson atlas (Paxinos and Watson, 1998) and corresponds to Fig. 1.

These punches of tissue were then deposited in previously weighed Eppendorf tubes and placed back in the freezer at −80 °C. Photos were taken throughout to ensure that the samples were taken from a similar plane each time. The tubes containing the samples were cautiously weighed again the day of the biochemical analysis (Dellu-Hagedorn et al., 2017) and 100 µl of perchloric acid ($HClO_4$ 0.1N, 4 °C) was added. Then, the samples were sonicated with ultrasound for about 6 seconds and centrifuged at 13,000 rpm for 30 min at 4 °C. A volume of 10–20 µl of the supernatant was injected into the high-pressure liquid chromatography (HPLC) system.

### 2.3. HPLC analysis and electrochemical detection

The tissue concentrations of monoamines were measured by HPLC coupled to the coulometric detection system. The mobile phase of the HPLC system was composed of methanol (7%), NaH2PO4 (70 mM), triethylamine (100 µl/l), EDTA (0.1 mM), sodium octyl sulphate (100 mg/l) diluted in deionized water (pH 4.2, adjusted with orthophosphoric acid) as previously reported (De Deurwaerdere et al., 1995). It was filtered (0.22 µm) before its installation in the system. The mobile phase was delivered through the HPLC column (Hypersyl, C18, 15 cm × 4.6 mm, particle size 5 µm, C.I.L.) at a flow rate of 1.2 mL/min using an HPLC pump (LC10Ad Vp, Schimadzu, France). The column was protected by a Brownlee-Newgard precolumn (RP-8, 15 × 3.2 mm, 7 µm; C.I.L.). The injection of the samples (10–20 µL) was carried out by a manual injection valve (Rheodyne, model 7725i, C.I.L.) equipped with a loop of 20 µl. The monoamines exit the column at different retention times (approximately NA: 3'40"; DA: 8'; 5-HT: 18') and passed into the coulometric detection cell (Cell 5011, ESA, Paris, France) equipped with two electrodes. The potential of these two electrodes was fixed via the coulometric detector (CoulochemII, ESA, Paris, France) at +350 mV (oxidation) and −270 mV (reduction), respectively. The coulometric detector was connected to a computer through an interface (Ulyss, Azur system, Toulouse, France).

The calibration curves were performed once the pics in a standard solution (1 ng/10 µl) were well separated in the chromatogram. The calibration curves were adapted according to the brain areas investigated, because the quantities of monoamines are heterogeneous (Fitoussi et al., 2013), requiring different gains set at the level of the detector using a timeline method. The gains used ranged from 5 nA (for the DA in the hippocampus) to 1 µA (for DA in the striatum). Standard solutions were used before each series of 10/12 samples to verify the good correspondence of the chromatographic conditions to both the elution time and quantities calculated from the calibration curves. Similarly, the quantities of monoamines in the standards varied according to the brain region investigated. The overall sensitivity for the compounds ranged from 2 pg/10 µl for DA to 7 pg/10 µl for 5-HT with a signal/noise ratio of 3:1.

### 2.4. Pharmacological treatment and experimental design

WAY-163909 (7bR,10aR)-1,2, 3,4,8,9,10,10a-octahydro-7bH-cyclopenta-[b][1,4]diazepino[6,7, 1hi]indole]), freshly diluted as free base in NaCl 0.9%, was injected i.p. (0.3 or 3 mg/kg). For each experimental group, the animals were randomized and received either the drug or appropriate vehicle (in 1 ml/kg).

WAY-163909 is one of the most selective 5-$HT_{2C}$ receptor agonists available on the market, it is brain penetrant and summarizes the main behavioral effects elicited by 5-$HT_{2C}$ receptor agonists in rodents (Dunlop et al., 2005, 2006). The doses of 0.3 and 3 mg/kg, i.p. have been chosen based on previous data showing that it reduced locomotor activity and induced purposeless oral movements (Marquis et al., 2007; Navailles et al., 2013). These studies reported that the behavioral effects elicited by WAY-163909 were observed 30–45 min after its injection. Therefore, we choose to observe the behavioral effect of WAY-163909 at 45 min after its injection with the aim of monitoring the abnormal oral movements and penile grooming. Then, quickly we collected the brain areas in order to get the monoamine status at a time corresponding to the highest behavioral activity. Their brains were finally stored at −80 °C for successive neurochemical post-mortem analysis.

### 2.5. Statistical analysis of the data

The tissue levels of NA, DA, and 5-HT for each structure were expressed in pg/mg. These levels are presented as the mean ± the standard error of the mean (SEM) according to their treatment group. Outlier data were discarded on the basis of the value outside the range of the average mean ± two standard deviations (Dellu-Hagedorn et al., 2017). The values for NA, DA, and 5-HT were compared between experimental groups (saline, WAY-163909 at 0.3 or 3 mg/kg, i.p.) with a one-way ANOVA, followed by the Fisher protected least significant difference (PLSD) post-hoc test. A similar analysis was performed for the weight of the tissue between groups for each structure. In all comparisons, $p < 0.05$ was used as the criterion for significance.

The qualitative analysis corresponds to multiple correlative analyses using the Bravais-Pearson's correlation coefficient. These analyzes were performed within and between NA, DA and 5-HT systems in the 29 brain regions investigated. The correlations have been separately performed in rats receiving saline, 0.3 mg/kg WAY-163909 and 3 mg/kg WAY-163909 to estimate monoamine connectivity in each group with no attempt to statistically compare the profiles. As previously reported (Fitoussi et al., 2013), p-values were adjusted using the False Discovering Rate (FDR) controlling procedures (Benjamini and Hochberg, 1995). Correlations were then considered as significant at the 5% level and were reported in the corresponding figures.

## 3. Results

### 3.1. Quantitative analysis of monoamine tissue content after WAY-163909 administration

All rats treated with WAY-163909 exhibited classical behaviors induced by 5-$HT_{2C}$ receptor agonists including abnormal oral movements

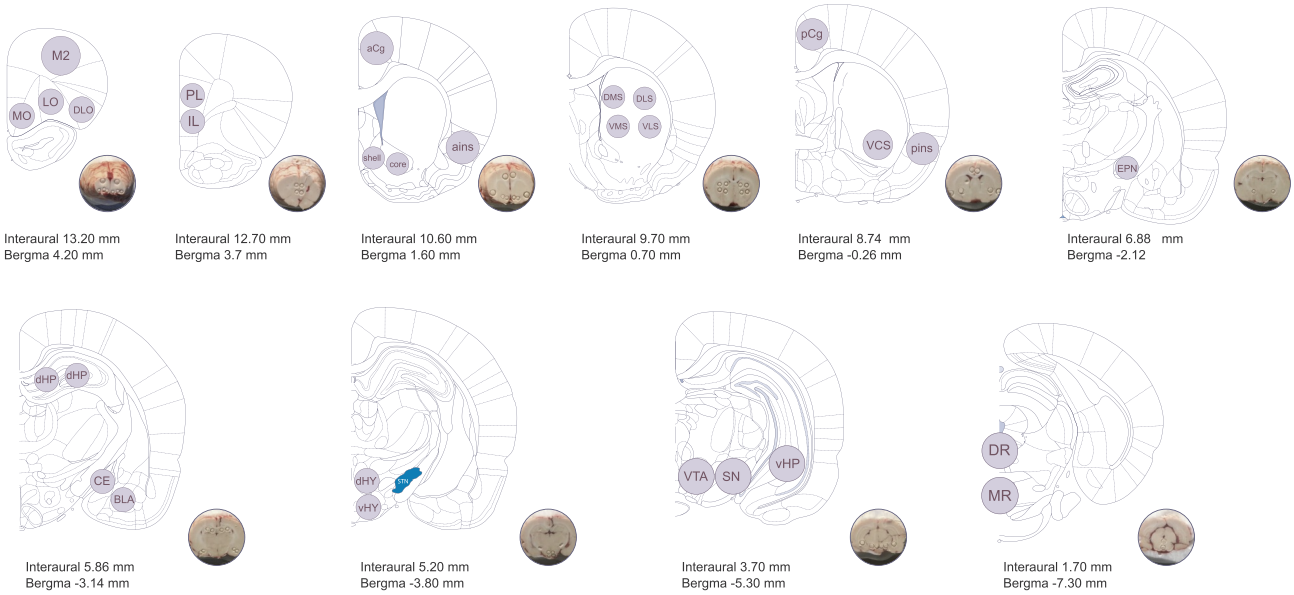


**Fig. 1. The approximate position of punched tissue aliquots from coronal sections of the rat brain** (adapted from (Paxinos and Watson, 1998)). Tissue extracts were taken from left and right cerebral hemispheres in a cryostat. Cortical areas: medial (MO), lateral (LO) and dorsolateral (DLO) orbitofrontal cortex, prelimbic (PL), and infralimbic (IL) cortices, anterior (aCg) and posterior (pCg) cingulate cortices, anterior (aIns) and posterior (pIns) insular cortices; motor cortex (M2) and subcortical areas including striatum (dorsomedial (DMS), ventromedial (VMS), ventrolateral (VLS), dorsolateral (DLS) striatum, ventrocaudal (VCS) striatum; nucleus accumbens (shell and core), dorsal and ventral hippocampus (dHP and vHP), amygdala [basolateral nucleus (BLA) and central nucleus (CE)], entopeduncular nucleus (EPN), dorsal and ventral parts of the hypothalamus (dHY and vHY), substantia nigra (SN), ventral tegmental area (VTA), dorsal raphe nucleus (DR); median raphe nucleus (MR), subthalamic nucleus (STN). This latter region was the only one selected without punching due to its shape and its small size. Two punched tissue in each side were taken from the dHP regions to be able to measure the concentrations of DA. The photomicrographs illustrate punched brain region.

and few episodes of penile grooming (Navailles et al., 2013b). WAY 163909 was administered at 0.3 or 3 mg/kg i.p. and the results of tissue monoamines of the 29 brain areas of interest were compared to those of saline-treated rats. The size of the tissue and the number of observations kept after removing outliers (or simply non-detected parameters) are indicated in Table 1. For all brain regions, the size of the tissue did not significantly vary between groups. The quantitative analysis of the effects of WAY 163909 for NA, DA, and 5-HT systems is reported in Fig. 2.

The tissue levels of DA were largely heterogeneous across the brain of the saline group. Briefly, DA levels were very high along the nigrostriatal and mesolimbic areas reaching up to 6118 ± 655 pg/mg of tissue in the DMS (almost equivalent in DLS). Conversely, it was very low and sometimes undetectable in the hippocampus (dHP and vHP with 3.4 ± 0.38 pg/mg and 3.8 ± 0.56 pg/mg of tissue, respectively) or LO. WAY 163909 treatments did not affect DA levels except for the dorsal hypothalamus (dHY) [one-way ANOVA $F(2,20) = 3.92$, $p = 0.039$], the CE amygdala [$F(2,23) = 5.38$, $p = 0.013$] and the MR [$F(2,19) = 4.07$, $p = 0.036$]. Precisely, DA levels were significantly decreased after the administration of 3 mg/kg WAY 163909 compared to saline treatment (Fig. 2). In the amygdala (CE), DA tissue content was significantly increased above values of the saline group after 0.3 mg/kg WAY-163909 only. DA content in the MR of 0.3 mg/kg WAY-163909 treated rats was higher compared to that of the 3 mg/kg group but not that of the saline group (Fig. 2).

Tissue levels of 5-HT were less heterogeneous compared to DA in investigated brain regions, although important and expected differences were reported. DA levels were highest in the SN and very high in DR, VTA, EPN, NAc shell and parts of the cortex. Conversely, 5-HT tissue content was low in the hippocampus (dHP and vHP), in the hypothalamus (dHY and vHY) and the lateral orbitofrontal cortices. WAY 163909 treatment did not alter 5-HT tissue content except for MO, DLO, and M2 [one-way ANOVA $F(2,23) = 6.32$, $p = 0.007$; $F(2,23) = 3.6$, $p = 0.045$ and $F(2,21) = 5.95$, $p = 0.01$ respectively]. The 5-HT content was increased in MO and M2 at the dose of 3 mg/kg WAY-163909 and reduced in DLO at the dose of 0.3 mg/kg WAY-163909.

Tissue levels of NA in the saline-treated group were heterogeneous as expected from previous works. The highest concentration was observed in the DR. High concentrations were observed in VTA, MR, SN, and an unexpectedly high level was found in the EPN. Some cortical territories (MO, M2), hippocampus, and hypothalamus had moderate values. The lowest concentrations were found in the striatal quadrants, LO and dHY. As illustrated in Fig. 2, WAY-163909 did not alter NA tissue content at the two doses used here in any of the brain regions considered (one-way ANOVA, ns for all comparisons).

### 3.2. Qualitative analysis of monoamine tissue content

#### 3.2.1. Within monoaminergic systems

To get a deeper analysis of the data profile, we then evaluated possible relationships of monoamines between the 29 investigated brain areas using a correlative approach. The main important point is to indicate that the correlations are few and their nature and number changed upon WAY-163909 administration at increasing doses (0.3 mg/kg and 3 mg/kg).

Correlations for DA content between brain structures upon saline administration appear to be limited and equilibrated between positive and negative correlations (15 and 14, respectively). Some brain regions differently from other established several correlations with the DA content at distal regions. Notably, DA content in the pins or the VMS had six correlations with the DA content in other regions; the vHP, the EPN, and the DR had five correlations. Conversely, DA content in the shell, DLS or VCS poorly correlated. The injection of WAY-163909 reduced the number of correlations at 0.3 mg/kg (19 comprising 4 negative correlations only) and 3 mg/kg (20 comprising 8 negative correlations). None of the correlations observed in saline-treated rats were present in WAY-163909-treated rats and they were different between 0.3 and 3 mg/kg condition. DA content in vHP and DR did not correlate after WAY-163909 injection. The pins and the VMS DA content still had a few correlations (1–3) though with different brain areas. The correlations of DA content after 3 mg/kg concerned mainly the cortex and the basal ganglia regions when compared to 0.3 mg/kg results.

Few correlations (17) appear for 5-HT content in the 29 brain areas

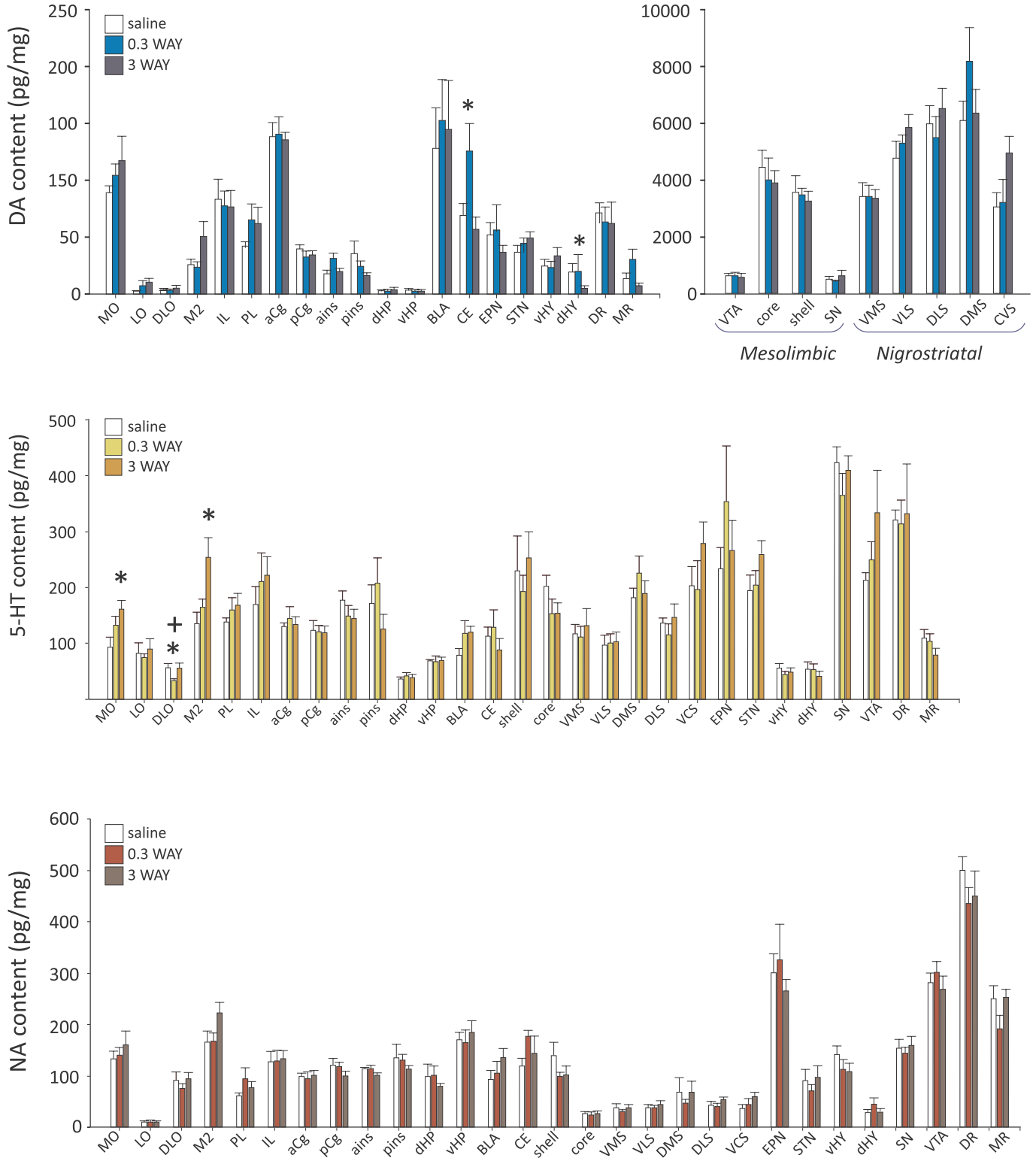


**Fig. 2. Effect of WAY-163909 on DA, 5-HT and NA across the brain**. Upper, middle and lower panels correspond to DA, 5-HT, and NA contents, respectively. The left, medial and right bars of histogram correspond to saline-, WAY-163909 0.3 mg/kg, and 3 mg/kg treated rats, respectively. The results correspond to the mean ± s.e.m of monoamine content (pg/mg tissue) in the 29 different rat brain regions. WAY-163909 has been intraperitoneally administered and the tissue values correspond to 45 min after the administration. WAY-163909's effects have been compared to saline-treated rats using a one-way ANOVA (see Table 1 for the number of observations). *$p < 0.05$ with respect to the saline group; +$p < 0.05$ with respect to the other group of WAY-163909 (PLSD's test).

upon saline administration, including 10 negative correlations. Several correlations concerned the NAc or the striatum with the orbitofrontal cortices or the hippocampus. The 5-HT content in the BLA, IL, aCg, LO, DR or VTA did not correlate. The profile of correlations was dramatically changed in WAY-163909-treated rats with very few correlations in saline-treated rats reported in WAY-163909 groups. WAY-163909 at 0.3 mg/kg induced a similar number of correlations (19) but they were mainly positive (15/19). The 5-HT content in MO and VCS

was correlated. The correlations were dispatched between prefrontal cortices, amygdala with other territories while 5-HT content of several brain regions (dHP, vHY, M2, VTA) did not correlate with other brain regions. At 3 mg/kg, the number of correlations increased (25 including 15 negative correlations). Several correlations were established between frontal cortices (particularly DLO, LO and M2) and basal ganglia, often positive, while correlations within the basal ganglia tended to be negative (Fig. 3).

Very few correlations (18 comprising an almost equal number of positive and negative correlations) were seen between the NA content of the 29 brain regions in the saline group. The profile of correlations was totally different from that of DA. This profile dramatically changed after WAY-163909 with a number of correlations reaching 29 and 16 after 0.3 and 3 mg/kg, respectively. NA content persisted in 0.3 mg/kg WAY-163909 between IL and LO. In addition, NA content in PL established correlations with NA content in seven other brain regions, all positive except with LO. NA content in pins, pCg, VLS, or MR also established some correlations (from 4 to 6). The number of correlations for NA content was reduced at 3 mg/kg WAY-163909. At variance with DA, it is marked by low cortical/basal ganglia associations. It is noteworthy that NA content in several brain regions including MO, M2, PL, ains or BLA did not correlate with other brain regions. However, NA content in the hypothalamus correlated more with that in the cortices, shell, and the STN.

### 3.2.2. Between monoaminergic systems

*3.2.2.1. 5-HT/DA tissue content.* A total of 48 correlations were observed between 5-HT and DA contents, including 8 in the same brain area (all positive) (Fig. 4). The proportion of negative correlations was slightly higher compared to positive correlations in saline-treated rats. One of the most striking patterns is the number of correlations between the 5-HT content in the EPN and the SN with DA content in diverse brain regions including within these two, and between the orbitofrontal cortices for the EPN and the frontal cortex for the SN. DA content in the SN negatively correlated with 5-HT content from the orbitofrontal cortices and the CE whereas DA content in the DR negatively correlated with 5-HT in the SN. DA and 5-HT contents strongly correlated within the shell and core. The STN or hypothalamic regions slightly correlated, including some striatal quadrants. The number of correlations after WAY-163909 was quite similar (48 after 0.3 and 42 after 3 mg/kg). However, the pattern was very different compared to saline-treated rats. The correlations were more positive, particularly in the case of 0.3 mg/kg. The number of correlations of 5-HT and DA content in single brain regions was increased to 14. In addition, the correlations reported at the level of the EPN and the SN were reduced. At 0.3 mg/kg, the DA content in the STN correlated with 5-HT content of some orbitofrontal/prefrontal regions. Contents (DA or 5-HT) were correlating more after 0.3 mg/kg between cortical regions or in the basal ganglia, particularly the VMS. No striking pattern was noticed after 3 mg/kg except a slightly higher proportion of correlations for both DA or 5-HT content within brain regions of the basal ganglia and the lower number of correlations of 5-HT and DA content in the same brain regions.

*3.2.2.2. DA/NA tissue content.* DA and NA contents correlated in 39 brain areas including 6 positive correlations within the same regions (shell, core, VLS, VCS, EPN, and SN) in saline-treated rats (Fig. 4). The number of positive and negative correlations were approximately even in distal comparisons. The brain regions in which a higher number of correlations is found for DA and/or NA content are the VCS, the SN, the EPN, the core. NA or DA content in MO negatively correlated with DA and NA content in the EPN, respectively. Interestingly, the content of NA in the SN, DR, and VTA had correlations with the CE, BLA, or dHP. WAY-163909 drastically modified the pattern of correlations, slightly increasing the number of correlations to 49 and 53 at 0.3 and 3 mg/kg respectively. The most noticeable changes at 0.3 mg/kg concern the numerous (5–9) correlations of DA contents in the LO, IL, or STN with NA content of diverse cortices and subcortical areas for the two later. NA content in the core or the aCg had multiple correlations with DA content of 4–6 areas. Finally, 0.3 mg/kg WAY-163909 increased NA/DA correlations in regions belonging to basal ganglia and decreased them in amygdala/hippocampus. At 3 mg/kg WAY-163909, DA/NA tissue correlations were observed between subcortical structures belonging to basal ganglia and limbic regions including amygdala and hippocampus. Of note, vHY NA or DA content correlated with the core, CE or M2. In fact, NA content in M2 displayed 5 correlations with DA content in other brain regions. The NA/DA correlativity of STN was low at this dose.

*3.2.2.3. 5-HT/NA tissue content.* NA and 5-HT contents diffusively correlated in saline-treated rats with 51 correlations including 7 positive ones within the same regions (Fig. 4). The correlations were found between cortical regions, between cortical and striatal regions, and slightly with the mesencephalon. The regions establishing the most distal correlations were pins and ains (6), LO, VLS, dHP and VCS (5) followed by MO, SN, core, EPN, BLA, DLS, DMS, MR, and the STN (4 each). No correlation was reported with NA or 5-HT content of aCg. Again, WAY-163909 changed the pattern of correlations with 58 and 53 correlations at 0.3 and 3 mg/kg, respectively. In both cases, a net, higher proportion of positive correlations were reported. The most evident change at 0.3 mg/kg was the number of NA/5-HT correlations in cortices involving LO, IL, PL, pCg towards the other cortices, hippocampus/amygdala, NAc, and striatum. The second important change is the increase of correlations within same the brain regions (from 7 to 13), including the shell, the core, VMS, DMS, VLS, or STN. Orbitofrontal/frontal cortex also displayed few correlations with the basal ganglia (notably the EPN). The hypothalamus regions displayed more correlations compared to saline-treated rats. At 3 mg/kg, fewer correlations between the two parameters (10) were observed within brain regions notably in the cortex and striatum (when compared with the pattern obtained at 0.3 mg/kg). Cortical 5-HT and NA content correlated with NA and 5-HT content in subcortical regions. In other words, the strong correlations centered in the cortex at 0.3 mg/kg were no longer present at 3 mg/kg.

## 4. Discussion

Peripheral administration of the 5-$HT_{2C}$ receptor agonist WAY-163909 affected 5-HT and DA tissue contents only in a few brain areas without altering the NA tissue content in rats. However, WAY-163909 produced numerous changes in the correlations profiles within and between monoamines across the various regions analyzed. These changes in the connectivity of the monoaminergic system likely suggest new interactions between neurobiological networks which are postulated to be simultaneously altered by central 5-$HT_{2C}$ receptor stimulation.

The tissue concentrations of endogenous monoamines here measured are comparable with the density of monoaminergic fibers in the brain (Aston-Jones, 2004; Björklund and Lindvall, 1986; Steinbusch, 1984; Steinbusch et al., 1981) with the highest heterogeneity for the DA system (Fitoussi et al., 2013). In addition to DA, 5-HT, and NA fibers coming from SN/VTA, DR/MR and LC, local neuronal systems likely contribute for tissue DA in the hypothalamus and DR (Bjorklund and Dunnett, 2007; Cho et al., 2017) whereas NA tissue content in the hypothalamus (dHY notably) also originate from A5 and A7 (Aston-Jones, 2004). The NA content in the dHY was low compared to the expectations (Brownstein and Palkovits, 1984) and it could come from the punching procedure (see below).

We report that monoamines tissue correlates in few instances within a monoaminergic system across multiple brain areas corresponding here to about 10% of the entire analyses for each monoamine content. This finding is consistent with previous findings (Fitoussi et al., 2013),

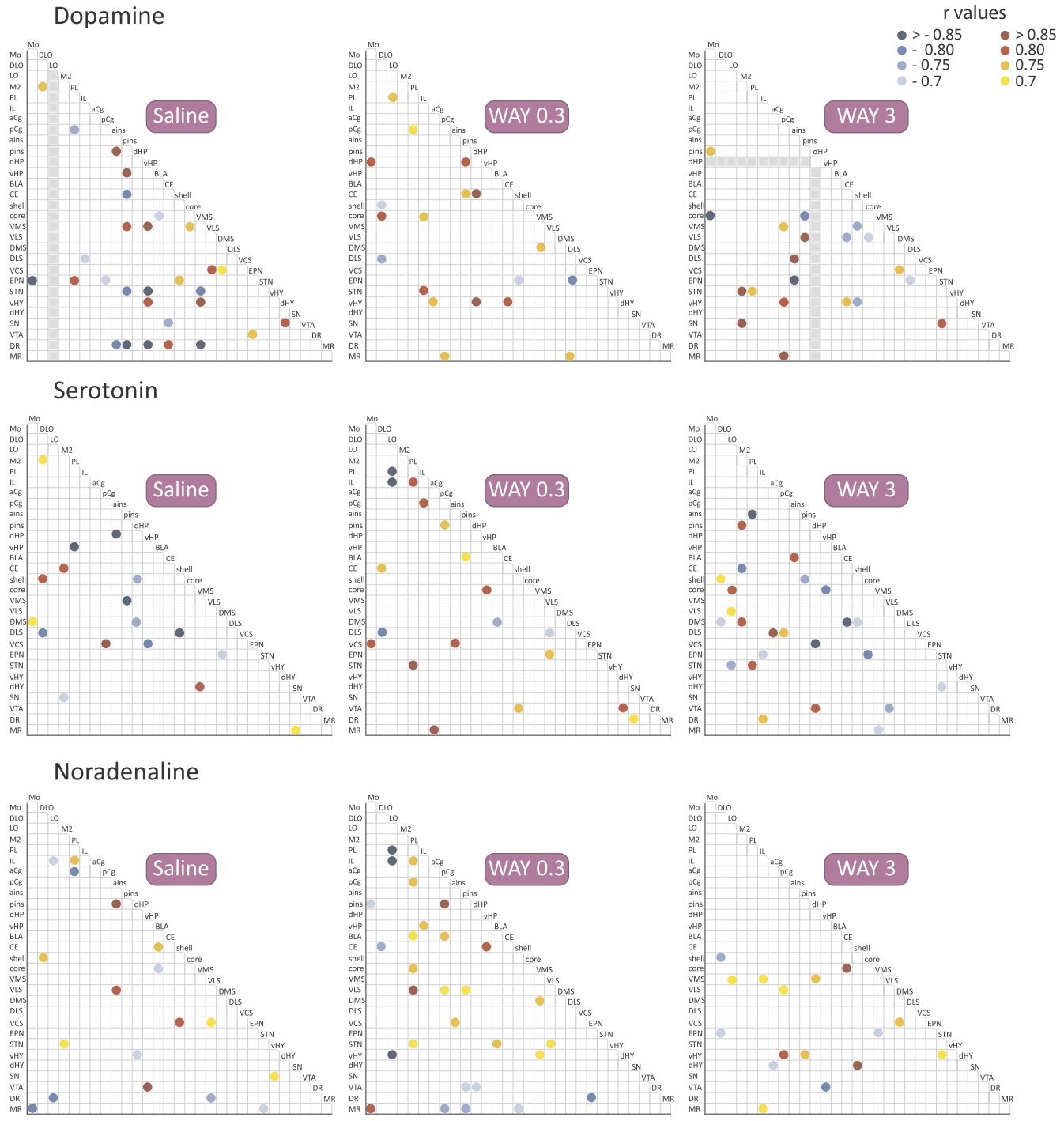


**Fig. 3. Correlative analysis of monoamine content across rat brain regions**. Representation of the range of Pearson's *r* values for each linear regression, of dopamine, serotonin, and noradrenaline tissue content (pg/mg) between the 29 brain areas in saline, WAY-163909 0.3 mg/kg, and 3 mg/kg treated rats. Colored boxes correspond to the existence of a correlation between the two parameters (red: positive; blue: negative) considered after correction for multiple comparisons. The grey boxes correspond to values that were not studied due to the small numbers of values (see Table 1).

although the proportion of negative correlations reported here is higher. Nonetheless, the two studies are not comparable on numerous criteria (distinct strain, handling, age, depth of tissue punched). Thus, we had no specific expectation as regards the pattern of monoamines connectivity in control animals, as it should probably depend on the experimental conditions that are always different, and the inter-individual differences which are important for monoamine tissue content (Dellu-Hagedorn et al., 2018). As postulated previously (Fitoussi et al., 2013), the content of one monoamine in the terminal region does not correlate or rarely with the content of the same monoamine at the level of the cell bodies, at least for DA and 5-HT. Nevertheless, the lack of correlations could be due to the punching procedure. Indeed, we used the larger punches for the SN, VTA, DR and the MR, with higher depth for raphe region, with the aim of to abolishing the anatomo-functional specificities of midbrain monoaminergic centers toward projections sites (Bjorklund and Dunnett, 2007; Björklund and Lindvall, 1986; Hale and Lowry, 2011; Kiyasova et al., 2011). On the other hand, the lack of correlations with midbrain contents strengthens the idea that the biochemical regulation of the content of one monoamine in terminal fields is in part independent from the incoming activity from

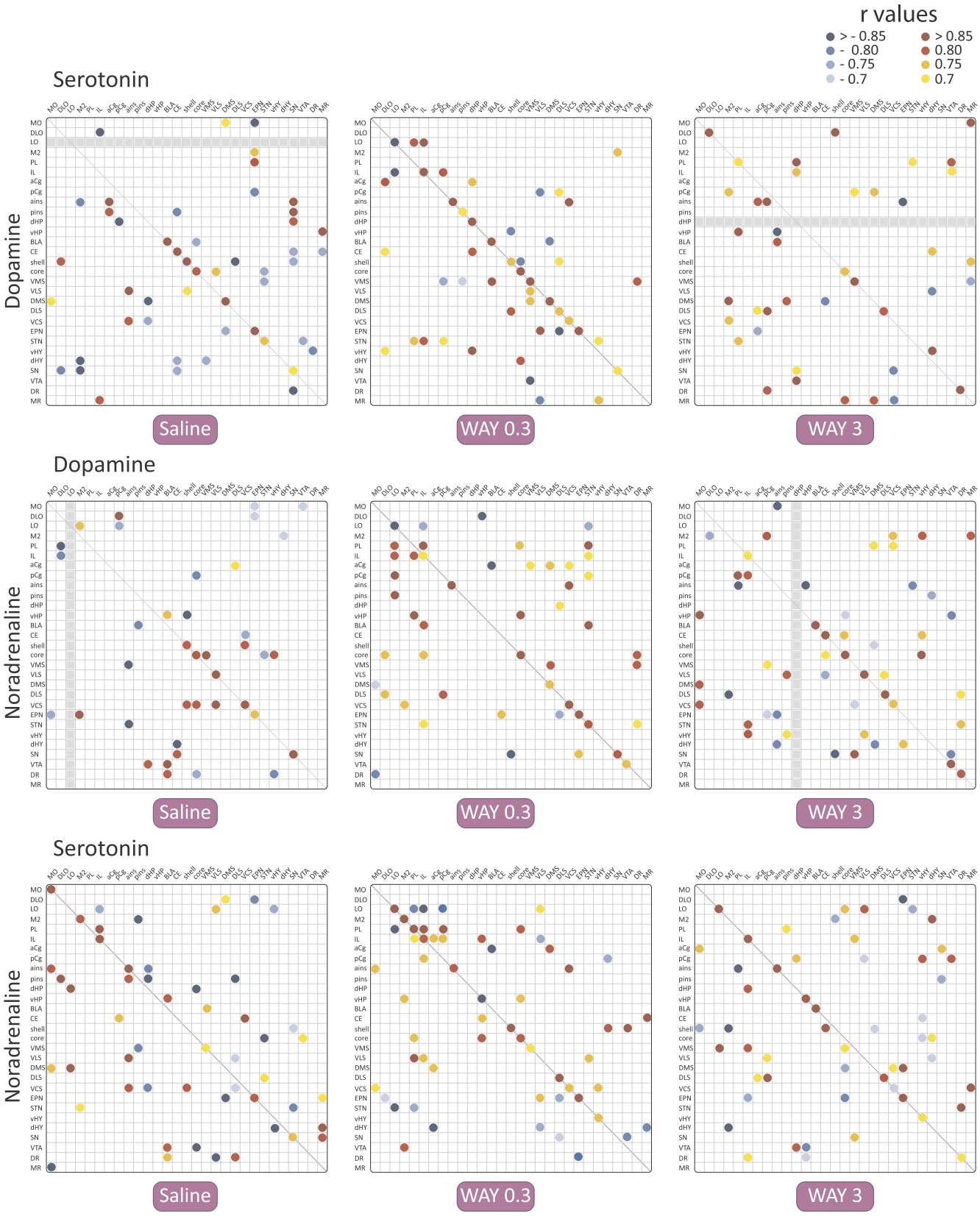


**Fig. 4. Correlative analysis of monoamine content across rat brain regions**. Representation of the range of Pearson's r values for each linear regression of serotonin/dopamine contents (first row), dopamine/noradrenaline contents (second row) and serotonin/noradrenaline contents (third row) within and between the 29 brain areas in saline (first column), WAY-163909 0.3 mg/kg (second column) and 3 mg/kg (third column) treated rats. Colored boxes correspond to the existence of a correlation between the two parameters (red: positive; blue: negative) considered after correction for multiple comparisons. The grey boxes correspond to values that were not studied due to the small numbers of values (see Table 1).

cell bodies, and dependent on local interaction with neighboring cells (Fitoussi et al., 2013).

WAY-163909 did not dramatically change the quantities of monoamines in numerous brain regions analyzed but it revealed a specific pattern of effect on 5-HT content tissue content in the orbitofrontal cortex. The lack of effect in other brain regions is consistent with a study in mice reporting that the 5-$HT_{2B/2C}$ agonist Ro60-0175, over a large range of doses, did not modify 5-HT tissue content taken from the hippocampus, dorsal frontal part of the cortex, NAc and VTA/SN (Mongeau et al., 2010). A pronounced effect in the orbitofrontal cortex is congruent with the ability of 5-$HT_{2C}$ receptor agonists to trigger compulsive forms of behaviors including grooming (Graf, 2006; Graf et al., 2003), penile grooming (Chagraoui et al., 2003; Millan et al., 1997), and purposeless oral movements (Gong and Kostrzewa, 1992; Kreiss et al., 2013; Navailles et al., 2013b; Stewart et al., 1989), the latter which could model compulsive emanation (Kreiss and De Deurwaerdere, 2017) rather than dyskinesia (Kostrzewa et al., 2007; Kreiss and De Deurwaerdere, 2017). The compulsive property of 5-$HT_{2C}$ receptor agonists has been hypothesized to be located in the OFC by appropriate behavioral tests results (Alsio et al., 2015; Flaisher-Grinberg et al., 2008).

The low effect of WAY-163909 on DA tissue content agrees with the data in the literature. Indeed, although the tissue measurement is considered less sensitive compared to the extracellular levels measured by microdialysis, our results are consistent with the finding that WAY-163909 did not modify DA release at 0.3, 1 or 3 mg/kg in the NAc or the striatum (Lagière et al., 2017; Marquis et al., 2007). Other 5-$HT_{2C}$ receptor agonists have been shown to inhibit DA release in the NAc and the firing rate of DA neurons in the VTA (De Deurwaerdere and Di Giovanni, 2017; Di Giovanni and De Deurwaerdere, 2016; Di Matteo et al., 2000; Prisco et al., 1994). Inhibitory effects of these agonists have been also reported at the level of the striatum (De Deurwaerdere et al., 2004; Di Giovanni and De Deurwaerdere, 2016; Gobert et al., 2000), without inhibition of SNc presumed-DA neurons electrical activity (Di Giovanni et al., 2000; Di Matteo et al., 2000). The lack of parallel outcomes between the firing rate and the extracellular levels at terminal fields, even described in the NAc, is in part due to the existence of several populations of 5-$HT_{2C}$ receptors which could impact, sometimes oppositely, the biochemical activity at terminals (Di Giovanni and De Deurwaerdere, 2016; Leggio et al., 2009a; Leggio et al., 2009b; Navailles et al., 2006). Moreover, some of the agonists previously used including Ro60-0175 are less selective compared to WAY-163909 and trigger non-selective effects (Fletcher et al., 2006; Navailles et al., 2013a).

Overall, the quantitative analysis revealed a poor influence of WAY-163909 on DA and 5-HT system and a total absence of effect on NA content over the regions analyzed. It is noteworthy that the tissue content of biogenic amines represents a complex balance between synthesis, storage capacity and degradation (Commissiong, 1985; De Deurwaerdere et al., 2017; Eisenhofer et al., 2004). Tissue measurement of metabolites could also witness changes in synthesis/metabolism of monoaminergic terminals in some regions. For instance, we succeed to measure homovanillic acid, a metabolite of DA, in some brain regions and found a specific increase in MO at both 0.3 and 3 mg/kg WAY-163909 (data not shown). On the other hand, the correlative analyses revealed profound changes for all monoamines within and between monoaminergic systems including the NA one. Even though the number of non-correlating parameters remains the great majority and somehow similar, WAY-163909 dramatically changed the pattern of correlations of DA content toward low (0.3 mg/kg) to the high proportion (3 mg/kg) of the cortico-striatal pattern. The general profile for 5-HT content was similar with several correlations found in the basal ganglia. The content of NA followed an opposite pattern. The comparisons between pairs of monoamines highlight different points. The relationships between 5-HT content in the EPN and SN with cortical DA content in saline-treated rats were absent in WAY-163909. The DA content in the STN established correlations with diverse brain regions at 0.3 mg/kg whereas DA/5-HT pattern of correlations at 3 mg/kg was more cortico-striatal. Therefore, without quantitative modifications of DA tissue content or DA release at the regimen used in this study, one may suggest that the changes in cortico-striatal connectivity could also narrow DA tone. In line with this evidence, both Ro 60-0175 and WAY-163909 (1 mg/kg) have been reported to enhance the electrophysiological response of the cortico-striatal indirect pathway on SNr neuronal activity (Beyeler et al., 2010; Lagière et al., 2017), basically opposed to DA tone (Nambu, 2008).

The absence of dose-dependent effect (quantitative and correlative through a hypothetical reinforcement of correlations) is compatible with the recruitment of behavioral effects as the dose of the 5-$HT_{2C}$ receptor agonist increases (Lagiere et al., 2013). WAY-163909 preferentially affects oral movements/penile grooming at low doses (i.e., 0.3 mg/kg) and reduces food intake at higher doses (i.e., 3 mg/kg) (Dunlop et al., 2005; Marquis et al., 2007; Navailles et al., 2013b). Interestingly, we report a quantitative reduction of DA content in the dHY at 3 mg/kg while the correlations for all monoamines changed for dHY and vHY depending on the doses.

The change in connectivity induced by WAY-163909 across the brain regions is widespread. It is in line with accumulating evidence that several brain regions mediate the efficacy of 5-$HT_{2C}$ receptor agonists in food intake (D'Agostino et al., 2018; Xu et al., 2017; Xu et al., 2008), drug addiction (Filip and Cunningham, 2002, 2003; Fletcher and Higgins, 2011; Fletcher et al., 2004; Higgins and Fletcher, 2015; Higgins et al., 2012; Howell and Cunningham, 2015), impulsive/compulsive behavior (Anastasio et al., 2015; Carli and Invernizzi, 2014; Fletcher et al., 2007) or anxiety (Di Giovanni and De Deurwaerdere, 2016; Heisler et al., 2007; Millan, 2003). The circuits overlap and interact which is likely the meaning of the different patterns of correlations obtained at the two doses. Speculatively, the level of activity of monoaminergic terminals could be kept almost constant (homologous and heterologous controls), but the changes of activity of neurobiological networks induced by WAY-163909 impact local partners of monoaminergic terminals. The changes of correlations profile of monoamines tissue content would witness distinct modes of activities of neurobiological networks triggered by WAY-163909. Unfortunately, we cannot propose now a specific fingerprint of the pattern of WAY-163909's correlations, and additional studies are warranted with other 5-$HT_{2C}$ receptor agonists to determine if there is a complete change of the correlation profile with respect to saline-treated rats and between the doses. Therefore, the neurochemical approach is still interesting in view of the poor ability of 5-$HT_{2C}$ receptor agonists to trigger c-Fos expression to map their brain effect (De Deurwaerdere et al., 2013; Devroye et al., 2015; Lagière et al., 2017).

In conclusion, WAY 163909 slightly modified 5-HT and DA, but not NA tissue content in a very few regions. However, it completely changed the pattern of correlations of monoamines in the brain. This result suggests that the change of brain excitability induced by WAY-163909 impacts the organization of monoaminergic connectivity rather than inducing quantitative changes.

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