Università degli Studi di Cagliari

PHD DEGREE in

NEUROSCIENCE

Cycle XXXII

Title of the PhD Thesis

Behavioural and Neurochemical characterization of the effects of Fipronil in the rat brain

Scientific Disciplinary Sector(s)

M-PSI/02 – Psicobiologia e Psicologia Fisiologica

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Final exam. Academic Year 2018 – 2019

Thesis defence: February-2020 Session

Acknowledgments

I wish to convey a deep sense of gratitude to my academic tutor Prof.ssa **Maria Rosaria Melis** and to respected coordinator of Neuroscience Ph.D. course, Professor **Antonio Argiolas** for giving me opportunity to be a part of their research group. In particular for PART-1 of the thesis, I really feel glad to seek their esteemed guidance, untiring support and kind cooperation throughout the tenure. Their encouragement has made it easier to accomplish my research activities successfully.

I also give my sincere gratitude to **Dr. Fabrizio Sanna** for his constant motivation, supervision and guidance during each and every step of the study. I really thank him for his all-time support, collaborative help in organizing and performing behavioral experiments and neurochemical assays. He always stood as a troubleshooter and gave personal care.

I acknowledge my sincere thanks to Dott.ssa **Carla Lobina** for helping me with behavioural experiments and for her always cooperating attitude.

I am really grateful to Prof.ssa **Critina Cocco** and Dott.ssa **Giulia Corda** for their great support in performing the immunohistochemical assays for my experiments.

I extend my thankfulness to my friend **Jessica Bratzu**, **Alice Boi** and **Andrea Contini** for their help during the experiments and kind behaviour during the working time.

For PART-2 of my thesis, I wish to give my wholehearted gratitude to my supervisor, Professor **Philippe De Deurwaerdère**, Universite de Bordeaux, France, for giving me the opportunity to work in his research lab. I really thank him for his valueable guidance, support and sharing his deep knowledge which has helped me in developing my scientific skills and for taking care throughout my stay in Bordeaux. I am also grateful to Ms. **Salome De Deurwaerdère** for her kind help during my research project.

I would like to pay my deep gratitude to Prof.ssa **Micaela Morelli** for her kindness and encouragement. I also thank to Dott.ssa **Tiziana Cubeddu** and Dott.ssa **Ester Loi** from Euraxess office for their kind support in all administrative processes in Cagliari and in Europe.

I would also thank Amit Kumar, Amitash Ojha, Barnali Choudhary, Swapneel Thakker, Hema Sekhar Reddy Rajula, Vijay Sonar, Venkata Krishnan Ramaswamy, Susruta Samanta, Suchithra, Rajesh etc etc…. for their valueable friendship, get-together, celebrations, travelling and food which always made me feel close to home. I really enjoyed their companionship in Cagliari.

The words at my command are inadequate both in the form and spirit to express my deep-felt appreciation and gratitude towards my respected **Parents,** my loving wife **Rashmi** and my beautiful daughter **Aaradhya** for their continuous stream of love, encouragement and unselfish support. They have been my biggest source of strength and motivation. I also thank my other family members and friends for their wishes and blessings.

Last, but not the least, I express my gratitude to Prof.ssa **Maria Collu**, **Barbara Tuveri, Marta, Elena** and all other people in Neuroscience department for their kind cooperation and generous support in the mean course of time.

La presente tesi è stata prodotta durante la frequenza del corso di dottorato in **NEUROSCIENZE** dell'Università degli Studi di Cagliari, **A.A 2016/2017 - XXXII ciclo**, con il supporto di una borsa di studio finanziata con le risorse dell'Università degli Studi di Cagliari .

Rahul Bharatbhushan Bharatiya also acknowledges the Institut de Neurosciences Cognitives et Intégratives d'Aquitaine (INCIA) and Centre National de la Recherche Scientifique (Unité Mixte de Recherche 5287), Université de Bordeaux, France, for supporting him to carry out his research activities abroad.

ABSTRACT

Parkinson's Disease (PD) is a chronic neurodegenerative disorder characterized by motor dysfunction (including resting tremor, muscle rigidity and bradykinesia) and non-motor symptoms (i.e., cognitive deficits such as learning and memory impairments, dementia, pain and others) that affects 0.5-1% of the population aged 65 year and over. The main cause is a selective and progressive degeneration of nigrostriatal dopaminergic neurons originating in the substantia nigra (SN) and projecting to the striatum, which leads to a marked decrease in striatal dopamine (DA) content. It is well accepted that environmental factors may play a main role in the initiation and progression of the disease. Among these are the pesticides as significative associations between some of these and the incidence of the disease have been identified. The aim of my thesis is to better characterize the neurotoxic effect of fipronil (FPN), a phenylpyrazole pesticide which is used to prevent insects such as fleas and ticks from plaguing cats, dogs and other pets as well as in agriculture for repelling a variety of insects from crops and homes (termites, fire ants, etc.).

For this purpose, two types of experiments were performed in adult male rats: the first experiment (Part 1 of the thesis) was aimed at studying the effect of FPN injected unilaterally, directly into the right substantia nigra (SN) of male rats on different tests (i.e., open field, rotarod test, tail flick test, novel object recognition test, social interaction test) 7 and 14 days after the injection, accordingly, to study that a deficit in the performance in these tests well correlate to a degeneration of the nigrostiatal dopaminergic system. Rats unilaterally injected with vehicle (DMSO) into right SN were used as controls. Unilaterally SN FPN-treated and control rats were also treated with a systemic challenge dose of the DA-agonist apomorphine to study the presence of a rotational behaviour, possibly correlated to a degeneration of the nigrostriatal dopaminergic system. Fifteen days after the injection, right SN FPN-treated and SN control DMSO-rats were sacrificed, their striata was removed for the determination of DA content in the ipsilateral and in the intact controlateral striatum as well as the SN was used for tyrosine hydroxylase (TH) immunoreactivity (by immunohistochemistry) in order to correlate behavioral changes to the neurodegeneration of the nigrostriatal dopaminergic system. The results confirm that FPN unilateral injection into the right SN caused a degeneration of the nigrostiatal dopaminergic neurons which leads to a decrease around 50% in striatal DA content and SN TH imunoreactivity with respect to control values. These changes are usually, but not always, correlated with

changes in motor activity and coordination, in nociception and cognition, and resemble those found in rats treated with other neurotoxins in the SN (6-hydroxydopamine, rotenone, MPTP and MPP^+).

The second type of experiment (part 2 of the thesis) was aimed at studing the effect of FPN chronically administered by oral gavage for 21 days to adult male rats on brain monoaminergic systems in order to evaluate possible alteration in the content of monoamines [noradrenaline (NA), DA, serotonin (5-HT) and their metabolites] and thus on monoaminergic connectivity across different brain areas induced by the exposure to the pesticide. FPN was able to significantly decrease DA and its metabolites levels in most striatal territories including the core of the nucleus accumbens and the substantia nigra (SN). The pesticide also diminished 5-HT levels in some striatal regions including the core, anterior and ventral striatum, and in SN. The indirect index of the turnover 3,4-dihydroxyphenylacetic acid (DOPAC)/DA ratio and 5-hydroxyindole-3-acetic acid (5-HIAA)/5-HT ratio were correspondingly increased in numerous brain regions. FPN reduced NA content only in the core of the nucleus accumbens. Using the Bravais-Pearson test to study the neurochemical organization of monoamines through multiple correlative analyses across the brain, fewer correlations for NA, DOPAC/DA ratio and 5-HIAA/5-HT ratio, and an altered pattern of correlations within and between monoamine systems were found. This suggests that chronic exposure to FPN quantitatively reduces DA and 5-HT content in some brain regions and qualitatively leads to changes in the whole central monoamine connectivity.

Together, the results of my thesis confirm that FPN exerts neurotoxic effects in the rat brain, that are accompanied by a decrease in DA and 5-HT content in the striatal territory, supporting the hypothesis that exposure to this pesticide may also contribute to the initiation and/or progression of Parkinson’s disease in humans.

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AIM OF THE THESIS

The aim of my thesis is to better characterize the neurotoxic effect of fipronil (FPN), a phenylpyrazole pesticide used to prevent insects such as fleas and ticks from plaguing cats, dogs and other pets as well as in agriculture for repelling a variety of insects from crops and homes (termites, fire ants, etc.), in order to ascertain whether rats treated with this compound may be considered useful as a living rat model of PD. For this purpose two types of experiments were performed in male rats: the first (Part 1 of the thesis) was aimed at studying the effect of FPN unilaterally injected directly into the right substantia nigra (SN), the second (Part 2 of the thesis) was aimed at studying the effect of FPN administered chronically by gavage for 21 days to male rats on brain monoaminergic systems in order to evaluate possible alteration in the content of monoamines [noradrenaline (NA), dopamine (DA), serotonin (5-HT), and their metabolites)] and thus on monoaminergic connectivity across different brain areas induced by the exposure to the pesticide.

When FPN was administered directly into the substantia nigra of male rats, two increasing doses of FPN (15 µg and 25 µg) were unilaterally microinjected into the right SN and spontaneous locomotor activity and rotarod test were performed 7 and 14 days later, accordingly, to study that a deficit in these performances well correlate to a degeneration of the nigrostiatal dopaminergic system. The rats were also treated with a systemic challenge dose of the DA-agonist apomorphine to study the presence of a rotational behaviour, possibly correlated to a degeneration of the nigrostriatal dopaminergic system. In order to correlate the behaviour to the neurodegeneration of the nigrostriatal dopaminergic system, the content of DA was measured in homogenates of the striatal tissue of these animals either by High Pressure Liquid Chromatography (HPLC) and immunohistochemistry (IHC) was performed by counting nigral TH immunoreactive neuronal cells. Moreover, since cognitive deficits as well as algesia are present in patients affected by PD, it was also studied in the rats unilaterally injected with FPN the presence of either cognitive/social interaction deficits or algesia by Novel object-recognition test, Social interaction test or Tail flick test.

When FPN was administered chronically by oral gavage for 21 days to male rats, its effect on brain monoaminergic systems (NA, DA, 5-HT and their metabolites) and thus on monoaminergic connectivity across different brain areas was investigated. The results of these

experiments show that FPN significantly decreased DA levels and its metabolites in most striatal territories including the core of the nucleus accumbens, and the SN. The pesticide also diminished 5-HT levels in some striatal regions including the core, anterior and ventral striatum, and in SN. The indirect index of the turnover of DA (DOPAC/DA ratio) and 5-HT (5-HIAA/5-HT ratio) were correspondingly increased in numerous brain regions. FPN reduced NA content only in the core of the nucleus accumbens. Using the Bravais-Pearson test to study the neurochemical organization of monoamines through multiple correlative analyses across the brain, the pattern of correlations within and between monoamine systems was analyzed.

Abbreviations

aCd (anterior caudate); **aCg** (anterior cingulate cortex); **ains** (anterior insular cortex); **BBB** (blood brain barrier); **BLA** (basolateral nucleus of amygdala); **CE** (central nucleus of amygdala); CNS (central nervous system); **COMT** (catechol-O-methyltransferase); **core** (core of the nucleus accumbens); COX (cyclooxygenase); **DA** (dopamine); **6-OHDA** (6-hydroxy dopamine); **dHP** (dorsal part of hippocampus); **dHY** (dorsal part of hypothalamus); **DLS** (dorsolateral striatum); **DMS** (dorsomedial striatum); **DMSO** (dimethyl sulphoxide); **DOPAC** (3,4-dihydroxyphenylacetic acid), **DRN** (dorsal raphe nucleus); **EPN** (entopeduncular nucleus); **FPN** (fipronil); GABA (γ-aminobutyric acid); **$GABA_A$** receptor (γ-aminobutyric acid A receptor); **GFAB** (glial fibrillary acidic protein); **GLU-Cl receptor** (Glutamate-gated chloride receptors); **GPe** (the globus pallidus pars externa); **Hb** (habenula); **HPLC** (high pressure liquid chromatography); **HVA** (homovanillic acid); **Iba-1** (Ionized calcium Binding adaptor molecule); **IFN-γ** (interferon-gamma); **IL** (infralimbic cortex); **iNOS** (inducible nitric oxide synthase); **M2** (motor cortex M2); **MAO** (monoamine oxidase); **MPP^+** (1-methyl-4-phenylpyridinium); **MPTP** (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine); **MRN** (median raphe nucleus); **NA** (noradrenaline); **NAc** (nucleus accumbens); **OFC** (orbitofrontal cortex); **PBS** (Phosphate-buffered saline); **PD** (Parkinson's disease); **PGE2** (prostaglandin E2); **PL** (prelimbic cortex); **ROS** (reactive oxygen radical species); **rpm** (revolutions per minute); **5-HIAA** (5-hydroxyindole-3-acetic acid); **5-HT** (5-hydroxytryptamine; serotonin); **shell** (shell of the nucleus accumbens); **SN** (substantia nigra); **SN1** (medial part of substantia nigra); **SN2** (lateral part of substantia nigra); **SNpc** (substantia nigra pars compacta); **SNpr** (substantia nigra pars reticulata); **STN** (subthalamic nucleus); **Th** (thalamus); **TH** (tyrosine hydroxylase); **TNF-α** (tumor necrosis factor-alpha); **VCS** (ventro-caudal striatum); **vHP** (ventral part of hippocampus); **vHY** (ventral part of hypothalamus); **VLS** (ventrolateral striatum); **VMS** (ventromedial striatum); **VTA** (ventral tegmental area).

PART-1

Behavioural and neurochemical characterization of the effects of Fipronil- unilaterally injected into right substantia nigra of the rats

This part of the thesis was done under the supervision of ***Prof.ssa*** **Maria Rosaria Melis**, Ph.D., Professor of Psychobiology and Physiological Psychology, at the Department of Biomedical Sciences, Section of Neuroscience and Clinical Pharmacology, **University of Cagliari, Italy**.

1. Introduction

Parkinson Disease: State of the art

1.1 Parkinson disease is a neurodegenerative disorder characterized mainly by motor dysfunction

Parkinson's disease (PD) is a chronic neurodegenerative disorder, second only to Alzheimer's disease, the other most common neurodegenerative disease, first described as a neurological syndrome by James Parkinson in 1817. PD is a complex disorder characterized by various motor, sensory, autonomic, and psychiatric signs and symptoms. Among risk factors, the age is the greatest risk factor for the development of PD since the incidence of PD increases nearly exponentially with age and peaks after 80 years of age (Beitz et al., 2014; De Virgilio et al., 2016; Driver et al., 2009; Pringsheim et al., 2014; Hirsch et al., 2016). Since the life expectance is supposed to increase worldwide with important health implications, the number of people with PD is supposed to increase more than 50% by 2030 (Dorsey et al., 2007). It is estimated that approximately 5–10% of cases are caused by inheritable genetic mutations and the remaining 95% of newly diagnosed PD cases are of idiopathic origin (Toulouse and Sullivan 2008). Idiopathic PD has been associated with risk factors including aging, family history, environmental chemicals, drug of abuse, traumatic brain injury as well as pesticide exposure (Ascherio and Schwarzschild al., 2016; Beitz et al., 2014; De Virgilio et al., 2016; Driver et al., 2009; Pringsheim et al., 2014). Accordingly, recent publications suggest that environmental stress and aging itself may promote neuropathology. Specifically, exposure to environmental toxins (e.g. pesticides), drugs of abuse, or the stress of the aging promotes a chronic inflammatory process that over time generates cellular senescence in brain neurons (Brown et al., 2006; Ceccatelli, 2013; Chinta et al., 2013). Many other risk factors have been suggested although epidemiologic evidence is not as robust. These include: use of well-water, milk consumption, excess body weight, exposure to hydrocarbon solvents, living in rural areas, farming or agricultural work, living in urban areas or industrialized areas with exposure to copper, manganese and lead, high dietary intake of iron, history of anemia and even higher levels of education (Breckenridge et al., 2016; Jankovic et al., 2019).

PD is characterized mainly by a motor dysfunction which includes resting tremor (initially unilateral), bradykinesia (slow movements), muscle rigidity, shuffling gait, and postural

instability. The onset is insidious when individuals may attribute the symptoms to aging processes. PD symptoms are progressive but rates of motor progression are highly variable (Fritsch et al., 2012). Also, subtypes of PD occur wherein tremor, muscle rigidity, or postural instability dominate (Chou, 2013). In addition to the "classic" motor symptoms previously described, other motor manifestations are observed. These include masked facial expression (hypomimia), decreased eye blink rate, blurred vision, impaired upward gaze, dystonia, stooped posture, difficulty turning in bed, kyphosis, scoliosis, "freezing" (inability to move) and speech impairment, such as hypophonia (increasingly soft voice), or palilalia (repetition of word or phrase) and dysarthria (Chou, 2013; Gazewood et al., 2013).

1.2 Parkinson's disease is also accompanied by many non-motor symptoms

It is known that almost 90% of PD patients experience non-motor symptoms during the course of the disease (Löhle et al., 2009). Notably, a number of non-motor features can precede the motor symptoms of PD by years, even decades, i.e. olfactory deficits and sleep abnormalities. The usual non-motor symptoms include olfactory deficits, sleep disorders and insomnia, depression, anxiety, cognitive impairment and dementia, psychosis, autonomic dysfunction, gastrointestinal, orthostatic hypotension, sweating, urologic, sexual dysfunction and pain syndrome (Adler, 2005; Chaudhuri and Schapira, 2009).

Regarding to olfactory deficits, there is good evidence that most PD patients develop impairment of olfaction (hyposmia) 4–6 years before they start to present motor impairment (Müller et al., 2003). Accordingly, Lewy bodies and Lewy neurites are present in the olfactory bulb even when the patients were still in the premotor phase of PD and there is a good correlation between α-synuclein pathology and the clinical symptom of hyposmia (Braak et al., 2004). Several studies have been performed to test the hypothesis that hyposmia may be a valuable tool for the detection of early pre-motor PD (Reichmann, 2017). Hyposmia is detected in more than 90% of PD patients. Most affected PD patients are usually unaware of the deficit. Olfactory testing helps with differential diagnosis of idiopathic PD versus other Parkinsonian Syndromes (Löhle et al., 2009; Reichmann, 2017). There is a good correlation between α-synuclein pathology and the clinical symptom of hyposmia in early state of PD (Reichmann, 2017).

Sleep disorders also belong to the most frequent non-motor symptoms in PD and can be observed in almost two-thirds of PD patients (Tandberg et al., 1999). Rapid eye movement sleep behavioural disorder is a frequently observed sleep disorder in PD patients (Comella et al. 1998) and may even precede the onset of motor symptoms by several years (Löhle et al., 2009; Schenck et al. 1996). Most commonly, early morning awakening and frequent waking during the night are reported. Rest tremor may act to awaken the patient during light sleep (Chou, 2013).

Daytime somnolence is also a problem; PD patients may be sleepy or experience "sleep attacks" (unintended sleep episodes). Whether they are due to PD or PD therapy is uncertain with obvious problems to driving a car. Fatigue can be related to sleep issues or occur independently (Chou, 2013). Research suggests that sleep disorders seriously lower quality of life for PD patients affected (Opara et al., 2012).

Cognitive (dementia) and neurobehavioral deficits such as mood disorders likely depression, anxiety, apathy and autonomic dysfunction (e.g., orthostasis and hyperhidrosis and erectile dysfunction) can be also present (Beitz, 2014) impairing further the quality of life in PD patients.

Depression is the most commonly explored mood disorder influencing quality of life in PD and has been found to be the best predictor overall for quality of life in several studies (Opara et al., 2012). Besides depression, anxiety disorders are a clinically significant problem in patients with PD and anxiety is the most frequent psychiatric mood disorder in PD; accordingly, the prevalence of anxiety has been typically found to affect 20–46% of PD patients.

Anxiety is thought to have an important impact on motivation, treatment compliance, and cognition and can exacerbate parkinsonian symptoms (Opara et al., 2012). Apathy (loss of motivation) and abulia (loss of ability to think or act) can also occur and, together with anxiety, have a big impact on the quality of life (Chou, 2013).

Cognitive dysfunction and dementia are common in PD, but develop over time. The dementia of PD is subcortical with altered personality, psychomotor retardation, and memory problems. Problems with decision-making, multiple tasks, memory retrieval and visuospatial perception are present (Palmeri et al., 2017). Dementia in PD occurs later in the disease. On the other hand, early onset dementia is associated with the Parkinsonian Syndrome and Dementia with Lewy Bodies. A six-fold increased risk for developing dementia accompanies PD (Aarsland

et al., 2017a). Dementia is more common in PD patients with a strong family association of PD. However, up to 60 percent of PD patients develop dementia within 12 years of diagnosis (Gazewood et al., 2013), and higher plasma α-synuclein levels and cognitive impairment have been found to have a correlation in PD patients (Aarsland et al., 2017b).

Psychosis with hallucinations can also occur in PD patients. Visual hallucinations are the most common psychotic symptoms. Positive symptoms in PD vary across its course. Early in the disease, symptoms experienced include passage hallucinations (where a person, animal or indefinite object is seen briefly passing in the peripheral visual field), illusions (for example, seeing the branch of a tree as a cat), and presence hallucinations (a feeling that someone is nearby). Later in PD, formed visual hallucinations, typically of animals or people, occur (Ffytche and Aarsland, 2017; Ffytche et al., 2017; Löhle et al., 2009). Up to 40 percent of drug-treated PD patients demonstrate some form of psychosis. This is a serious problem as all anti-parkinsonian medications have demonstrated able to cause induction of psychosis (Chou, 2013).

Psychosis spectrum symptoms can be predictors of future cognitive decline in PD: a range of factors have been identified, the interrelationship of which has yet to be clarified, likely a correlation with amyloid and tau pathology (Ffytche and Aarsland, 2017; Ffytche et al., 2017).

Cognition and visual hallucinations are closely related. Subsequent studies have replicated the finding of limbic pathology associated with visual hallucinations. Visual hallucinations are linked to Lewy bodies presence in cortical regions (Ffytche et al., 2017). Unlike patients with PD psychosis who have dementia, those without dementia usually do not have cortical Lewy body involvement (Ffytche et al., 2017).

Autonomic disturbance or aberration is manifested in multiple body systems in such conditions as orthostasis, constipation, fecal incontinence; lower urinary tract symptoms are high in PD patients negatively affecting their quality of life. The quality of life in PD patients is also compromised by dysphagia and sialorrhea (excessive salivation). Seborrhea, hyperhidrosis (excessive sweating especially at night) and increased risk for malignant melanoma, and non-melanoma skin cancers can also be present (Löhle et al., 2009; Beitz, 2013). The risk of dysfunctions and greater disease severity increases with higher age.

Sexual dysfunction is another non motor symptom that compromises the quality of life of PD patients; as men with PD may develop erectile dysfunction.

Additionally, PD patients suffer from painful sensations such as musculo-skeletal pain due to parkinsonian rigidity and skeletal deformity, radicular-neuropathic pain, dystonic pain, central neuropathic pain (Young Blood et al., 2016). Painful sensory symptoms can be localized or general and have been described as burning, tingling or lancinating (Chou, 2013). Estimated to affect about 2/3 of PD patients, the pain is likely due to musculoskeletal pain, radicular pain and other pains (non-radicular low back pain, arthritic, and visceral pain), central neuropathic pain and dystonic pain (Valkovic et al., 2015). All types of pain were more prevalent in advanced-stage PD patients than in early-stage PD patients, except for arthritic pain (subclassified under "other pain"). The frequency and intensity of actual, average, and worst experienced pain were significantly more severe in advanced-stage patients. Some hypothesize that there is abnormal processing of nociceptive inputs in PD patients. Health-related quality of life can be enormously affected by the chronic pain (Löhle et al., 2009).

PD patients with general pain and in advanced stages were more depressed and had poorer quality of life. Depression correlated with worst pain in the last 24 hours and with pain periodicity (the worst depression score in patients with constant pain) (Valkovic et al., 2015). Pain is a frequent problem in PD patients, and it worsens during the course of the disease (Valkovic et al., 2015).

1.3 Eziopathological features of PD

The major pathologic feature of PD is the profound loss of pigmented neurons, mainly the dopaminergic neurons of the substantia nigra pars compacta of (SNpc) of the midbrain with gliosis and a decrease in striatal dopamine (DA) level (Fahn, 2003; Postuma et al., 2009; Jankovic et al., 2013). Associated with this neuronal loss is the presence of large eosinophilic inclusions, called Lewy bodies, within the remaining pigmented neurons, made up of a series of proteins, including neurofilaments, α-synuclein fibrils, ubiquitin, parkin, and proteasomal elements, and although the diagnosis of PD is entirely clinical, histopathology on autopsy is the only way to definitively confirm the diagnosis (Ascherio and Schwarzschild, 2016; Beitz et al., 2014; De Virgilio et al., 2016; Driver et al., 2009; Pringsheim et al., 2014). However, a certain level of dopaminergic degeneration of approximately 50% in SNpc and 80% in striatum must be reached for PD symptoms to become apparent (Bernheimer et al., 1973; Hauber, 1998; Postuma et al., 2009; Toulouse and Sullivan 2008; Jankovic et al., 2013). Up to this level, changes in

residual DA neurons are thought to compensate for the loss of DAergic innervation. These compensatory mechanisms include the increase in DA synthesis, metabolism, release and a decrease in reuptake (Blesa et al., 2017). In addition to motor dysfunctions, PD can also be associated with cognitive impairment (dementia) and the no appearance of memory loss in all PD patients can be due to compensatory mechanisms occurring in the putamen (Hornykiewicz, 1993; Poston et al., 2016; Blesa et al., 2017).

Depigmentation can also occur on noradrenergic neurons of the locus ceruleus as well as on dopaminergic neurons of the ventral tegmental area (Bogerts et al., 1983; Postuma et al., 2009; Seidel et al., 2015; Jankovic et al., 2013; see also Beitz et al., 2014). Neuromelanin plays a toxic role in neurodegenerative conditions like PD. Extracellular neuromelanin deposits release toxic metals inducing oxidative stress, microglial activation and chronic local neuroinflammation (Martin-Bastida et al., 2017).

It has been hypothesized that an imbalance between the cytoprotective and cytotoxic action of the pigment melanin, may cause neuronal death via mitochondrial oxidative stress, inhibition of ubiquitine-proteasome system and α-synuclein accumulation (Stepień et al., 2007). Extraneuronal melanin may contribute to chronic inflammation by excessive secretion of cytokines and nitric oxide due to prolonged microglia activation (Bogerts et al., 1983; Stepień et al., 2007). Genetic mutations that code proteins of the central nervous system play also a role in neuronal death by causing abnormal forms of α -synuclein, which self aggregate either in the SN or the ventral tegmental area (VTA) (Fasano and Lopiano, 2008) as well as in noradrenergic neurons of the locus ceruleus (Mills et al., 2017). As previously reported, these insoluble aggregates of α -synuclein are considered a marker of nerve cell degeneration in PD (Fasano and Lopiano, 2008; Seidel et al., 2015; Jankovic et al., 2013; but see also Beitz et al., 2014; De Virgilio et al., 2016; Driver et al., 2009; Pringsheim et al., 2014). In addition, systems designed to break down abnormal proteins like the ubiquitin – proteasome system also become impaired (Fasano and Lopiano, 2008). Other impaired processes that may play a role in PD are mitochondrial dysfunction or abnormal oxidative stress through reactive oxygen species (ROS) causing neuronal degeneration (Fasano and Lopiano, 2008; Jankovic et al., 2013).

Interestingly, PD has a more benign course in females than in males, speculated to be caused by higher estrogen activity, which leads to higher dopamine levels in the striatum (Haaxma et al., 2007).

At the moment, neuroimaging plays a small role in PD diagnosis and is not used routinely. Studies like magnetic resonance imaging (MRI), ultrasonography, positron emission tomography (PET) scan, etc., lack evidence in diagnosing PD. At best, they may help distinguishing PD from Multiple System Atrophy or Essential Tremor but not idiopathic PD itself (Fahn et al., 2010). Despite the best of contemporary medical and surgical therapies, PD steadily worsens over time in both motor and non-motors aspects. Mortality rates are higher in PD patients versus matched controls. The mean age at death is about the same (mid-70s) regardless of age of onset and quality disease management (Fahn et al., 2010).

1.4 Treatments of PD

Recent decades have witnessed a proliferation of medical pharmacologic therapies and innovative surgical interventions like deep brain stimulation (DBS). The combination of carbidopa and levodopa (L-DOPA, 3,4-dihydroxyphenylalanine) is the most effective agent available for the treatment of motor symptoms. However, its early use is associated with earlier development of dyskinesias (abnormal involuntary movements) and non motor symptoms of PD; likely, psychosis, orthostatic hypotension and sleep attacks may relate to L-DOPA dosing or side effects (Lim and Lang, 2010; Gazewood et al., 2013).

Dopamine receptor agonists are less effective than L-DOPA in treating motor symptoms of PD, but have a lower incidence of dyskinesias. Ergot-derived DA receptors agonists such as cabergoline, lisuride and pergolide should not be used as first-line treatments because of the risk of serosal fibrosis and cardiac valvulopathies. Monoamine oxidase-B (MAO-B) inhibitors are less effective than either carbidopa/ L-DOPA or DA agonists in treating motor symptoms of PD but cause less dyskinesia than carbidopa/ L-DOPA, and generate fewer adverse effects than DA agonists (Gazewood et al., 2013).

Besides motor symptoms, non-motor symptoms can represent some of the greatest challenges to the quality of life and appropriate management of PD patients, since these symptoms usually do not respond to DA therapy as well as motor symptoms do (Lim and Lang, 2010; Gazewood et al., 2013).

While the most important contemporary PD therapy is still medical (pharmacological), DBS provides therapy to some PD patients, but adverse effects can be more frequent in these patients; these included surgical site infection, falls, and depression. DBS does not slow disease

progression, and patients eventually develop treatment-resistant symptoms such as gait freezing (Gazewood et al., 2013). Although at the moment a definitive disease-modifying therapy is still lacking and experimental therapies are being developed and tested with limited results, experimental therapies other than DBS offer hope and a possible treatment option. These include stem-cell-based therapies (Loewenbruck and Storch, 2011), bright light therapy for PD-related sleep disorders (Rutten et al., 2012) and gene-therapy (Berry and Foltynie, 2011).

Other non-pharmacological alternative therapies like exercise, education, support groups, speech therapy and nutrition, while not slowing the inexorable course of PD, each offers benefit to some aspects of the disease and/or deal with its pathophysiologic impact. It is important to begin their usage early in the disease course (Beitz, 2014; Gazewood et al., 2013). Knowledge of strategies to promote optimal quality of life for PD patients is of paramount importance for caregivers, health providers and patients themselves.

1.5 Inflammation and PD

Inflammation is a response of a tissue to injury, ischemia, autoimmunity, toxic metabolites, or infectious agents. It is closely related to the initiation and progression of neuronal cell damage and plays a significant pathological role in the development of neurodegenerative diseases (Hunot et al., 1997; Slemmer et al., 2008; Sofroniew 2009; Sofroniew and Vinters, 2010; Stojkovska et al., 2015). It is well known that during an inflammatory response in the brain, microglia become activated, releasing interleukin-1b (IL-1b), IL-6, IL-8, tumor necrosis factor- α (TNF- α), and superoxide ions. Post-mortem studies examining the SN of PD patients have revealed the presence of reactive microglia. Activated microglia is prevalent not only in acute traumatic and toxic lesions, but also appears with increasing frequency with age in the human brain. Increased expression of numerous pro-inflammatory enzymes and molecules, such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), prostaglandin E2 (PGE2), TNF-α and interferon-gamma (IFN-γ), has been reported in reactive glial cells, and these pro-inflammatory and cytotoxic factors derived from the activation of microglia induced by damage of the central nervous system, can lead to dopaminergic neuronal cell death. One of these pro-inflammatory enzymes, COX-2, plays a key role in the inflammatory process and is increased in the ventral midbrain of PD subjects compared to controls (Teismann et al., 2003; Okuno et al., 2005; Block and Hong, 2005; Park et al., 2016b).

Inflammatory component in PD not only involves deregulation of inflammatory pathways resulting from genetic vulnerability (De Virgilio et al., 2016) but also immune alterations associated with aging and with primary activation of glia in the presence of neuronal injury (Ceccatelli, 2013; Chinta et al., 2013). Accordingly, exposure to environmental toxins has been identified as a substantial causal risk factor for the majority of age-dependent diseases, and not only for diabetes, heart disease, stroke, but also for neurodegenerative disorders such as Alzheimer's disease and PD (Chinta et al., 2013). In particular, neuronal damage is believed to involve the induction of neuroinflammatory events mediated by glial cell activation. Senescent cells accumulate with age, and they express a senescence associated secretory phenotype (SASP) connected to the secretion of many inflammatory cytokines, growth factors and proteases. Senescent glia contributes to age-related neurodegeneration by creating a chronically inflamed milieu (Ceccatelli, 2013). Accordingly, proliferative glial cells (i.e. astrocytes, oligodendrocytes and microglia), which normally provide structural, metabolic and trophic support to neurons, can also have detrimental effects on neighboring neurons due to chronic production of pro-inflammatory factors, including ROS and leukocyte-attracting cytokines, which occurs with increasing frequency during ageing (Chinta et al., 2013). Environmental stressors associated with PD, likely chemicals such as pesticides and 1-methyl-4-phenyl-1,2,3,6-tétrahydropyridine (MPTP) may act in part by eliciting senescence and senescence associated secretory phenotype expression by glial cells in the aging brain, thereby contributing to the characteristic decline in neuronal integrity that occurs in PD (Ceccatelli, 2013). Accordingly, the incidence of diabetes, heart disease, stroke, Alzheimer's disease and PD, which are major causes of death in both industrialized and developing countries, has risen markedly in the last century, largely due to the increase in life-expectancy as well as environmental stressors. The human brain is very susceptible to oxidative stress and PD is one of the most prominent neurodegenerative diseases in which environmental exposure to chemicals plays a significant role. In agreement to what above reported, experimental data in animal models have confirmed that environmental exposure to toxins is capable of inducing senescence and an accompanying senescence associated secretory phenotype, which could contribute to neurodegeneration associated with both normal brain ageing and neurodegenerative disease (Chinta et al., 2013).

1.6 Pesticides, Inflammatory Processes and PD

Positive associations have been found between Parkinson disease risk and exposure to pesticides known to affect mitochondrial complex I (including rotenone) or to cause oxidative stress (including paraquat). Overall, evidence that pesticide exposure increases PD risk is substantial, but the risk associated with specific compounds remains uncertain (Ascherio and Schwarzschild, 2016). It has been reported that pesticides are able to induce PD development if they have effects on the SN dopaminergic neurons to induce a decrease in DA levels and/or an increase in DA turnover as a short term compensatory mechanism, which would be identified by an increase in metabolites or the enzyme tyrosine hydroxylase (TH) as well as mechanistic effects (for example, on oxidative stress, mitochondrial dysfunction/complex I inhibition, and α-synuclein levels and aggregation (Brown et al., 2006). Among pesticides that have been found able to induce inflammation at central level the most studied are rotenone, paraquat, cyclodienes, pyrethroids and fipronil. However, only for rotenone, paraquat and, to a lesser extent, fipronil, evidence has been accumulated that these compounds may be involved significantly in the induction of PD.

1.6.1 Rotenone

Rotenone is a naturally occurring insecticide and acaricide with broad spectrum of action, isolated from the roots of tropical plants of the Legumes families (*Derris elliptica, Derris involuta, Lonchocarpus utilis, lonchocarpus urucu*, etc.) that is extensively used as herbicide or pesticide and is a well-characterized high-affinity specific inhibitor of complex I (NADH-dehydrogenase). It induces toxicity and apoptosis through the selective inhibition of complex I in mitochondria. Due to its hydrophobic structure, rotenone crosses biologic membranes with a easily access to the cytoplasm. It is likely that rotenone produces systemic inhibition of complex I and oxyradical generation accompanied by lesion of nigrostriatal dopaminergic neurons (Betarbet et al. 2000, 2002; Jenner, 2008). Rotenone-lesioned rats also present cytoplasmic inclusions containing α-synuclein in the nigral neurons, which resembled the precursors to Lewy bodies found in humans with PD. Erbaş et al. (2012, 2013) have used selective inhibitors of the electron transport chain (e.g., complex I), such as MPTP and rotenone, to induce selective toxicity on dopaminergic neurons as *in vitro* and/or *in vivo* experimental models of PD. Regarding rotenone, animals treated with this compound also develop motor and postural deficits

characteristic of PD. The severity of these deficits correlated with the extent of the pathologic lesions, even after cessation of the rotenone treatment. The presence of activated microglia in the brain has been implicated in rotenone neurotoxicity (Brown et al., 2006; Erbaş et al., 2012, 2013; Von Wrangel et al., 2015). Chronic environmental rotenone exposure in laboratory animals was also studied by these authors. In particular, in contrast with earlier results showing that inhalation of rotenone for 30 days in mice failed to produce clinical signs of Parkinsonism (Rojo et al., 2007), in the studies of Erbaş et al. (2012, 2013), rotenone injection directly into the SN successfully produced a model of PD either for motor and postural and/or neurophatological disturbances.

1.6.2 Paraquat

Paraquat is a nonselective contact herbicide with high pulmonary toxicity (Corasaniti et al., 1998). Paraquat is a charged molecule, which may not cross the blod brain barrier (BBB) and it is not metabolized to a species more likely to gain access to the brain (Sanchez-Ramos et al., 1987). Paraquat did not appear to induce a major neurotoxicologic risk in brain areas with a functional BBB (Shimizu et al., 2001), but further experiments suggested the involvement of the neutral amino acid transporter in the passage of paraquat from blood into the brain, followed in turn by transportation into striatal, possibly neuronal cells, in a Na^+-dependent manner (Shimizu et al., 2003). Although not directly relevant to human exposure pathways, paraquat has been shown to be neurotoxic after direct injection into brain areas, and depending on the brain region into which the compound was injected, it produced different behavioral patterns. In particular, an increase in dopaminergic neuronal death was observed in the SNpc of intranigral paraquat-treated rats, with no depletion in striatal DA but enhanced DA synthesis, as indicated by an increased activity of striatal TH (McCormack et al. 2002). Moreover, depending on the brain area injected with paraquat, increased locomotor activity and convulsions were also observed; these effects were accompanied by neuronal cell death and led to suggest that paraquat neurotoxicity was not specific to the dopaminergic nigrostriatal system, because the above effects were observed when paraquat was injected into regions of the brain rich in neurotransmitters other than DA. On the other hand, no neurotoxic effects or changes in brain DA levels were observed (Brown et al., 2006). However, in rats treated intravenously with paraquat, the brains were found to have lower complex I activity and higher levels of lipid

peroxides (which indicate free radical activity) and a lower level of DA in the striatum (Tawara et al., 1996).

1.6.3 Cyclodienes

Bloomquist and colleagues (1999) have carried out studies examining possible effects of the organochlorine cyclodiene pesticides, in particular, dieldrin and heptachlor, on possible biomarkers of PD. Heptachlor increased the maximal rate of striatal DA uptake suggesting that this compound, and perhaps other organochlorine pesticides exert selective effects on striatal dopaminergic neurons and may play a role in the etiology of PD. There is some evidence that dieldrin may interfere with electron transport (complex I) and increases the generation of superoxide radicals (Stedeford et al., 2001). In one study, dopaminergic receptors were reported to be unusually sensitive to the action of endosulfan, an organochlorine cyclohexan pesticide (Seth et al., 1986).

1.6.4 Pyrethroids

Mice treated with the pyrethroid permethrin showed increased DA uptake at low doses whereas at higher doses DA uptake was reduced (Karen et al., 2001). Reduced mitochondrial function was also observed in *in vivo* synaptosome preparations, and although striatal DA levels were not decreased, there was an increased DA turnover and decreased motor activity. In particular, these results suggested that although frank Parkinsonism was not observed, dopaminergic neurotransmission can be affected by exposure to permethrin. When the pyrethroid insecticide fenvalerate was given orally to rats, a pronounced, but not dose-related, inhibition of DA and its metabolites and a decreased DA binding were found in several brain regions, including the striatum (Husain et al., 1991). In another study, a significant increase in DA and muscarinic receptors was found in striatal membranes of the rat pup, suggesting an alteration of both the dopaminergic and cholinergic pathways in the striatum (Malaviya et al., 1993).

1.6.5 Fipronil

Fipronil (FPN) is a phenylpyrazole pesticide used to prevent insects such as fleas and ticks from plaguing cats, dogs and other pets as well as in agriculture for repelling a variety of insects from crops and homes (termites, fire ants, etc.) (Tingle et al., 2003). Poultry (including egg-

laying hens) in the Netherlands and other EU countries were inadvertently exposed to FPN in 2017, and violative residues of the pesticide were detected in eggs across Europe and in China. Because FPN is highly lipophilic, it can become sequestered in tissues with a high lipid for an extended period of time (Stafford et al., 2018). FPN acts on ionotropic GABA ($GABA_A$) receptors of the insect nervous system and is a potent toxicant for mammals though less powerful than for insects. FPN can cause disruption of thyroid function in rats, in human hepatocytes and produce developmental neurotoxicity; moreover, FPN can bind to mammalian $GABA_A$ and GLU-Cl receptors and may concern its risk to human health (Mohamed et al., 2004; Varrò et al., 2009; Lee et al., 2010; Lee et al., 2011). Due to its antagonism at the $GABA_A$ receptor, FPN can cause effects such as convulsions (Woodward, 2012).

FPN has been also reported to be a potent disruptor of neuronal cell development in PC12 cells, used as a model of neuronal development, which lack the $GABA_A$ receptor (Lassiter et al., 2009), suggesting that this compound may cause neuronal cell toxicity through a different pathway than $GABA_A$ receptors. Accordingly, due to the increase in oxidative stress reported during the pesticide poisoning, including increased lipid peroxidation, diminished energy metabolism and decreased cytochrome oxidase activity, dopaminergic neurons may be preferentially targeted by the pesticide since, compared to other neuronal cells, dopaminergic cells are much more sensitive to oxidative injury (Lee et al., 2011) In line with this possibility, in *in vitro* studies FPN was found able to induces cell death of human dopaminergic SH-SY5Y cells (Lee et al., 2011; Park et al., 2016a), an in vitro model of dopaminergic neurons used in PD research. In particular, FPN acts on SH-SY5Y cells by increasing ROS generation and furthermore activating the caspase-3 apoptotic pathway system via cytochrome c release from mitochondria. FPN-induced apoptotic cell death in SH-SY5Y cells was due to the alteration of several proteins including Bcl2, p53, Akt and GSK_3 as a result of oxidative stress, confirming that the protein GSK_3 plays a key role in this apoptotic cell death, as found also for other pesticides, likely for rotenone. The cytotoxic effect of FPN to these cells was concentration- and time-dependent from the cell viability. Moreover, FPN treatment induced a decrease of TH expression but not that of glutamic acid decarboxylase (GAD65) expression. This suggests that FPN-induced cytotoxicity affects only the dopaminergic neuronal component among the other neuronal components of SH-SY5Y cells.

Interestingly, FPN injected into the SN of male rats was found able to induce an inflammatory response and a concomitant loss of nigrostriatal dopaminergic neurons (Park et al., 2016b). The inflammatory responses caused by FPN were revealed by increased levels of proinflammatory factors such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor-alpha (TNF-α) in the SN and in the striatum, by the up-regulation of glial fibrillary acidic protein (GFAP) expression and by the activation of microglia demonstrated in turn by an increase in Ionized calcium Binding adaptor molecule-1 (Iba-1) immunoreactivity in the striatum and SN. The increase in proinflammatory mediators in FPN-treated rats was inversely correlated to the loss of nigrostriatal dopaminerigc neurons, shown by the decrease in tyrosine hydroxylase (TH) immunoreactive neurons in the striatum and SN, decrease also confirmed by western blot analysis expression (Park et al., 2016b). These results are in line with those of many other studies supporting the roles for a number of environmental agents, such as rotenone, MPTP, and paraquat, in inducing PD-like syndromes and loss of dopaminergic neurons in the SN (Spencer et al., 1992; Ridet et al., 1997; Corrigan et al., 2000; McGeer et al., 2003; Block and Hong, 2005; Watanabe et al., 2008; Pan-Montojo et al., 2010; Walsh et al., 2011) as well as of 6-hydroxy-dopamine (6-OHDA), the highly potent catecholaminergic neurotoxin, extensively used to realize an animal model of PD (Mogi et al., 1994; Marinova-Mutafchieva et al., 2009; Lee et al., 2012).

2. MATERIALS and METHODS

2.1 Animals

Adult male Sprague Dawley rats, weighing 250-300 g (at the beginning of the experiments), were used in the study. Animals were housed in groups of 4 per cage, and maintained under standard conditions with 12-h light/dark cycles at room temperature (22 ± 2°C, 60 ± 5% humidity). They were fed with standard pellet diet and tap water ad libitum along the study. The rats were handled once daily in order to avoid the stress induced by the experimental procedures during the surgery and the experimental session, and also to familiarize them with the experimental operators. The behavioural experiments were performed between 09:00–16:00 h, according to the guidelines of the European Communities Directive of September 22, 2010 (2010/63/EU) and the Italian Legislation (D.L. March 4, 2014, n. 26), and approved by the Ethical Committee for Animal Experimentation of the University of Cagliari.

2.2 Drugs and reagents

Fipronil, apomorphine-HCl, dimethyl sulphoxide (DMSO) were purchased from Sigma Aldrich, (Dusseldorf, Germany), normal saline (0.9% NaCl) was prepared before experiments. All other reagents were from available commercial sources.

2.3 Microinjections into right substantia nigra (SN)

After a preliminary examination of the performance of the animals on the Rotarod test, on the locomotor activity apparatus, and the control of the body weight, rats were divided into three groups: group-1 is control-DMSO, group-2 is FPN 15 µg and group-3 is FPN 25 µg. For microinjections into the SN, rats were positioned in the stereotaxic apparatus (Stoelting® Co., Wood Dale, IL, USA) under isoflurane anesthesia (1.5–2.0%), the skin over the skull was cut with a lancet, the two edges of skin separated with pincers and a small hole was made in the skull with a dentist's drill, at the following coordinates relative to the bregma: AP: -5.3 mm; ML: -2.0 mm; DV: -8.0 mm (Paxinos and Watson, 2007). The solution of FPN was prepared in DMSO and diluted to the used concentrations (FPN 15 µg and FPN 25 µg) and infused unilaterally into the right SN in a volume of 1 µL/site by means of a 10 µL Hamilton microsyringe mounted on the holder of the stereotaxic apparatus (Stoelting® Co., Wood Dale, IL, USA) and driven by hand. Injection speed was 1 µL/min and the needle of the microsyringe was kept in the injection place for additional 3 min to allow a better diffusion of the injected solution before being slowly

retracted. Group 1 (Control group) rats received an unilateral injection of 1 µL DMSO into the right SN (since now DMSO-treated rats), Group 2 (FPN 15) and Group 3 (FPN 25) rats received an unilateral injection of 15 µg and 25 µg of FPN in 1 µL of DMSO into the SN (since now FPN 15- and FPN 25-treated rats), respectively. All rats were given 5 days to recover after the microinjections and behavioural tests were performed thereafter. The body weight of all animals was recorded after the recovery period daily up to 15-days post-intranigral microinjection. The Rotarod test was performed after 7 days of surgery once daily until day 15 and other behavioural tests such as locomotor activity, apomorphine-induced rotation test, tail flick test, novel object recognition test and social interaction test were performed 14-16 days after FPN microinjection.

2.4 Behavioural tests

2.4.1 Locomotor activity

Locomotor activity was measured as already described (Angioni et al., 2016). Before the beginning of the experiment, rats were daily handled for at least one week to avoid stress due to manipulation during the experimental sessions. At the end of this period, each rat underwent one habituation session that lasted for 1 hour in order to prevent the influence of novelty factors linked to the experimental procedure and motility apparatus during the experimental sessions. Rats were individually tested for motor activity under standardized environmental conditions (in a soundproof room with a light level of 30 lux) with a Digiscan Animal Activity Analyzer (Omnitech Electronics, Columbus, Ohio). Each cage (42 cm x 42 cm x 63 cm) had two sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor and a further set of 16 horizontal beams whose height was adapted to the size of the animals (20 cm). Horizontal and vertical activities were measured as total number of sequential infrared beam breaks (counts) in the horizontal or vertical sensors, recorded every 5 minutes, beginning immediately after placing the animals into the cage, over a test period of 60 minutes.

2.4.2 Rotarod test

Motor performance was checked using the Rotarod apparatus according to a procedure already described (Hoffman and Tabakoff, 1984). All animals were trained for 7 days before microinjection on the Rotarod apparatus in order to reach a stable performance. During the training phase and experimental sessions, the rats were placed perpendicular to the rotating axis

and the head against the direction of the rotation; the animal must therefore move forwards in order to stay on the rod. The rats were tested under the following protocol: beginning at the constant rotation speed of 2 rpm for the first 5 min, then progressively accelerated from 2 to 20 rpm for the next 5 min (acceleration phase) and finally under the constant speed of 20 rpm for other 5 min. The latency to fall was recorded at the beginning of the acceleration phase in a total 10 min (600 sec) test period. Only the rats which successfully completed the training sessions (600 sec) were selected. (Lobina et al., 2005; Loi et al., 2010).

2.4.3 Apomorphine-induced rotation test

FPN-treated rats were compared to control rats for rotational behavior with a challenge dose of apomorphine-HCl (0.5 mg/kg). This test should be able to reveal the extent of the FPN-induced lesion at the level of the nigrostriatal dopaminergic neurons (Fornaguera et al., 1993). The rats were placed in a circular Plexiglas apparatus located in a dimly-lit, quiet room before test, partially filled with sawdust, to acclimatize with it. Apomorphine hydrochloride was dissolved in bidistilled water and injected subcutaneously (s.c.) at a dose of 0.5 mg/kg (Fornaguera et al., 1993). Two minutes after apomorphine injection, the rats were placed in the rotameter chamber and full 360-degree rotations (either ipsilateral or contralateral) were recorded for 30 min test period by means of a digital video-camera and the video-files stored in a backup device for following analyses.

2.4.4 Tail flick test

The animal's response to phasic pain was tested by measuring the latency of tail flick to a high intensity light beam. The test was performed with a Tail Flick instrument that gives an automatic recording of tail flick latency to radiant heat (TSE Systems, Bad Homburg, Germany). The animal was placed on the recording platform of the apparatus with its tail placed on the radiant heat window. A beam of light at 56°C was projected and the latency to removal of the tail in response to the noxious stimulus was recorded. For each animal, the thermal stimulus was applied on three different parts of the tail and the latency to removal of the tail was considered as the mean of the three measurements. A cut-off exposure time of 20 seconds was set to prevent tissue damage (Tassorelli et al., 2003).

2.4.5 Novel object-recognition test

The novel object-recognition test was used to assess cognitive alterations associated with FPN treatment. Training in the object recognition task took place in open field arena, with the floor covered with sawdust. Rats (always one animal per time) were first habituated individually in the open field (45 x 45 x 30 cm), in a dimly-lit quiet room, for 10 min to acclimatize with the arena. The rats were then removed from the open field and placed in their home cage, and two identical objects were positioned in two adjacent corners of the open field 9 cm apart from the walls and the rats were placed in remaining corner, facing towards the wall of the apparatus and familiarized with the object for 10 min (Vedovelli et al., 2011**)**. At least 1 h after familiarization, rats explored the open field for 10 min in presence of a familiar object (F) and a novel object (N) positioned in similar way. The exploration of the objects (defined as sniffing or touching the object with the nose and/or forepaws) was recorded by means of a digital video-camera, and video-files stored in a backup device for the following analyses. The time spent exploring the test objects (e.g., the duration of exploration) was measured. A recognition index expressed by the ratio TN/(TF+TN) [TF = time spent exploring the familiar object F; TN = time spent exploring the novel object N] was calculated for each animal (de Lima et al., 2005, 2008). Between trials, the objects were washed with 70% ethanol solution.

2.4.6 Social interaction test

The test rats (always one animal per time) were first habituated individually as described in the novel object recognition test. On the test day, to minimize transfer effects and avoid possible visual or olfactory influences, test rat and pairing rat, were transferred to testing room and allowed to acclimate for 10 min with the surrounding environment. For this, the rat pairs were weight-matched (a difference of no more than ± 5 g) and made up of rats that were unknown to each other (i.e. rats that did not share the same homecage from birth to single housing). Prior to the test, each test rat was marked by a vertical line on the back for differentiation between the two. Each pair of rats was placed in the open field arena and the behavior (such as sniffing, genital investigation, crawling under and climbing over) was recorded for 10 min test period by means of a digital video-camera and video-files stored in a backup device for following analyses (Loi et al., 2010, Slamberova et al., 2016).

2.5 Brain tissue dissections

Once the behavioral studies were completed for each animal, the rats were deeply anesthetized and sacrificed by decapitation. The brain was quickly removed and washed with ice-cold saline. After cooling, the right and left striatum were micro-punched (Palkovits et al., 1983) and transferred in 2 mL pre-weighed centrifuge tubes. The tissues were weighed and immediately stored at -80°C until processing. Briefly, the striatal tissues were weighed, homogenized by sonication in 0.1 mM perchloric acid ($HClO_4$) (1:20 weight tissue per solvent volume) (Devoto et al., 2015) and centrifuged at 23,000 rpm for 30 min. Then the supernatant obtained was filtered using microspin centrifuge tubes (0.22-µm nylon filter) at 10,000 rpm for 10 min. The filtered supernatant was stored at -80°C until the determination of DA content in the striatum.

2.6 Brain Histology

In order to verify the injection site in right SN, the remaining portion of the brain was immediately put in 4% paraformaldehyde solution until processing. The brain portion was then transferred in 30% sucrose solution stored at 4°C for at least 48 hours before the sectioning procedure. Brain sections of 40 µm were made by using a cryostat maintained at -20°C, placed on a glass slide, stained with Neutral Red solution, then allowed to dry overnight under dark and on next day observed with a contrast phase microscope. Only the rats which showed the track of the microinjection needle positioned correctly in the right SN (Figure 1) have been considered for the statistical analysis of the results.

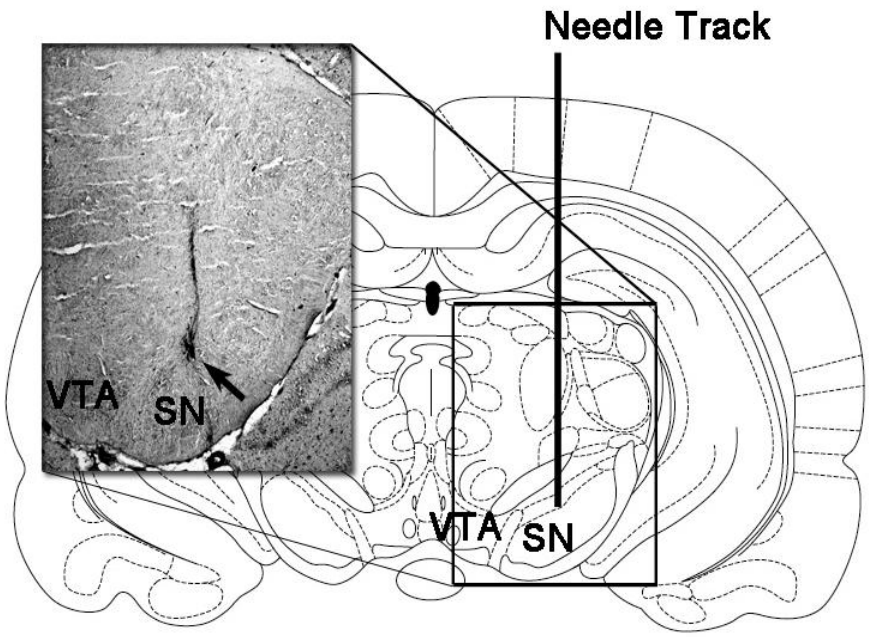


Figure 1. Schematic representation of a coronal section of the rat brain showing the tip of the microinjection needle in the SN (Paxinos and Watson, 2007). The portion of the Neutral Red-stained section showing the tip of microinjection needle into the SN (marked by the black arrow) is magnified in the insert. Abbreviations: SN = substantia nigra; VTA = ventral tegmental area.

2.7 Measurement of striatal dopamine concentration

DA was measured by injecting 20 µL aliquot of the supernatant obtained from striatal homogenates (as described above) by using high pressure liquid chromatography (HPLC) coupled to electrochemical detection using a 4011-dual cell (Coulochem II, ESA, Cambridge, MA, USA) as already described (Melis et al., 2003, Sanna et al., 2017). Detection was performed in reduction mode at +350 and −180 mV. The HPLC was equipped with a Supelcosil C18 column (7.5 cm × 3.0 mm i.d., 3 µm particle size; Supelco, Supelchem, Milan, Italy), eluted with 0.06 M citrate/acetate pH 4.2, containing methanol 20% *v/v*, 0.1 mM EDTA (Ethylene-diamine-tetra-acetic acid), 1 µM triethylamine, and 0.03 mM sodium dodecyl sulfate as a mobile phase, at a flow rate of 0.6 mL/min and room temperature. The sensitivity of the assay was 0.125 pg.

2.8 Immunohistochemistry

Animals were deeply anesthetized with chloral hydrate (400 mg⁄kg, *i.p.*) and transcardially perfused-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2–7.4. Brains were rapidly removed; washed overnight in PBS containing 7% sucrose and 0.01% NaN_3, and orientated in aluminium foil moulds in cryo-embedding medium (in g/ L: polyvinyl alcohol, 80; polyethylene glycol, 42.6; Tween-20, 10; and NaN_3, 0.5) (Cocco et al., 2003), and frozen in melting freon (cooled with liquid nitrogen). Coronal cryosections (10 µm) comprehensive of the whole SN obtained from the midbrain (starting from section with AP≈−6.5 up to section with AP≈−4.5) (Paxinos and Watson, 2007), were collected onto poly-L-lysine-coated slides and stored in the vapour phase of a liquid nitrogen tank until used.

For immunohistochemistry, sections prepared as reported above obtained from the rats of each group, were brought to room temperature and washed in Triton X-100 (1mL/L, in PBS solution). Adjacent serial sections (10 µm) of the SN were selected and then labeled with polyclonal sheep anti-TH (Millipore, Darmstadt Germany, AB_373131, 1:600), followed by the incubation with the corresponding species-specific donkey secondary antibody conjugated with Cy3 (Jackson Immunoresearch Laboratories, West Grove, PA) to reveal the immunoreactivity of the primary antibody. Primary and secondary antibodies were routinely diluted in PBS containing 30 ml/L of normal donkey serum, 30 ml/L of normal rat serum and 0.02 g/L NaN_3, in order to prevent the non specific binding. Sections were finally washed with PBS, coverslipped with PBS-glycerol and visualized using an Olympus BX41/BX51 fluorescence microscope (Milan, Italy), equipped with a Fuji FinePix S2 and S3 Pro digital camera (Fujifilm, Milan, Italy). Controls included negative controls (replacement of the primary antibody by antibody diluent alone). In order to obtain a semi-quantitative determination of the immunofluorescent signal, the FIJI image processing package, based on ImageJ (NIH) was used. The values of three sections (encompassing the initial, medial and final portion of the SN) acquired in order to cover the entire SN, were then summed and the means calculated to obtain SN density values for each animal. The means ± SEM of density values for TH, were then calculated for each group of experimental animals (control DMSO, FPN 15 µg and FPN 25 µg), and changes among groups reported as percent of control DMSO-treated rats (PBS IR = 100%) (Angioni et al., 2016).

2.9 Statistical analysis

Data are presented as mean values ± SEM and were analyzed by means of one- or two-way ANOVAs with the treatment as between subjects factor and the time (i.e., day test or test fraction) or brain side (in the case of neurochemical and immunohistochemical analyses) as within subjects factor. When ANOVAs revealed statistically significant main effects and/or interactions, pairwise comparisons were performed by using the Tukey's multicomparison test. Statistical analyses were all carried out with PRISM, Graph Pad 6 Software (San Diego, USA) with the significance level set at $P < 0.05$.

3. RESULTS

3.1 Body weight

As shown in Figure 2, there was no significant difference in the body weight pattern between rats unilaterally injected into the right SN either with vehicle (DMSO, control rats) or FPN at the dose of either 15 µg or 25 µg. A slight decrease in body weight was observed in the first week after surgery in the majority of rats. The weight gain pattern, from 8 to 12 days after the microinjection, was progressive and slightly higher in control DMSO-treated rats as compared to FPN-treated rats, but the difference was not statistically significant (all $P > 0.05$) Thereafter, the weight gain pattern seemed nearly similar until 15 days of the test period in all the rats.

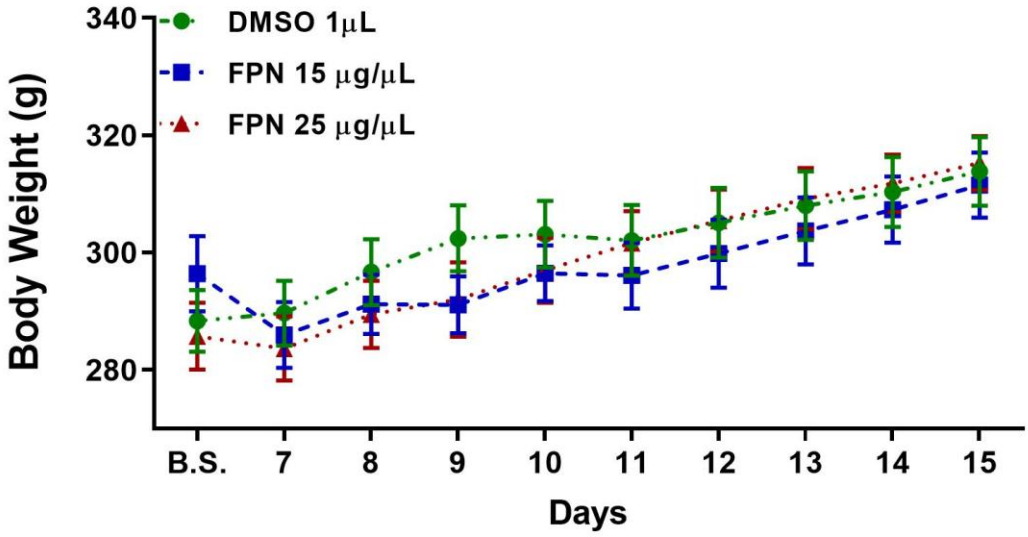


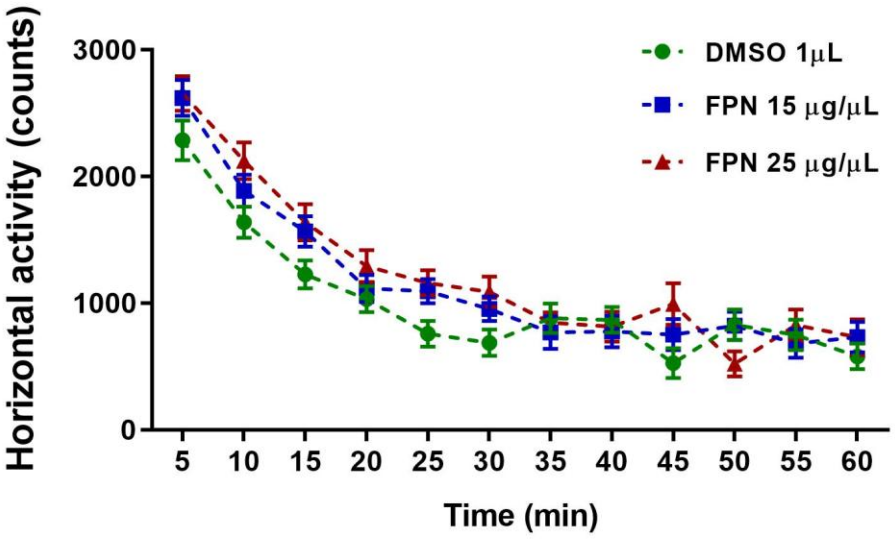
Figure 2. Body weight of fipronil (FPN, 15 µg and 25 µg)- and control DMSO-treated rats. Values are expressed as Mean ± SEM (n = 15 rats per group) (two-way ANOVA followed by Tukey's post hoc test). B.S. = Before surgery.

3.2 Locomotor activity

As shown in Figure 3, FPN 15 µg and 25 µg-treated rats did not show significant difference in horizontal activity (Figure 3a) or vertical activity (Figure 3b) in the first 30 min of the test as compared to control DMSO-treated rats, although a tendency to increase either in total horizontal (Figure 3a) or vertical activity (Figure 3b) was observed in the first 30 min of the test with

respect to control DMSO-treated rats, but without reaching a statistical significance (*P > 0.05*). The horizontal and vertical activities began to decrease in the next 30 min of the experiment in a similar way in all the three groups of rats.

(a)



(b)

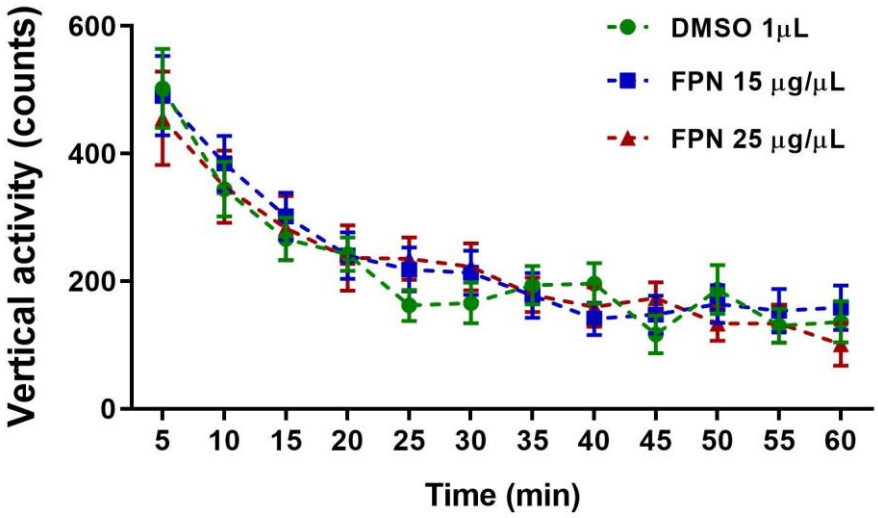
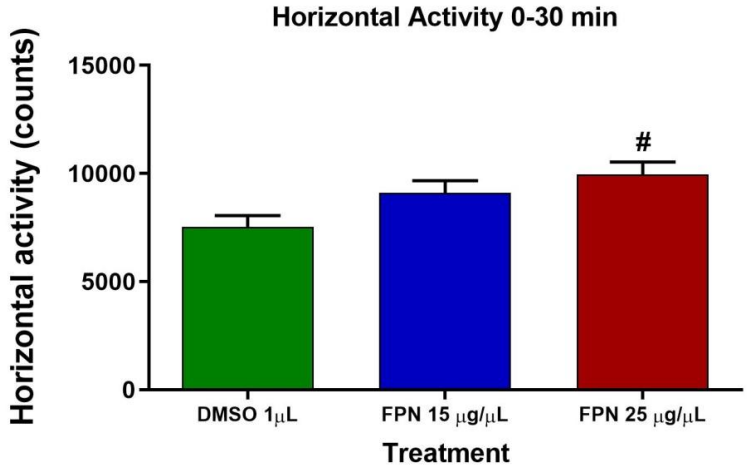


Figure 3. Horizontal (a) and vertical (b) locomotor activity of fipronil (FPN, 15 µg and FPN 25 µg)- and control DMSO-treated rats. FPN and DMSO were injected into the right SN 14 days before the experiment. Rats were put individually inside the apparatus and locomotor activity was recorded for 60 min (12 consecutive periods of 5 min). Values are expressed as Mean ± SEM (*n* = 15 rats per group) (two-way ANOVA followed by Tukey's post hoc test).

In line with the results shown in Figure 3, further analyses revealed that FPN 25 µg-treated rats show an increase in the total counts of horizontal activity (Figure 4a) but not of vertical activity, when compared to control DMSO-treated rats in the first 30 min of the test ($P < 0.05$); however, this difference was no longer detectable when considering the whole 60 min duration of the test (Figure 4b).

(a)



(b)

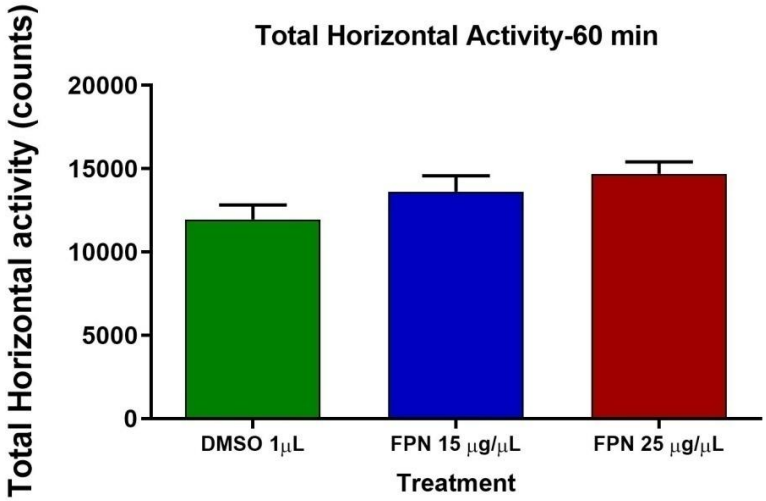


Figure 4. Total counts of horizontal locomotor activity in the first 30 min (a) and (b) in the whole 60 min test of fipronil (FPN, 15 µg and 25 µg)-treated rats and control DMSO-treated rats. FPN and DMSO were injected into the right SN 14 days before the experiment. Values are expressed as Mean ± SEM (n = 15 rats per group). $^{\#}P < 0.05$, FPN 25 µg-treated rats vs. control DMSO-treated rats (one-way ANOVA followed by Tukey's post hoc test).

3.3 Rotarod test

As shown in Figure 5, FPN-treated rats (FPN 15 µg and FPN 25 µg) displayed a marked impairment of motor performance as compared to control DMSO-treated rats in the Rotarod task. It clearly shows a significant impairment of motor performance in FPN-treated rats as compared to control rats in all days of test after intranigral microinjection. The degree of impairment is higher in rats treated with 25 µg than 15 µg on days 7 and 8 of the test. But, after day 8, the degree of impairment is higher in rats treated with FPN 15 µg than 25 µg until day 15 of the test. Some impairment in motor performance is also seen in control DMSO-treated rats, which may be attributed to the habituation on the Rotarod apparatus that may cause a gradual decrease in motor performance per se (Figure 5).

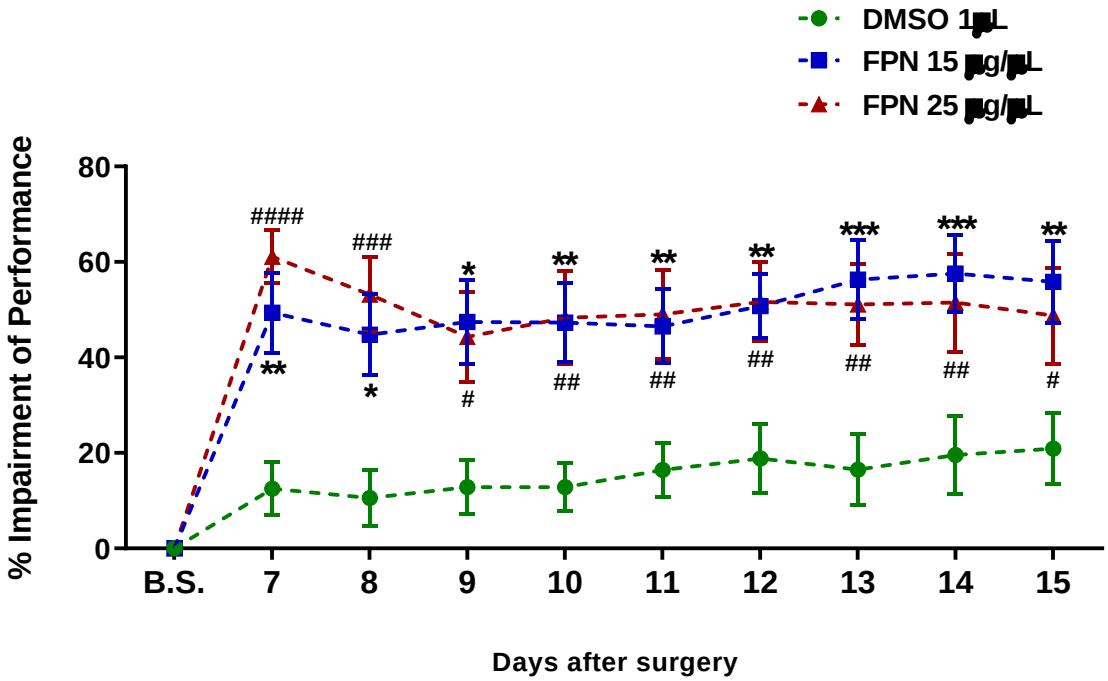


Figure 5. Percent impairment of motor performance of rats in Rotarod task after unilateral SN microinjections of fipronil (FPN, 15 µg and 25 µg). Percent impairment of motor performance is defined as [(T1-T2)/T1] x 100%, where T1 and T2 are the amount of time each rat remained on the rotating drum in the two trials conducted before and after microinjections, respectively. Values are expressed as Mean ± SEM (n = 15 rats per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, FPN 15 µg-treated rats vs. Control DMSO-treated rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, FPN 25 µg-treated rats vs. Control DMSO-treated rats (two-way ANOVA followed by Tukey's post hoc test). B.S. = Before surgery.

3.4 Apomorphine-induced rotation test

As shown in Figure 6, apomorphine (0.5 mg/kg s.c.) induces a greater number of ipsilateral than contralateral rotations to the FPN injected side in FPN-treated rats. Accordingly, FPN (15 µg and 25 µg)-treated rats showed significantly higher number of ipsilateral rotations to the FPN-injected side (ipsilateral) as compared to control DMSO-treated rats (both $P < 0.001$**)**.

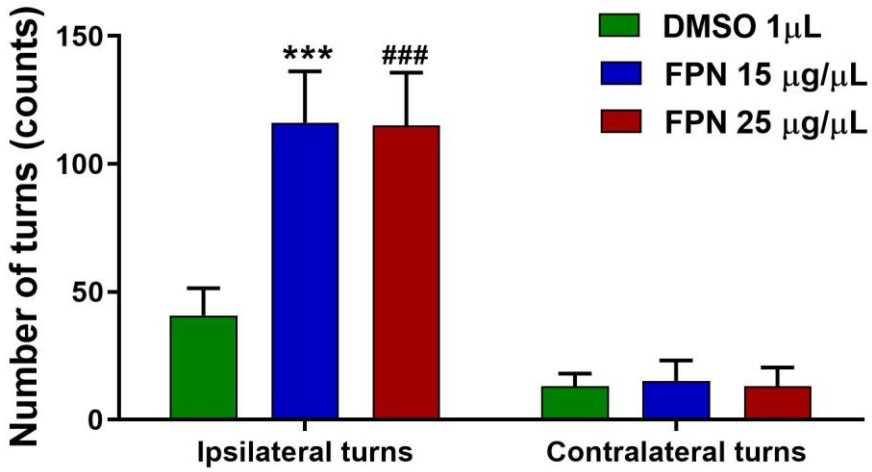


Figure 6. Apomorphine (0.5 mg/kg, s.c.) induces rotations mainly ipsilateral to the injection site in fipronil (FPN, 15 µg and 25 µg)-treated rats as compared to control DMSO-treated rats. Fipronil and DMSO were injected into the right SN 14 days before the experiment. Values are expressed as Mean ± SEM (n = 15 rats per group). ***$P < 0.001$, FPN 15 µg-treated rats vs. Control DMSO rats, $^{###}P < 0.001$, FPN 25 µg-treated rats vs. Control DMSO-treated rats (two-way ANOVA followed by Tukey's post hoc test).

3.5 Tail flick test

The algesia tail flick test was used to evaluate pain sensitivity of FPN (15 µg and 25 µg) and control DMSO-treated rats in presence of acute noxious stimuli. FPN (15 µg and 25 µg)-treated rats show significant less time to flick the tail ($P < 0.001$) when exposed to the acute noxious thermal stimuli on tail as compared to control DMSO-treated rats (Figure 7). This suggests that FPN-treated rats have a lower threshold to pain when compared to DMSO-treated rats.

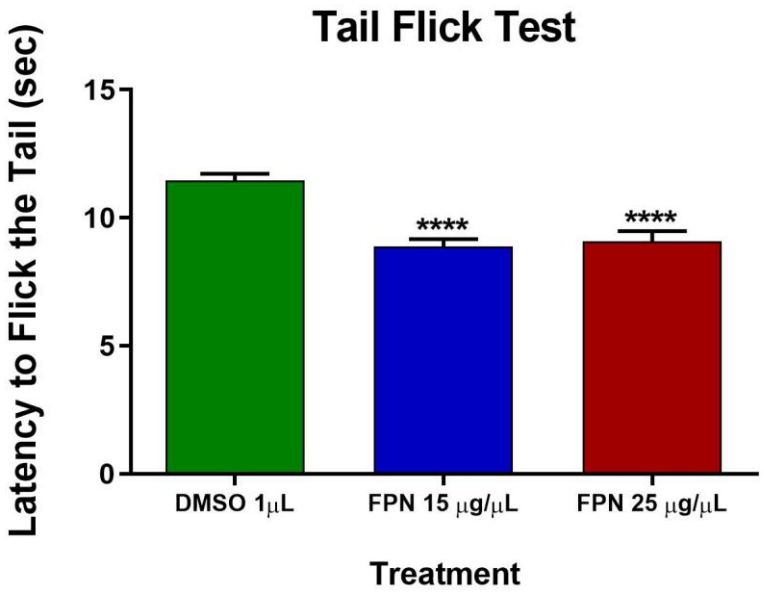


Figure 7. Pain sensitivity of fipronil (FPN, 15 µg and 25 µg)- and DMSO-treated rats in presence of acute thermal noxious stimuli, evaluated using tail flick test. FPN and DMSO were injected into the right SN 14 days before the experiment. Values are expressed as Mean ± SEM (n = 15 rats per group). ****$P < 0.001$, FPN (15 µg or 25 µg)-treated rats vs. control DMSO rats (one-way ANOVA followed by Tukey's post hoc test).

3.6 Novel object-recognition test

As shown in Figure 8 and Figure 9, no significant difference was found in recognition index (calculated for each rat by the ratio TN/(TF+TN) [TF = time (T) spent exploring the familiar object (F); TN = time (T) spent exploring the novel object (N)] (Figure 8) ($P > 0.05$) as well as latency (seconds) to approach the objects (Figure 9) ($P > 0.05$) between the FPN (15 µg and 25 µg)- and control DMSO-treated rats. On the other hand, an increase in the number of approaches to a novel object was observed in control DMSO- and FPN 15 µg-treated rats as compared to a familiar object (both $P < 0.05$), but not in FPN 25 µg- treated rats (Figure 10).

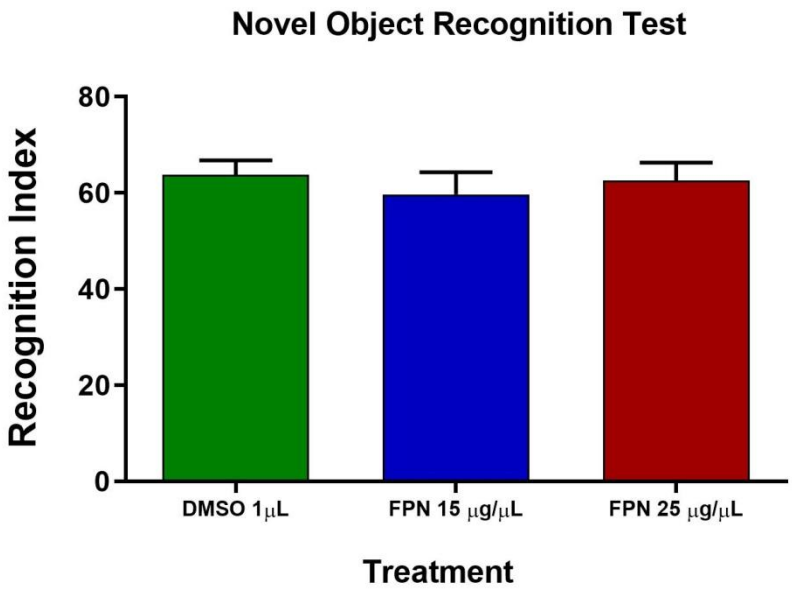


Figure 8. Novel object recognition task performed on fipronil (FPN, 15 µg and 25 µg)- and control DMSO-treated rats: effect on the recognition index (calculated for each rat by the ratio TN/(TF+TN) [TF = time spent exploring the familiar object (F); TN = time spent exploring the novel object (N)]. FPN and DMSO were injected into the right SN 14 days before the experiment. Values are expressed as Mean ± SEM (n = 15 rats per group) (one-way ANOVA followed by Tukey's post hoc test).

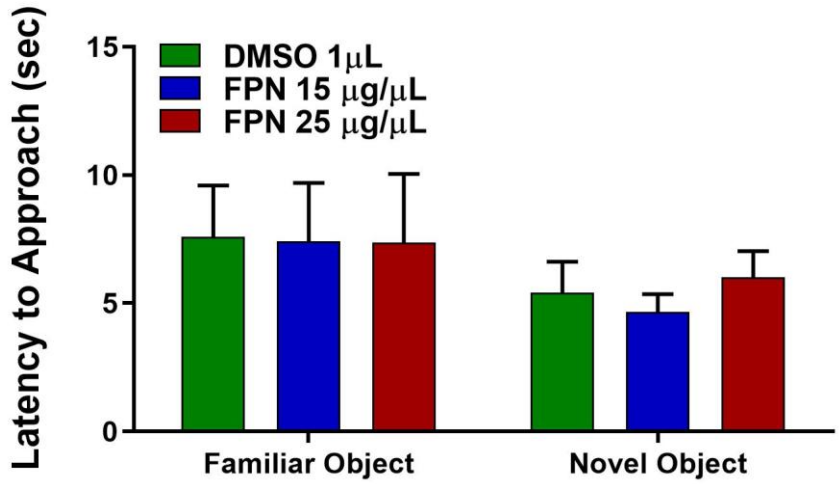


Figure 9. Novel object recognition task performed on fipronil (FPN, 15 µg and 25 µg)- and DMSO-treated rats: latency to approach objects (sec). FPN and DMSO were injected into the right SN 14 days before the experiment. Values are expressed as Mean ± SEM (*n* = 15 rats per group) (one-way ANOVA followed by Tukey's post hoc test).

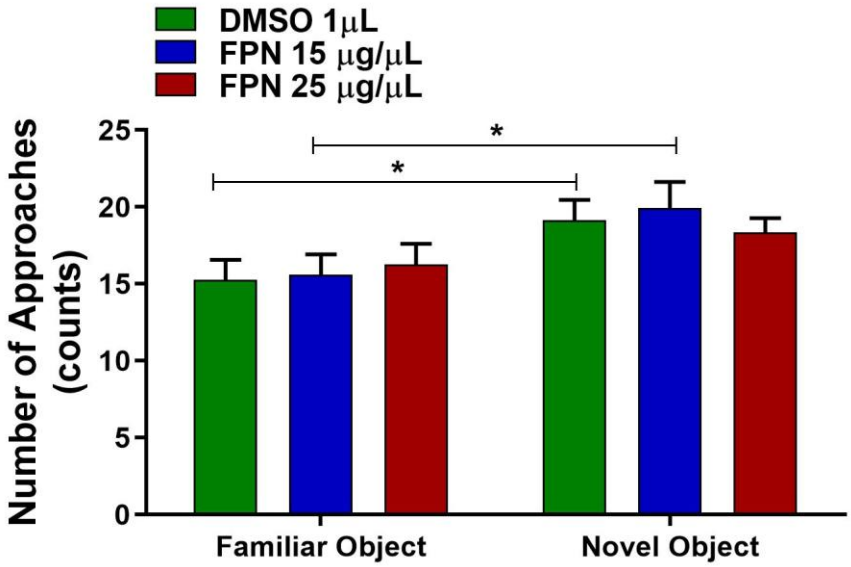
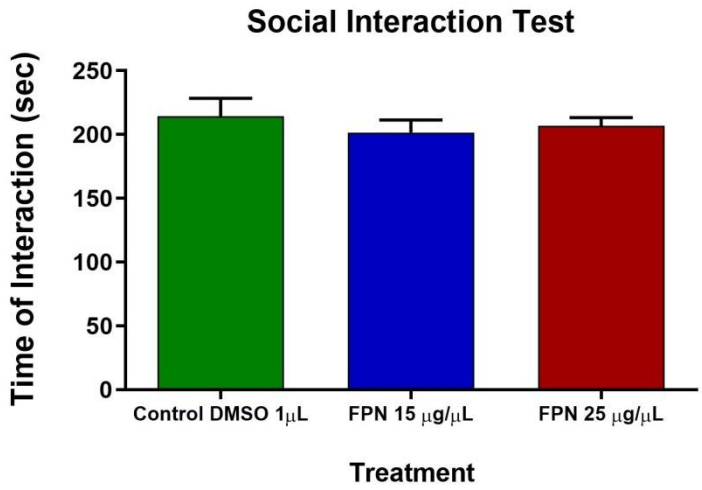


Figure 10. Novel object recognition task performed on fipronil (FPN, 15 µg and 25 µg)- and DMSO-treated rats: number of approaches on objects. FPN and DMSO were injected into the right SN 14 days before the experiment. Values are expressed as Mean ± SEM (*n* = 15 rats per group). **P < 0.05* (one-way ANOVA followed by Tukey's post hoc test).

3.7 Social interaction test

The social interaction test failed to show significant differences in the time of interaction (contact) (Figure 11a) and number of contacts (Figure 11b) between FPN 15 µg and 25 µg and control DMSO-treated rats (all $P > 0.05$).

(a)



(b)

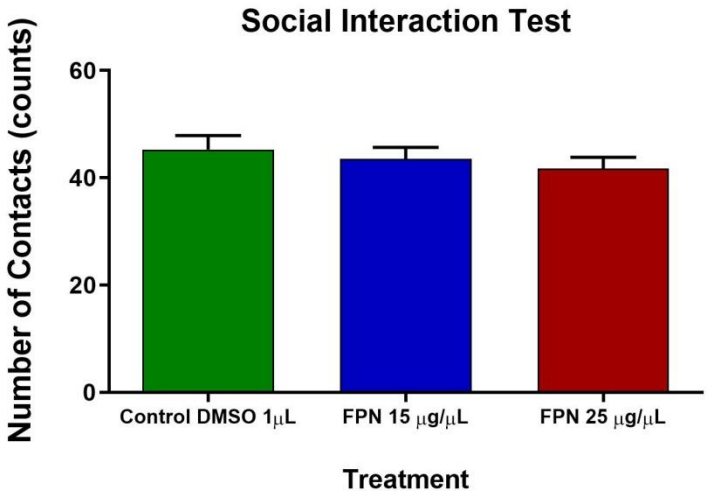


Figure 11. Social interaction test in fipronil (FPN, 15 µg and 25 µg)- and control DMSO-treated rats. a) Time of interaction (sec), b) Number of contacts. FPN and DMSO were injected into the right SN 14 days before the experiment. Values are expressed as Mean ± SEM (n = 15 rats per group) (one-way ANOVA followed by Tukey's post hoc test).

3.8 Neurochemistry: striatal DA concentration

As shown in Figure 12, FPN 15 µg and 25 µg microinjected into the right SN induced both a marked decrease of about 40-50% in the concentration of DA in the striatum ipsilateral to the injected (right) SN when compared to control DMSO-treated rats ($P < 0.0001$, two-way ANOVA followed by Tukey test). This indicates a significant neurodegenerative/neurotoxic effect of FPN injected in the SN as compared to DMSO treatment. In both FPN (15 µg and 25 µg)-treated rats, the decrease in DA content in the striatum ipsilateral to the injected SN was also highly significant when compared to the DA content in the intact contralateral (left) striatum (two-way ANOVA followed by Tukey test, $P < 0.0001$).

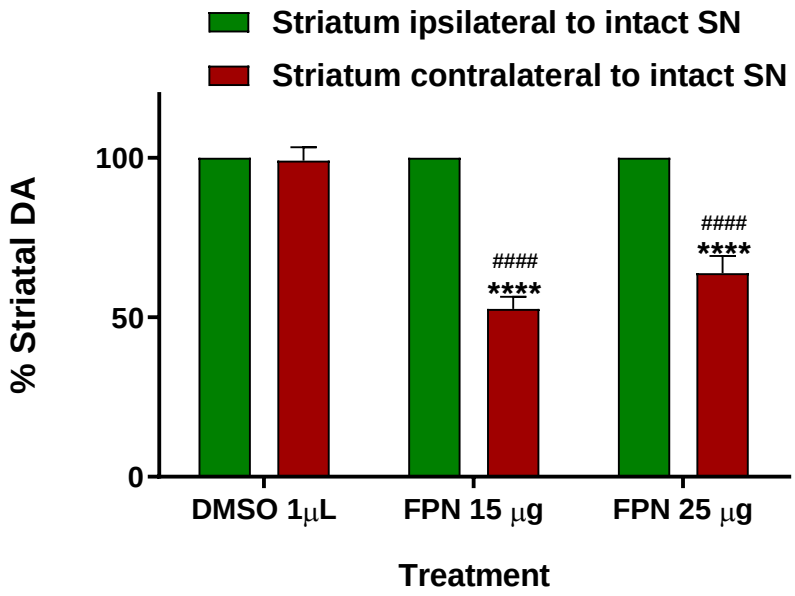


Figure 12. Percent (%) dopamine (DA) in the right (injected) and left (intact) striatum of Fipronil (FPN, 15 µg and 25 µg)-treated rats compared to control DMSO-treated rats. FPN and DMSO were injected into the right SN 14 days before the experiment. Values are expressed as Mean ± SEM (n = 6 rats per group). ****$P < 0.0001$ FPN treated rats vs. Control DMSO, $^{\#\#\#\#}P < 0.0001$ right striatum vs. left striatum, (two-way ANOVA followed by Tukey's post hoc test).

3.9 Immunohistochemistry

Differences in TH immunostaining between the treated (right) and untreated (left) SN were found using the TH antibody. Accordingly, as shown in Figure 13 and Figure 14, dopaminergic neurons were reduced in number in the substantia nigra after FPN treatment. In particular, a similar decrease was observed after injection of FPN 15µg and 25µg. In both cases, the decrease was extended through the different sections examined (Figure 13).

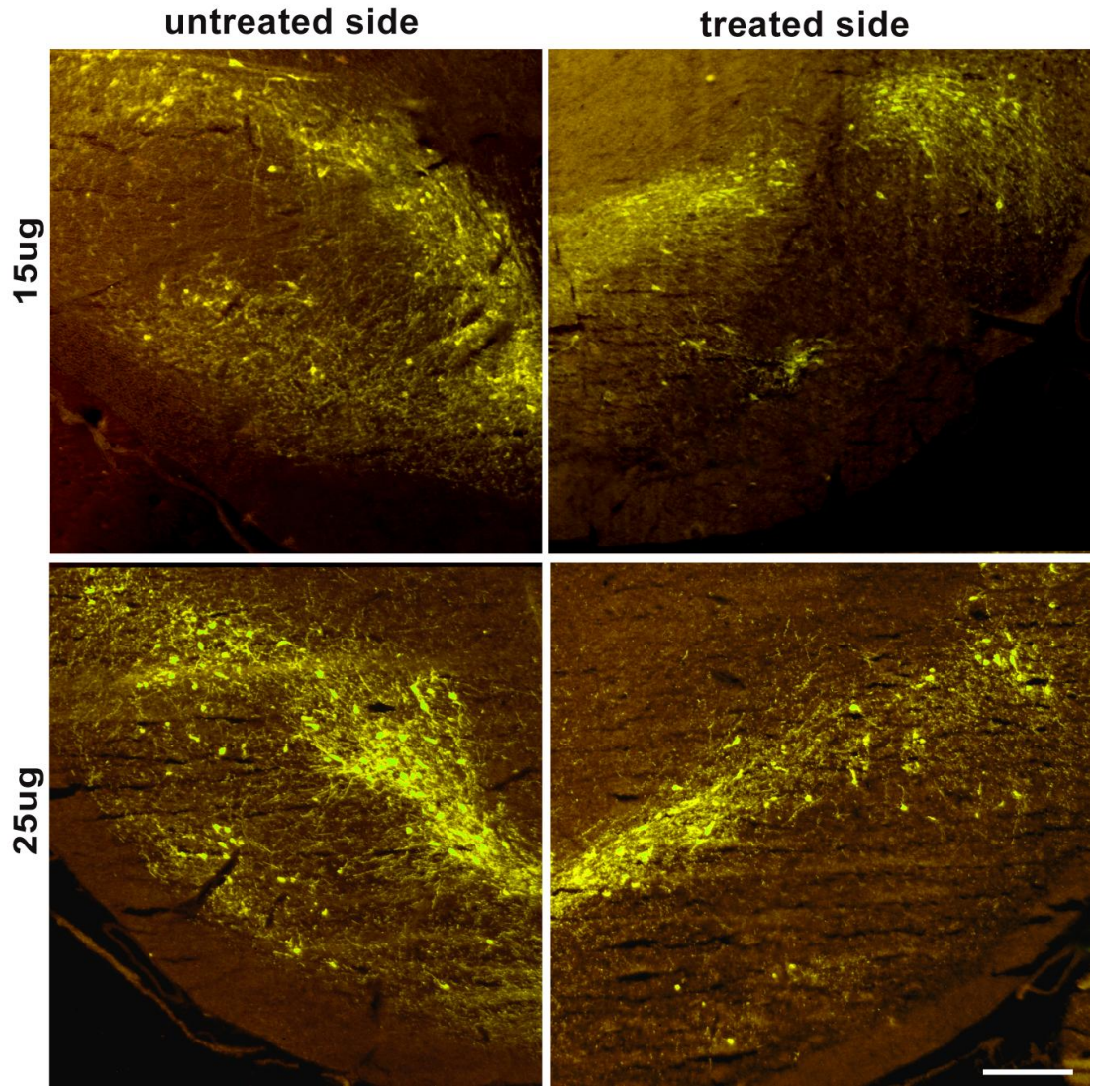


Figure 13. Tyrosine hydroxylase (TH) staining in the substantia nigra of treated and untreated side. Representative treated vs. untreated sides in which TH immunostaining (Cy3, yellow labeling, which appears yellow because of the filter used to magnify immunostaining vision) is similarly decreased after intranigral injection of fipronil at both concentrations (15 - upper pictures, and 25 µg – lower pictures). Scale bar: 100µm.

The differences in TH immunostaining between FPN- and DMSO-treated groups were found significant after ImageJ analysis (Figure 14). Accordingly, FPN, 15 µg and 25 µg microinjected into the right SN induced both a marked decrease of about 40-50% in the TH immunofluorescence in the injected SN when compared to control DMSO-treated rats ($P < 0.001$, two-way ANOVA followed by Tukey's post-hoc test). Apparently FPN 25 µg induces a similar decrease in TH immunofluorescence when compared to FPN 15 µg ($P < 0.05$). This indicates a significant neurodegenerative/neurotoxic effect of FPN injected in the SN as compared to DMSO treatment. In both FPN (15 µg and 25 µg)-treated rats, the decrease in the TH immunofluorescence in the injected SN was also very evident when compared to the TH immunofluorescence in the intact contralateral SN (two-way ANOVA followed by Tukey's post-hoc test, $P < 0.001$).

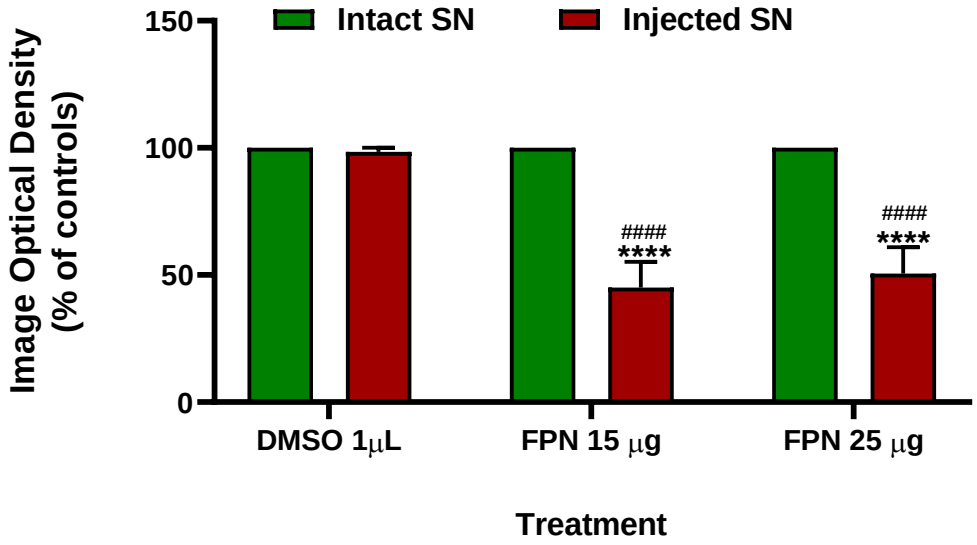


Figure 14. Percent (%) of TH immunofluorescence in the right (injected) and left (intact) SN of Fipronil (FPN, 15 µg and 25 µg)-treated rats compared to control DMSO-treated rats. FPN and DMSO were injected into the right SN 14 days before the experiment. Values are expressed as Mean ± SEM (n = 12 samples, 3 sections for each of 4 rats per group). ****$P < 0.0001$ FPN treated rats vs. Control DMSO, $^{\#\#\#\#}P < 0.0001$ right SN vs. left SN (two-way ANOVA followed by Tukey's post hoc test).

4. DISCUSSION

The experimental data reported in my thesis confirms the neurotoxic effects of FPN on the nigrostriatal dopaminergic system when injected into the SN (Lee et al., 2011; Park et al., 2016a,b). Accordingly, the pesticide injected unilaterally, at the doses of 15 µg and 25 µg, directly into the right SN induces a significant 40-50% decrease in the content of DA in the righ striatum when compared to the right striatum of control rats, injected in the right SN with vehicle alone (DMSO) and to the left striatum when the animals were sacrificed 15 days after FPN microinjection. The decrease in striatal DA content of FPN-treated rats was parallel to a 40-50% decrease in right SN TH-immunoreactivity. TH is the rate-limiting DA synthesizing enzyme usually used as a classical marker of nigrostriatal DA neurons in immunohistochemistry. This neurochemical and immunohistochemical changes occurred with only minor effects on body weight pattern of right SN FPN-treated rats when compared to control DMSO-treated rats during the 15 days of the experiment. These findings resemble to those obtained in rats with lesions of the nigrostriatal dopaminergic system induced by neurotoxins such as 6-hydroxy-dopamine (6-OHDA), (Ungersted, 1971; Su et al., 2018), MPTP and its oxidation product MPP^+ (believed to be responsible for MPTP neurotoxicity, at least in rats) (Ferro et al., 2005; Sindhu et al., 2006; Geed et al., 2014) or rotenone (Sindhu et al., 2006; Fagotti et al, 2019), injected in the SN or in the medial forebrain bundle, and which are considered as reliable preclinic animal models of PD (Truong et al., 2006; Su et al., 2018). All these neurotoxins cause lesions of the nigrostriatal dopaminergic neurons, whose extent is usually evaluated post-mortem, by measuring DA content in striatal tissue homogenates or by measuring the decrease in nigral TH-immunoreactivity in brain sections, as described above, and were also found in right SN FPN-treated rats.

The SN FPN-treated rats also show changes in locomotor activity and in motor coordination when studied in the open field and rotarod tests as well as in rotational circling when tested with a dopaminergic drug (i.e., apomorphine). These findings are also in line with those of earlier studies in living rats with a lesioned nigrostriatal dopaminergic system by 6-OHDA, MPTP, MPP^+, and rotenone, which have shown that the extent of the loss of nigrostriatal dopaminergic neurons originating in the SN may be predicted by studying changes in locomotor activity in rats with unilateral or bilateral lesions of the nigrostriatal dopaminergic system or by analyzing the rotations/circling induced by dopaminergic drugs (i.e., apomorphine, a direct DA

receptor agonist, or d-amphetamine, which facilitates dopaminergic neurotransmission by inhibiting DA reuptake and facilitating synaptic DA release) in rats with the nigrostriatal dopaminergic system lesioned unilaterally (see references below).

As to the studies showing changes in locomotor activity in rats with lesions of the nigrostriatal dopaminergic system induced by the above neurotoxins, these studies have revealed that changes in locomotor activity are associated with alterations of the dopaminergic function in PD. Accordingly, decreased DA levels in rats with unilateral or bilateral lesions of the nigrostriatal system induced by 6-OHDA leads to a marked reduction in horizontal and vertical locomotor activity measured starting at seven days after the neurotoxin injection (Deumens et al., 2002; Jackson et al., 1983; Ungerstedt et al., 1974). In these rats, the reduction in locomotor activity was parallel to a decrease in striatal DA and TH immunoreactivity higher than 80%. In contrast, in rats with bilateral lesions of the nigrostriatal dopaminergic system induced by MPTP, which produced about a 50-60% decrease in striatal DA and TH immunoreactivity, an increase in locomotor activity was found in the open field at 6 days after injection of the neurotoxin, a finding that resembles that we found in this study with SN FPN-treated rats in which a tendency to increase, rather than a decrease, in horizontal locomotor activity has been observed 15 days after the pesticide injection. However, a decrease in locomotor activity was found in MPTP-treated rats 18 days after the drug injection in the above study (Ferro et al., 2005). A decrease in locomotor activity revealed by a decrease in the distance travelled and by an increase in total time of immobility in the open field test, was also found in rats treated bilaterally in the SN with rotenone (Fagotti et al., 2019) and MPP^+ (Geed et al., 2014), showing a decrease in striatal DA content and TH immunoreactivity around 60-65%, thus much lower than that found in 6-OHDA-treated rats. Further experiments are required to ascertain whether right SN FPN-treated rats also show a decrease in locomotor activity at times longer than 15 days after the injection, although the difference between FPN-treated rats and the other neurotoxins (MPTP, MPP^+, rotenone and also 6-OHDA) may be also secondary to the fact that FPN was given unilaterally into the right SN while the other compounds were usually given bilaterally.

Irrespective of the differences in locomotor activity between unilaterally SN FPN-treated rats and bilaterally SN 6-OHDA-, MPTP-, MPP^+- and rotenone-treated rats discussed above, in this study unilaterally SN FPN-treated rats show a marked impairment of motor performance in the rotarod test when compared to control DMSO-treated rats that was already evident at 7 days

after treatment at both doses of FPN tested, impairment that lasted for all the time of the experiment (15 days after injection). The rotarod test is approved for the evaluation of neurological impairments in rodents. It can be applied repeatedly to individual rats in order to determine muscle strength, force and hindlimb motor coordination and the balance (Lobina et al. 2005; Loi et al., 2010; Urbach et al., 2010). It has been extensively used in the last decades to determine the efficacy of drugs as potential PD therapeutics (Bové and Perier, 2012; Lee et al., 1996; Meredith and Kang, 2006; Su et al., 2018). In particular, the significant impairment of SN FPN-treated rats in the rotarod test is in line with earlier experiments reporting a marked decrease in the latency to fall in the rotarod test in 6-OHDA- or MPP^+- treated rats starting two weeks after the neurotoxin injection either in the striatum or the SN. Such a motor impairment in the rotarod occurred together with a marked decrease in locomotor activity in the open field test (Su et al., 2018; Hwang et al., 2016), and with the decline of motor performance in the rotarod test found in rats systemically treated with the rotenone (Rahimmi et al., 2015).

As to the studies on the rotational circling induced by DA agonists in rats with the nigrostriatal dopaminergic system lesioned unilaterally, these studies have revealed that in unilaterally lesioned rats, systemically administered apomorphine can induce either no turning/circling or ipsilateral or controlateral turning/circling to the injection side while systemic d-amphetamine usually induces rotational turning/circling ipsilateral to the lesion side,. Although these studies are complicated by the different sites of injection of the neurotoxin used to lesion nigrostrialtal dopaminergic neurons (i.e., 6-OHDA, MTPT, MPP^+, rotenone injected in the SN, VTA, striatum or middle forebrain bundle), the different effects found with apomorphine, which stimulates directly DA receptors of the D1 and D2 type, has been explained by assuming that, while d-amphetamine-induced ipsilateral turning is always due to the fact that the drug increases the amount of extracellular DA in the intact striatum, which in turn works more effectively then, thus prevealing on the lesioned one (Carman et al., 1991; Kirik et al., 1998; Hudson et al., 1993). The controlateral turning can be induced by apomorphine only when an almost complete, usually higher than 90% decrease in striatal DA content is present. As the total lesion produces DA receptors hypersensitivity in the lesioned side, it leads to favor controlateral versus intact site under apomorphine challenge (Ungerstedt and Arbuthnott, 1970; Ungerstedt, 1971, 1976; Thal et al., 1979; Hudson et al., 1993). The hypersensitivity of DA receptors does not occur when DA content in the lesioned striatum of rats treated with 6-OHDA, MPP^+, or rotenone (Sindhu et al.,

2006) is still present at amounts that reach 50-60% of the neurotransmitter values of the intact striatum, as these rats when tested with apomorphine are found to show either no turning or more frequently ipsilateral turning (Blesa et al., 2017; Deumens et al., 2001; Schwarting and Huston, 1996, Jávor-Duray et al., 2017). This is similar to what we found in right SN FPN-treated rats of our study, which show a decrease in striatal DA and SN TH immunoreactivity of around 50% of the control values and ipsilateral turning/circling when challenged with systemic apomorphine. The failure of apomorphine to induce controlateral turning in these conditions has been explained by assuming that when DA content is still around 50% of the control values, compensatory mechanisms occur in the striatum making turning/circling after apomorphine challenge either not visible or even ipsilateral. These mechanisms may be secondary to changes in DA activity in lesioned areas (i.e. increased DA synthesis in or DA release from surviving neurons) or to changes in the activity of other neurotransmitter systems in the basal ganglia (i.e., GABA, glutamic acid, serotonin and others) (Angioni et al., 2016; see part 2). These compensatory responses by the surviving dopaminergic neurons and also by the postsynaptic cells in the striatum may help mitigate the progressive loss of dopaminergic innervation. Accordingly, compensatory responses by afferents to the dendrites of dopaminergic neurons in the SN have been reported (Costall et al., 1976; Funk and Westermann, 1979; Anglade et al., 1995). This may be relevant for FPN as it is well known that this pesticide decreases GABA neurotransmission by acting on $GABA_A$ receptors in insects, although this is thought to occur with lower efficacy in mammalian $GABA_A$ receptors. However, despite the lower affinity of FPN to the native mammalian hetero-oligomeric $GABA_A$ receptor, a recent report has shown that FPN has a similar high affinity to the human receptor $GABA_A$ subunit β3 as to the insect $GABA_A$ receptors. The human $GABA_A$ receptor β3 subunit has been linked to neurodevelopmental disorders such as autism, Angleman syndrome and epilepsy (Vasylieva et al., 2015, 2017).

In this study, the right SN FPN-treated rats also show significantly lesser time (sec) to flick the tail when exposed to thermal stimuli as compared to control DMSO-treated rats in a classical tail flick test used for testing drug-induced analgesia. This finding is in line with experimental evidence implicating a role of the basal ganglia in general and of DA in particular, in the regulation of nociception. For example, the destruction of DA-producing neurons in the SN may impair natural analgesia by disrupting the DA-mediated descending pathways that block

neurotransmission of ascending nociceptive signals from the spinal cord (Fil et al., 2013). Accordingly, in rats with unilateral injection of 6-OHDA into the medial forebrain bundle inducing a depletion of striatal DA around 80%, 6-OHDA-lesioned animals exhibited lower thermal thresholds than sham control rats although the response latency of the tail flick test was only lightly shorter in the 6-OHDA-lesioned rats respect to sham control rats (Gee et al., 2015). Interestingly, chronic pain is one of the most common non-motor symptoms occurring in 60–85% of PD patients (Sung et al., 2018; Thompson et al., 2017). Although pain in PD patients is often secondary to motor symptoms, such as from rigidity or abnormal posturing, pain often appears early in the development of PD and may be present years before PD clinical diagnosis, that is prior to the onset of motor symptoms. Pain certainly contributes to PD-related disability, sleep disturbance, and impaired quality of life and abnormal nociceptive information processing may be considered a feature of PD (Sung et al., 2018; Thompson et al., 2017).

This study also shows for the first time how unilateral SN FPN-treated rats behave in the novel object recognition test and in the social recognition test. These two tests are used to evaluate the cognitive domain of memory, which is often affected in PD patients. The novel object recognition test allows the evaluation of recognition memory and the estimation of the cognition state of an animal by measuring the time spent/dedicated to a new object presented to the animal when compared to that dedicated to a familiar object (Mathiasen and Di Camillo, 2010). Surprisingly, although no significant difference was found in the recognition index as well as in the latency to approach the objects between FPN (both doses, 15 µg and 25 µg)- and control DMSO-treated rats, an increase in the number of approaches to the novel object was observed in all rats as compared to familiar object, although the difference was significant only between FPN 15 µg- and control DMSO-treated rats. This finding, which does not support a cognitive impairment in SN FPN-treated rats, is in contrast with earlier studies showing that in rats bilaterally injected with rotenone in the SN, which causes a decrease of around 50% of TH immunoreactivity in the SN and a significant decrease in locomotor activity in the open field test, induced also a significant decrease in the time spent in exploring novel objects when compared to familiar objects in the novel object recognition test, a finding that suggests a deterioration of the cognitive memory recognition ability (Fagotti et al., 2019). If this difference is due to the fact that rotenone was injected bilaterally, while in this study FPN was given unilaterally in the right SN, is unknown. However, the increase in the number of approaches to the novel object of FPN-

treated rats may be related to the fact that SN FPN-treated rats, showed a tendency to increased locomotor (horizontal and vertical) activity in the open field when compared to control SN DMSO treated rats, while the rotenone-treated rats in the above study showed a decrease in locomotor activity in the open field when compared to control rats.

In contrast to the results obtained with the novel object recognition test discussed above, no difference was found between right SN FPN-treated rats and control SN DMSO-treated rats in the social interaction test, as indicated by the absence of significant differences in the number of contacts between and the time of interaction with an unknown rat between the animals of the two groups of rats. This finding is in contrast with the ability of chronic systemic rotenone, given at a dose that reduces DA content by 45% in the striatum and in the hippocampus, to exert a negative impact on social interaction test, the forced swimming test, the open field test or the sucrose preference test, suggesting that this kind of treatment may induce a depressive-like behavior, as it occurs in the prodromic phase of PD in humans (Madiha and Haier, 2019). Anhedonia and depressive like behavior are also found, but with a temporal dissociation (anhedonia appears first and depression-like defense behavior later, when anhedonia has disappeared) in rats with partial lesion of the nigrostriatal dopaminergic system induced by bilateral SN 6-OHDA that produces a 70% decrease in striatal and medial prefrontal cortex TH immunoreactivity, with social isolation being evident only after 20 days of 6-OHDA injection when anehedonia was already disappeared (Matheus et al., 2016).

5. CONCLUSION

My work confirms that the pesticide FPN unilaterally injected in the right SN exerts a neurotoxic effect on nigrostriatal dopaminergic neurons, as revealed by a decrease in DA content in the ipsilateral striatum and SN TH immunoreactivity around 50% of the control values. These neurochemical and immunoistochemical results resemble those of earlier studies showing that other neurotoxins (i.e., 6-OHDA, MPTP, MPP^+) including pesticides (i.e., rotenone) when injected into the SN, the striatum, medial forebrain bundle or VTA, induce marked lesions of the nigrostriatal dopaminergic neurons (originating in the SN and projecting to the striatum, but also to the nucleus accumbens and medial prefrontal cortex). This has led to the use of animals, mainly rodents, with their nigrostriatal dopaminergic system lesioned monolaterally or bilaterally by these compounds, to be used as living animal models of PD. Among these animal models of PD, the most well known is certainly the 6-OHDA model, which allows to obtain animals in which DA depletion in the striatum may be in the range from modest to moderate and to almost total depending on the dose of neurotoxin injected into the brain areas relevant for dopaminergic neurons. However, the 6-OHDA model of PD is considered to be very artificial, as it does not satisfy the environmental theory of PD, one of the most accepted eziopathological theories of this neurodegenerative disease (Nandipati and Litvan, 2016).

According to this theory, dopaminergic neurons are destroyed by accumulating neurotoxic agents that behave as environmental stressors, which include different pesticides (i.e. rotenone, FPN) and MPTP (MPP^+). These stressors damage dopaminergic neurons mainly by inhibiting mitochondrial complex I, leading to a decrease in cellular ATP levels and cell death (Blum et al., 2001, Ferro et al., 2005), whereas 6-OHDA acts mainly by generating ROS due to its oxidation, which can occur spontaneously or can be catalyzed by monoaminooxydase (MAO) or iron (Blum et al., 2001; Cadet and Brannock, 1998; Ferro et al., 2005). Moreover, the abnormal activation of glia cells observed in inflammation and neuronal loss induced by MPP^+, FPN and rotenone is usually not observed in 6-OHDA-lesioned rats with loss of dopaminergic neurons. This suggests that other pathways such as cytokines or inflammatory factors may also be involved in the process, which may not be associated with morphological microglia activation (Depino et al., 2003; Ferro et al., 2005), although the retrograde degeneration induced also by a intrastriatal injection of a small dose of 6-OHDA has been reported to lead to an astroglial and microglial reaction in the nigrostriatal dopaminergic pathway (Rodrigues et al., 2001).

The similarity of the neurochemical and immunoistochemical results between FPN, rotenone, MPTP and MPP^+ (i.e., about 50% decrease in striatal DA and TH immunoreactivity) suggests that also SN FPN-treated rats may be used as a living rodent model of PD, like rotenone, MPTP- and MPP^+- treated rats. Interestingly, the decrease in striatal DA and SN TH immunoreactivity found in FPN-treated rats are also accompanied by changes in locomotor activity (measured in the open field test), motor coordination (evaluated in the rotarod test), nociception (measured with the tail flick test), in the novel object recognition test, but not in the social interaction test. These behavioral changes are only in part coincident with those found with rotenone, MPTP and MPP^+ when available. This suggests that FPN acts in the SN to induce loss of DA neurons by mechanisms that may be only partially similar to those induced by the above compounds. However, it cannot be ruled out that a main reason for these behavioral differences is that in my experiments FPN was injected unilaterally into the right SN of rats whereas bilateral injections were often used in the studies reporting the behavioral responses of rotenone-, MPTP- and MPP^+-treated rats. Although studies with bilateral injections of FPN are necessary to clarify the above discrepancies, the results of my study suggest that unilaterally SN FPN-treated rats, which show a 50% decrease in striatal DA and SN TH immunoreactivity, may be considered a preclinical model of the early stage of PD, as already suggested not only for rotenone-, MPTP- and MPP^+-treated rats, but also for 6-OHDA-treated rats, when given at small doses that induce a 50% decrease of DA (Su et al., 2018). Moreover, although FPN is generally considered safe for humans, the results of my thesis confirming the ability of FPN to damage the nigrostriatal dopaminergic neurons make this compound a possible reason of concern for human health. Accordingly, its increasing use as insecticide may lead to an increased chronic exposure to FPN not only as it occurs in the case of occupational exposure but also in human population in general.

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PART-2

Neurochemical alterations of monoaminergic systems after chronic administration of Fipronil in rats

This part of the thesis was done under the supervision of **Professor Philippe De Deurwaerdere**, Ph.D., Professor of Neuroscience and Neuropharmacology, at the Institut de Neurosciences Cognitives et Intégratives d'Aquitaine (INCIA) and Centre National de la Recherche Scientifique (Unité Mixte de Recherche 5287), **Université de Bordeaux, France**.

1. Introduction

Pesticides are widely used in agricultural and non-agricultural pest control but their use is a matter of Public health. Past pesticides such as rotenone have been associated with the development of neurological conditions including PD or Alzheimer's disease (see first part). Other toxic strategies toward insects have been developed leading to new generations of compounds. FPN, a type class-C of pesticides, is one of these drugs which has been extensively used. Although supported by very few data, FPN is suspected to produce noxious effects possibly leading to Parkinson-like and Alzheimer-like conditions.

To briefly recall the chapter 1 of the thesis, earlier report suggests that FPN blocks chloride ion cellular uptake in invertebrates, leading to uncontrolled central nervous system (CNS) hyper-excitation by blockage of $GABA_A$ receptors, convulsion, and cell death (Raymond-Delpech et al., 2005; Das et al., 2006**)**. Although considered less toxic toward mammals, its absorption is rapid and it easily crosses the blood brain barrier (Khalaf et al., 2019) and recent alarming reports suggest that FPN produces dramatic effects in the brain of rodents. Chronic treatment of FPN in rats caused memory impairment in part associated to the modulation of the GABAergic system (Godinho et al., 2016) and the deposition of amyloid plaques (Cam et al., 2018**)**. FPN exposure could trigger cytotoxicity and neurodegenerative effects due to oxidative stress and modulation of enzyme levels (Abdel-Daim et al., 2018a,b; Khalaf et al., 2019). Upon intra-nigral administration in rats, FPN triggered Parkinson-like conditions; it reduced DA content in the striatum as well as TH levels in the striatum and the SNpc **(**Park et al., 2016; Part 1 of the thesis**)**. It is not established whether the toxic effects of FPN are selective of striatal DAergic systems or, by extension, can alter other monoaminergic systems including the catecholamine noradrenaline (NA) in the brain.

A noxious action of FPN on monoaminergic systems is worth of investigation because it could predispose individuals to developing neuropsychiatric diseases. Indeed, the monoaminergic systems DA, NA, and serotonin (5-HT) have their cell bodies located in the substantia nigra/ventral tegmental area, the locus coeruleus, and the dorsal and medial raphe nuclei, respectively (see below). They innervate the whole central nervous system (CNS) at various degrees and exert complex neuromodulation on motor, cognitive, affective or neuroendocrine functions. It is possible to address the effect of FPN on monoamine systems by

measuring the content of the monoamines and their metabolites. The method is interesting because it allows one to determine possible regional and quantitative alterations of monoamine and the metabolites tissue content in various parts of the brain (Chagraoui et al. 2019; Pifl et al., 1991). In addition, using multiple linear regressions of monoamine content between pairs of brain regions (Fitoussi et al., 2013; Dellu-Hagedorn et al., 2017), it is possible to address possible re-organization of monoamines within and between systems across the brain. Considering the previous studies showing that FPN could impair motor and cognitive functions, it is hypothesized that FPN could alter monoaminergic function in various brain territories.

In the following parts, I will briefly recall the brain organization of monoaminergic systems, their biochemistry, and the possibility to address the tissue contents with descriptive analyses.

1.1 Monoaminergic systems

1.1.1 Dopamine system

DA neurons are a heterogeneous group of cells that are localized in the mesencephalic, diencephalic and the olfactory bulb regions of the brain. However, nearly all DA cells reside in the ventral part of the mesencephalon. The total neuronal population of DA neurons in the brain accounts for less than 1%, ranging from 40,000 in rats and 600,000 in humans (Bjorklund and Dunnett, 2007). The major DAergic pathways are the nigrostriatal, mesolimbic and mesocortical, which play a major role in DA functioning. They originate from mesodiencephalic DA neurons which correspond to A8, A9 and A10 group of cells. The nigrostriatal pathway includes DA neurons originating from the more lateral SNpc (A9 cell group) and projects its fibres into the striatum (caudate-putamen nucleus) and are the most studied due to their involvement in the pathogenesis of PD (Grace and Bunney, 1985). The mesolimbic and the mesocortical pathways comprise of DA neurons originating from VTA (A10 cell group) and extend towards structures closely associated with the limbic system and different cortices (Bannon and Roth, 1983; Deutch et al., 1987; Di Giovanni et al., 2009; Kalivas, 1993; White, 1996). The mesocorticolimbic DA systems appear critically involved in the modulation of emotion-related behaviour and cognitive function (Le Moal and Simon, 1991). The mesolimbic dopaminergic pathway includes VTA DA neurons that project mainly to accumbal areas, amygdala and poorly to the hippocampus (HP). Drugs of abuse, natural reward stimuli such as sexual stimuli and feeding behaviours exert their effects through actions in the mesolimbic system and DA contributes at least partially to their

reinforcing effects (Di Chiara and Imperato, 1988; Koob, 1992)**.** The selective lesioning of DA innervations in the nucleus accumbens (NAc) induces hypo-exploration, enhanced latency in the initiation of motor responses, disturbances in organizing complex behaviours and the inability to switch from one behavioural activity to another. The mesocortical system projection to the medial prefrontal cortex (mPFC) is generally associated with cognitive functions including working memory, planning and execution of behaviour, inhibitory response control and maintenance of focused attention (Le Moal and Simon, 1991). In addition, a variety of physical and psychological stressors activate the mesolimbic DA pathway (Horger et al., 1995). Thus, stress-induced activation of mesocortical DA neurons may be necessary for the behavioural expression of such stimuli (Morrow et al., 1999).

The substantia nigra (SN) also comprises of the substantia nigra pars reticulata (SNpr) area which mainly contains γ-amino-butyric acid (GABA)-ergic neurons that constitutes major efferences of the basal ganglia. There are also some less defined DA neurons in the A11 nucleus, responsible for the mere DA innervation of the spinal cord. Other DA centers can also be found in the hypothalamus (A12 nucleus), playing a role in neuroendrine processes via its projection in the pituitary gland (the tuberoinfundibular pathway). Some DA neurons have been reported in the dorsal raphe nucleus (DRN) (De Deurwaerdère and Di Giovanni, 2017).

1.1.2 Serotonin system

Almost all parts of the brain receive 5-HT innervation that is arising from cell bodies located in the medial raphe nucleus (MRN) and the DRN (Azmitia and Segal, 1978). The 5-HT-containing cell bodies of the raphe nuclei send projections to DA cells located both in the VTA, SNpc, the NAc, mPFC, amygdala and the striatum. The DRN nucleus contains 8,000 to 91,000 5-HT neurons in mice and humans, respectively. It represents 30-56% of 5-HT neurons in the CNS depending on the species (Jacobs and Azmitia, 1992). Interestingly, like DA neurons, 5-HT neurons may coexpress and release other neurotransmitters such as GABA, glutamate and nitric oxide and neuropeptides such as corticotropin-releasing factor (Jacobs and Azmitia, 1992; De Deurwaerdère and Di Giovanni, 2017).

Based on their anatomy and functional topography, six parts of the DRN have been described (Hale and Lowry, 2011). The dorsal part of the DRN send projections to the central and basolateral nuclei of amygdala, the dorsal hypothalamic area and the mPFC (Lowry et al.,

2008). The ventral part of the DRN innervates the sensorimotor cortex and the caudate putamen. The lateral part projects mainly to subcortical regions including the lateral hypothalamus or superior colliculus. The rostral parts send projections to the caudate putamen and the SN, whilst the caudal part sends projections to the amygdala, ventral hippocampus and thalamic nuclei. In mice, the 5-HT neurons innervating adjacent territories such as prelimbic and infralimbic cortices or SNpc and SNpr are distinct in an ontogenic point of view (De Deurwaerdère and Di Giovanni, 2017). The DRN also receives several afferences from the lateral habenula, the lateral dorsal and posterior hypothalamic nuclei, bed nucleus of the stria terminalis, amygdala, cingulate cortex and prefrontal cortex. In central and caudal levels, the DRN receives projections from the SNpr. These projections are organized and show synaptic specialization (Valentino et al., 2001).

The MRN contains less 5-HT cell bodies, comprising approximately 5% of the neurons in the nucleus that project in HP. Although the MRN innervates several brain regions, the projections from this nucleus to the basal ganglia are presumably not releasing 5-HT (Jacobs and Azmitia, 1992; De Deurwaerdère and Di Giovanni, 2017). On the other hand, MRN 5-HT projections innervate specific regions such as the HP or septum.

Due to the widespread distribution in the brain, 5-HT is virtually associated to all CNS functions (Jacobs and Azmitia, 1992) and largely interacts with DA functions in a complex manner (De Deurwaerdère and Di Giovanni, 2017). In terms of psychopharmacology, the 5-HT system has been federating for decades a high interest for the development of drugs ameliorating mood disorders including depression and anxiety. The selective 5-HT reuptake inhibitors are still the first-line treatment of depression in normal population as well as in PD patients (Di Giovanni et al., 2016). They participate also in the mechanisms of action of DBS, DBS inhibiting their activity (Temel et al., 2007; Navailles et al., 2010a), and L-DOPA, 5-HT neurons being responsible for the increase in DA extracellular levels induced by L-DOPA (Navailles et al., 2010b; Tanaka et al., 1999). 5-HT neurons are also altered in PD to a lesser extent than DA neurons (Kish et al., 2008) and, depending on the preclinical model of PD, 5-HT neurons are less altered than DA neurons, not altered, or sometimes activated (Navailles et al., 2012).

1.1.3 Noradrenaline system

The adrenergic cell bodies are dispersed across 7 distinct nuclei (A1 to A7) in the brainstem and innervate in almost all regions of the brain. The NA system corresponds mainly to the locus cœruleus (LC, A6). A1, A5 and A7 clusters are responsible for some ascending and ventral NA projections to the septum, the bed nucleus of the stria terminalis or the hypothalamus (HY). The LC, in which 50% of neurons are adrenergic, is present in all mammalian species and is located dorsally in the pons. It is mainly responsible for the adrenergic innervation of the telencephalon and represents the main source of NA within CNS (Aston-Jones, 2004). The widespread network of LC-derived adrenergic projections exerts a powerful modulatory influence on cortical and sub-cortical circuit and loss of LC-derived adrenergic input to the SNpc may accelerate the demise of dopaminergic cell bodies (Bo Xing, 2016). Nonetheless, the adrenergic innervation of the striatum and globus pallidus is very sparse (Aston-Jones, 2004; Fitoussi et al., 2013).

NA neurons are altered in PD, the depletion of NA being sometimes higher than that reported for DA (Bastide et al., 2015). According to the Braak et al. (2003) hypothesis, NA neurons would be destroyed before DA neurons. No specific NA drugs are used in the treatment of PD although tricyclic antidepressant (blocking the 5-HT and NA transporters) or selective NA reuptake inhibitors can be used as second line of treatment of depression in PD (Delaville et al., 2011).

1.2 Biochemistry of monoaminergic systems

DA is a catecholamine synthesized by the enzyme TH, which catalyzes the conversion of the amino acid L-tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine). L-DOPA is then converted to DA by the aromatic *L-amino acid decarboxylase* (AADC) (Lawlor, 2006). DA can be metabolized into NA by *dopamine β-hydroxylase* (DBH) enzyme. DBH is mainly present in vesicles of NA neurons and can be found in the cytosol as well (Gagnon et al., 1976). 5-HT is produced from L-tryptophan by the activity of *tryptophan hydroxylase* (TPH) and AADC (Di Giovanni et al., 2016).

The degradation of biogenic amines is a complex mechanism because it involves several enzymes and cell types. The *monoamine oxidase* (MAO) exists in two isoforms: MAO-A and MAO-B, which catalyzes the oxidative deamination and produce hydrogen peroxide, ammonia and the corresponding aldehyde. The catecholaminergic neurons mainly contain MAO-A,

whereas serotonergic neurons are rich in MAO-B. DA, NA and 5-HT can be degraded by MAO-A (Di Giovanni et al., 2016).

The other enzyme *catechol-O-methyltransferase* (COMT) can also metabolize DA and NA (Eisenhofer et al., 2004). Two isoforms have been described - soluble COMT (S-COMT) and membrane-bound COMT (MB-COMT) having different subcellular compartmentation. The S-COMT preferentially transforms exogenous catecholamines, whereas MB-COMT mainly inactivates catecholamines and derivatives originating from DA and NA neurotransmission (Myohanen et al., 2010; Tammimaki et al., 2010). COMT is not present in DA terminals (Schendzielorz et al., 2013), implying that glial cells or other neurons reuptake extracellular DA in tissues where the clearance of DA is low. COMT is also involved in the degradation of metabolites generated by MAO activities. The final product from these two distinct catabolic pathways is homovanillic acid (HVA) for DA metabolism and either vanillyl mandelic acid (VMA) for NA metabolism (Eisenhofer et al., 2004; Di Giovanni et al., 2016).

Hence, DA is metabolized to 3,4-dihydroxyphenylacetic acid (DOPAC) by the activity of MAO-A and aldehyde dehydrogenase enzymes which is further converted to HVA by COMT. Likewise, NA is metabolized to VMA by the activity of MAO-A and COMT. The 5-HT is metabolized to 5-hydroxyindole acetic acid (5-HIAA) by MAO-A and aldehyde dehydrogenase.

1.3 Correlative analysis

The monoaminergic systems interact in the mammalian brain and regulate the activity of neurobiological networks. The widespread innervation of DA, 5-HT, and NA systems in cortical and sub-cortical regions suggests that their biochemical interactions can occur in multiple regions directly or indirectly via neurobiological networks (De Deurwaerdere and Di Giovanni, 2017; Fitoussi et al., 2013). The tissue content for DA or 5-HT is often correlated in the same regions but poorly at distal regions. Such an approach may inform about qualitative changes of monoamine tissue contents that could occur independently from quantitative changes (Klouche et al., 2015). However, the tissue content of the neurotransmitter has a limited functional value. It represents mostly the monoamine stored at high concentrations in different vesicular compartments which vary between regions (Dellu-Hagedorn et al., 2017).

The relationship between the content of the neurotransmitter and its metabolite should give a positive correlation of their tissue content within one region in the inter-individual analysis. This

would be a good validation but the metabolism of monoamines is complex. The DA metabolites such as DOPAC, HVA, and 5-HT metabolite such as 5-HIAA are usually measured simultaneously with parent neurotransmitters. The metabolism for a monoamine varies across brain regions as exemplified by the ratios DOPAC/DA and 5-HIAA/5-HT (Fitoussi et al., 2013; Dellu-Hagedorn et al., 2017).

Aim of the study

The action of FPN towards monoamines has been poorly investigated. It could indirectly lead to Parkinson-like conditions by diminishing DA content in the striatum and related brain regions although it remained to be established. We hereby postulate that it may constitute a predisposition factor to develop brain pathologies by altering functional and biochemical organization of monoamines in the brain. The aim of this study was to determine the potential noxious effects of chronic exposure of FPN on monoamine systems in various regions of the rat brain. A moderate dose of FPN 10 mg/kg in our study was given chronically for a period of 21 days in rats by oral gavage. The monoamines were measured using HPLC-ECD in 30 parts of the brain including 5 cortical regions, the nucleus accumbens shell and core, 6 parts of the striatum and the other brain regions associated to the basal ganglia, the habenula, the thalamus, the subthalamic nucleus, the globus pallidus, the dorsal and ventral hypothalamus, dorsal and ventral hippocampus, the central and basolateral amygdala, SN, VTA as well as DRN and MRN.

2. Materials and methods

2.1 Animals

Adult male Sprague Dawley rats, weighing 300-350 g at the beginning of the experiments, were used in the study. Animals were housed in group, 2 per cage, and were maintained under standard conditions with 12-h light/dark cycles at room temperature (22 ± 2°C, 60 ± 5% humidity). They were fed by standard pellet diet and tap water ad libitum along the study. The rats were handled once daily in order to avoid the stress induced during handling and to familiarize them with the experimental person. The treatment was performed between 09:00–16:00 h. All the procedures used were in accordance to European Economic Community (86-6091 EEC) and the French National Committee guidelines (décret 87/848, Ministère de l'Agriculture et de la Forêt) for the care and use of laboratory animals and will be approved by the Ethical Committee of Centre National de la Recherche Scientifique, Région Aquitaine-Limousin.

2.2 Drug and reagents

Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile) (FPN) was provided by Sigma Aldrich, Dusseldorf Germany. All other reagents from available commercial sources were used.

2.3 Treatment and behavioural assessment

After handling during some days, the rats were divided in two treatment groups; the control group with drinking water and the FPN treated group (10 mg/kg) dissolved in drinking water (Kartheek and David, 2018). The rats were given chronic treatment once daily, by oral gavage, for 21 days. During the treatment period, the rats were assessed for their normal behaviour and body weight was taken on daily basis.

2.4 Tissue collection of brain regions

After 48 hours of the last dose, rats were brought to the experimental room in their home cage. After habituation, the rats were sacrificed by decapitation using a guillotine in a quiet room next door. The brain was rapidly removed, immediately immersed in isopentane maintained at -35°C for 3 minutes and then stored in deep freezer at -80°C until use.

On the day of dissection, the rat brain was placed in a cryostat maintained at -24°C and brain areas were collected with the help of a rat brain atlas (Paxinos and Watson, 1998). Using a magnifying glass, the discrete regions were taken out using stainless steel cannulae of 500 or 800 µm inner diameters. The left and right parts of one discrete brain region were pooled in one tube. For the STN, tissue has been scratched using blade in order to avoid the lateral hypothalamus. Samples were stored in labelled and pre-weighed small Eppendorf tubes (0.6 mL volume) and stored in deep freezer at -80°C until analysis.

The bilateral punches were taken from 30 distinct brain regions including motor cortex (M2), orbitofrontal cortex (OFC), prelimbic cortex (PL), the infralimbic cortex (IL), the anterior cingulate cortex (aCg), the anterior insular cortex (ains), the nucleus accumbens (NAc) shell (shell) and core (core), the anterior striatum (aCd), four quadrants of the striatum - dorsomedial striatum (DMS), dorsolateral striatum (DLS), ventromedial striatum (VMS), ventrolateral striatum (VLS), the ventro-caudal striatum (VCS), the globus pallidus pars externa (GPe), the entopeduncular nucleus (EPN), the central (CE) and basolateral (BLA) nucleus of the amygdala , the dorsal and ventral hippocampus (dHP and vHP), the thalamus (Th), the dorsal and ventral hypothalamus (dHY and vHY), the subthalamic nucleus (STN), the Habenula (Hb), the substantia nigra (medial SN1 and lateral SN2), the ventral tegmental area (VTA), the dorsal and median raphe nucleus (DRN and MRN, one punch only). A camera was used to capture pictures of punches of all brain regions (Figure 1).

2.5 Tissue processing and neurochemical analysis

On the day of the neurochemical analysis, the Eppendorf tubes containing the brain tissue of one region were retrieved from deep freezer and placed on ice. The tubes were quickly wiped and weighed on the same precision balance (Dellu-Hagedorn et al., 2017). In all cases, the size for each structure was not significantly different between the groups. The brain tissues were homogenized in 100 µL of 0.1 N $HClO_4$, sonicated, and centrifuged at 13,000 rpm for 30 min at 4°C. Aliquots (10 µL) of the supernatants were directly injected into the HPLC system coupled with electrochemical detection (HPLC-ECD).

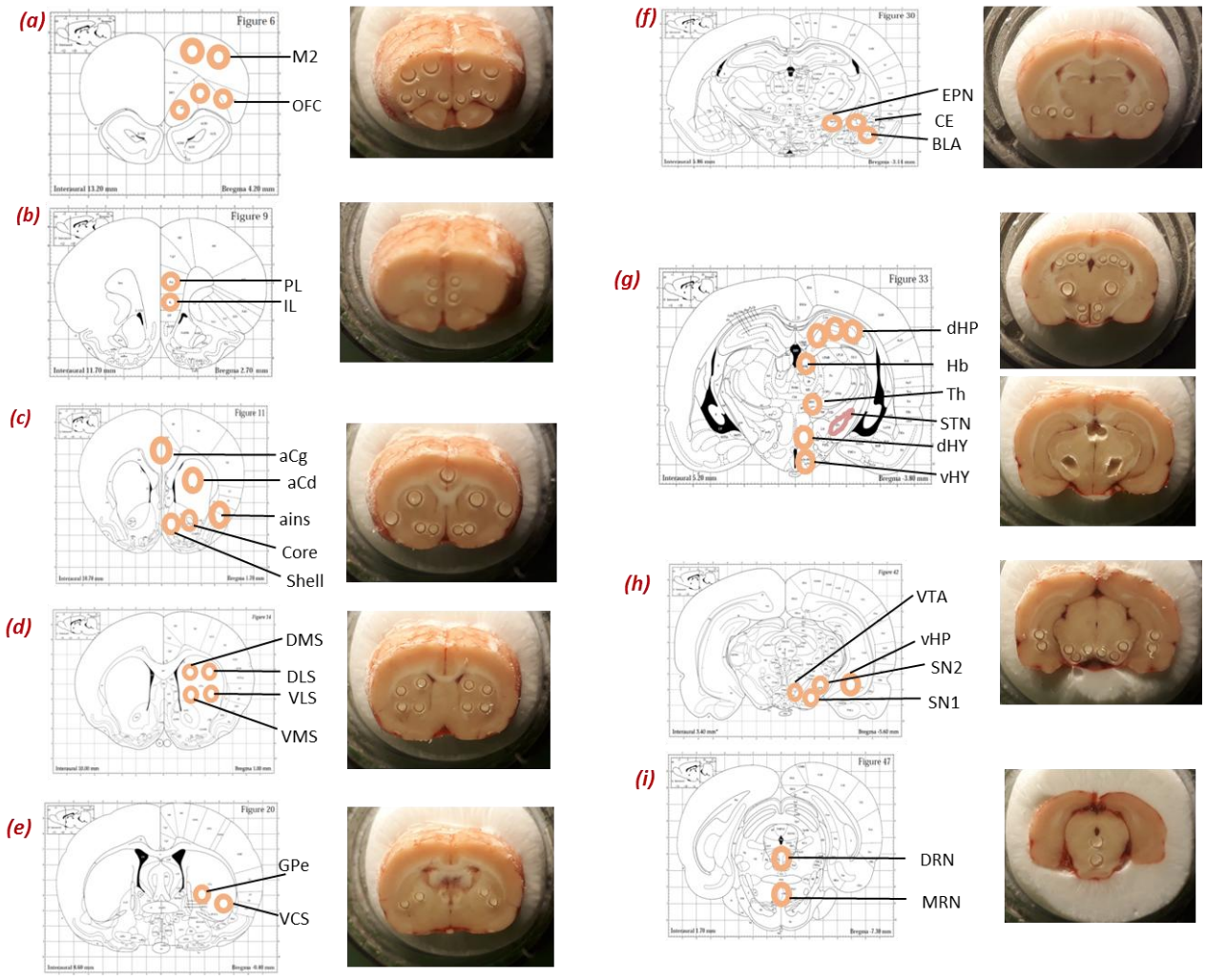


Figure 1. The approximate position of punched tissue aliquots from coronal sections of the rat brain (adapted from Paxinos and Watson, 1998). Tissue samples were taken from left and right cerebral hemispheres in a cryostat. Cortical areas: orbitofrontal cortex (OFC), motor cortex M2, prelimbic (PL) and infralimbic (IL) cortices, anterior cingulate cortex (aCg), anterior insular cortex (ains); Sub cortical areas: nucleus accumbens – shell and core, striatum – anterior caudate (aCd), dorsomedial (DMS), dorsolateral (DLS), ventromedial (VMS), ventrolateral (VLS) and ventrocaudal (VCS) striatum; globus pallidus pars externa (GPe), entopeduncular nucleus (EPN), dorsal and ventral hippocampus (dHP and vHP); habenula (Hb); Thalamus (Th); subthalamic nucleus (STN); amygdala [basolateral nucleus (BLA) and central nucleus (CE)]; dorsal and ventral parts of the hypothalamus (dHY and vHY), substantia nigra - medial part (SN1) and lateral part (SN2); ventral tegmental area (VTA); dorsal raphe nucleus (DRN) and median raphe nucleus (MRN). Three punched tissue in each side were taken from the dHP regions to be able to measure the concentrations of monoamines. The photomicrographs illustrate punched brain region.

2.6 Chromatographic analysis

Tissue concentrations of the monoamines NA, DA, 5-HT and their metabolites were measured using a HPLC-ECD system. Samples were kept on ice after the centrifugation (series of 8 or 10 samples at maximum). Supernatants were injected using a manual injector (Rheodyne 7725i, C.I.L.-Cluzeau, Sainte-Foy-La-Grande, France) into Equisil ODS (C18) HPLC column (150 x 4.6 mm, 5 µm; C.I.L.-Cluzeau) preceded by a Brownlee–Newgard precolumn (RP-8, 15 x 3.2 mm, 7 µm; C.I.L.-Cluzeau). The composition of the mobile phase was as follows (in mM): 70 mM NaH_2PO_4, 0.1 mM disodium EDTA, 2-Octane-sulfonic acid (concentration approximately corresponding to 130 mg/L of mobile phase and adjusted to obtain the best separation) in deionized water (18 $M\Omega.cm^{-2}$) containing 7% methanol. The pH was adjusted at approximately 4 with orthophosphoric acid to get a good separation of the eluents in the chromatogram. The mobile phase was filtered using 0.22 mm Millipore filter. The temperature of the column was maintained at 40°C. The mobile phase was delivered at 1.200 mL/min flow rate using a HPLC pump (LC20-AD, Shimadzu, France).

The monoamines eluted from the column at different retention times (approximately: NA: ≈2.5 min; DOPAC: ≈3.7 min; DA: ≈4.9 min; 5-HIAA: ≈6.2 min; HVA: ≈8.2 min and 5-HT: ≈12.1 min), which then entered the coulometric detection cell (Cell 5011, ESA, Paris, France) equipped with two electrodes (Figure 2). The potential of the two electrodes was fixed at +350 mV and -270 mV on the coulometric detector (Coulochem II, ESA, Paris, France). In return, the detector detects the electrons at the level of the electrodes, the current generated by a compound being directly proportional to its injected quantity (quantitative method) over the tested ranges of concentrations. The coulometric detector was connected to a computer via an interface (Ulyss, Azur system, Toulouse, France). The Azur system allows visualizing the elution time and the amplitude of the different neurotransmitters and their metabolites.

Calibration curves were performed using a range of concentrations of eluents compatible with the expected quantities (ng range for DA in the striatum; pg range for the hippocampus). The changes of gain programmed during the acquisition were precisely set to increase the sensitivity of detection (Chagraoui et al., 2019). Standard solutions containing all the compounds of interest at known concentrations were systematically injected each day before and after a series of samples.

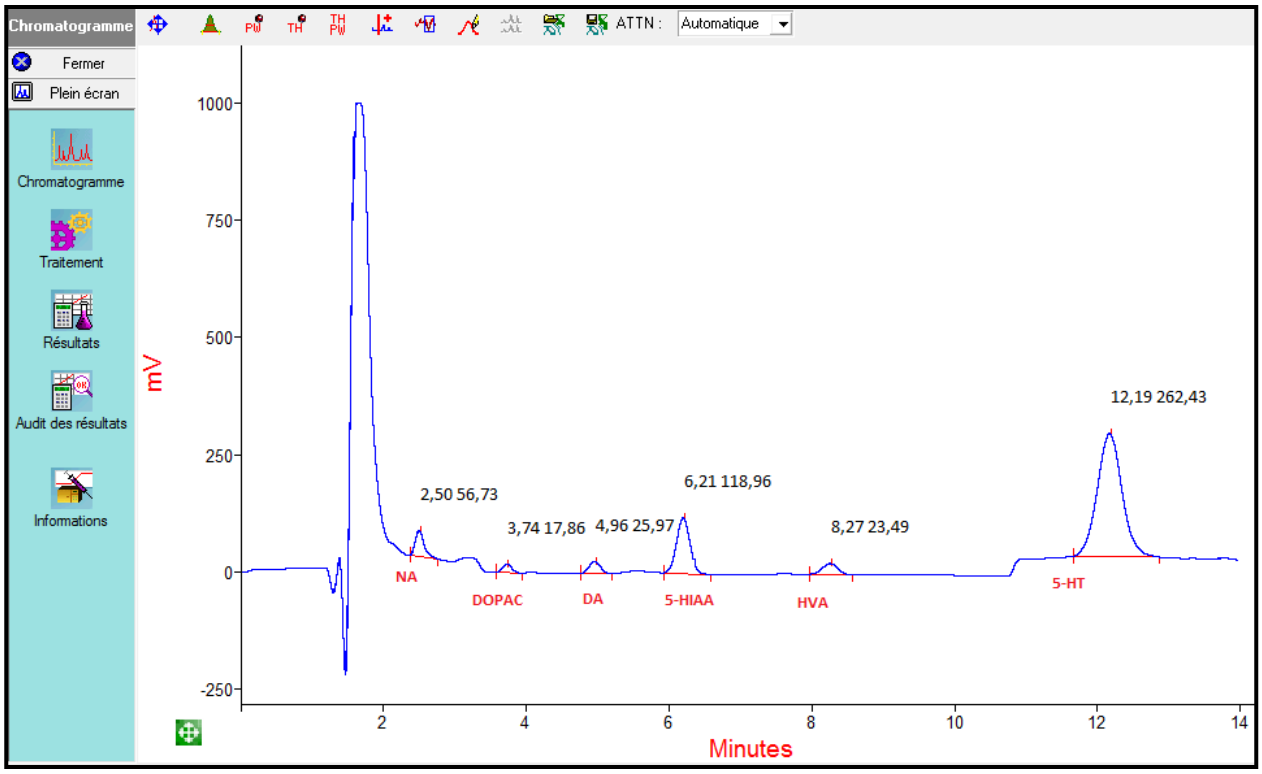


Figure 2. Chromatogram of analytical tissue sample showing 6 corresponding peaks namely NA, DOPAC, DA, 5-HIAA, HVA and 5-HT in the order of their elution from the column.

2.7 Statistical data analysis

The tissue levels of monoamines (NA, DA and 5-HT) and respective metabolites were expressed in pg/mg of tissue. The index of the turnover corresponding to the ratio between the metabolite and its parent neurotransmitter (DOPAC/DA and 5-HIAA/5-HT) was also calculated. For each brain region, the data are presented as the mean ± SEM of values. Aberrant data were discarded on the basis of the value outside the range of the average mean ± two standard deviations (Fitoussi et al., 2013).

The statistical analysis was performed using a Student's t-test comparing the data obtained in control and FPN treated rats for all eluents or the ratios. Correlations was performed using Bravais-Pearson's R correlation test for the content of each monoamine (n=12/group before outliers). For each kind of multiple comparison analysis (Fitoussi et al., 2013), within and between monoamine systems, p-values were adjusted using the False Discovering Rate (FDR) controlling procedures (Benjamini and Hochberg, 1995). Correlation was considered as significant at the 5% level.

3. Results

3.1 Body Weight

The changes in body weight gain under control rats and those exposed to chronic treatment of FPN (10 mg/kg) for 21 days are given in Figure 3. The result showed no significant difference in body weight gain pattern between FPN treated- and the control water-treated rats. A slight decrease in body weight was observed in FPN-treated rats. This indicated that FPN did not hamper on the feeding behavior of rats.

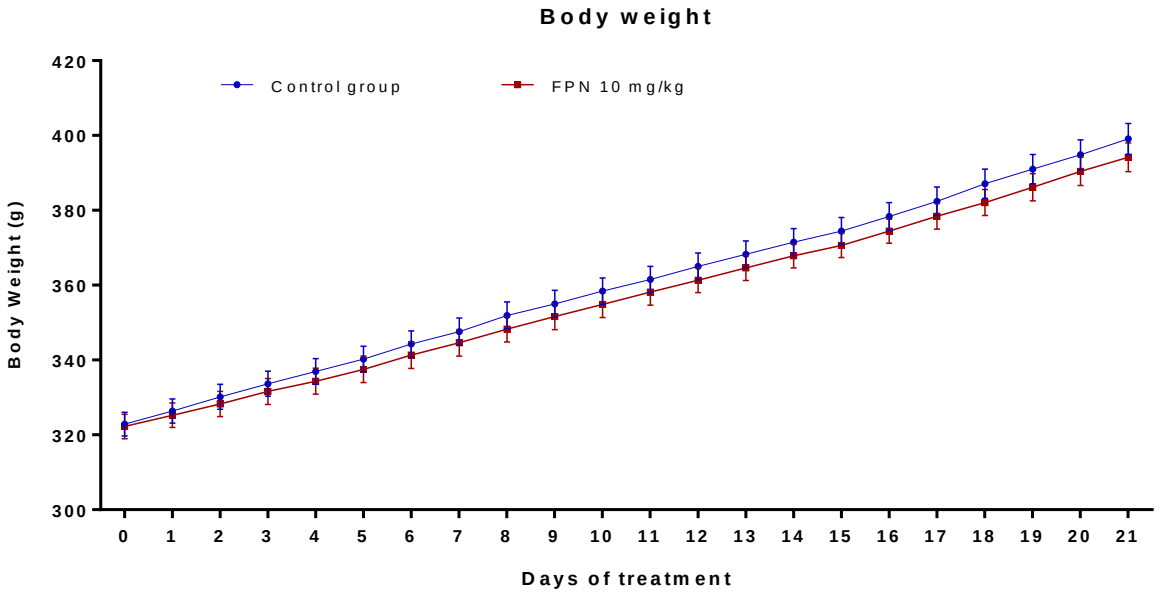


Figure 3. Changes in the body weight gain of control water-treated rats and FPN (10 mg/kg)-treated rats. Values are expressed as mean ± SEM (n = 12 rats per group).

3.2 Quantitative analysis of monoamine tissue contents

We studied the effect of chronic treatment of FPN (10 mg/kg, oral gavage) for 21 days on quantitative distribution of NA, DA and 5HT neurochemical indices in 30 brain regions and compared it with control (water treated) rats. For all brain regions, the size of the tissue did not significantly vary between groups (Student's t-test). The quantitative analysis of the effects of FPN on NA, DA, and 5-HT systems is reported in Table-1.

3.2.1 Quantitative analysis of DA system

The tissue levels of DA were largely heterogeneous across the brain regions of the rats (Table 1a). Briefly, DA levels were very high along the nigrostriatal and mesolimbic areas reaching up to 5039 ± 435 pg/mg of tissue in the aCd; almost equivalent in the striatum (DMS, DLS and VMS except in VLS) reaching over 2800 pg/mg of tissue. The DA levels were also elevated in the NAc shell and core and lower in the amygdala, STN, SN and VTA. Conversely, it was very low in the hippocampus (dHP and vHP with 2.33 ± 0.50 pg/mg and 3.82 ± 1.49 pg/mg of tissue, respectively) and low in the thalamus (8.39 ± 1.28 pg/mg of tissue). The chronic treatment of FPN significantly decreased DA levels in all regions of the striatum (DMS, DLS, VMS, VLS, aCd, and VCS) by 30 to 60% with respect to control rats. Lower levels of DA were also reported in both medial and lateral SN (by ≈50%), in motor cortex M2 (by ≈50%), and in NAc core (by ≈27%). Conversely, the DA level was strongly elevated in the PL by approximately 75% in FPN treated rats. Moreover, FPN did not significantly affect DA levels in other brain regions including cortex (OFC, IL, aCg, ains), the GPe, amygdala, hypothalamus, and hippocampus, VTA, DRN, and MRN.

The distribution of the two main metabolites of DA, namely DOPAC and HVA, was similar to the parent neurotransmitter, marked by very high concentrations in the striatum subdivisions and the NAc shell and core, moderately high in the SN or VTA, and poorly present in the hippocampus (dHP and vHP) (Table 1a). In FPN-treated rats, the DOPAC and HVA levels were significantly decreased in all striatal regions except the VLS when compared to control rats. The levels were also decreased in the medial substantia nigra (SN1) but not significantly in its lateral part. The levels of DOPAC, but not HVA, were substantially decreased in motor cortex M2 of FPN-treated rats. On the other hand, the levels of DOPAC were higher in the hippocampus (dHP and vHP) after chronic FPN treatment, and the levels of HVA levels were

higher only in the vHP. In other brain regions, the levels of DOPAC and HVA were not significantly different between water-treated and FPN-treated rats.

The chronic FPN treatment affects DA turnover (DOPAC/DA ratio) in few brain regions. The DOPAC/DA ratio was significantly decreased in PL of FPN-treated rats which was evident from significant elevation of DA levels. On the contrary, the DA turnover was substantially increased in DLS as compared to control rats. The FPN treatment also enhanced the DA turnover in VLS, VCS and lateral SN (SN2) apparently with considerable decrease in DA levels, but not altering DOPAC levels, in these regions. It was noteworthy that FPN dramatically increased DOPAC/DA ratio in VTA and MRN without significantly modifying the DOPAC or DA levels in these areas (Table 1a).

3.2.2 Quantitative analysis of 5-HT system

Unlike DA, the tissue levels of 5-HT were less heterogeneous across all 30 brain regions. The 5-HT levels were high in DRN (1150 ± 151 pg/mg), MRN (1183 ± 101 pg/mg), SN (845.4 ± 46.6 pg/mg in medial part SN1 and 500.1 ± 54.6 pg/mg in lateral part SN2), and the VTA (688.2 ± 60.8 pg/mg). The levels of 5-HT were lower in other brain regions though easily detectable (Table 1b). The chronic treatment of FPN showed significant effect on 5-HT levels in nucleus accumbens and striato-nigral regions. The levels were significantly decreased in NAc shell and core (by ≈34% and ≈29% respectively) when compared to control group. An important decrease was observed among striatal territories including the aCd (by ≈27%), the ventral striatum (VLS and VMS by ≈42% and ≈37% respectively), and the VCS (by ≈38%), but not in the two dorsal parts of the striatum of FPN-treated rats. As for the DA level, FPN significantly reduced the 5-HT level in the medial SN (SN1, by ≈40) but not in its lateral part. Despite some trend toward a decrease (M2, IL, EPN, CE, BLA, STN, and VTA) or an increase (GPe, SN2, vHP, DRN, and MRN), FPN did not significantly altered 5-HT content in the other brain regions (Table 1b).

The chronic treatment of FPN also modified the pattern of 5-HIAA levels in few brain regions (Table 1b). The tissue levels of 5-HIAA in FPN-treated rats were significantly reduced in the EPN. Like 5-HT, the 5-HIAA levels were decreased in SN1 with respect to control rats. It was noteworthy that the 5-HIAA levels were significantly increased in aCg and DMS after FPN treatment.

Table 1a. Tissue content of Dopamine and its metabolites (pg/mg) in various brain regions of control-water treated rats and FPN-treated rats.

	DA		DOPAC		HVA		DOPAC/DA	
Brain region	**Control**	**FPN**	**Control**	**FPN**	**Control**	**FPN**	**Control**	**FPN**
OFC	16.62 ± 3.99	12.93 ± 2.17	38.96 ± 4.65	37.54 ± 3.43	19.16 ± 2.89	15.84 ± 2.15	2.59 ± 0.37	3.09 ± 0.29
M2	28.20 ± 4.60	13.19 ± 0.98*	57.28 ± 9.03	32.24 ± 3.01*	16.13 ± 2.33	11.58 ± 2.22	2.39 ± 0.15	2.18 ± 0.22
PL	20.37 ± 2.71	35.56 ± 3***	66.65 ± 6.49	85.35 ± 7.00	28.42 ± 3.45	28.54 ± 2.73	3.66 ± 0.25	2.73 ± 0.25*
IL	34.42 ± 8.14	29.02 ± 3.64	83.49 ± 6.36	109.7 ± 16.8	31.93 ± 5.25	36.85 ± 5.56	3.30 ± 0.33	3.65 ± 0.39
aCg	16.74 ± 2.15	16.62 ± 2.29	63.14 ± 8.34	75.58 ± 15.9	49.62 ± 5.96	60.72 ± 13.0	3.64 ± 0.26	4.24 ± 0.52
ains	38.46 ± 6.14	33.44 ± 4.75	77.47 ± 8.51	92.29 ± 13.59	81.96 ± 7.18	92.48 ± 10.3	2.05 ± 0.23	3.11 ± 0.55
Shell	1096 ± 92.6	845.9 ± 86	1643 ± 157	1581 ± 103	262.6 ± 17.9	286.6 ± 24.9	1.75 ± 0.26	1.85 ± 0.15
Core	1291 ± 80.3	941.0 ± 117*	1601 ± 121	1194 ± 159	325.5 ± 26.4	260.5 ± 28.1	1.33 ± 0.08	1.45 ± 0.13
aCd	5039 ± 435	2432 ± 317***	2829 ± 128	1832 ± 186***	696.9 ± 44.33	435.9 ± 40.16***	0.58 ± 0.07	0.74 ± 0.09
DMS	2816 ± 220	1643 ± 129***	1523 ± 134	874.4 ± 71.6***	291.1 ± 20.4	178.9 ± 13.1***	0.53 ± 0.05	0.52 ± 0.03
DLS	2820 ± 206	1230 ± 160***	1159 ± 96.4	729.8 ± 63.5***	370.1 ± 22.5	261.2 ± 18.2**	0.42 ± 0.04	0.63 ± 0.08*
VMS	2835 ± 164	1124 ± 147***	1921 ± 199	1242 ± 120**	432.9 ± 43.6	277.2 ± 24.5*	0.79 ± 0.09	1.19 ± 0.17
VLS	2057 ± 274	1207 ± 162*	1135 ± 127	1082 ± 113	357.3 ± 25.6	412.1 ± 37.5	0.54 ± 0.05	0.94 ± 0.15*
VCS	1799 ± 139	1276 ± 170*	689.6 ± 90.3	651.7 ± 65.9	199.7 ± 20.3	174.9 ± 15.0	0.38 ± 0.04	0.52 ± 0.05*
GPe	66.24 ± 9.38	69.35 ± 11.1	101.9 ± 11.2	101.4 ± 10.8	64.18 ± 6.08	71.45 ± 9.26	1.51 ± 0.13	1.45 ± 0.14
EPN	88.01 ± 9.03	68.08 ± 8.54	58.89 ± 7.73	47.93 ± 8.55	16.44 ± 2.05	16.78 ± 3.69	0.62 ± 0.05	0.59 ± 0.05
STN	100.4 ± 7.34	92.42 ± 13.18	63.37 ± 4.43	65.97 ± 6.73	12.18 ± 1.08	12.48 ± 1.78	0.59 ± 0.05	0.59 ± 0.04
Th	8.39 ± 1.28	6.22 ± 1.01	13.20 ± 1.12	12.73 ± 1.30	4.43 ± 0.72	4.85 ± 0.75	1.75 ± 0.25	2.20 ± 0.19
Hb	16.19 ± 2.63	12.47 ± 1.53	23.45 ± 4.06	19.14 ± 3.86	7.76 ± 1.07	7.13 ± 1.08	1.41 ± 0.29	1.61 ± 0.16
CE	878.5 ± 90.6	645.7 ± 116	324.5 ± 22.2	244.9 ± 32.7	48.91 ± 3.81	51.22 ± 9.57	0.37 ± 0.04	0.40 ± 0.03
BLA	601.8 ± 99.3	575.1 ± 148	308.3 ± 35.6	435.3 ± 67.1	84.80 ± 8.90	97.84 ± 14.0	0.55 ± 0.06	0.81 ± 0.14
dHP	2.33 ± 0.50	2.06 ± 0.42	5.90 ± 1.01	9.34 ± 0.94*	4.82 ± 0.79	4.94 ± 0.64	2.18 ± 0.33	4.88 ± 1.25
vHP	3.82 ± 1.49	2.87 ± 0.75	6.93 ± 0.45	8.56 ± 0.55*	3.44 ± 0.33	4.96 ± 0.39**	2.91 ± 0.65	3.34 ± 0.59
dHY	72.06 ± 9.62	81.76 ± 9.32	42.97 ± 6.17	44.79 ± 3.99	4.90 ± 0.95	5.01 ± 0.42	0.59 ± 0.05	0.55 ± 0.04
vHY	45.43 ± 7.24	33.79 ± 3.92	44.94 ± 5.48	43.97 ± 8.65	5.51 ± 0.49	6.85 ± 0.71	0.84 ± 0.09	1.23 ± 0.28
SN1	318.5 ± 52.2	157.6 ± 27.5*	157.8 ± 16.1	93.94 ± 12.5*	55.25 ± 5.41	28.68 ± 4.65**	0.52 ± 0.04	0.62 ± 0.06
SN2	227.2 ± 33.5	139.3 ± 16.8*	86.04 ± 13.3	64.97 ± 8	27.00 ± 4.07	22.18 ± 2.87	0.36 ± 0.02	0.48 ± 0.04*
VTA	319.3 ± 58.8	291.8 ± 50.6	534.5 ± 97.5	689.3 ± 127	142.8 ± 13.1	124.9 ± 13.2	1.63 ± 0.09	2.28 ± 0.19**
DRN	53.78 ± 8.23	51.09 ± 7.30	34.64 ± 2.93	30.31 ± 3.40	9.38 ± 1.29	10.35 ± 1.27	0.69 ± 0.07	0.61 ± 0.06
MRN	43.85 ± 4.64	34.31 ± 3.95	32.41 ± 2.63	27.29 ± 2.02	25.41 ± 2.53	22.53 ± 2.49	0.61 ± 0.05	0.79 ± 0.07*

Results are expressed as mean ± SEM values (pg/mg of tissue) in various brain regions of control-water treated and FPN-treated rats, except for DOPAC/DA ratio. Starting from 12 rats per group, the final number of observations/group after the outliers is 11 for each parameter and brain regions, except in vHY and Hb where n = 10. *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-test).

The 5-HT turnover (5-HIAA/5-HT ratio) was markedly increased in few brain regions after FPN treatment including the NAc shell, the aCd, the VLS, and the VCS. Moreover, the ratio was also increased in the DMS. The 5-HT turnover was substantially increased in IL, the DLS, DRN, and MRN of FPN-treated rats without significant changes on 5-HT or 5-HIAA tissue levels (Table 1b).

3.2.3 Quantitative analysis of NA tissue contents

The tissue levels of NA are reported in Table 1b. The highest levels were observed in hypothalamus (dHY, vHY), VTA, DRN, and MRN. The levels were lower in the other brain regions. They were not detected in some parts of the cortex (including M2 and OFC), the striatum (DMS, DLS, VMS, VLS, aCd) as well as the GPe. Even if the levels are reputedly low in these brain regions, the elution time of NA in our conditions was too close to the solvent front impairing a good determination of the electrochemical signal corresponding to NA in these brain regions.

As compared to control group, the chronic treatment of FPN significantly decreased NA level in the NAc core only (by ≈33%). The NA levels were slightly, though not significantly, decreased by FPN in other regions such as EPN, BLA, hypothalamus (dHY, vHY) and SN1 (Table 1b).

Table 1b. Tissue content of Noradrenaline, Serotonin and its metabolites (pg/mg) in various brain regions of water-treated and FPN-treated rats.

	NA		5-HT		5-HIAA		5-HIAA/5-HT	
Brain region	**Control**	**FPN**	**Control**	**FPN**	**Control**	**FPN**	**Control**	**FPN**
OFC	nd	nd	89.67 ± 15.79	73.27 ± 7.68	594.3 ± 65.1	560.4 ± 68.1	6.99 ± 0.86	7.62 ± 0.74
M2	nd	nd	170.5 ± 24.3	140.8 ± 19.2	675.5 ± 82.6	628.3 ± 46.8	4.14 ± 0.70	5.73 ± 0.75
PL	43.15 ± 6.33	36.07 ± 3.83	55.77 ± 7.74	49.76 ± 5.67	362.8 ±38.4	404.9 ± 32.2	6.60 ± 0.51	8.67 ± 1.04
IL	52.20 ± 10.4	53.94 ± 6.99	83.63 ± 16.8	67.84 ± 8.41	415.8 ± 50.6	516.9 ± 73.1	5.16 ± 0.67	7.50 ± 0.88*
aCg	66.20 ± 5.14	69.99 ± 8.57	45.75 ± 4.69	44.06 ± 6.67	348.3 ± 43.3	543.4 ± 80.0*	7.87 ± 1.09	10.34 ± 0.94
ains	98.14 ± 14.4	93.90 ± 7.73	174.4 ± 19.5	184.3 ± 16.7	645.9 ± 40.9	629.2 ± 68.4	3.79 ± 0.45	4.09 ± 0.53
Shell	120.4 ± 15.3	115.3 ± 15.4	136.1 ± 16.0	89.27 ± 10.4*	300.4 ± 20.78	310.1 ± 24.57	2.25 ± 0.29	3.62 ± 0.49*
Core	75.38 ± 4.91	50.29 ± 7.38*	114.3 ± 5.98	80.13 ± 6.81**	276.6 ± 21.2	244.7 ± 36.4	2.58 ± 0.17	2.91 ± 0.22
aCd	nd	nd	97.66 ± 7.04	70.03 ± 9.71*	410.2 ± 18.5	347.4 ± 32.8	4.12 ± 0.21	5.21 ± 0.42*
DMS	nd	nd	59.45 ± 4.89	57.12 ± 8.68	199.2 ± 16.8	405.57 ± 46.4***	3.18 ± 0.23	7.03 ± 0.45***
DLS	nd	nd	68.68 ± 6.04	56.19 ± 9.29	264.7 ± 24.0	244.6 ± 28.7	3.57 ± 0.17	4.47 ± 0.33*
VMS	nd	nd	199.8 ± 17.4	115.9 ± 15.0*	459.1 ± 50.5	352.0 ± 30.4	2.83 ± 0.15	3.08 ± 0.32
VLS	nd	nd	67.55 ± 8.54	42.08 ± 4.76*	242.0 ± 19.1	227.6 ± 28.6	3.56 ± 0.28	5.58 ± 0.44***
VCS	49.96 ± 3.77	37.16 ± 5.16	184.5 ± 15.9	113.3 ± 18.2**	235.3 ± 20.7	182.7 ± 21.2	1.27 ± 0.11	1.64 ± 0.09*
GPe	nd	nd	181.7 ± 11.9	211.5 ± 29.7	666.7 ± 80.4	602.4 ± 64.3	3.40 ± 0.41	2.84 ± 0.37
EPN	95.32 ± 9.49	80.36 ± 13.05	219.1 ± 22.3	158.2 ± 22.7	627.9 ± 62.8	414.5 ± 43.4*	2.71 ± 0.12	2.67 ± 0.20
STN	284.9 ± 18.72	266.6 ± 30.3	359.2 ± 30.5	267.1 ± 38.1	846.7 ± 62.9	741.2 ± 63.4	2.27 ± 0.19	2.43 ± 0.12
Th	185.5 ± 8.99	172.2 ± 15.3	100.0 ± 16.8	84.82 ± 7.43	530.9 ± 51.9	507.4 ± 41.2	5.78 ± 0.76	5.69 ± 0.56
Hb	53.9 ± 7.66	46.58 ± 6.13	122.8 ± 22.3	103.2 ± 17.5	390.7 ± 66.1	412.7 ± 50.1	3.31 ± 0.85	3.42 ± 0.55
CE	184.9 ± 20.9	184.4 ± 19.1	250.1 ± 32.6	201.7 ± 24.5	442.3 ± 63.2	382.8 ± 40.5	1.74 ± 0.16	1.93 ± 0.17
BLA	126.0 ± 9.29	99.37 ± 11.1	214.5 ± 18.1	181.3 ± 28.9	428.2 ± 27.6	451.3 ± 37.3	1.97 ± 0.21	2.72 ± 0.33
dHP	218.9 ± 21.1	223.5 ± 16.1	133.3 ± 15.9	121.3 ± 14.2	428.1 ± 46.8	480.6 ± 35.3	3.21 ± 0.40	4.19 ± 0.68
vHP	191.8 ± 22.5	231.1 ± 18.3	165.3 ± 19.9	187.9 ± 21.8	684.9 ± 50.7	830.4 ± 92.8	3.91 ± 0.51	5.23 ± 0.59
dHY	702.6 ± 143	645.9 ± 137	253.6 ± 34.4	219.2 ± 22.3	522.9 ± 71.5	509.0 ± 48.9	2.03 ± 0.14	2.28 ± 0.11
vHY	636.9 ± 111	510.5 ± 55.9	171.4 ± 19.2	143.5 ± 16.1	392.2 ± 43.7	503.6 ± 71.7	2.27 ± 0.19	3.46 ± 0.56
SN1	119.4 ± 13.4	95.23 ± 19.4	845.4 ± 46.6	501.4 ± 62.3***	941.5 ± 35.1	771.2 ± 72.4*	1.14 ± 0.05	1.52 ± 0.17
SN2	121.3 ± 13.3	109.3 ± 9.69	500.1 ± 54.6	544.6 ± 52.5	574.1 ± 74.9	731.7 ± 54.1	1.11 ± 0.05	1.36 ± 0.11
VTA	474.1 ± 38.9	370.9 ± 47.2	688.2 ± 60.8	494.2 ± 79.0	885.5 ± 88.9	728.3 ± 86.1	1.24 ± 0.09	1.50 ± 0.09
DRN	568.9 ± 83.8	501.9 ± 70.9	1150 ± 151	1228 ± 193	2119 ± 186	2279 ± 317	1.65 ± 0.07	1.87 ± 0.06*
MRN	352.4 ± 30.6	302.8 ± 38.4	1183 ± 101	1125 ± 121	2960 ± 262	3206 ± 370	2.47 ± 0.09	2.84 ± 0.07**

Results are expressed as mean ± SEM values (pg/mg of tissue) in various brain regions of control-water treated and FPN-treated rats, except for 5-HIAA/5-HT ratio. Starting from 12 rats per group, the final number of observations/group after the outliers is 11 for each parameter and brain regions, except in vHY and Hb where n = 10. *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-test). nd – not detected.

3.3 Qualitative and correlative analysis of monoamine tissue contents

3.3.1 Within monoaminergic systems

To get a deeper analysis, we then evaluated possible relationships of monoamines between the 30 investigated brain areas using a correlative approach in order to evaluate the pattern and the number of correlations after chronic treatment of FPN (10 mg/kg).

3.3.1.1 Correlative analysis of DA system

The number of correlations for DA content in the control group rats was 25 including positive (20) and negative (5) correlations. The DA content in IL and DRN mostly correlated with that in other brain regions (5 and 6 respectively), while the GPe and ains showed 3 and 4 correlations respectively. The DA content in the OFC, PL, DLS, VMS, Hb, and MRN did not correlate with other brain regions. The FPN (10 mg/kg) treatment did not alter number of correlations for DA content (23, comprising 19 positive and 4 negative correlations) but modified the pattern. At variance with control water-treated rats, FPN treatment induced correlations for DA content of the OFC, Th and MRN (4-6) and slightly for DLS (2). Conversely, no correlations were observed for the DA content of the NAc shell and core, DMS, BLA, the two parts of the hypothalamus, and Hb (Figure 4a).

Likewise, DOPAC correlated in 32 brain areas (comprising 27 positive and 5 negative correlations) in control rats. The pattern of correlations seemed balanced across the brain regions. The DOPAC content in NAc shell showed higher number of correlations (7) with that in other brain regions, while that in the striatal territories, EPN, dHY, vHY, VTA, and SN2 showed few correlations (2-4). The number of correlations slightly decreased after FPN 10 mg/kg treatment (25 comprising 17 positive and 8 negative correlations). FPN treatment decreased the correlations in NAc shell (2), the striatal regions (except DMS), the EPN, dHY, SN2 (1 in each), and the VTA (0). Conversely, the correlations for DOPAC content increased in IL and core (4 and 3 respectively) after FPN treatment. The number of correlations remained similar in other brain regions between the two groups. In terms of general pattern, the numerous correlations (13) for DOPAC inside the basal ganglia of the control group were reduced to only one in FPN treated rats (Figure 4b).

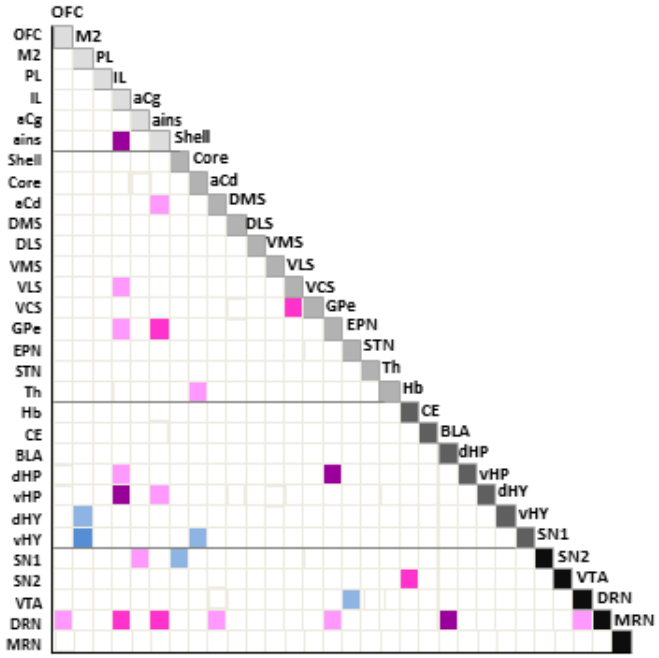
The HVA content correlated in 25 brain areas, mostly positive (only 1 negative correlations) in control group rats while number of correlations almost remained similar in FPN (10 mg/kg) treated rats (26, 18 positive and 8 negative correlations) (Figure 4c). In control group rats, the HVA content in aCg and Th correlated with few other brain regions (2-4) which were remarkably decreased in FPN-treated rats. The HVA content of GPe correlated with that in aCg, NAc shell, and aCd; DMS HVA with that in CE and dHP of control group rats were diminished in FPN-treated rats. Similarly, the correlations of HVA content present in VLS, dHP, vHY, and CE were completely lost after FPN treatment. On the other hand, the FPN treatment enhanced the correlations of HVA content in PL with 6 other brain regions (aCg, NAc shell, VLS, EPN, BLA, and SN2), in dHY with 3 other brain regions (BLA, vHP, and MRN) as well as in BLA with 4 other brain regions (dHY, SN2, vHP, and MRN).

We also evaluated the effect of chronic administration of FPN (10 mg/kg) on DA turnover (DOPAC/DA ratio) (Figure 4d). As expected (Dellu-Hagedorn et al., 2017), we observed a higher number of correlations for DOPAC/DA ratio compared to DA or DOPAC alone (44 significant including 40 positive and 4 negative correlations). Regions such as IL, aCd, EPN, and CE established greater number of correlations with other brain regions (6-8). The pattern of correlation for DA turnover was more prominent in cortico-striatal region, striatal with amygdala (CE, BLA), EPN with frontal cortex. The chronic treatment of FPN (10 mg/kg) decreased the number of correlations for the DA turnover (31, 23 positive and 8 negative correlations). FPN treatment dramatically reduced the correlations for the DA turnover between cortical and basal ganglia, striatum with amygdala (CE, BLA), EPN with frontal cortex. Conversely, FPN treatment enhanced the correlation for DOPAC/DA ratio in Th, vHY, and Hb. The pattern of correlation for DOPAC/DA ratio in vHP was restricted to IL, NAc shell, EPN and CE of control rats while it extended to VCS, Th, and STN of FPN-treated rats.

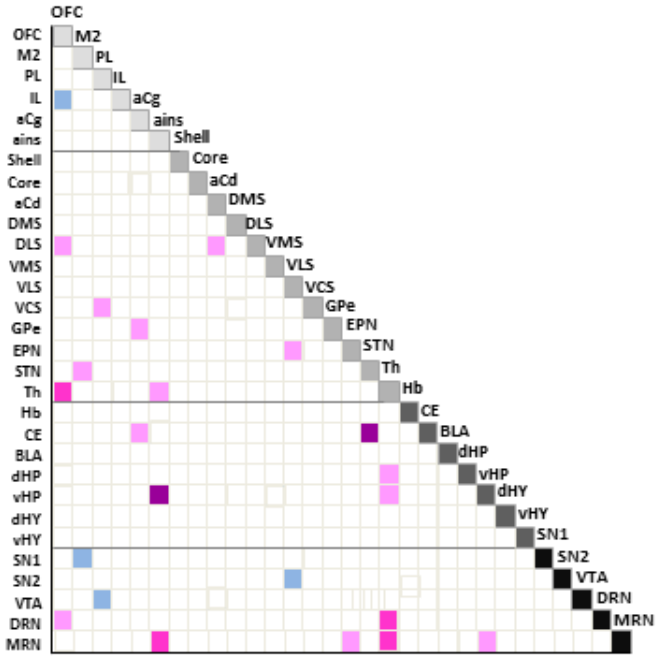
a) Dopamine

Positive correlations	
R > 0.60	
R > 0.70	
R > 0.80	

Negative correlations	
R > 0.60	
R > 0.70	
R > 0.80	



Control group

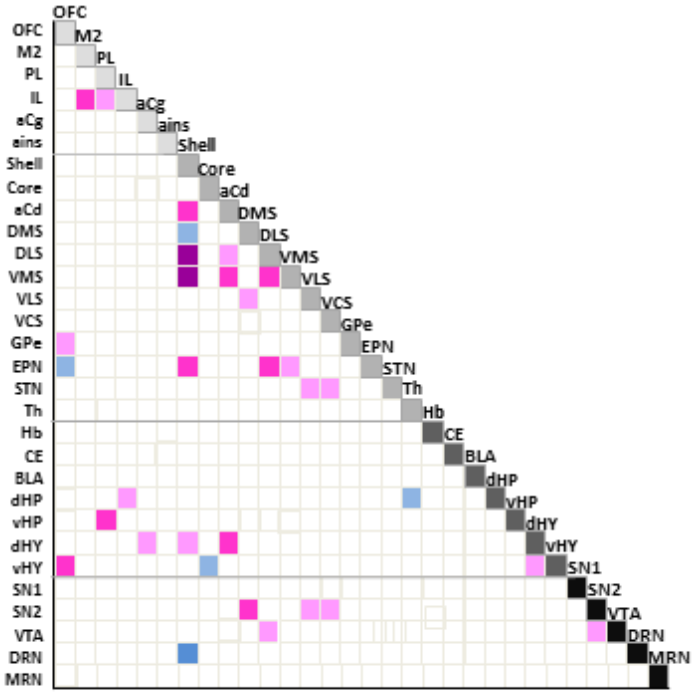


FPN 10 mg/kg

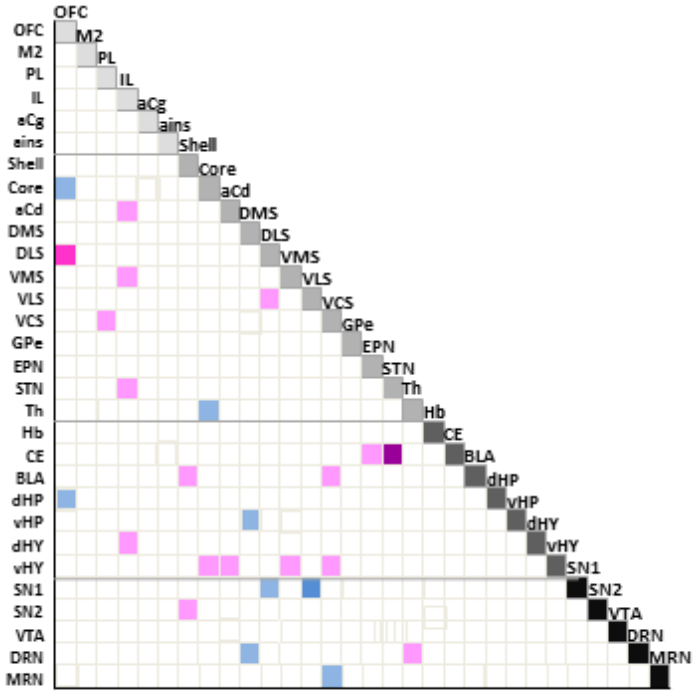
b) DOPAC

Positive correlations	
R > 0.60	
R > 0.70	
R > 0.80	

Negative correlations	
R > 0.60	
R > 0.70	
R > 0.80	



Control group

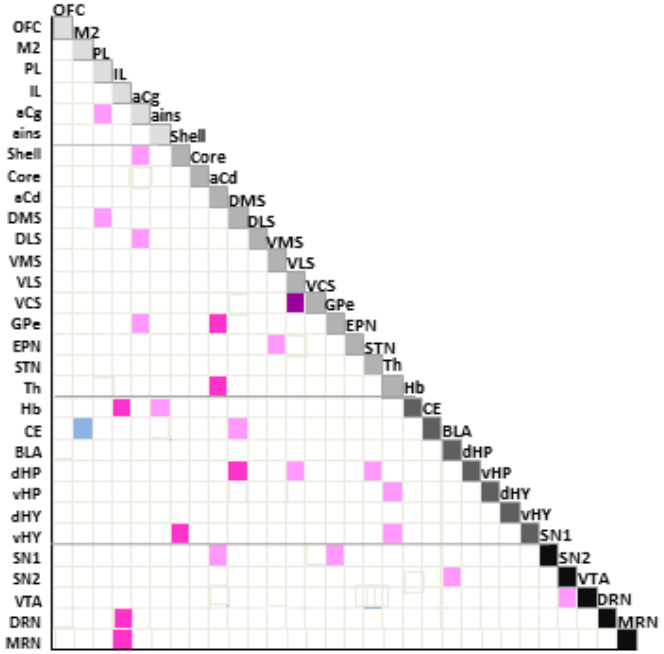


FPN 10 mg/kg

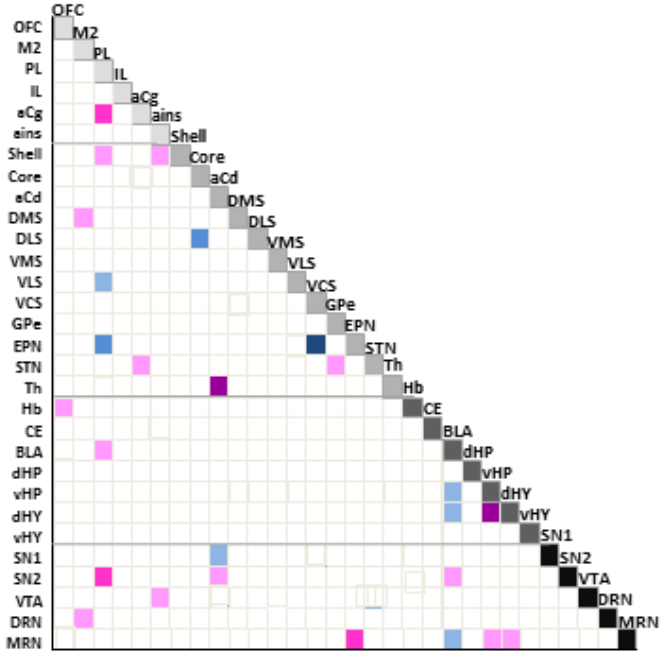
c) HVA

Positive correlations	
R > 0.60	
R > 0.70	
R > 0.80	

Negative correlations	
R > 0.60	
R > 0.70	
R > 0.80	



Control group



FPN 10 mg/kg

d) DOPAC/DA ratio

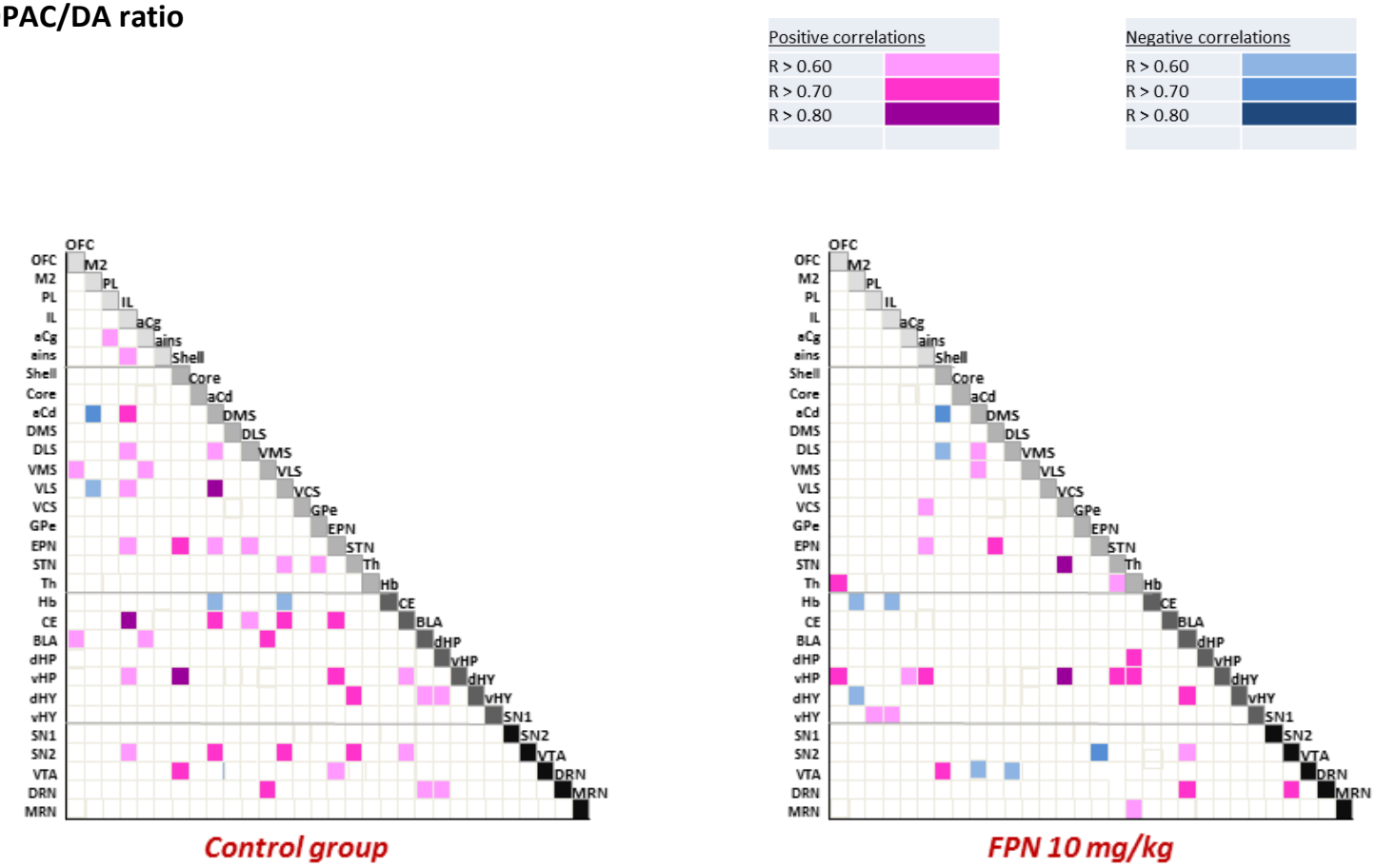


Figure 4. Correlative analysis of DA content across rat brain regions. Representation of the range of Pearson's R values for each linear regression of DA (a), DOPAC (b), HVA (c) tissue contents (pg/mg) as well as DOPAC/DA ratio (d) between the 30 brain areas in control water-treated rats (first column) and FPN 10 mg/kg treated rats (second column). Colored boxes correspond to the existence of a correlation between the two parameters (pink: positive; blue: negative) considered after correction for multiple comparisons.

3.3.1.2 Correlative analysis of 5-HT system

We then analyzed the effect of chronic treatment of FPN (10 mg/kg) on pattern of correlations of 5-HT and 5-HIAA contents along with its turnover (5-HIAA/5-HT ratio).

The number of correlations for 5-HT content was slightly different between the control group (27, just 2 negative correlations) and FPN (10 mg/kg) treatment group (23, comprising 15 positive and 8 negative correlations) (Figure 5a). The correlations of 5-HT content, more prominent in the cortical region (M2, OFC, IL, ains) and the NAc of control group rats were decreased in FPN treated rats. In contrast, the correlations enhanced in dorsal striatum (DMS and DLS) and NAc core with other brain regions in FPN treated rats. FPN treatment also reduced the correlations for 5-HT content between the cortex (PL, IL) with STN, DRN and MRN, and the vHP.

We observed higher number of correlations for 5-HIAA content in control groups (38, all positive) compared to FPN group (29, containing 22 positive and 7 negative correlations). The correlations were numerous in NAc shell, DLS, VLS, and STN with other brain regions (6-7) of control rats. The pattern was more confined between the striato-striatal region, striatum with cortical (aCg, ains) and NAc shell of control rats which was considerably reduced in FPN treated rats. Likewise, the correlations of the 5-HIAA content in SN2, EPN, and vHP were lost after FPN treatment. FPN also reduced the correlations of 5-HIAA content in hypothalamus (dHY and vHY), the STN, vHP and VTA; on the contrary the correlations were increased with M2, OFC, NAc core, and DRN (Figure 5b).

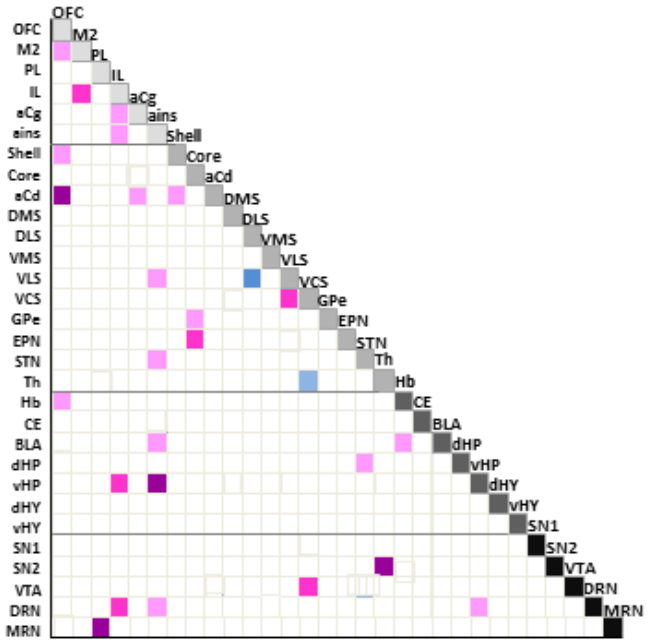
We also evaluated the effect of chronic treatment of FPN (10 mg/kg) on 5-HT turnover (5-HIAA/5-HT ratio) (Figure 5c). It was noticeable that the number of correlations for 5-HIAA/5-HT ratio was dramatically higher than 5-HT or 5-HIAA. The control group rats showed 83 strong correlations for 5-HT turnover (just 1 negative). The number of correlations was very high with GPe, STN, and vHP (12-14), and still high with M2, aCg, NAc shell, BLA, Hb, and MRN (8-10). FPN treatment decreased the number of correlations for 5-HT turnover to 55 (49 positive and 6 negative correlations). Numerous correlations cited above were reduced in FPN-treated rats. On the contrary, FPN treatment enhanced the correlations of the 5-HT turnover involving the frontal cortices OFC and PL, NAc core, and Th. FPN treatment also elevated correlations of the 5-HIAA/5-HT ratio in VLS. The number of correlations for 5-HT turnover remained similar

for ains, DMS, hypothalamus (dHY, vHY), dHP in the two groups even if the pattern was slightly modified.

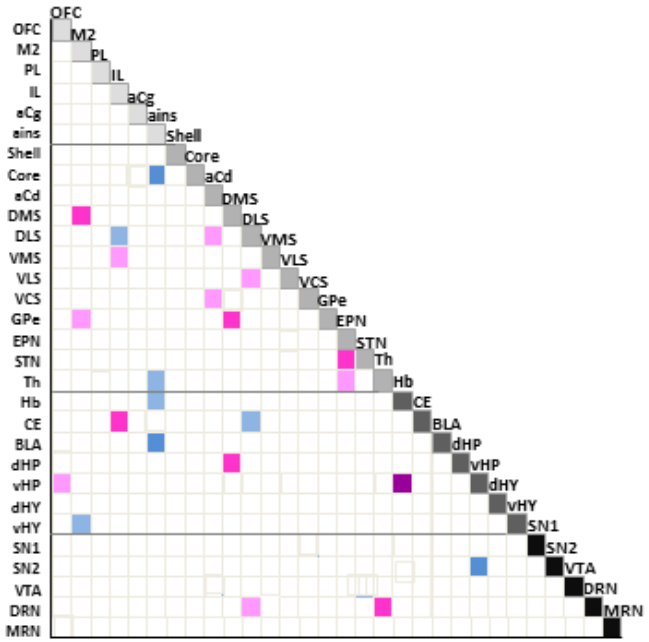
a) Serotonin

Positive correlations	
R > 0.60	
R > 0.70	
R > 0.80	

Negative correlations	
R > 0.60	
R > 0.70	
R > 0.80	



Control group

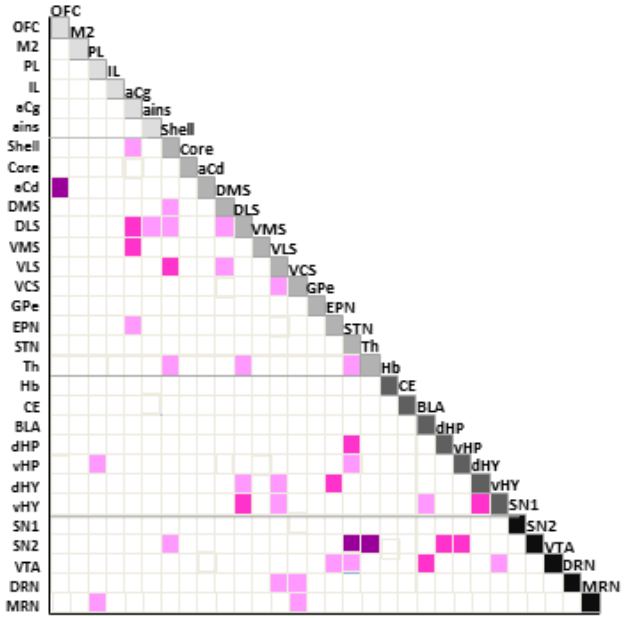


FPN 10 mg/kg

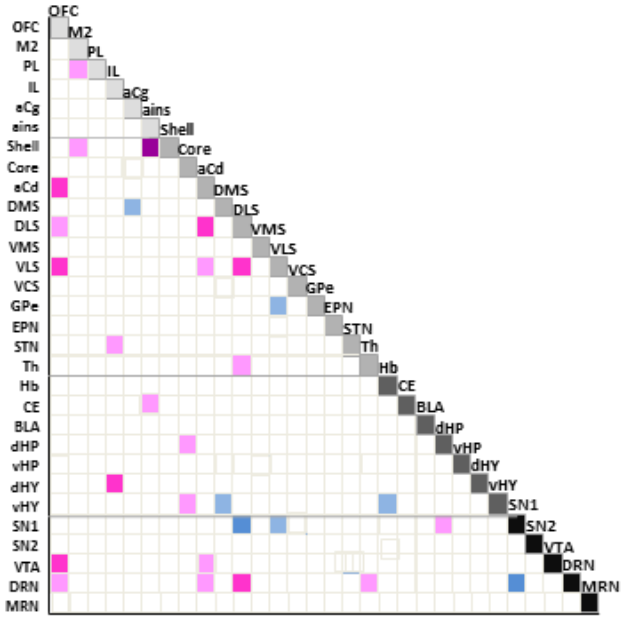
b) 5-HIAA

Positive correlations	
R > 0.60	
R > 0.70	
R > 0.80	

Negative correlations	
R > 0.60	
R > 0.70	
R > 0.80	



Control group



FPN 10 mg/kg

c) 5-HIAA/5-HT ratio

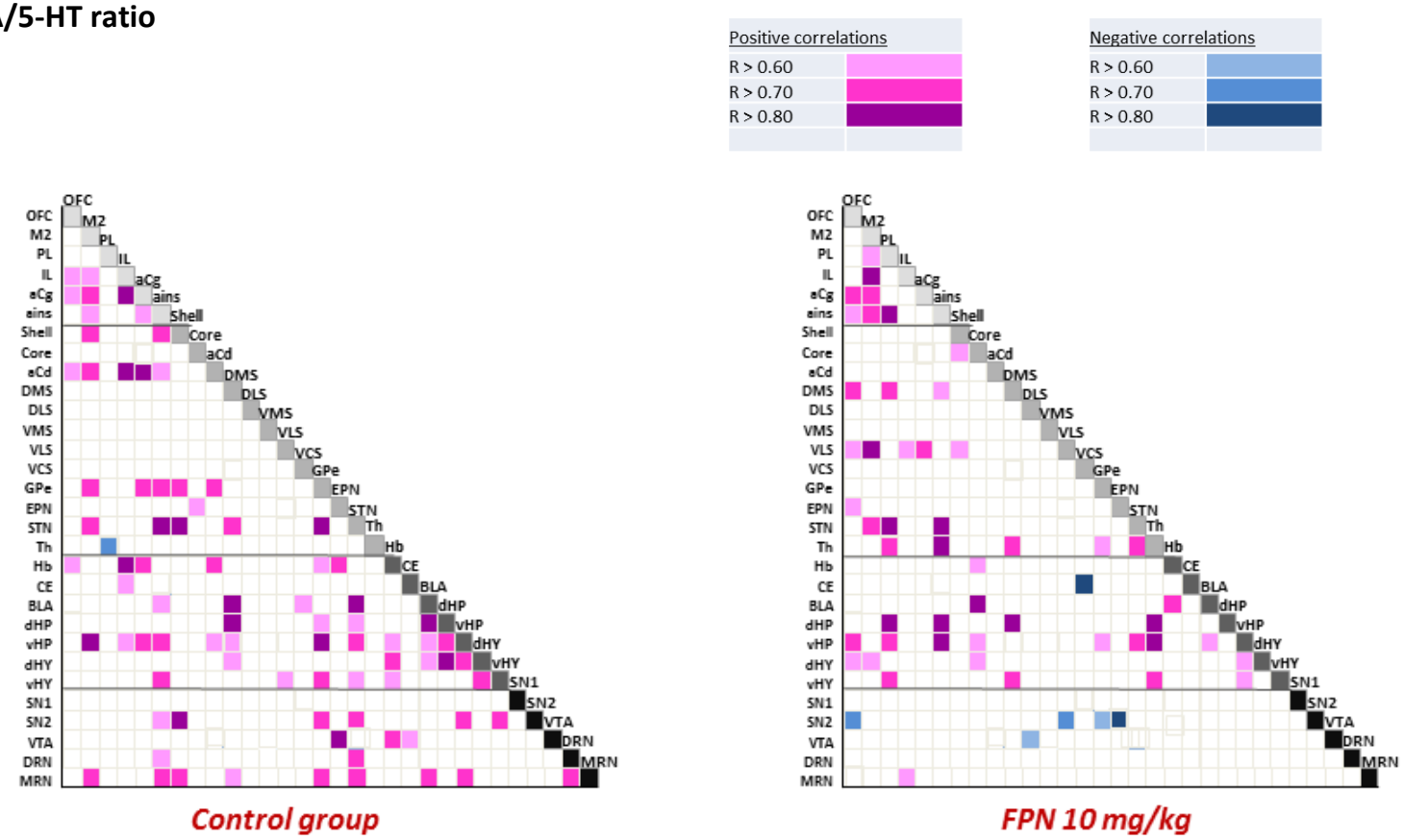


Figure 5. Correlative analysis of 5-HT content across rat brain regions. Representation of the range of Pearson's R values for each linear regression of 5-HT (a), 5-HIAA (b) tissue contents (pg/mg) as well as 5-HIAA/5-HT ratio (c) between the 30 brain areas in control water-treated rats (first column) and FPN 10 mg/kg treated rats (second column). Colored boxes correspond to the existence of a correlation between the two parameters (pink: positive; blue: negative) considered after correction for multiple comparisons.

3.3.1.3 Correlative analysis of NA tissue contents

Noradrenaline

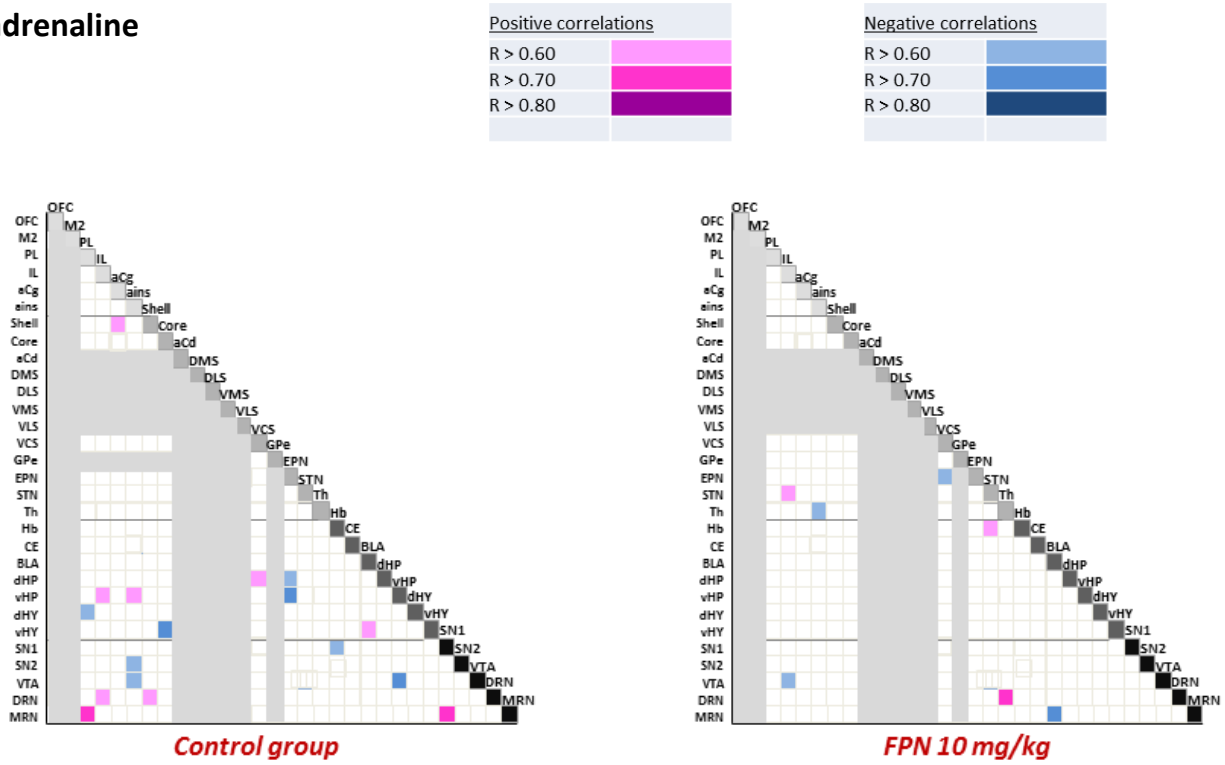


Figure 6. Correlative analysis of NA content across rat brain regions. Representation of the range of Pearson's R values for each linear regression of NA tissues content (pg/mg) between the 30 brain areas in control water-treated rats (first column) and FPN 10 mg/kg treated rats (second column). Colored boxes correspond to the existence of a correlation between the two parameters (pink: positive; blue: negative) considered after correction for multiple comparisons. The gray boxes correspond to undetected values for NA.

We observed very few correlations between the NA content (17, comprising 9 positive and 8 negative correlations) in keeping in mind that the correlations were done a limited number of regions compared to the other parameters (see figure). The FPN treatment decreased the number of correlations for NA content (7, comprising 3 positive and 4 negative correlations). In control group rats, the NA content in the NAc shell correlated with that in IL and DRN which was completely lost in FPN treated rats. Likewise, NA content in the hippocampus (dHP, vHP) and hypothalamus (dHY, vHY) was absent in FPN treated rats. FPN treatment decreased the correlations in IL, ains, VCS, EPN, and DRN as compared to control water-treated rats (Figure 6).

3.3.2 Between monoaminergic systems

We further extended our analysis to understand effects of chronic FPN treatment on possible correlations between the monoamines using the combinations of DA with 5-HT, NA with 5-HT, NA with DA and/or the DOPAC/DA with 5-HIAA/5-HT ratios across the 30 brain regions under consideration.

3.3.2.1 Correlative analysis between DA and 5-HT tissue contents

The DA and 5-HT tissue contents correlated in 78 brain areas (comprising 61 positive and 17 negative correlations), including 19 in the same brain area (all positive) in control rats. The regions establishing the most correlations were IL (10), ains (8), VLS and vHP (7 each); followed by OFC, aCd, VCS, GPe, CE, SN2 (5-6) and few in other brain regions (1-4). The general pattern showed that the cortical content of 5-HT correlated with the DA content in the cortex, the basal ganglia, the limbic regions and the mesencephalon. Similarly, the cortical DA content correlated with the content of 5-HT in subcortical regions. The number of correlations decreased after FPN treatment (63, comprising 44 positive and 19 negative correlations). The FPN enhanced the correlations within aCg, ains, VLS, GPe and Th. The main change corresponded to the substantial reductions of correlations involving cortical 5-HT and DA content within the cortex and toward the subcortical areas. Conversely, there was an increase in the correlations involving DA in the basal ganglia and 5-HT in limbic regions (Figure 7).

3.3.2.2 Correlative analysis between NA and DA tissue contents

A total of 56 correlations were observed between NA and DA contents, including 10 in the same brain area (all positive). The proportion of negative correlations was low (17) compared to positive correlations (39) in control group rats. FPN 10 mg/kg treatment decreased the number of correlations (42 comprising 31 positive and 11 negative correlations), including 9 in the same brain area. NA content in the cortex correlated less with DA in the limbic and mesencephalon regions in FPN-treated rats. Similarly, cortical DA correlated less with NA in the limbic and mesencephalic regions in FPN-treated rats. The correlations of NA content in the DRN with the DA content of 8 different brain regions were reduced in FPN treated rats (Figure 8).

Between DA and 5-HT tissue contents

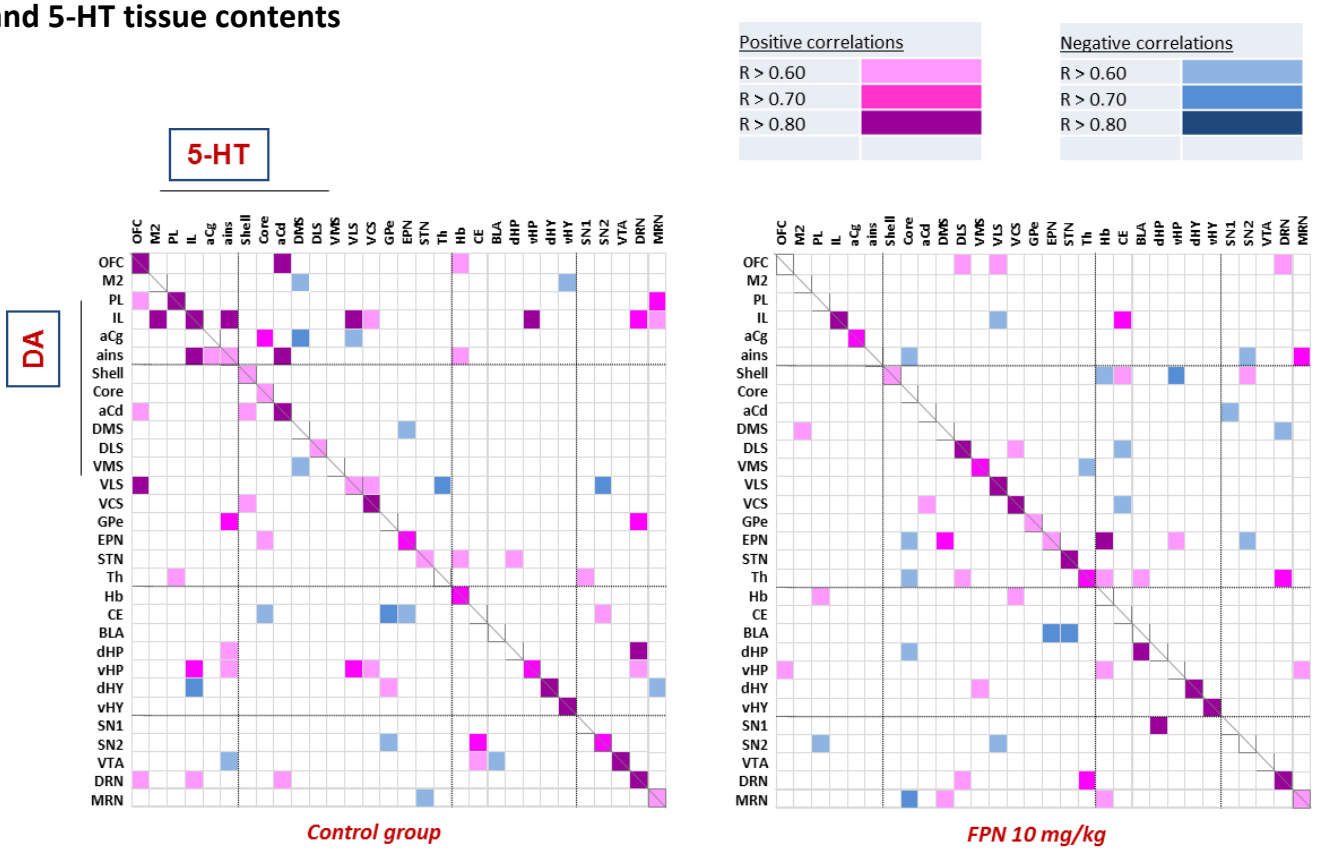


Figure 7. Correlative analysis between DA and 5-HT tissue contents across rat brain regions. Representation of the range of Pearson's R values for each linear regression between DA and 5-HT tissue contents (pg/mg) between the 30 brain areas in control water-treated rats (first column) and FPN 10 mg/kg treated rats (second column). Colored boxes correspond to the existence of a correlation between the two parameters (pink: positive; blue: negative) considered after correction for multiple comparisons.

Between NA and DA tissue contents

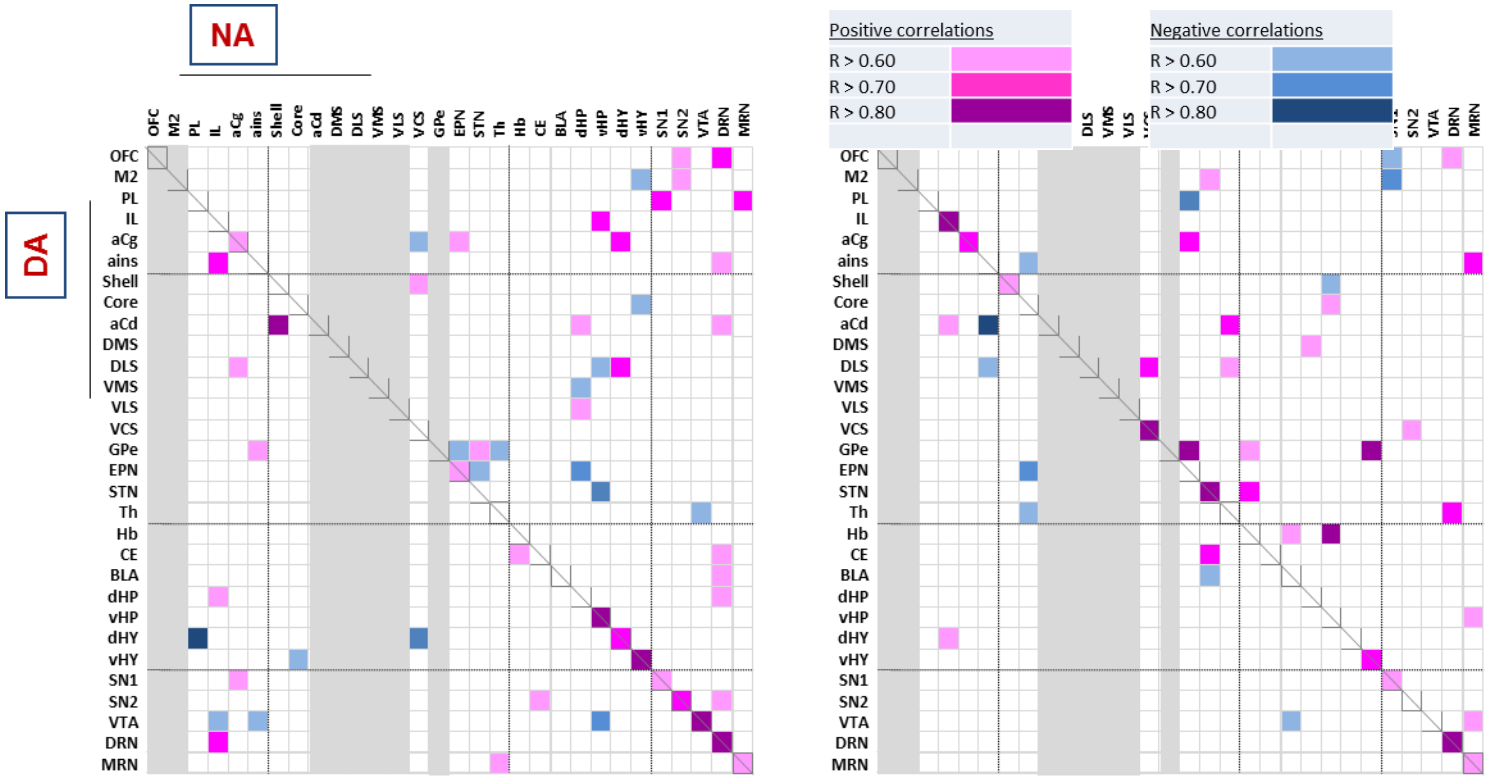


Figure 8. Correlative analysis between NA and DA tissue contents across rat brain regions. Representation of the range of Pearson's R values for each linear regression between NA and DA tissue contents (pg/mg) between the 30 brain areas in control water-treated rats (first column) and FPN 10 mg/kg treated rats (second column). Colored boxes correspond to the existence of a correlation between the two parameters (pink: positive; blue: negative) considered after correction for multiple comparisons. The gray boxes correspond to undetected values for NA.

3.3.2.3 Correlative analysis between NA and 5-HT tissue contents

The NA and 5-HT tissue contents correlated in 39 brain areas (comprising 29 positive and 11 negative correlations), including 13 in the same brain area (all positive) of control-group rats. The brain regions in which a higher number of correlations found for NA and 5-HT contents were the PL, ains, and vHP (6-7); the aCg, SN1, SN2 and MRN (5 each). The 5-HT content in PL negatively correlated with NA content in hypothalamus (dHY and vHY) while EPN 5-HT content negatively correlated with NA content in ains and hippocampus (dHP and vHP).

The number of correlations slightly decreased after FPN treatment (34 comprising 24 positive and 11 negative), including 9 in the same brain area. There was no correlation of NA content in the mesencephalon with 5-HT in the mesencephalon or limbic regions compared to control rats. A main difference also was the increase of correlations of NA and 5-HT content in the basal ganglia in FPN-treated rats (Figure 9).

Between NA and 5-HT tissue contents

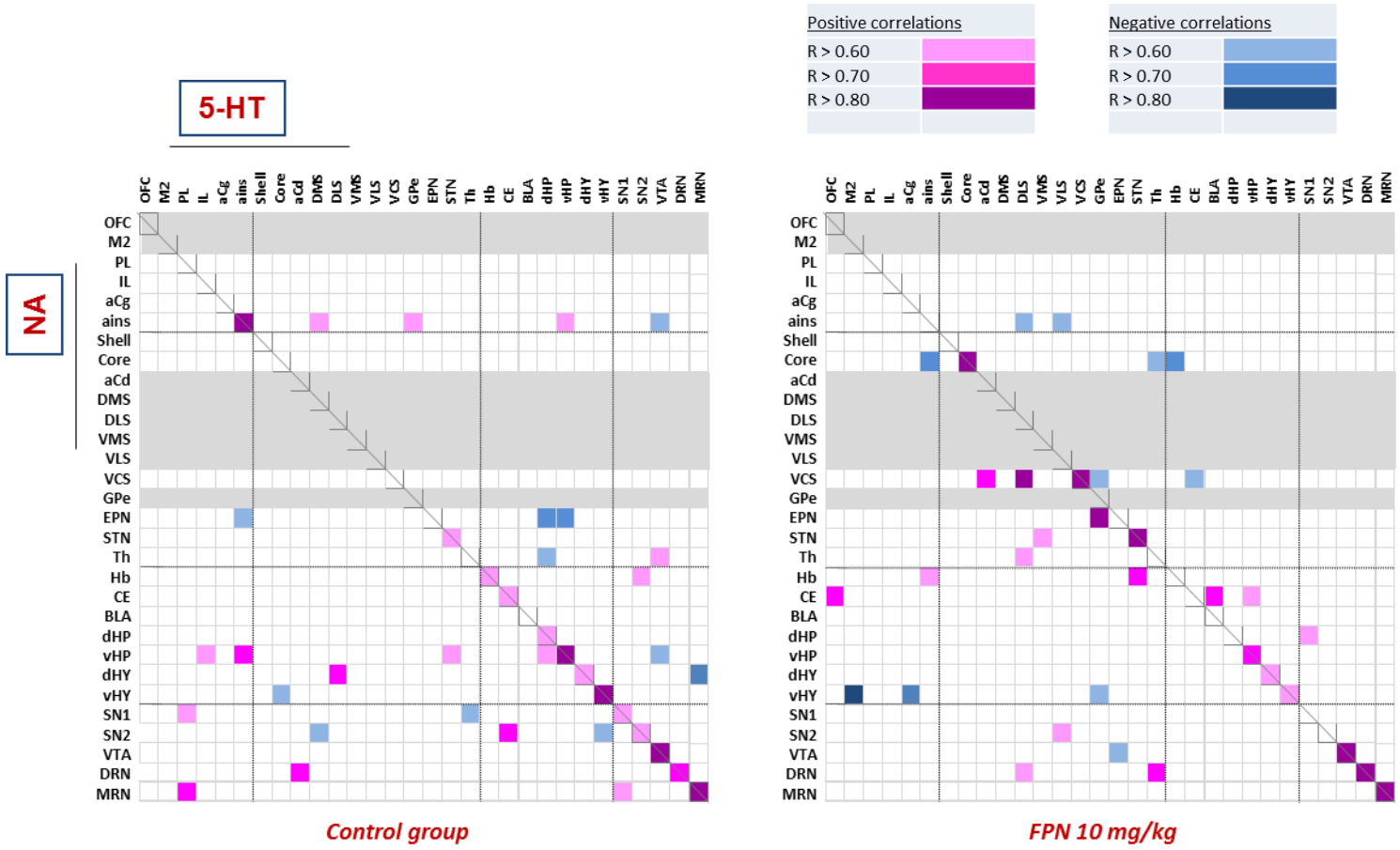


Figure 9. Correlative analysis between NA and 5-HT tissue contents across rat brain regions. Representation of the range of Pearson's R values for each linear regression between NA and 5-HT tissue contents (pg/mg) between the 30 brain areas in control water-treated rats (first column) and FPN 10 mg/kg treated rats (second column). Colored boxes correspond to the existence of a correlation between the two parameters (pink: positive; blue: negative) considered after correction for multiple comparisons. The gray boxes correspond to undetected values for NA.

3.3.2.4 Correlative analysis between DOPAC/DA ratio and 5-HIAA/5-HT ratio

We looked at the correlations established between DA metabolism (DOPAC/DA ratio) and 5-HT metabolism (5-HIAA/5-HT ratio) across selected brain regions (Figure 10). Interestingly, we observed a large number of correlations between DA turnover (DOPAC/DA ratio) and 5-HT (5-HIAA/5-HT ratio) turnover in both groups. The DOPAC/DA ratio and 5-HIAA/5-HT ratio significantly correlated in 124 brain areas of control group rats, including 18 in same brain region. The proportion of positive correlations was high (119). The number of correlations was higher in vHP and NAc shell (22 and 17 respectively), moderately high in aCg, aCd, ains, EPN, CE and Hb (9-12). As regard the pattern, 5-HIAA/5-HT ratio in the cortices established several correlations with the ratio DOPAC/DA from the cortex to the mesencephalon. Both ratios established also numerous correlations within the basal ganglia, and within limbic regions.

FPN treatment decreased the number of correlations as compared to control group. We observed 89 strong correlations between DA turnover and 5-HT (5-HIAA/5-HT ratio) equilibrated between 71 positive and 18 negative correlations, including 15 in same brain region. FPN 10 mg/kg reduced the number of correlations established by 5-HIAA/5-HT with DOPAC/DA in the brain. Also, 5-HIAA/5-HT in the mesencephalon region did not correlate with any DOPAC/DA ratio from the cortex or the limbic regions. Finally, both ratios correlated less within the basal ganglia, with a distinct pattern, and in limbic regions. Conversely, there was a noticeable increase in the correlations established by cortical DOPAC/DA ratio with 5-HIAA/5-HT in the basal ganglia or the limbic regions.

Between DA and 5-HT turnovers

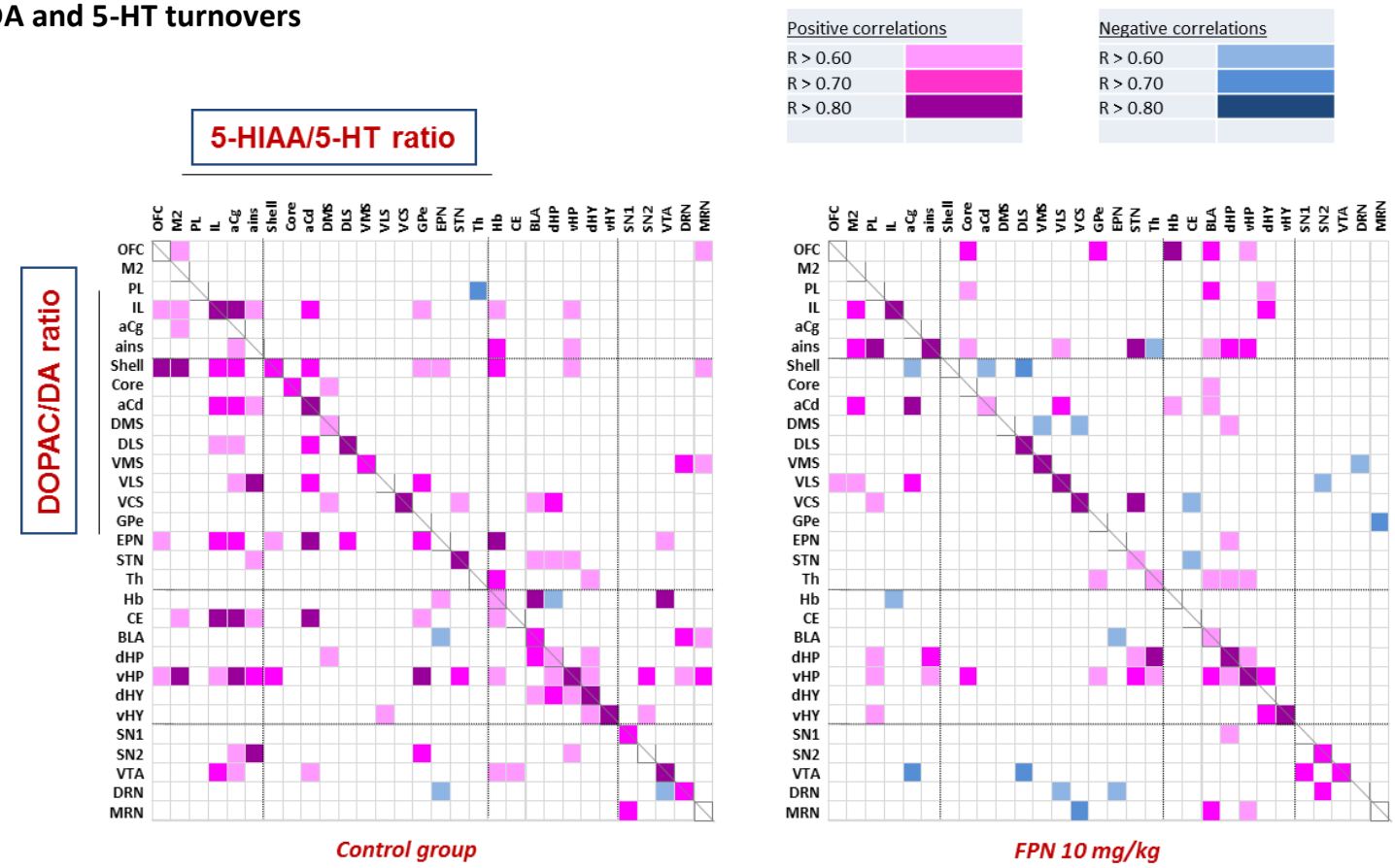


Figure 10. Correlative analysis between DA turnover (DOPAC/DA ratio) and 5-HT turnover (5-HIAA/5-HT ratio) across rat brain regions. Representation of the range of Pearson's R values for each linear regression between DA and 5-HT turnovers between the 30 brain areas in control water-treated rats (first column) and FPN 10 mg/kg treated rats (second column). Colored boxes correspond to the existence of a correlation between the two parameters (pink: positive; blue: negative) considered after correction for multiple comparisons.

4. Discussion

In this study, we investigated the effect of chronic exposure of FPN (10 mg/kg) on monoamine tissue content in 30 distinct brain regions of rat. FPN administration caused a marked deficit in the DA and 5-HT tissue contents in some brain regions, with very effects on NA tissue levels. The qualitative analysis using correlations between pairs of brain regions revealed that FPN dramatically modified the pattern and balance of monoamines between brain regions.

We show that chronic administration of FPN significantly reduced DA levels in a region-dependent manner and more particularly along the mesoaccumbal and mesostriatal tracts with the alterations heterogeneously reported at the level of cell bodies and terminals. It also altered 5-HT tissue content. Previous studies showed that FPN dose-dependently changed the activity of various enzymes involved in oxidative stress and inflammation in the brain when chronically administered at 5-30 mg/kg range of doses (Godinho et al., 2016; Abdel-Daim et al., 2018a,b; Khalaf et al., 2019; Kartheek and David, 2018). The main finding of the study is to show that the dose of 10 mg/kg, considered as a moderate dose, profoundly alters the neurochemistry of monoamines, particularly DA and 5-HT.

Our results extend the demonstration of previous studies in which the authors found a decrease in striatal DA and TH tissue content upon the intra-nigral administration of FPN (Park et al., 2016). In the accompanying study of the thesis, we also report after the intra-nigral administration of FPN (15 and 25 µg), a decrease in striatal DA tissue levels reaching about 50% decrease and a loss in nigral TH immune-labelling compared to vehicle treated rats (Study-1 of the thesis). The DA alterations were similar between the two doses of FPN suggesting that the toxicity toward DA neurons reached already its maximum at the lower dose administered. These data strongly suggest that the decrease in DA tissue content and metabolites after chronic oral gavage is related to neurodegeneration of some DA neurons, possibly related in part to an effect at the level of the SN.

A marked heterogeneity of the DA alteration was reported from the shell of the nucleus accumbens to the ventrocaudal striatum, paralleled by the heterogeneity reported at the level of regions housing DA cell bodies. Briefly, FPN had low, not significant impact on DA and its metabolites content in the VTA and the NAc shell which is exclusively innervated by VTA DA

neurons (Bjorklund and Lindvall, 1986). FPN had also lower noxious effects, notably for the DA metabolites, in the lateral SN, VLS and VCS, the DA innervation of these latter striatal territories originating mainly from the lateral SN (Bjorklund and Lindvall, 1986). The main noxious effects are found at the level of the medial SN and its corresponding innervated striatal territories including the anterior, mediolateral part of the striatum and part of the NAc core. The heterogeneity of the effects, at least with respect to the mesoaccumbens versus mesostriatal territories, has been previously reported with other toxins and notably MPTP (Pifl et al., 1991). Yet, it might not only be due to specific, deleterious actions of FPN on the DA neurons of the medial SN. In fact, using the ratio DOPAC/DA, an indirect index of the DA turnover (Eisenhofer et al., 2004), we found that the turnover was increased more specifically in the VTA, the lateral SN, the VCS, and the VLS, exactly the territories that were less affected by FPN for DA. Whether or not reductions of DA markers are present, these data indicate that VTA and lateral SN neurons are metabolically more active in these regions as well as in the ventrolateral striatum. It is tempting to speculate that these territories are engaged in a neurodegenerative process as well, though delayed with respect to the medial SN. Factors other than a direct, poisoning action of FPN on medial SN DA neurons are likely involved in this intriguing pattern of degeneration. The idea is also supported by the fact that the DA content in other brain regions is poorly altered by FPN, and even increased.

Serotonin is obviously one of these external factors involved in the outcome of DA neurons. A decrease in 5-HT concentrations is observed in some brain regions and notably in the brain regions where DA is altered. However, the ratio 5-HIAA/5-HT is largely enhanced in these brain regions suggesting that the activity of 5-HT neurons is increased. This parameter is important because it is known that 5-HT can enhance the activity of DA neurons in particular when DA neurons are activated (Spampinato et al., 1985; De Deurwaerdere and Di Giovanni, 2017). Such an excitatory effect has been well illustrated in the mechanism of action of the toxin MDMA and has been also shown in the mechanism of action of haloperidol and amphetamine (De Deurwaerdère and Di Giovanni, 2017). It basically involves the stimulation of the 5-HT_{2A} receptor subtype (Schmidt et al., 1992a,b; 1995; Lucas et al., 2000a,b), sustaining the enhanced synthesis of DA in the striatum and less in the NAc. Other state-dependent influences involving 5-HT_3 and 5-HT_4 receptors have been also reported, principally acting at the level of DA cell bodies (Imperato A 1989; Di Chiara and Imperto, 1988; Lucas et al., 2001; Porras et al., 2002)

but that can be observed in the striatum and the NAc in case of co-activation of DA and 5-HT releases (De Deurwaerdère et al., 2005). While we can postulate that the metabolic activity of 5-HT neurons is enhanced in some brain regions, we cannot really affirm that the decrease in 5-HT tissue content reported in the striatum or the SN corresponds to a loss of 5-HT terminals in these regions. In fact, this loss of 5-HT content is poorly associated with corresponding changes of 5-HIAA, which can be itself enhanced in specific brain regions such as the DMS. In addition, we have no evidence that the number of 5-HT cell bodies could be decreased by FPN. Rather, there is a tendency toward an increase of both 5-HT and 5-HIAA in the DRN and the MRN and a significant increase in the 5-HIAA/5-HT ratio in both regions. Despite the existence of some anatomical specificities concerning the location of the 5-HT cell bodies innervating the brain (Hale and Lowry, 2011; Stansley and Yamamoto, 2014), the neuronal origin of 5-HT cell bodies innervating the striatum is quite similar, thereby merely accounting for the heterogeneity of the responses to FPN reported in adjacent striatal territories.

The qualitative analysis is designed to address changes in the pattern of correlations within and between neurotransmitters systems. As expected from previous study, the number of correlations for a single neurotransmitter system between paired of brain regions was quite low for monoamines and their metabolite(s) and higher for the ratios (Dellu-Hagedorn et al., 2017), reaching unexpected high level of correlations for the 5-HIAA/5-HT ratio. The meaning of these correlations is not firmly established, starting from the different pattern of correlations for the control groups from different experiments. Several reasons could be evoked to account for the lack of consistency of these patterns in controls including the species, the age, the handling or training in distinct behavioral experiments, the number of animals/per group, the chromatographic analysis, and the size and location of the punch (Dellu-Hagedorn et al., 2017; Chagraoui et al., 2019; Puginier et al., 2019). Regarding the last aspect, the size of the STN sampled was too large compared to previous punching procedures. The “STN” in our experiment represented only part of the tissue sampled which surely leads to modification of both quantitative and qualitative assessments. In any case, there are some interesting patterns that could be extremely pertinent for the understanding of the actions of FPN in the brain. While the profile of correlations obtained on DA itself is not drastically modified by FPN, the one obtained on DOPAC clearly shows that the correlations of DOPAC content for striato-striatal regions, previously reported for the ratio DOPAC/DA (Dellu-Hagedorn et al., 2017) are almost

suppressed in FPN-treated rats. Moreover, the correlations of the DOPAC/DA ratio between cortical regions and cortico-basal ganglia regions are surprisingly low in FPN-treated rats and, more generally, the number of correlations of the DOPAC/DA ratio in the whole brain was lower in FPN-treated rats. We previously reported a lower number of correlations in the R6/1 mouse model of Huntington's disease compared to the wild-type counterpart at a pre-symptomatic stage (Puginier et al., 2019). It preceded dramatic decreases in striatal DA contents at later, symptomatic stages in those mice. Nonetheless, the reduction in the DOPAC/DA ratio does not constitute a signature of ongoing degenerative process since we observed it with the 5-HT_{2C}R agonist lorcaserin (De Deurwaerdère et al., submitted), a drug known to oppose the behavioral consequences associated with an increase in DA transmission (De Deurwaerdère and Di Giovanni, 2017). The data with FPN suggest that the functional mapping of DA transmission between cortical-subcortical regions is less efficacious, favoring other types of regulatory processes.

The correlative profile obtained with the 5-HT markers is also changing with FPN. There was a major loss of correlations of the 5-HT content or the 5-HIAA/5-HT ratio of the mesencephalic regions toward other brain regions. When looking at the 5-HT/DA correlations or the correlations of their ratio in the same region, there was a decrease in the SN and an increase in striatal quadrants. To further the comparison with previous examples found in the lab, the reduction in DA correlations and/or the DOPAC/DA ratio in the brain induced by the 5-HT_{2C} agonists WAY-163909 and lorcaserin occurred in a context of increased correlations for 5-HT content and even of increased for NA content in the whole brain (Chagraoui et al., 2019; De Deurwaerdère et al., submitted). 5-HT and NA are the prototypical neuromodulatory systems of the brain and their inability to counteract the loss of DA connectivity can be part of a broader picture of a neurodegenerative process. This aspect could be studied with known neurotoxins altering DA neurons integrity including 6-OHDA, MPTP or rotenone.

The alterations reported suggest that FPN can predispose to developing numerous devastating neuropsychiatric disorders, including PD. It is noteworthy that neither upon intra-nigral administration, nor oral gavage, animals exhibited classical signs of PD except the loss of motor coordination and the higher pain sensitivity (Study-1). In animals receiving FPN chronically by oral gavage, no visible signs (rigidity, purposeless oral movements, grip ability)

could be detected. However, in both cases, it seems clear that dopaminergic neurons are altered which makes our study extremely interesting for the prodromal, pre-symptomatic phase of PD.

5. Conclusion

Our study shows that chronic oral administration of Fipronil modified DA and 5-HT levels in few brain regions without significant effect on NA tissue levels. Thus, it confirms that FPN quantitatively induced marked effects on nigrostriatal DAergic systems as well as raphe-striatal and raphe-nigral 5-HTergic systems. It also tends to increase the DA and 5-HT metabolism. It completely changed the pattern of correlations of monoamines in the brain, more prominently the DA and 5-HT turnovers. The loss of connectivity could confer a higher predisposition of dopaminergic neurons to the neurotoxic influence of FPN. In terms of environmental pollution, it is likely that the dose used was perhaps excessive with respect to its concentrations that could be absorbed. Nonetheless, FPN has a long half-life and can stay in the tissue for days. In addition, the noxious effects of environmental toxins are rarely isolated and the deleterious effects are presumably always linked to a combination of several factors, some being endogenous (stress), most of them being exogenous (heavy metals, pesticides). FPN should be considered with additional care by the Public Health systems.

6. References

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