Stereological investigation of serotonin type 3 receptors in the substantia nigra and dorsal raphe nucleus in the rat

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Abstract

Serotonin (5-hydroxytryptamine, 5-HT) is a common neurotransmitter in mammals, and the serotonergic system plays an essential role in the regulation of various behaviours such as sleep, perception, and cognitive and autonomic functions in the mammalian central nervous system (CNS). Previous studies have investigated the distribution pattern of the 5-HT type 3 (5-HT_3) receptor in the brain. Moreover, it has been demonstrated that 5-HT_3 receptors are expressed in both the substantia nigra (SN) and the dorsal raphe nucleus (DRN). Here, we determine the quantitative distribution of 5-HT_3 receptors in the SN and DRN of the rat.

Six rats were deeply anaesthetised with 4% isofluorane in 100% O_2 and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Double immunofluorescence immunohistochemistry was performed on coronal sections (40 μm) of rat brain covering the entire rostro-caudal extent of the SN and DRN with an antibody specific to the 5-HT_{3A} receptor subunit in combination with antibodies targeting the monoaminergic markers tyrosine hydroxylase (TH) and serotonin transporter (SERT). Then, the number of 5-HT_3-, TH- and SERT-positive neurons were counted either in the SN or in the DRN using stereological techniques.

We found that TH- and 5-HT_{3A}-positive cells are present in the SN, with a higher number of TH- positives cells compared to 5-HT_{3A}-positive cells. We also found that SERT- positive cells are present in the DRN in a higher proportion. In contrast, no 5-HT_{3A}-positive cells were found in the DRN. The present results support the presence of 5-HT_3 receptors in the SN, but not in the DRN, and do not support their expression on dopaminergic nor serotonergic cells.

Résumé

La sérotonine (5-hydroxytryptamine, 5-HT) est un neurotransmetteur répandu chez les mammifères. En effet, le système sérotoninergique joue un rôle essentiel dans la régulation de plusieurs comportements, dont le sommeil, la perception, ainsi que les aspects cognitifs et autonomes du système nerveux central des mammifères. Des études antérieures ont montré la distribution du récepteur 5-HT de type 3 (5-HT_3) dans le cerveau et d'autres ont rapporté son expression dans la substance noire (SN) et le noyau dorsal du raphé (NDR). Dans cette étude, nous déterminons quantitativement la distribution des récepteurs 5-HT_3 dans la SN et le NDR dans le rat.

Six rats ont été anesthésiés avec 4% d'isofluorane dans 100% d'O_2 et ont été perfusés transcardialement avec de la saline 0.9% suivi par 4% de paraformaldehyde dans du tampon phosphate salin (pH 7.4). De l'immunohistochimie à double immunofluorescence a été performée sur des coupes coronales (40 μm) de cerveau de rat couvrant la distribution rostro-caudale de la SN et du NDR avec un anticorps spécifique à la sous-unité 3A du récepteur 5-HT_3 (5-HT_{3A}) en combinaison avec des anticorps ciblant les marqueurs monoaminergiques tyrosine hyroxylase (TH) et le transporteur de sérotonine (SERT). Ensuite, le nombre de neurones marqués avec 5-HT_3, TH et SERT a été compté dans la SN ou le NDR en utilisant des technique stéréologiques.

Nous avons trouvé que les cellules positivement marquées avec la TH et 5-HT_{3A} sont présentes dans la SN. Le nombre de cellules marquées avec TH était plus élevé comparativement aux cellules marquées avec 5-HT_{3A}. Nous avons aussi rapporté la présence de cellules marquées positivement avec SERT dans le NDR, sans la présence de cellules marquées positivement pour le récepteur 5-HT_3. Les résultats ci-présents soutiennent la présence du récepteur 5-HT_3 dans la SN, mais pas dans le NDR. De plus, l'absence d'expression du récepteur 5-HT_3 sur les cellules dopaminergiques et sérotoninergiques a été observé.

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List of abbreviations

5-HT: 5-Hydroxytryptamine

5-HT$_{1A}$: Serotonin type 1A receptor

5-HT$_{1E}$: Serotonin type 1E receptor

5-HT$_{1}$: Serotonin type 1 receptor

5-HT$_{2A}$: Serotonin type 2A receptor

5-HT$_{2B}$: Serotonin type 2B receptor

5-HT$_{2C}$: Serotonin type 2C receptor

5-HT$_{2}$R: Serotonin type 2 receptor

5-HT$_{3A}$: Subunit 3A of the serotonin type 3 receptor

5-HT$_{3B}$: Subunit 3B of the serotonin type 3 ……..receptor

5-HT$_{3E}$: Subunit 3E of the serotonin type 3 ……..receptor

5-HT$_{3}$: Serotonin type 3 receptor

5-HT$_{4}$: Serotonin type 4 receptor

5-HT$_{5}$: Serotonin type 5 receptor

5-HT$_{6}$: Serotonin type 6 receptor

5-HT$_{7}$: Serotonin type 7 receptor

5-HTRs: Serotonin receptor

6-OHDA: 6-Hydroxydopamine

ADHD: Attention deficit hyperactivity …….disorder

AR: Antigen retrieval

BG: Basal ganglia

CaBP: Calbindin-positive

cAMP: Cyclic adenosine monophosphate

CE: Coefficient of error

CM: Centromedian nucleus of the thalamus

CNS: Central nervous system

Cys: Cysteine

D$_{1}$: Dopamine type 1 receptor

D$_{2}$: Dopamine type 2 receptor

DAPI: 4′,6-diamidino-2-phenylindole

DAT: Dopamine transporter

DMT: N,N-dimethyltryptamine

DRN: Dorsal raphe nucleus

FITC: fluorescein isothicyanate

GABA: γ-aminobutyric acid

GABA$_{A}$: γ-aminobutyric acid type A ……...receptor

GFP: Green fluorescent protein

GP: Globus pallidus

GPCR: G-protein coupled receptor

GPe: Globus pallidus, external segment

GPi: Globus pallidus, internal segment

GPv: Globus pallidus, ventral segment

Hb: Habenula

HD: Huntington's disease

IF: Immunofluorescence

IHC: Immunohistochemistry

L-DOPA: L-3-4-dihydroxyphenylalanine

LDTg: Laterodorsal tegmental nucleus

LP: Lateroposterior nucleus of the thalamus

LSD: lysergic acid diethylamide

LTD: Long-term depression

m-CPBG: m-chlorophenylbiguanide

MD: mediodorsal nucleus of the thalamus

MDD: Major depressive disorder

MDMA: 3,4-
……...methylenedioxymethamphetamine

MFB: Medial forebrain bundle

MnR: Median raphe nucleus

mRNA: messenger ribonucleic acid

MSN: Medium spiny neuron

NAc: Nucleus accumbens

nACh: Nicotinic acetylcholine receptor

NCS: Nucleus centralis superior

NGS: Normal goat serum

NTS: Nucleus of the solitary tract

OB: Olfactory bulb

OCT: Optimal cutting temperature

OT: Olfactory tubercule

PBS: Phosphate-buffered saline

PD: Parkinson's disease

PFA: Paraformaldehyde

PKC: Protein kinase C

PNS: Peripheral nervous system

PPN: Pendunculopontine nucleus

REM: Rapid eye movement

ROI: Region of interest

RT: Room temperature

S.D.: Standard deviation

S.E.M.: Standard error of the mean

SERT: Serotonin transporter

SN: Substantia nigra

SNc: Substantia nigra pars compacta

SNr: Substantia nigra pars reticulata

SRS: Systematic random sampling

SSRI: Selective serotonin reuptake inhibitor

STN: Subthalamic nucleus

TALENs: Transcription activator-like
effector nucleases

TH: Tyrosine hydroxylase

VA: Ventroanterior nucleus of the thalamus

VGluT1: Vesicular glutamate transporter,
……type 1

VGluT2: Vesicular glutamate transporter,
……type 2

VGluT3: Vesicular glutamate transporter,
……type 3

VL: Ventrolateral nucleus of the thalamus

VMH: Ventromedial hypothalamus

VP: Ventroposterior nucleus of the thalamus

VTA: Ventral tegmental area

ZFNs: Zinc finger nucleases

Preface

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Introduction

1.1. General introduction

Serotonin, also known as 5-hydroxytryptamine (5-HT) is one of the most phylogenetically ancient neurotransmitters. It is therefore involved in a plethora of behaviours and processes in the brain, hence being of critical importance in the day-to-day functioning of animals and humans (Mohammad-Zadeh *et al.*, 2008). Although 14 5-HT receptors distributed across 7 families have been discovered to date, only one subtype, the 5-HT_3 receptor, is a ligand-gated ion channel. All others are G-protein coupled receptors (GPCRs) (Nichols and Nichols, 2008). Therefore, contrary to other 5-HT receptors, 5-HT_3 receptors can directly modulate rapid synaptic transmission (Férézou *et al.*, 2002). This renders them an attractive target for pharmacotherapy of disorders associated with abnormal 5-HT transmission, as they may offer increased potency when compared to the more modulatory role of GPCRs in synaptic transmission (Andrade and Beck, 2010). Indeed, a number of drugs with 5-HT_3 antagonistic properties are used clinically to treat diarrhoea-predominant irritable bowel syndrome, depression, psychosis, malaria and chemotherapy-induced emesis, amongst other uses (Zoldan *et al.*, 1995; Nakagawa *et al.*, 1998; Gill and Hatcher, 2000; Thompson and Lummis, 2008; Lewis, 2010; Hesketh *et al.*, 2017). In addition, ondansetron, a selective $5HT_3$ antagonist, may reduce visual hallucinations in patients with advanced Parkinson's disease (PD), without interfering with mainstay L-3-4-dihydroxyphenylalanine (L-DOPA) treatment (Butler *et al.*, 1988; Zoldan *et al.*, 1993; Zoldan *et al.*, 1995). Conversely, excitation of 5-HT_3 receptors proved to be anticonvulsant in a mouse seizure model (Gholipour *et al.*, 2009). This brief summary highlights how 5-HT_3 receptors and 5-HT_3 modulation play broad roles within the nervous system.

At the pharmacological level, studies have shown that modulation of 5-HT_3 receptors can alter the release of dopamine and 5-HT, both of which could explain the effects of 5-HT_3 ligands

described above (Blandina *et al.*, 1989; Martin *et al.*, 1992; Blier and Bouchard, 1993; Zazpe *et al.*, 1994; Haddjeri and Blier, 1995; MacDermott *et al.*, 1999). Whereas several anatomical studies have been performed using various techniques such as *in situ* hybridisation and autoradiographic receptor binding, it is striking that there are few, if any, studies aiming at precisely determining the cellular localisation of 5-HT_3 receptors within two key monoaminergic structures of the brain, the substantia nigra (SN) and the dorsal raphe nucleus (DRN) (Kilpatrick *et al.*, 1987; Kilpatrick *et al.*, 1988; Waeber *et al.*, 1988; Barnes *et al.*, 1989a; Kilpatrick *et al.*, 1989; Waeber *et al.*, 1989; Barnes *et al.*, 1990; Pratt *et al.*, 1990; Waeber *et al.*, 1990; Gehlert *et al.*, 1991; Jones *et al.*, 1992; Laporte *et al.*, 1992b; Morales *et al.*, 1996b; Morales and Bloom, 1997; Morales *et al.*, 1998; Morales and Wang, 2002; Koyama *et al.*, 2017). If it were demonstrated that 5-HT_3 receptors are expressed by monoaminergic neurons of both the SN and the DRN, then one might suspect that 5-HT_3 ligands have a direct effect on monoaminergic neurons, as opposed to an indirect effect via actions on interneurons. Here, we seek to determine if monoaminergic neurons of the SN and the DRN harbour 5-HT_3 receptors using immunohistochemistry (IHC) and stereological counting.

1.2 The basal ganglia

1.2.1 Anatomical organisation

The basal ganglia (BG) are a collection of subcortical nuclei with pivotal roles in movement, motivation, affect and cognitive functions (Parent and Hazrati, 1995a, b; Stathis *et al.*, 2007). There are four main constituents of the classic model of the basal ganglia, all of which except one have subdivisions which can be considered nuclei in and of themselves. These include, the striatum (putamen and caudate nucleus), the globus pallidus (GP), SN and the subthalamic nucleus (STN) (Koprich *et al.*, 2009). Figure 1 shows the anatomical organisation of the basal

ganglia and related structures identified on coronal section. For this thesis, the SN is our nucleus of interest within the BG.

1.2.1.1 Substantia nigra

The SN is located in the midbrain, playing an important role in reward and movement (Ikemoto, 2007; Ikemoto *et al.*, 2015). The SN can be subdivided into two areas which have different cell types (Francois *et al.*, 1985). The most ventral area is referred to as the SN pars reticulata (SNr) and the more dorsal portion the SN pars compacta (SNc) (Francois *et al.*, 1985). Certain authors describe a third distinct subregion, the SN pars lateralis (SNl), however this is generally included in the SNr. The large dopaminergic neuronal population of the SNc is darkly pigmented by neuromelanin, which instills a grey hue to the SNc with age (Beck, 2011). The SNc projects to the striatum and, according to the classic model of the BG, determines the valence, *i.e.* excitation or inhibition, of striatal projections to the GP based on the activation of dopamine type 1 (D_1) or dopamine type 2 (D_2) receptors (DeLong and Wichmann, 2007). The SNr is a much less dense structure than the SNc, with efferents which are largely inhibitory projections which release γ-aminobutyric acid (GABA) (Parent, 1990; Parent and Hazrati, 1995a). Similarly, cells in the SNr tend to be thinner and smaller than their dopaminergic counterparts in the SNc, resembling more closely pallidal GABAergic neurons (Cooper and Stanford, 2000). The SNr, on the other hand, is adjacent (caudal) to the GP pars interna (GPi) and projects to the thalamus (Stathis *et al.*, 2007; Koprich *et al.*, 2009; Reed *et al.*, 2013).

The SNc is composed of three subgroups of cells which together form its classic bilateral structure. The primary group is the densocellular (β) group, but there is also a ventral (γ) group, known as the cell column group, as well as the dorsal (α) group (Haber, 2014). The dorsal group merges indiscriminately with ventral tegmental area (VTA) cells medially, however can be

differentiated from the more ventral densocellular group owing to their horizontal rather than oblique orientation (Haber, 2014). An important distinction exists between the VTA and SNc, the former belonging to the A10 dopamine neuron developmental group, and the latter belonging to the A9 group (Björklund, 2011). This difference in ontogeny has important functional implications, notably the more motivational role of VTA dopaminergic projections and the broader role of SNc dopaminergic projections (Ikemoto, 2007; Ikemoto *et al.*, 2015). Together, the dorsal SNc group and VTA are known as the dorsal tier midbrain dopamine cells, whereas the densocellular and cell column groups are known as the ventral tier (Haber, 2014). These two tiers can also be chemically distinguished by calbindin-positive (CaBP) staining in the former, while calbinding staining is absent from the latter. Furthermore, unlike the dorsal tier, ventral tier dendrites extend into the SNr, having important functional consequences on BG parallel-loop processing. Although the SNr is primarily a GABAergic signalling structure, there are some endogenous dopaminergic neurons which do not differ from SNc ones, as well as the aforementioned projections from the ventral tier of the SNc (Richards *et al.*, 1997; Björklund, 2011).

1.2.1.2 Striatum

The striatum is one of the most diversified of the nuclei of the BG, comprised of the dorsal and ventral striatum (Stathis *et al.*, 2007). The ventral striatum is composed of the nucleus accumbens (NAc) and the deep portions of the olfactory tubercle (OT), both of which play roles in reward learning, decision making and motivated behaviour (Ikemoto *et al.*, 2005; Ikemoto, 2007; Tremblay *et al.*, 2009; Ikemoto *et al.*, 2015). The dorsal striatum is comprised of the putamen and caudate nucleus, both of which are having various functions, with motor control being of particular importance (Voorn *et al.*, 2004).

1.2.1.3 Globus pallidus

The GP has an internal and external segment (GPi and GPe, respectively), the former being the primary output nucleus of the BG, projecting to the thalamic relay nuclei, the latter modulating this output via the indirect pathway of the basal ganglia (Koprich *et al.*, 2009). The amalgamation of the GP and putamen is known as the lentiform nucleus, which does not bear any specific physiological function, but is rather an anatomical entity. A white matter tract known as the internal capsule separates the caudate nucleus from the lentiform nucleus, defining the medial boundaries of the latter (Russmann *et al.*, 2003). There are some differences in the anatomy of the GP between primates and "lower" species such as rodents or felines which have functional implications. For instance, in primates there is the presence of a ventral GP (GPv) proper which is distinct from the GPe/GPi (Parent and Hazrati, 1995a, b). Furthermore, in rodents and cats, the entopeduncular nucleus, which appears to serve similar functions as the GPi, is generally considered homologous to the latter, although not entirely (Parent and Hazrati, 1995a, b). For instance, there is a more diffuse distribution of pallidotegmental projections, *i.e.* to the pedunculopontine nucleus (PPN), however it is unclear as to whether these differences are due to variable development of target structures or to differences in the organisation of motor systems (Parent and Hazrati, 1995a). Anatomical differences such as these are important to ponder when studying brain circuitry using translational models. Nevertheless, a conserved feature is that most of the neurons in the GP use a GABA signal as their primary neurotransmitter (Parent, 1990). Similarly, the GP receives its primary inputs from the striatum and STN, as well as secondary inputs from the SNc, DRN and PPN (Parent, 1990).

1.2.1.4 Subthalamic nucleus

Lastly, there is the STN, distinguishable as being the only nucleus of the BG with, in contrast to all of the structures mentioned above, primarily glutamatergic efferents (Lévesque and Parent, 2005). Because of this distinctive feature, it is believed to be a key modulator of BG outputs by activating GABAergic pallidothalamic projections, hence inhibiting thalamocortical pojections and movement in turn (DeLong and Wichmann, 2007). The STN projects primarily to the GPi (Figure 2) (Lévesque and Parent, 2005).

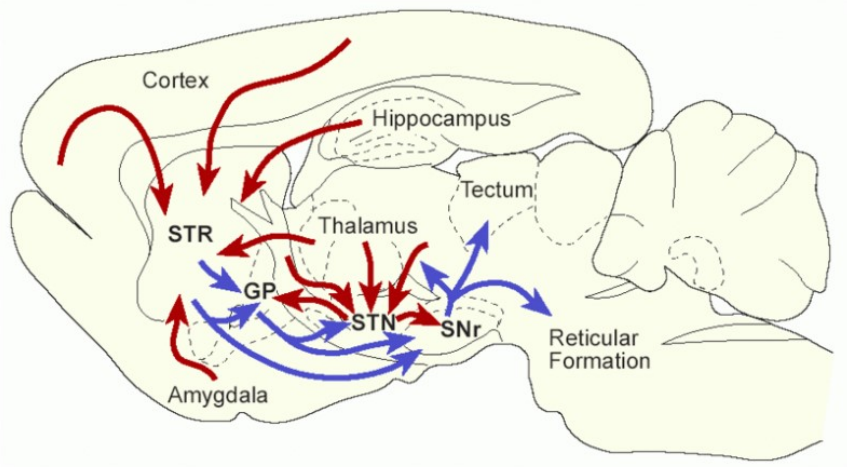

Figure 1. Organisation of the basal ganglia and surrounding structures. The striatum (STR), composed of the caudate nucleus and putamen, the globus pallidus (GP), with its internal and external segments, the subthalamic nucleus (STN), and substantia nigra (SN) are the main nuclei of the BG. Red arrows indicate excitatory projections; blue ones indicate inhibitory projections Reproduced from Redgrave (2007).

1.2.2 Basal ganglia pathways

Owing to the complex anatomy of the BG, it is useful to use models of BG connectivity to understand their functional anatomy. In the BG circuit, there are two pathways controlling movements, namely the direct and indirect pathways, although there is sometimes mention of a

third, hyperdirect, pathway (DeLong and Wichmann, 2007). Figure 2 shows the classic illustration of the organisation of the BG.

1.2.2.1 Direct pathway

The direct pathway plays a role in the initiation and execution of voluntary behaviours (Calabresi *et al.*, 2014). The nigrostriatal pathway consists of dopaminergic projections from the SNc to the dorsal striatum. This pathway is the primary input to both the direct and indirect movement pathways of the BG, depending on whether these dopaminergic efferents activate D_1 or D_2 receptors (Koprich *et al.*, 2009; Haber, 2014). These SNc efferents activate D_1 dopamine receptors in the striatum, the medium spiny neurons (MSNs) of which project to the GPi and SNr, inhibiting these structures. (Parent, 1990; Parent and Hazrati, 1995a; DeLong and Wichmann, 2007). The SNc also projects to the cortex, more generally via collaterals to layer I, and more specifically to layers V-VI of regions which project to the striatum themselves (Haber, 2014). Hence, these projections are seen as generally modulatory or modulating an indirect nigrocorticostriatal pathway, respectively (Haber, 2014). The SNc also projects directly to the thalamus, the amygdala, the hippocampus and the GP (Haber, 2014). It also receives reciprocal innervation not only from the striatum, but also from the cortex (Bunney and Aghajanian, 1976). The SNc is influenced by a variety of neurotransmitters, notably 5-HT projections from the DRN, but also cholinergic, glutamatergic and GABAergic ones from other nuclei such as the PPN, the rostromedial tegmental nucleus, the superior colliculus as well as the GPe and thalamic ventroposterior (VP) nucleus (Fibiger and Miller, 1977; Oertel and Mugnaini, 1984; Weiner *et al.*, 1990; Gervais and Rouillard, 2000; Chen and Rice, 2002; Forster and Blaha, 2003; Wooltorton *et al.*, 2003; Haber, 2014; Miguelez *et al.*, 2014). Apart from nigral afferents, the striatum receives substantial input from the cortex and the thalamus, mainly from the intralaminar nuclei of the

centromedian (CM)/parafascicular complex and, to a lesser extent, from relay nuclei such as the ventrolateral (VL), ventroanterior (VA), lateroposterior (LP), mediodorsal (MD) and pulvinar nuclei (Parent and Hazrati, 1995a). The striatum also receives inputs from the midbrain raphe nuclei (Brigitte and André, 1990; Parent and Hazrati, 1995a). The striatum has a much greater ratio of projection to interneurons compared to most brain structures, with a 9:1 ratio in rats, and a 3:1 ratio in primates (Graveland and Difiglia, 1985; Parent and Hazrati, 1995a). Most striatal efferents from GABAergic MSNs project to both segments of the GP, with those to the GPi releasing thalamocortical projections from inhibition, permitting movement or other behavioural outputs (Parent *et al.*, 1989; Parent, 1990; Parent and Hazrati, 1995a, b). The GPi sends massive projections to the CM, VA and VL nuclei of the thalamus, as well as the habenula (Hb) and PPN (Parent and Hazrati, 1995a). In primates, these projecting fibres are highly collateralised, especially with respect to thalamic and PPN efferents (Harnois and Filion, 1982; Parent and De Bellefeuille, 1982, 1983; Fénelon *et al.*, 1990; Hazrati and Parent, 1991). Conversely, pallido-habenular projections, which are more prominent in rodents, arise largely from a distinct, non-collateralising population of neurons (Parent and Hazrati, 1995a). The GPi also sends projections to the SNr, the efferents of which, in combination with the aforementioned pallidothalamic projections, constitute the outputs of the direct pathway (DeLong and Wichmann, 2007). In the rat, up to 40% of striatal efferents send collateral projections to both the GP and SNr, whereas projections to different nuclei originate from separate populations in primates and cats, however the functional significance of this disparity remain unclear (Féger and Crossman, 1984; Parent *et al.*, 1984; Loopuijt and Van der Kooy, 1985; Beckstead and Cruz, 1986; Parent *et al.*, 1989; Parent, 1990).

1.2.2.2 Indirect pathway

The indirect pathway is initiated by an inhibitory dopaminergic projection from the SNc to the striatum. This pathway helps to prevent unwanted muscle contractions from interfering with voluntary and involuntary movements and consists of SNc efferents activating D_2 dopamine receptors in the striatum, thereby inhibiting GABA-producing neurons in the GPe, which releases the inhibition of the GPe on the STN (Parent, 1990; Parent and Hazrati, 1995a, b; DeLong and Wichmann, 2007). This is known as the pallidosubthalamic pathway (Parent and Hazrati, 1995b; DeLong and Wichmann, 2007). The STN is the only excitatory glutamatergic structure of the BG and provides excitatory projections to the output neurons of the BG (Parent and Hazrati, 1995b). These fibres synapse in the dorsal portion of the STN, and the STN then sends glutamatergic projections to the GPi and SNr, reinforcing the GABA-mediated inhibition of thalamocortical projections from thalamic nuclei, *eg.* the VA and VL nuclei, thereby inducing a negatively-valenced shift in BG outputs to the cortex (Parent, 1990; Parent and Hazrati, 1995a, b; DeLong and Wichmann, 2007). There is also presence of a hyperdirect pathway, which involves cortical projections directly to the STN, activating GPi/SNr projections to the thalamus, thereby inhibiting thalamic signalling to the cortex (Figure 2B).

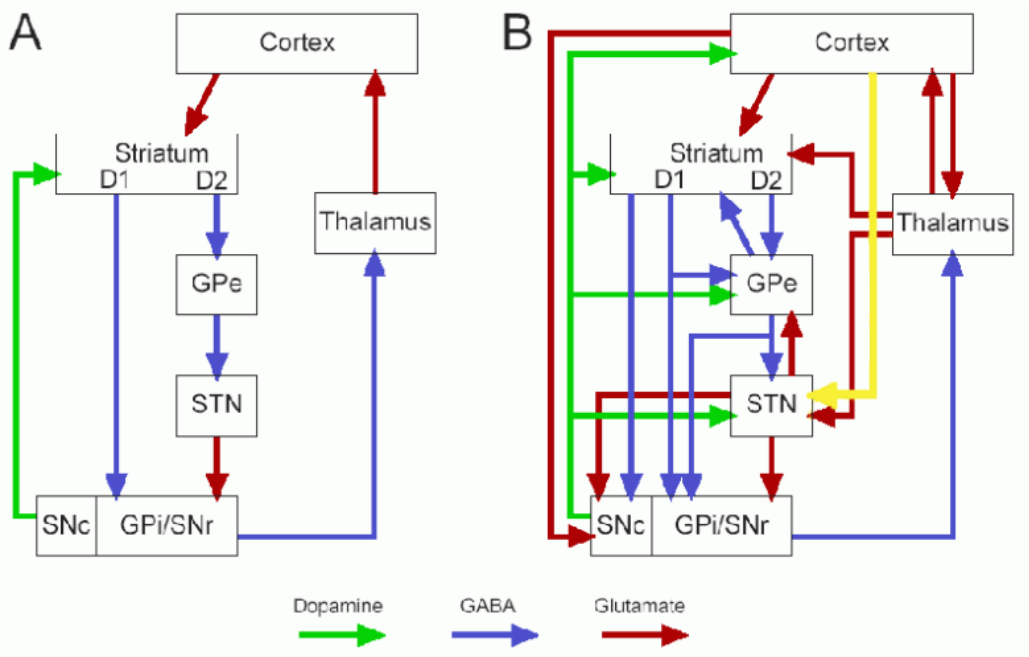

Figure 2. Schematic representation of the classic model of basal ganglia. A. The direct (excitatory) pathway consists of the activation of D_1 receptors in the striatum such that striatopallidal cells release GABA in the GPi and SNr, disinhibiting the thalamus. The indirect (inhibitory) pathway consists of the activation of D_2 receptors in the striatum so that striatopallidal cells release GABA in the GPe, which releases the STN of inhibition, increasing the activity of GPi and SNr GABAergic projections to the thalamus. **B.** More detailed representation of anatomical connections, illustrating the presence of multiple parallel pathways, feedback and feedforward loops, as well as the addition of the glutamatergic hyperdirect pathway in yellow. Adapted from Redgrave (2007).

1.2.3 Function of the basal ganglia

The BG are a network of complexly organised connections, with specific nuclei and pathways activated to achieve different functions under different circumstances (Obeso *et al.*, 2008). The BG play a key role in the mediation of a large number of behaviours, including movement, associative learning, planning, working memory and emotional regulation (Selemon and Goldman-Rakic, 1985; Parent, 1990; Alexander *et al.*, 1991; Parent and Hazrati, 1995a, b; Haber *et al.*, 2000; Haber, 2003; Zahm, 2006; DeLong and Wichmann, 2007; Obeso *et al.*, 2008; Tremblay *et al.*, 2009; Miguelez *et al.*, 2014; Ikemoto *et al.*, 2015; Kim and Hikosaka, 2015). By

integrating diffuse cortical inputs conveying motor, cognitive (*i.e.* associative) and limbic information, the BG potentiate or inhibit behavioural outputs to address stimuli in the environment (Parent, 1990; Alexander *et al.*, 1991; Parent and Hazrati, 1995a, b; Tremblay *et al.*, 2009; Haber, 2014; Ikemoto *et al.*, 2015; Kim and Hikosaka, 2015). This is most readily seen in the case of motor outputs, however there are subtler elements such as motivation and affect mediated by the BG which strongly influence behaviour (Parent, 1990; Alexander *et al.*, 1991; Parent and Hazrati, 1995a, b; Haber *et al.*, 2000; Zahm, 2006; DeLong and Wichmann, 2007; Tremblay *et al.*, 2009; Haber, 2014; Miguelez *et al.*, 2014; Ikemoto *et al.*, 2015; Kim and Hikosaka, 2015). Hence, the BG act less as an output system *per se* and rather as a broader gating network. Dysfunction of the BG results in a plethora of diseases, many of which having grave personal and societal consequences (DeLong and Wichmann, 2007; Fox *et al.*, 2009; Lees *et al.*, 2009; Huot *et al.*, 2011). Two of the most known of these are PD and Huntington's disease (HD), representing the two extremes of the spectrum of motor deficits associated with the BG (Steward *et al.*, 1993a; Yang *et al.*, 2008). The former consists of a hypokinetic disorder, whereas the latter is a hyperkinetic disorder (Steward *et al.*, 1993a; Yang *et al.*, 2008).Although their motor characteristics are arguably their most evident symptoms, both have accompanying neurocognitive changes which highlight the profound role the BG play in daily life (Steward *et al.*, 1993a; Yang *et al.*, 2008; Fox *et al.*, 2009). Beyond these classical BG disorders, other associated diseases include addiction, attention deficit hyperactivity disorder (ADHD), cerebral palsy, major depressive disorder (MDD), Tourette's syndrome, anxiety disorder and many more (DeLong and Wichmann, 2007; Fox *et al.*, 2009; Drysdale *et al.*, 2016; Heller, 2016; Pariyadath *et al.*, 2016).A better appreciation of how BG circuits are modulated, for instance through $5HT_3$ receptors, may have profound impacts on the lives of many individuals as well as society in a broader sense.

1.3 The raphe nuclei

1.3.1 Anatomical organisation

The raphe nuclei are part of the medial portion of the reticular formation, an interconnected network of nuclei in the brainstem, forming a ridge along the sagittal plane (Hornung, 2012). They are a collection of seven nuclei located in the brainstem, often separated into a rostral and a caudal portion (Istvan, 1990). These nuclei are the primary producers of the 5-HT in the central nervous system (CNS) (Adell, 2015).

The caudal part of raphe is subdivided in three nuclei: the nucleus raphe obscurus (B2 cell group), the nucleus raphe pallidus (B1 cell group) and the nucleus raphe magnus (B3 cell group) (Istvan, 1990). The rostral nuclei represent the primary portion of 5-HT neurons, with the caudal nuclei being substantially smaller (Hornung, 2012).

The rostral part of the raphe is divided as follows. Two nuclei are found in the pontine reticular formation: the raphe pontis (B5 cell group) and raphe centralis inferior, also known as the nucleus linearis. Two nuclei are located in the midbrain reticular formation: the raphe centralis superior (NCS), also known as the median raphe (MnR) formed of the B8 cell group and the DRN formed of the B6 and B7 cell groups (Istvan, 1990; Jacobs and Azmitia, 1992). The rostral nuclei represent approximately 85% of all 5-HT neurons in the brain (Hornung, 2003; Adell, 2015). The DRN is one of the largest raphe nuclei and can be divided into nine sub-regions in rodents (Hale and Lowry, 2011; Commons, 2016). Histologically, the DRN is a dense nucleus, composed of sparse cells with many unmyelinated fibres and short, spiny dendrites of 5-HT neurons, consistent with neuropil (Adell *et al.*, 2002; Beliveau *et al.*, 2015). It is also the largest 5-HT nucleus and provides a substantial proportion of the 5-HT innervation of the forebrain.

1.3.2 Raphe nuclei connections

Given the focus of the present thesis being on the potential contribution of 5-HT_3 receptors to modulation of BG function, the sections below will focus primarily on the rostral raphe, and the DRN in particular.

1.3.2.1 Afferents

Although primary role of the raphe nuclei in the brain is to provide 5-HT input to other structures, there are nonetheless important modulatory inputs to the raphe nuclei as well (Soiza-Reilly and Commons, 2011). For instance, the DRN receives reciprocal dopaminergic innervation from midbrain dopaminergic centres (Ferré and Artigas, 1993). Moreover, the MnR and DRN both receive inputs from limbic cortices, as well as the lateral and medial preoptic areas (Vertes and Linley, 2008). Sub-cortically, these structures receive inputs from the lateral Hb and various nuclei of the hypothalamus (perifornical, lateral and dorsomedial) (Figure 3) (Vertes and Linley, 2008). They also receive input from several brainstem areas including the central grey at both the midbrain and pontine levels, the locus coeruleus, the laterodorsal tegmental nucleus (LDTg) and the caudal raphe nuclei (Figure 3) (Vertes and Linley, 2008). In addition to these, the DRN more specifically receives inputs from the lateral septum the bed nucleus of the stria terminalis, the tuberomammillary nucleus and the diagonal band nuclei (Vertes and Linley, 2008). Hence, the general pattern of forebrain modulation of the rostral raphe nuclei is provided by limbic areas.

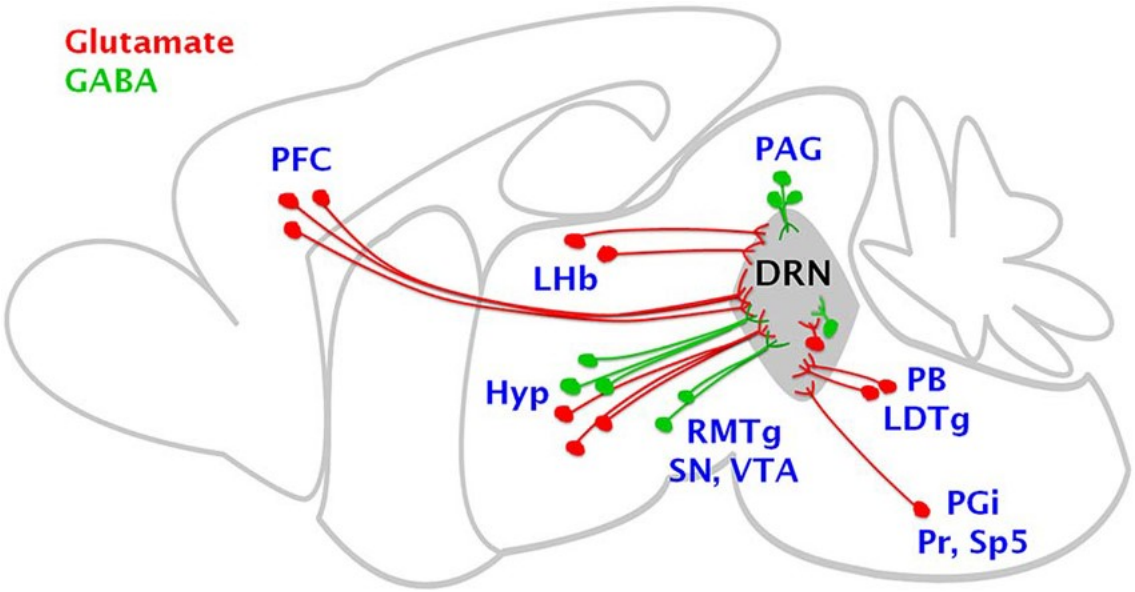

Figure 3. Afferent glutamatergic and GABAergic projections to the dorsal raphe nucleus. Illustration of main excitatory (*i.e.* glutamatergic) and inhibitory (*i.e.* GABAergic) projections to the DRN. Glutamatergic projections are in red, GABAergic projections are in green. PFC: prefrontal cortex, Hyp: hypothalamus, LHb: lateral habenula, SN: substantia nigra, RMTg: rostromedial tegmental nucleus VTA: ventral tegmental area, PAG: periaqueductal gray, DRN: dorsal raphe nucleus, LDTg: laterodorsal tegmental nucleus, PB: parabrachial nucleus, PGi: paragigantocellular nucleus, Pr: prepositus hypoglossal nucleus, Sp5: spinal trigeminal nucleus. Reproduced from Soiza-Reilly & Commons (2014).

1.3.2.2 Efferents

Generally speaking, the caudal raphe nuclei project to the spinal cord, brainstem and cerebellum, whereas the more rostral nuclei of the pons and midbrain project to higher brain areas such as the striatum and across the cortex (Figure 4) (Istvan, 1990; Jacobs and Azmitia, 1992; Mohammad-Zadeh *et al.*, 2008). One example of caudal pathways is the activation of gastric motility via the vagal nerve by the nucleus raphe obscurus, an effect partially mediated by 5-HT_3 receptors (Krowicki and Hornby, 1993). This nucleus also regulates expiration via the phrenic nerve through a 5-HT receptor type 1A (5-HT_{1A})-mediated mechanism and plays a role in hypoglossal nervous output (Lalley *et al.*, 1997; Peever *et al.*, 2001). Of the rostral nuclei, the

MnR, sends 5-HT projections to more medial forebrain regions than the DRN (Commons, 2016). The former projects mainly to more lateral brain regions such as the cortex, amygdala, lateral hypothalamus, thalamus, midbrain dopaminergic centres and the striatum (Sawyer *et al.*, 1985; Commons, 2016). These projections arise from outbranchings of the medial forebrain bundle (MFB) (Park *et al.*, 1982). Of particular interest for the present study, the DRN is known to synapse in the SNc of rats, primates, and humans, and can therefore provide 5-HT modulation to this key input nucleus to the BG (Fibiger and Miller, 1977; Nicolaou *et al.*, 1979; Hornung and Celio, 1992; Jacobs and Azmitia, 1992; Andrade and Beck, 2010; Sharp, 2010). Similarly, 5-HT terminals have been reported in the ventromedial SNr (Steinbusch, 1981). These 5-HT projections appear to tonically inhibit the SN, however this effect is not uniform (Dray *et al.*, 1978; Gervais and Rouillard, 2000). In contrast, area B6, *i.e.* the caudal DRN, projects to more medial areas such as the hippocampus, the septum, the retromamillary nucleus, suprachiasmatic nucleus, the medial and lateral Hb, and the paraventricular hypothalamus (Commons, 2016). Strikingly, these connectivity patterns, reinforced by developmental and genetic evidence, share remarkable similarities with the MnR (Jensen *et al.*, 2008; Fox and Deneris, 2012; Alonso *et al.*, 2013; Commons, 2016).

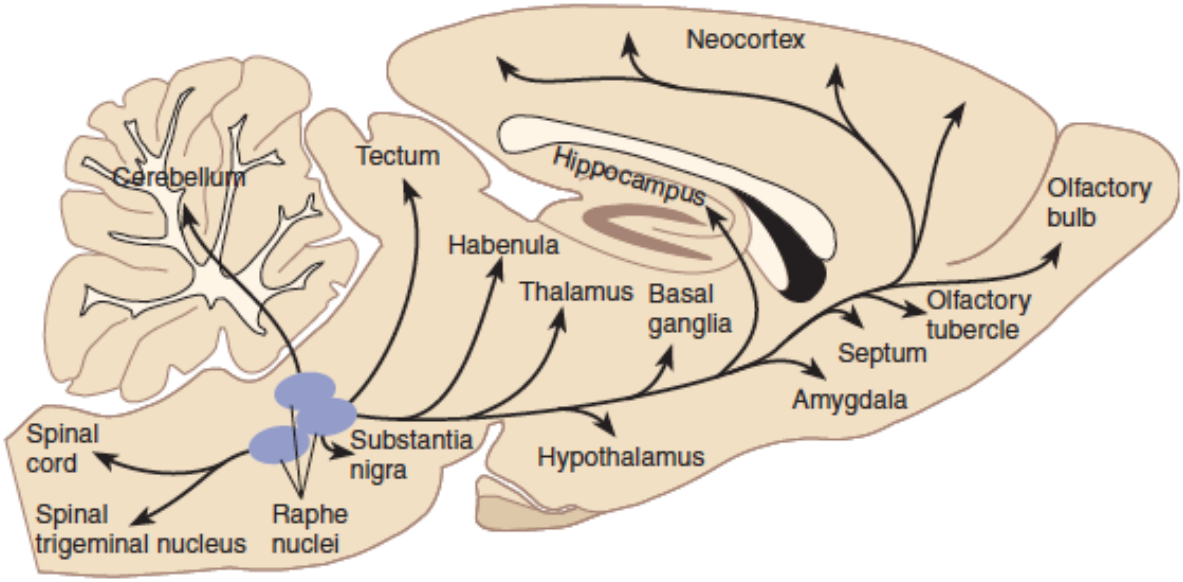

Figure 4. Mid-sagittal section showing the neuroanatomy of the 5-HT system in rat. Schematic of the raphe nuclei and their major projections in the rat brain, showing the broad range of targets spanning the entirety of the CNS. Reproduced from Carlson (2013).

1.3.3 Functions of the raphe nuclei

The primary function of the raphe nuclei is to provide 5-HT innervation to the rest of the brain (Tõrk, 1990). As mentioned previously, the nucleus raphe obscurus is in the medulla and activates gastric motility via the vagal nerve (Krowicki and Hornby, 1993). The raphe pallidus is rostral to the raphe obscurus, and is primarily involved in sympathetic responses such as tachycardia, thermogenesis and pyrexia (Nakamura *et al.*, 2002; Zaretsky *et al.*, 2003; Tupone *et al.*, 2011). The most rostral of the caudal nuclei is the raphe magnus, which is primarily associated with pain modulation through inhibition of peripheral nociceptive circuits in the dorsal horn of the spinal cord, as well as cardiorespiratory, sexual, autonomic and thermoregulatory functions (Mason and Gao, 1998; Mason, 2001; Hellman *et al.*, 2007; Hellman *et al.*, 2009). The nucleus raphe pontis is the most caudal of the rostral nuclei, playing a role in narcotic anaesthesia-associated muscle rigidity and having a putative interaction with the vascular system (Scheibel *et*

al., 1975; Broekkamp *et al.*, 1984; Blasco *et al.*, 1986). Similarly, the nucleus centralis inferior is also involved in analgesia through 5-HT projections to the spine (Oliveras *et al.*, 1975; Guilbaud *et al.*, 1977; Oliveras *et al.*, 1977). As mentioned previously, the MnR, sends 5-HT projections to more medial forebrain regions than the DRN (Commons, 2016). This nucleus has been found to be involved in hallucinogenesis, as well as in memory consolidation through 5-HT modulation of hippocampal rhythms (Trulson *et al.*, 1984; Wang *et al.*, 2015). As mentioned previously, the raphe nuclei are the main source of 5-HT innervation to both the CNS and peripheral nervous system (PNS), roles served primarily by the rostral and caudal nuclei, respectively. Although generally seen as a modulatory neurotransmitter, 5-HT can play a more direct role in neural processing through its sole ionotropic receptor, the $5HT_3$ receptor (Sugita *et al.*, 1992; Férézou *et al.*, 2002; Varga *et al.*, 2009). Given the massive scope of these projections in terms of downstream nuclei, 5-HT plays a role in almost every function of the brain (Trulson *et al.*, 1984; Mohammad-Zadeh *et al.*, 2008; Berger *et al.*, 2009; Larson *et al.*, 2015; Wang *et al.*, 2015). With this plethora of functions, dysfunctional 5-HT signalling can result in a number of pathologies. Two of the most common being MDD and other mood disorders (Hornung, 2003; Mohammad-Zadeh *et al.*, 2008; Berger *et al.*, 2009; Andrade and Beck, 2010; Zhang *et al.*, 2012). However, the exact role of 5-HT in these disorders, as well as how to treat its dysfunction, remain elusive and the subject of intensive research. One of the main therapeutic venues is to administer selective 5-HT reuptake inhibitors (SSRIs), a class of molecules which block the reuptake of 5-HT by the 5-HT transporter (SERT) (Adell *et al.*, 2002). Given that 5-HT signalling appears to be decreased in MDD, by blocking SERT, the hope is to increase the efficiency of 5-HT transmission by keeping it in the cleft longer (Hornung, 2003; Mohammad-Zadeh *et al.*, 2008; Berger *et al.*, 2009; Andrade and Beck, 2010; Zhang *et al.*, 2012). More intimately related to the BG, abnormal 5-HT signalling has

been proposed as one of the main causative factors in L-DOPA induced dyskinesia in PD (Carta *et al.*, 2008; Shin *et al.*, 2012). Another illness associated with abnormal 5-HT activity is the Serotonin Syndrome (Turkel *et al.*, 2001; Boyer and Shannon, 2005). This syndrome, caused by a hyperactivation of the 5-HT system, occurs in 14-16% of patients overdosing SSRIs, or being administered SSRIs alongside other 5-HT-modulating therapies (Turkel *et al.*, 2001; Boyer and Shannon, 2005). The syndrome results in a plethora of symptoms including tremor, abnormal heart rhythms, shivering, excessive sweating, hypertension, diarrhoea, seizures, renal failure, delirium, neuromuscular rigidity, anxiety, and hyperthermia, highlighting the truly profound, global role 5-HT plays in mammalian physiology (Boyer and Shannon, 2005).

1.4 Neurotransmitter systems

Although there are numerous neurotransmitters involved in the physiology of the BG and the raphe nuclei, an exhaustive review is beyond the scope of the present work. Hence, we will focus on the two most pertinent to the SN and DRN: dopamine and 5-HT.

1.4.1 Serotonergic system

As previously mentioned, there are 14 5-HT receptors distributed across 7 families which have been discovered to date, with only one subtype, the 5-HT_3 receptor, being a ligand-gated ion channel. All others are GPCRs (Nichols and Nichols, 2008). Given the involvement of 5-HT in almost every function of the brain, an in-depth review of the functionality and localisation of all of its receptors is beyond the scope of the present work (Mohammad-Zadeh *et al.*, 2008; Berger *et al.*, 2009; Larson *et al.*, 2015). Nonetheless, an in-depth review of $5HT_3$ receptors and key characteristics of the GPCR 5-HT receptors are presented below.

1.4.1.1 5-HT$_3$ receptors

1.4.1.1.1 Pharmacological properties

5-HT$_3$ receptors belong to the cysteine (Cys)-loop superfamily of ligand-gated ion channels and have similar characteristics to nicotinic acetylcholine (nACh), glycine and GABA type A ($GABA_A$) receptors (Hannon and Hoyer, 2008; Nichols and Nichols, 2008; Lummis, 2012). They are non-specific cation-selective channels, depolarising the membrane primarily through Na^+ or K^+ influx, but also Ca^{2+} and other small organic cations (Lummis, 2012). Interestingly, 5-HT$_3$ receptors can share ligands with other receptors as is the case with tropisetron, a 5-HTR antagonist, and dopamine in N1E-115 cells (Neijt *et al.*, 1986; Hannon and Hoyer, 2008). In addition, 5-HT$_3$ receptors can be allosterically modulated by compounds such as ethanol and trichloethanol to increase the effect of 5-HT$_3$ agonists (Lovinger, 1991; Bentley and Barnes, 1995; Downie *et al.*, 1995). 5-HT$_3$ receptors are pentamers, the internal boundary of which forms the channel through which cations can pass (Hannon and Hoyer, 2008). Each receptor requires at least one 5-HT$_{3A}$ subunit to function, meaning that the 5-HT$_{3A}$ subunit is the only one capable of forming functional homomers. There are two versions of the 5-HT$_{3A}$ subunit, a short and a long form, the former having a six amino acid deletion. Both variants appear to function similarly (Doucet *et al.*, 1999; Lummis, 2012). There are four additional subunits: 3B, 3C, 3D and 3E (Niesler *et al.*, 2007; Hannon and Hoyer, 2008; Kapeller *et al.*, 2011; Lummis, 2012). The latter three have so far only been identified based on genetic data in humans (Niesler *et al.*, 2007; Hannon and Hoyer, 2008; Kapeller *et al.*, 2011; Lummis, 2012). Subunit 3B has been show to increase channel conductance when coupled to a 5-HT$_{3A}$ subunit, representing what appears to be the predominant wild-type 5-HT$_3$ receptor (*i.e.* 5-HT$_{3AB}$ heteromer) in tissue when compared to previous *in vitro* studies using

cloned 5-HT_{3A} homomers (Lummis, 2012). The 5-HT_{3B} and 5-HT_{3E} subunits have been found to have three and five isoforms, respectively (Lummis, 2012).

1.4.1.1.2 Anatomical distribution

Given the need to have at least one 5-HT_{3A} subunit for 5-HT_3 receptors to function, most specific studies mapping 5-HT_3 receptor distribution in the body have focussed on mapping the 5-HT_{3A} subunit (Morales *et al.*, 1996b; Morales *et al.*, 1998; Doucet *et al.*, 1999; Morales and Wang, 2002; Huang *et al.*, 2004; Koyama *et al.*, 2017). Several studies have also been conducted using autoradiographic binding for 5-HT_3 receptors or pharmacological assays (Kilpatrick *et al.*, 1987; Kilpatrick *et al.*, 1988; Waeber *et al.*, 1988; Barnes *et al.*, 1989a; Kilpatrick *et al.*, 1989; Waeber *et al.*, 1989; Barnes *et al.*, 1990; Pratt *et al.*, 1990; Waeber *et al.*, 1990; Gehlert *et al.*, 1991; Jones *et al.*, 1992; Laporte *et al.*, 1992a; Bufton *et al.*, 1993a; Gehlert *et al.*, 1993; Steward *et al.*, 1993b; Morales *et al.*, 1996a; Morales and Bloom, 1997). There is significant disparity in the apparent distribution of 5-HT_3 receptors between species (Bentley and Barnes, 1995). For instance, in humans there are high levels of 5-HT_3 receptors in the caudate, putamen, amygdala, NAc and hippocampus, whereas there are low levels found in the cortex, GP and SN (Barnes *et al.*, 1989a; M *et al.*, 1989; Waeber *et al.*, 1989; Abi-Dargham *et al.*, 1993; Bufton *et al.*, 1993b; Barnes and Sharp, 1999). Conversely, in rodents, there have been reports of significant 5-HT_3 receptor presence in the cortex (Barnes and Sharp, 1999). Moreover, in humans, it seems that 5-HT_3 receptors are not located on dopaminergic cells as they appear to be unaffected by nigrostriatal degeneration associated with PD (Steward *et al.*, 1993a). In contrast, there is significant depletion of 5-HT_3 receptors accompanying the neurodegeneration associated with HD, which affects striatal MSNs (Steward *et al.*, 1993a; Barnes and Sharp, 1999).

In the rat, brainstem areas such as the dorsal vagal complex, area postrema (believed to mediate the antiemetic effects of 5-HT_3 antagonists), nucleus of the solitary tract (NTS), trigeminal nucleus and the dorsal horn of the spinal cord generally display robust 5-HT_3 receptor expression (Pratt *et al.*, 1990; Gehlert *et al.*, 1991; Gehlert *et al.*, 1993; Steward *et al.*, 1993b; Huang *et al.*, 2004). However, forebrain areas show mixed patterns of expression depending on the methods used by experimenters, *e.g*. pharmacological experiments, radioligands, mRNA probes or antibodies (Barnes *et al.*, 1990; Gehlert *et al.*, 1991; Gehlert *et al.*, 1993; Tecott *et al.*, 1993; Morales *et al.*, 1996a; Morales *et al.*, 1996b; Maswood *et al.*, 1997; Morales and Bloom, 1997; Morales *et al.*, 1998). Studies have found weak 5-HT_3 expression across the cortex, in the amygdala, subiculum, striatum, OT, NAc, glomerular layer of the olfactory bulb (OB), anterior olfactory nucleus, hippocampus, ventromedial hypothalamus (VMH) and very low expression in the thalamus (reticular and paraventricular nuclei), cerebellum, SN and DRN (Kilpatrick *et al.*, 1988; Barnes *et al.*, 1990; Gehlert *et al.*, 1991; Laporte *et al.*, 1992b; Gehlert *et al.*, 1993; Morales *et al.*, 1996a; Morales *et al.*, 1996b; Morales and Bloom, 1997; Morales *et al.*, 1998; Doucet *et al.*, 1999; Spier *et al.*, 1999; Geurts *et al.*, 2002; Puig *et al.*, 2004). Furthermore, the ultrastructural pattern of expression analysed using electron microscopy has varied considerably depending on the detection method used, ranging from largely axonal, to pre-synaptic, to dendritic, to somatic (Doucet *et al.*, 1999; Miquel *et al.*, 2002; Huang *et al.*, 2004). Non-visual methods of detection such as membrane binding assays, *in vivo* electrophysiology or behavioural assays have detected or implied the presence of 5-HT_3 receptors in the SN (Sorensen *et al.*, 1989; Palfreyman *et al.*, 1993; Maswood *et al.*, 1997; Alex and Pehek, 2007). Conversely, other studies have provided evidence refuting the presence of 5-HT_3 receptors in the SNc (Rasmussen *et al.*, 1991; Prisco *et al.*, 1992).

A similar distribution to that seen in rats was found in the marmoset, *i.e.* strong signals in the hindbrain and medium binding in the hippocampus (Jones *et al.*, 1992). As for the rat, patterns in the forebrain were less conclusive, with low levels of detection in the cortex, the medial SNc, the amygdala, thalamus, septum, interpenduncular nucleus, and hypothalamus (Jones *et al.*, 1992). More recently, Carrillo and colleagues (2010) found 5-HT_{3A}-positive fibres in the caudal SN, as well as fibres and cell bodies in the caudal DRN of the Syrian hamster. In the mouse, data from the Allen Brain Institute (http://mouse.brain-map.org/experiment/show/74724760) show higher levels of mRNA expression in hindbrain regions, low-level expression in various regions throughout areas similar to rats (*e.g.* the hippocampus, cortex, etc.), very low levels in the SNr, and undetectable levels in the DRN (Lein *et al.*, 2006). Alternatively, Koyama and colleagues (2017) used a transgenic mouse model expressing green fluorescent protein (GFP) under the control of the promoter for the gene encoding the 5-HT_{3A} subunit to visualise the transcription of the receptor. They found very strong labelling in the OB, OT, NTS, trigeminal nucleus, moderate to high levels in the cortex, hippocampus, amygdala, caudate and putamen, NAc, but no signal in the SN, cerebellum, or the DRN (Koyama *et al.*, 2017). Clearly, there are many similarities – and differences – in 5-HT_3 receptor expression depending on the species examined and methods employed, which require judicious interpretation.

1.4.1.1.3 Functional and physiological properties

As mentioned previously, 5-HT_3 receptors modulate rapid synaptic transmission, and this can occur both pre and post-synaptically (Chen *et al.*, 1991; Kidd *et al.*, 1993; Morales *et al.*, 1996b; Bloom and Morales, 1998; MacDermott *et al.*, 1999). Studies have shown that 5-HT_3 receptors can modulate GABAergic transmission, particularly in hippocampal and cortical interneurons, but also in the medial preoptic area and posterior hypothalamus (Ferraro *et al.*, 1996;

Morales *et al.*, 1996a; Morales and Bloom, 1997; Puig *et al.*, 2004). Multiple studies have shown that 5-HT_3 receptors act as facilitating autoreceptors for 5-HT release when excited, creating a positive feedback loop (Martin *et al.*, 1992; Blier and Bouchard, 1993; Haddjeri and Blier, 1995). Similarly, there is substantial *in vitro* and *in vivo* evidence that these receptors also play a role in dopamine release (Blandina *et al.*, 1989; Schmidt and Black, 1989; Lian Hai *et al.*, 1990; Chen *et al.*, 1991; Su-Jin *et al.*, 1991; Benuck and Reith, 1992; Zazpe *et al.*, 1994). A study found that there was an increase of 5-HT_3 receptors following injection of 6-hydroxydopamine (6-OHDA) to the MFB of rats, which depletes the striatum of midbrain dopaminergic afferents, leadin them to conclude that 5-HT_3 receptors effects on dopaminergic transmission may not be due to direct effects on striatal dopaminergic terminals, but rather is mediated by non-monoaminergic projections to the striatum (Kidd *et al.*, 1993). Moreover, excitation of 5-HT_3 receptors enhances the release of a third monoamine, noradrenaline, in the rat (Mongeau *et al.*, 1994). Whether these increases in monoaminergic transmission require a carrier-mediated mechanism, are due to direct effects of 5-HT_3 receptors on monoaminergic cells, or effects on interneurons or other glial cells remains uncertain (Morales *et al.*, 1996b; Morales and Bloom, 1997; Morales *et al.*, 1998; Alex and Pehek, 2007). Similarly, there is evidence of 5-HT_3 receptors modulating acetylcholine release (Barnes *et al.*, 1989b). Hence, further investigation into the potential expression of 5-HT_3 receptors on dopaminergic and 5-HT neurons would address a lacuna in the current literature, given the importance of these monoamines in virtually every function of the brain.

5-HT_3 receptors have been investigated as potential targets for a number of illnesses including anxiety, schizophrenia-related psychosis, on top of their well-known anti-emetic effects (Barnes *et al.*, 1990; Bentley and Barnes, 1995). Indeed, as previously stated, a number of 5-HT_3 antagonists are used clinically to treat gastric diseases, affective disorders, psychosis, malaria and,

especially, chemotherapy-induced emesis, amongst other uses (Zoldan *et al.*, 1995; Nakagawa *et al.*, 1998; Gill and Hatcher, 2000; Thompson and Lummis, 2008; Lewis, 2010; Hesketh *et al.*, 2017). More relevant to the present work, ondansetron, a selective $5HT_3$ antagonist, may reduce visual hallucinations in patients with advanced PD, without interfering with mainstay L-DOPA treatment (Butler *et al.*, 1988; Zoldan *et al.*, 1993; Zoldan *et al.*, 1995).

1.4.1.2 Other 5-HT receptors

As mentioned previously, there are 7 families of 5-HT receptors, all of which apart from 5-HT_3 receptors are GPCRs (Nichols and Nichols, 2008). 5-HT type 1 (5-HT_1) receptors are autoreceptors which inhibit the release of 5-HT from 5-HT cells, or act as inhibitory postsynaptic heteroreceptors on non-5-HT cells through a $G_{i/o}$-coupled mechanism (Nichols and Nichols, 2008). 5-HT_1 receptors contribute to a plethora of phenomena, including cerebrovascular regulation, migraines, learning and memory, anxiety, addiction, schizophrenia, PD and depression (Blier and De Montigny, 1987; Sprouse and Aghajanian, 1987; Lucki, 1991; Heisler *et al.*, 1998; Parks *et al.*, 1998; Nilsson *et al.*, 1999; Bantick *et al.*, 2001; Kannari *et al.*, 2002; Prinssen *et al.*, 2002; Blier and Ward, 2003; Buhot *et al.*, 2003; Ramadan *et al.*, 2003; Toth, 2003; Åhlander-Lüttgen *et al.*, 2003; Assié *et al.*, 2005; Kleven *et al.*, 2005; Rutz *et al.*, 2006; Eskow *et al.*, 2007; Müller *et al.*, 2007; Stark *et al.*, 2007; Muñoz *et al.*, 2008; Nichols and Nichols, 2008). 5-HT type 2 (5-HT_2) receptors act through a $G_{\alpha q}$-coupled mechanism to increase protein kinase C (PKC) signalling and intracellular Ca^{2+} concentrations, therefore acting as excitatory receptors (Nichols and Nichols, 2008). 5-HT_2 receptors, particularly 5-HT type 2A (5-HT_{2A}) receptors and to a lesser degree 5-HT type 2C (5-HT_{2C}) receptors, are notable for being the consensus locus of hallucinogenic activity in the brain caused by drugs such as lysergic acid diethylamide (LSD). N,N-dimethyltryptamine (DMT), and psilocybin, amongst others (Titeler *et al.*, 1988; Sadzot *et al.*, 1989; Branchek *et al.*,

1990; Egan *et al.*, 1998; Krebs-Thomson *et al.*, 1998; Smith *et al.*, 1998; Vollenweider *et al.*, 1998; Aghajanian and Marek, 1999; Nelson *et al.*, 1999; Smith *et al.*, 1999; Scruggs *et al.*, 2000; Ebersole *et al.*, 2003; Nichols and Nichols, 2008). Outside of this context, 5-HT_{2A} receptors have also been implicated in weight regulation, hearing, depression, suicide, schizophrenia, anxiety, spatial memory, analgesia at the level of the spine, and in dyskinesia, a side-effect of chronic L-DOPA treatment for PD, with mixed effects on psychosis-like behaviour (Niswender *et al.*, 2001; Sodhi *et al.*, 2001; Berthoud, 2002; Schmauss, 2003; Sommer, 2004; Iwamoto *et al.*, 2005; Nitanda *et al.*, 2005; Hackler *et al.*, 2006; Du *et al.*, 2007; Tadros *et al.*, 2007; Van Steenwinckel *et al.*, 2008; Hamadjida *et al.*, 2018a; Hamadjida *et al.*, 2018b; Hamadjida *et al.*, 2018c, d). 5-HT type 4 (5-HT_4) receptors are $G_{\alpha s}$-coupled receptors which upregulate cyclic adenosine monophosphate (cAMP) signalling, generally resulting in an excitatory response through cAMP-gated ion channels or in the upregulation of gene transcription through the cAMP response element binding protein (CREB) (Nichols and Nichols, 2008). 5-HT_4 agonists have been shown to positively affect learning and memory in animal models, with 5-HT_4 receptors mediating long term depression (LTD) in the CA1 region of the hippocampus (Terry Jr. *et al.*, 1998; Lamirault and Simon, 2001; Lelong *et al.*, 2001; Kemp and Manahan-Vaughan, 2005; Micale *et al.*, 2006; Nichols and Nichols, 2008). 5-HT_4 receptors also tonically upregulate 5-HT signalling in the raphe nuclei (Conductier *et al.*, 2006). Similarly to 5-HT_1 receptors, 5-HT type 5 (5-HT_5) receptors are $G_{i/o}$-coupled receptors (Nichols and Nichols, 2008). Although their distribution, apparently almost exclusively reserved to the CNS, is well described in humans and rodents, little is known about their functional role given the lack of selective agonists (Pasqualetti *et al.*, 1998; Grailhe *et al.*, 1999; Oliver *et al.*, 2000; Nichols and Nichols, 2008). However, based on localisation, they are hypothesised to be involved in circadian regulation, mood, and cognition, and activation thereof could act as an

antipsychotic for schizophrenia (Thomas, 2006; Nichols and Nichols, 2008). Similarly to the 5-HT_4 receptor, the 5-HT type 6 (5-HT_6) receptor is a $G_{\alpha s}$-coupled receptor (Nichols and Nichols, 2008). 5-HT_6 blockade upregulates cholinergic neurotransmission through an indirect mechanism as these receptors are not expressed on cholinergic neurons, which has proven to be beneficial to learning and memory in animal models (Riemer *et al.*, 2003; Lieben *et al.*, 2005; Hirst *et al.*, 2006; Marcos *et al.*, 2006). 5-HT_6 receptors have also been shown to influence glutamatergic, dopaminergic, adrenergic, noradrenergic and GABAergic transmission, and are therefore under investigation for enhancing cognition in Alzheimer's disease and schizophrenia, treating depression, and tackling obesity (Dawson *et al.*, 2000; Mitchell and Neumaier, 2005; Fisas *et al.*, 2006; Schechter *et al.*, 2007; Svenningsson *et al.*, 2007; Wesołowska and Nikiforuk, 2007). The last of the 5-HT receptors is the 5-HT type 7 (5-HT_7) receptor, which is also a $G_{\alpha s}$-coupled receptor (Nichols and Nichols, 2008). Little is known as to the function of 5-HT_7 receptors in the CNS owing to a lack of selective agonists, but studies with antagonists and knock-out mice have led to the hypotheses that 5-HT_7 receptors regulate sleep, circadian rhythms, body temperature and mood in general (Guscott *et al.*, 2003; Hedlund *et al.*, 2003; Thomas *et al.*, 2003; Hedlund and Sutcliffe, 2004; Guscott *et al.*, 2005).

1.4.1.3 SERT

As previously mentioned, SERT is classically responsible for the reuptake of 5-HT from the synaptic cleft following its vesicular release induced by an action potential (Hoffman *et al.*, 1998). SERT is a dynamic membrane protein, with expression beyond the pre-synaptic terminal, given the ability of 5-HT cells to release their transmitter by means of volume secretion anywhere in the cell (Descarries *et al.*, 1982; Kapadia *et al.*, 1985; Liposits *et al.*, 1985; Chazal and Ralston, 1987; Hoffman *et al.*, 1998; Adell *et al.*, 2002). Hence, this transporter is a good marker of 5-HT

cells (Hoffman *et al.*, 1998). SERT plays a pivotal role in determining the strength of 5-HT transmission. Too rapid reuptake of 5-HT will prevent appropriate binding of 5-HT to 5-HT receptors, whereas too slow reuptake results in a loss of temporal encoding of the signal or non-specific diffusion outside the synapse (Amara and Kuhar, 1993; Borowsky and Hoffman, 1995; Hoffman *et al.*, 1998). As seen previously, SERT is the primary target for drugs aiming to treat MDD, known as SSRIs (Blakely *et al.*, 1991; Hoffman, 1994; Tatsumi *et al.*, 1997; Adell, 2015). Moreover, it is also a target for psychostimulants such as fenfluramine and 3,4-methylenedioxymethamphetamine (MDMA) (Schuldiner *et al.*, 1993).

1.4.2 Dopaminergic system

Dopamine is involved in numerous functions, particularly with respect to motivation, reward, cognition, emotion and movement (Ikemoto, 2007; Govoni, 2009; Ikemoto *et al.*, 2015). Dopamine is a derivative of tyrosine, with tyrosine hydroxylase (TH) being the rate limiting enzyme of its synthetic pathway. Therefore, anti-TH antibodies are regularly used to label dopaminergic cells (Hökfelt *et al.*, 1976; S.Y. *et al.*, 1999). Considering that TH is involved in the synthesis of other catecholamines such as noradrenaline and adrenaline, staining for the dopamine transporter (DAT) may be a more selective approach to labelling dopaminergic neurons. Nonetheless, staining for TH has been shown to be an effective way to visualise dopaminergic neurons in the SN as other catecholamines are not present (S.Y. *et al.*, 1999).

Similarly to 5-HT, dopamine is a monoamine neurotransmitter, with multiple receptors and DAT, analogous to SERT, modulating its wide-ranging effects (Beaulieu *et al.*, 2015). However, a detailed description of these interactions is beyond the scope of this thesis. For a functional understanding of dopamine effects in the BG physiology, see section 1.2 (Haber, 2014; Ikemoto *et al.*, 2015). Outside of the BG, dopamine also plays role in the physiology of the raphe nuclei

(Ferre *et al.*, 1994). For instance, dopaminergic neurons are encountered alongside 5-HT neurons in the DRN and MnR (Ochi and Shimizu, 1978; Jahanshahi *et al.*, 2013). Indeed, it has been demonstrated that dopamine regulates 5-HT raphe-striatal projections, as well as extracellular 5-HT within the DRN itself (Ferré and Artigas, 1993; Ferre *et al.*, 1994). More surprisingly, the DRN also has dopaminergic projections which increase dopamine levels in the NAc of rats, potentially through a VTA-mediated mechanism (Stratford and Wirtshafter, 1990; Yoshimoto and McBride, 1992). Hence, although classically considered a 5-HT structure, this does not reflect the broad functions of the DRN.

1.5 Hypothesis and objectives

Better understanding of the distribution of 5-HT_3 receptors in the rat SN and DRN could have implications for our understanding of how the BG and the 5-HT system interact, notably to modulate monoamine release within these structures so that they perform their roles in normal movement, behaviour, and cellular physiology. Thus, the present study investigates the localisation of 5-HT_3 receptors in the SN and DRN in the BG of the rat. More specifically, the hypotheses are as follows:

- 5-HT_3 receptors are present in the SN of the rat, in which they are expressed by dopaminergic neurons.
- 5-HT_3 receptors are present in the DRN of the rat, in which they are expressed by 5-HT neurons.

To validate these hypotheses, we specifically aim to:

- Determine the quantitative distribution of cells expressing 5-HT_3 receptors and TH in the SN using IHC and unbiased stereology.
- Determine the quantitative distribution of cells expressing 5-HT_3 receptors and SERT in the DRN using IHC and unbiased stereology.

Materials & Methods

2.1 Antibodies used

In the SN, TH was labelled with an Alexa Fluor 488 (green) probe, whereas the 5-HT_{3A} subunit was labelled with an Alexa Fluor 594 (red) probe. 4′,6-diamidino-2-phenylindole (DAPI) [blue] was applied as a nuclear counterstain. The 5-HT_{3A} subunit of the 5-HT_3 receptor is the target antigen in the proposed experiments as all known functional 5-HT_3 receptors to date require at least one 5-HT_{3A} subunit to be functional (Hanna *et al.*, 2000; Hassaine *et al.*, 2014). Therefore, this subunit permits the identification of all 5-HT_3 receptors, whether they are homoreceptors or heteroreceptors.

In the DRN, 5-HT cells were labelled with an Alexa Fluor 488 probe targeting SERT, whereas 5-HT_{3A}-containing cells were labelled with the same Alexa Fluor 594 probe. Again, DAPI was applied as a nuclear counterstain. The use of Alexa Fluor 488 for the anatomical rather than experimental targets was decided upon as biological tissue exhibits increased autofluorescence under green wavelengths of light (Mosiman *et al.*, 1997; Billinton and Knight, 2001). As the anatomical distribution of TH and SERT within the SN and DRN are well characterised, whereas that of 5-HT_{3A} is not, this configuration was favoured to increase the signal-to-noise ratio of the experimental condition [*i.e*. the 5-HT_{3A} subunit] (Zhao *et al.*, 2003; Li *et al.*, 2016). The antibodies used in this set of experiments were selected based on available data pertaining to specificity and applicability for IHC in murine rodents (Carlsson *et al.*, 2009; Lu *et al.*, 2010; Shin *et al.*, 2012; Tronci *et al.*, 2012; Hitora-Imamura *et al.*, 2015; Schreglmann *et al.*, 2015; Van Rompuy *et al.*, 2015; Zharikov *et al.*, 2015; Chang *et al.*, 2016). Immunofluorescent (IF) methods were favoured over colourimetric IHC as multi-labelling is crucial to answer the hypotheses posited for this project. Although multi-labelling is possible with colourimetric approaches, the manipulations required (*e.g*. quenching different endogenous enzymes or peroxidases) can be problematic (Inc.,

2015; van der Loos, 2017). Moreover, the resolution between different colourimetric stains is lesser than is possible with IF microscopy (Biologicals, 2018; Inc., 2018). Lastly, IF permits the easy identification of overlapping signals, which will be critical in the analysis described below (Busceti *et al.*, 2012).

2.2 Animals

Six (2 male and 4 female) adult Sprague-Dawley rats (250 - 275 g, Charles River, Saint-Constant, Canada) were separately group-housed in a temperature, humidity, and light-controlled environment (under 12-h light/dark cycle, on 07:00) with free access to food and water. Upon arrival, rats were left undisturbed to acclimatise to the housing conditions for at least 3 days before they were euthanised. Experimental protocols were approved by McGill University Animal Care Committee in agreement with guidelines established by the Canadian Council on Animal Care.

2.3 Animal perfusion and tissue processing

Animals were deeply anaesthetised with 4% isofluorane in 100% O_2 (1L/min), and perfused transcardially with 0.9% NaCl (1 mL/g of body weight), followed by 4% paraformaldehyde (PFA) in 1X phosphate buffered saline (PBS, pH 7.4), both at a flow rate of 50 mL/min (Gage *et al.*, 2012). Then, brains were carefully extracted from the skull and transferred to 50 mL Eppendorf tubes containing approximately 15 mL of cryoprotective solution composed of 30% sucrose in 1X PBS at 4°C, until they sunk (Koyama *et al.*, 2017). Approximately two days later (once the brains sunk), the cryoprotected tissue was remove from the solution and quickly frozen at -56°C with methyl butane before being stored at -80°C.

One day before sectioning, frozen tissues were embedded with OCT (Sakura Finetek, Torrance, California, USA) and transferred to a cryostat set to -23 °C overnight, in which brains were cut in the frontal plane in a single series of 40 μm thickness. Sections were taken in a rostral to caudal direction and immediately transferred to 24-well plates containing approximately 1 mL of 1X PBS (pH 7.4) before proceeding with IHC.

2.4 Immunohistochemistry

Double IF IHC was performed for this study. Sections were rinsed 3 times with 1X PBS (pH 7.4) at room temperature (RT), followed by a 30-min antigen retrieval (AR) incubation in 10 mM sodium-citrate buffer (pH 8.5) heated to 80°C in a water bath, and then rinsed again 3 times at RT with 1X PBS (pH 7.4). AR was empirically determined to be necessary to unmask SERT antigens following the PFA-fixation process. After rinsing, sections were transferred to a blocking solution containing 5% normal goat serum (NGS) in 1X PBS for 30 minutes. NGS was used as a blocking agent against non-specific binding, as both secondary antibodies (Alexa Fluor 488 and Alexa Fluor 594, Jackson Immunoresearch, West Grove, Pennsylvania, USA) were raised in goats. Sections were then incubated overnight (20 h) at 4°C with the following primary antibodies: 1:1,200 rabbit anti-5-HT_{3A} antibody (Cat # ASR-031, Alomone Labs, Israel), 1:4,000 mouse anti-SERT antibody (Cat # MAB1564, EMD Millipore, Burlington, Massachusetts, USA), 1:8,000 mouse anti-TH antibody (Cat # MAB318, EMD Millipore,Burlington, Massachusetts, USA) in the same blocking solution (5% NGS, 1X PBS). After this primary incubation, sections were rinsed again 3 times at RT with 1X PBS (pH 7.4), and incubated with the following secondary antibodies: 1:1,000 Alexa Fluor 594 goat anti-rabbit antibody (Cat # 111-585-144, Jackson ImmunoResearch, West Grove, Pennsylvania, USA) and 1:1,000 Alexa Fluor 488 goat anti-mouse antibody (Cat #

115-545-146, Jackson ImmunoResearch, West Grove, Pennsylvania, USA) for one hour in the dark at RT, in 1X PBS (pH 7.4). Following secondary incubation, tissues were rinsed thrice at RT with 1X PBS (pH 7.4) in the dark, counter-stained with DAPI for 5 min in the dark and underwent one final round of 3 rinses with RT 1X PBS (pH 7.4) in the dark. Tissues were then mounted on gelatinised slides and allowed to air-dry (generally 1-2 h) in the dark. Slides were then coverslipped using ProLong™ Diamond antifade fluorescent mounting medium (Cat # P36965, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and allowed to air-dry overnight at RT in the dark. Once dried, slides were cleaned with gauze soaked in xylene to remove any mounting residue, hence improving visibility under the microscope. All reasonable precautions were taken to avoid photobleaching of samples, including working away from direct light when preparing secondary antibodies and during all following steps by covering samples with aluminium foil when possible. Excitation time was also minimised when using the microscope through the generation of Z-stack virtual images which were then used for digital analysis. All incubations other than AR were performed on a shaker plate set to 165 rpm.

2.5 Stereological estimations and image analysis

A neuroanatomical reconstruction system, consisting of a computer-interfaced microscope (Nikon Eclipse E800, Tokyo, Kantō, Japan) and associated software (StereoInvestigator, MicroBrightField Bioscience, Williston, Vermont, USA), was used to count TH-, 5-HT_{3A}- and SERT-positive cells in the SN and DRN (Glaser, 2007). Cell numbers in the SN and DRN of the rat were estimated, using a design-based stereology protocol (Schmitz and Hof, 2005; Benarroch *et al.*, 2008). A systematic random series (SRS) of 40 μm-thick coronal sections using a one-in-

six sampling scheme was used for all analyses in both the SN and DRN (Glaser, 2007; Ahmad *et al.*, 2008; Nair-Roberts *et al.*, 2008; Baquet *et al.*, 2009).

The total number of TH-positive cells in the SN and SERT-positive cells in the DRN was determined using SRS. We also estimated the number of 5-HT_{3A}-positive cells in both the SN and DRN. For each section, the region of interest (ROI) was outlined on the basis of the atlas by Paxinos & Watson (2007), over which a lattice in the X-Y plane was randomly superimposed for sampling. The size of this initial lattice was such that there were approximately 10 randomly-generated counting frames per ROI as determined by the StereoInvestigator software. This corresponded to a 250 μm × 300 μm grid for the SN, and a 234 μm × 155 μm grid for the DRN. A second, smaller (100 μm × 100 μm × 15 μm, X × Y × Z) randomly placed unbiased counting box was inserted in the initial field, representing the volume in which cells would be counted at 60× magnification (Glaser, 2007). The size of the initial SRS grid and sampling box were empirically determined during preliminary experiments such that dopaminergic and 5-HT cell estimates for the SN and DRN were similar to those previously published in the literature (Descarries *et al.*, 1982; Nair-Roberts *et al.*, 2008). We only counted immunoreactive cells that could be clearly identified by the presence of a soma, dendritic processes, or both in sequential focal planes. Accuracy of cell counts were evaluated using Gundersen's m=1 CE. This formula evaluates the variance of samples owing to noise, as well as that due to SRS. CE values less than 0.1 are deemed satisfactory, whereas values of 0.06 and below are preferred for biological samples (Gundersen and Jensen, 1987; Mattfeldt, 1989; West *et al.*, 1991; Gundersen *et al.*, 1999; West, 2012).

Digital images were acquired using a digital camera (OPTRONICS Inc., Serial No. DG604048-H, Santa Barbara, California, USA) attached to a microscope (Nikon Eclipse E800, Tokyo, Kantō, Japan). The digitised images were then imported to Adobe Lightroom CC (Adobe

Inc., San Jose, California, USA) and adjusted for brightness and contrast to maximise visibility of positive signals.

2.6 Statistical analyses

TH-, 5-HT_{3A}- and SERT-positive cells counted in the SN and DRN are presented as the mean ± standard error of the mean (SEM) and were analysed using descriptive statistics. Statistical analyses (mean and SEM) were computed using Microsoft Excel (Microsoft Inc., Redmond, Washington, USA) and graphed with GraphPad Prism 7.03 (GraphPad Software Inc., La Jolla, California, USA).

Results

3.1 Validation of the anti-5-HT$_{3A}$ antibody

Figure 5 shows four positive control regions demonstrating the characteristics of 5-HT$_{3A}$-positive staining. Figure 5A shows the positive staining of OB glomerular cells, Figure 5B cells in the NTS, Figure 5C cells in the LDTg and Figure 5D a cell in the VMH with slight projection labelling. Notice the generally punctate appearance of fluorescent signals, as well as their orange hue under high fluorescein isothiocyanate (FITC) background (Figure 5A & B).

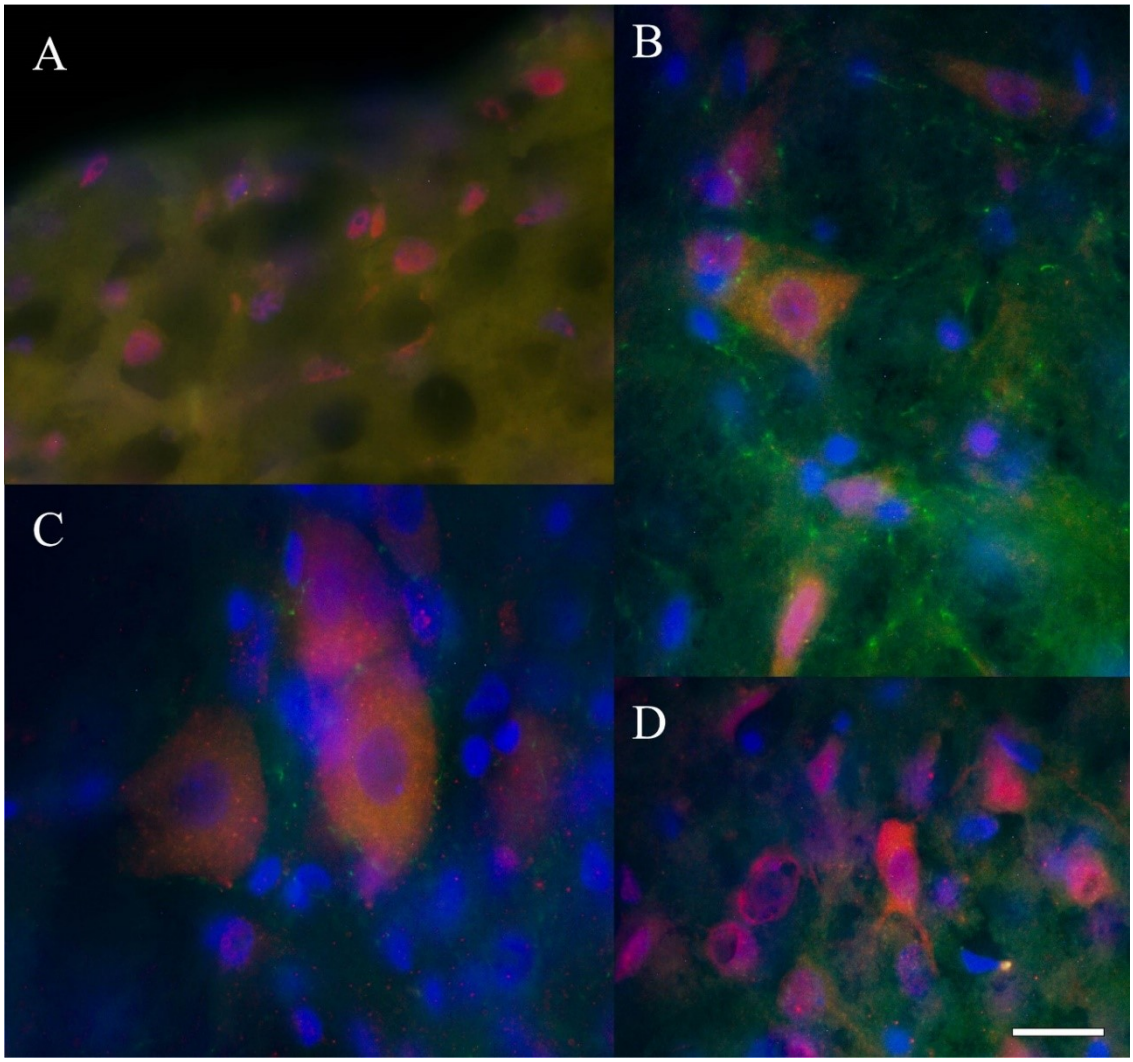

Figure 5. 5-HT$_{3A}$-positive cells in the olfactory bulb (A), nucleus of the solitary tract (B), laterodorsal tegmental nucleus (C) and ventromedial hypothalamus (D). 60× magnification, scale bar: 20 µm.

3.2 Immunohistochemistry in the substantia nigra

Figure 6 shows a whole section of the SN of the rat showing dopamine neurons stained for TH. The bilateral "wings" of the SNc are clearly visible, as are the more medial dopaminergic cells of the VTA. A few sparse dopaminergic cells are visible in the core of the SNr, as well as in its dorsolateral portion (*i.e.* the SNl). Figure 7 shows the localisation of TH-positive and 5-HT_{3A}-positive cells in the SN. Low magnification of the SN after TH and 5-HT_{3A} IHC are shown in Figure 7A and B. TH-positive cells of the SN were more densely and clearly visible (Figure 7A), whereas 5-HT_{3A}-positive cell in the SN were less apparent at low magnification (Figure 7B). Figure 7 C-E displays higher magnification of TH-positive and 5-HT_{3A}-negative cells found in the SNc. The differences in intensity of TH-positive signals compared to background 5-HT_{3A} signalling is apparent. No overlap is seen in the multichannel image (Figure 7E). Figure 7 F-H displays higher magnification of TH-negative and 5-HT_{3A}-positive cells found in the SNr. No overlap is seen in the multichannel image (Figure 7H), although the red signal shines through the FITC background.

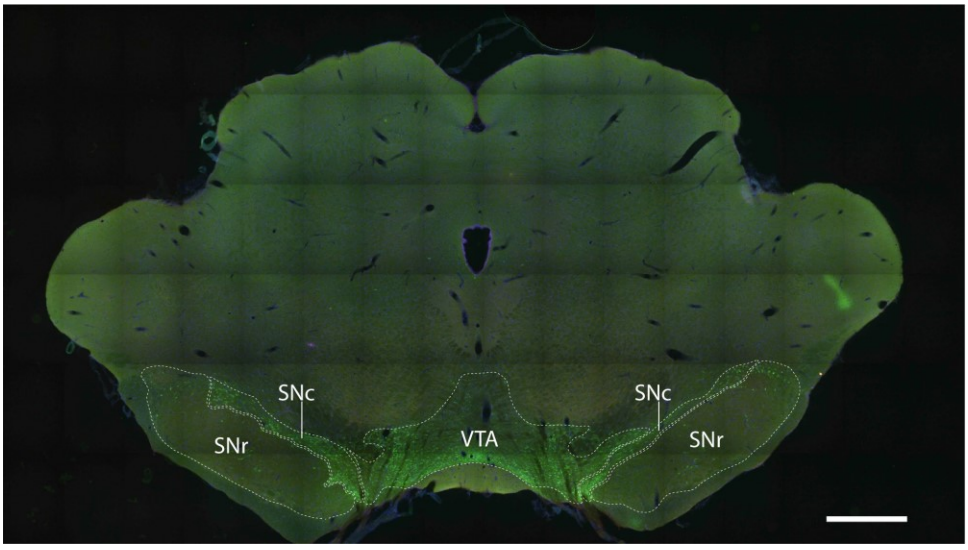

Figure 6. Representative section of the substantia nigra of the rats showing dopamine neurons stained for TH. Scale bar:1000 μm

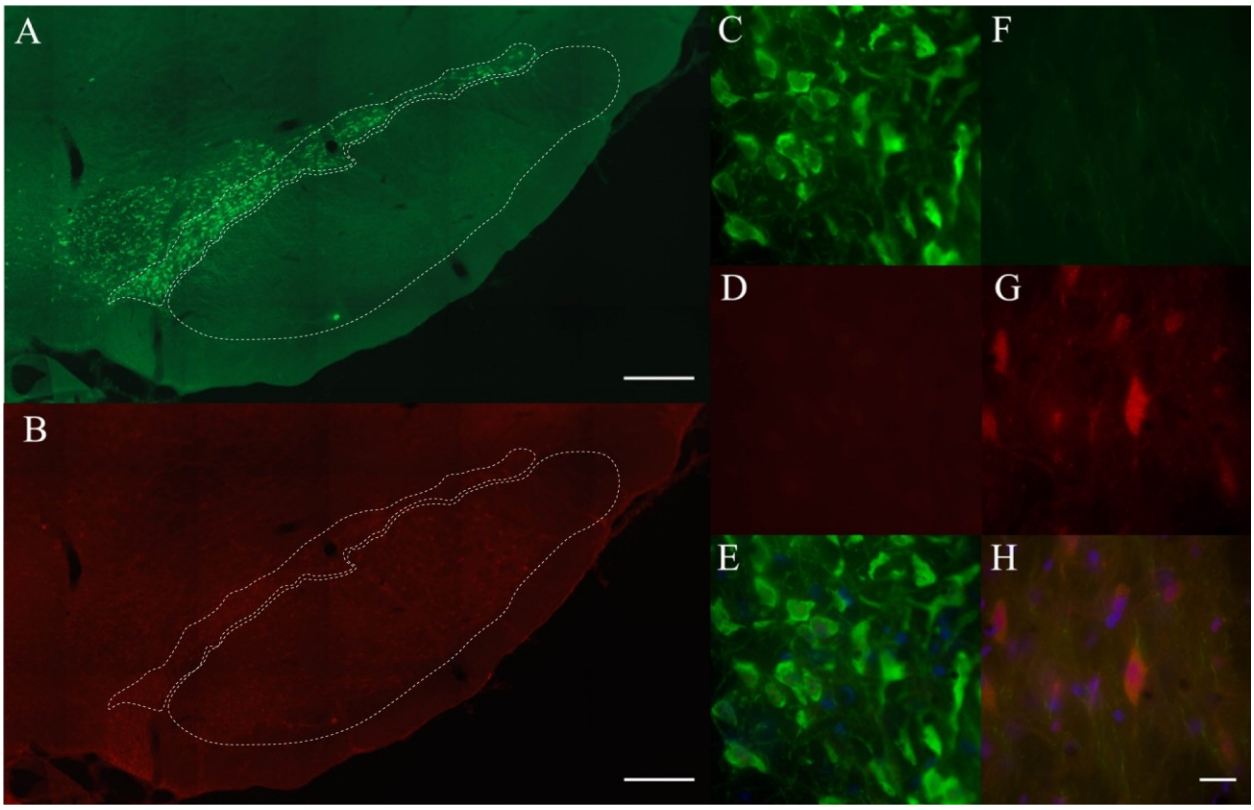

Figure 7. Multichannel staining of TH- and 5-HT$_{3A}$-positive cells in the substantia nigra. Double-labelled immunofluorescent staining was used to quantify TH (green) and 5-HT$_{3A}$ (red) expressing cells in the SN of rats, with delineation of the SNc and SNr. **A.** 10× magnification in FITC channel, showing robust staining of TH-positive neurons. **B.** 10× magnification in Texas Red channel, illustrating high background in SNc and faint positive signals in the SNr. **C.** 60× magnification of the SNc in FITC channel. **D.** 60× magnification of the SNc in Texas Red channel. **E.** 60× magnification multichannel image with FITC, Texas Red and DAPI (blue) staining in the SNc. **F.** 60× magnification of the SNr in FITC channel. **G.** 60× magnification of the SNr in Texas Red channel. **H.** 60× magnification multichannel image with FITC, Texas Red and DAPI staining in the SNr. Scale bars: 500 µm in A & B, 20 µm in C-H.

3.3 Quantitative distribution of TH- and 5-HT$_{3A}$-positive cells in the substantia nigra

The number of dopaminergic cell bodies within the SN of the rats is summarised in Table 1. The average estimate of TH-positive cells in the SN was of 28,428 ± 888 (CE = 0.05) (Figure 8). The vast majority of these cells were located in the SNc, with a few cells descending ventrally into the SNr or being part of the SNl (Figure 7A). TH-positive cell counts per section ranged from 0-120 cells. In contrast, the average estimate of 5-HT$_{3A}$-positive cells in the SN was of 1,250 ± 64

(CE = 0.24) (Figure 8). 5-HT_{3A}-positive cells were all observed in the SNr, and counts per section ranged from 0-10 cells, therefore being much more rarely observed.

animal ID	individual cell count estimate (TH)	individual cell count estimate (5-HT_{3A})
1	22,554	756
2	32,382	819
3	27,405	1,827
4	37,989	1,197
5	26,838	1,575
6	23,399	1,324
mean	28,428	1,250
SEM	888	64

Table 1. **Total number of TH- and 5-HT_{3A}-positive cells in the rat substantia nigra.** SEM: Standard error of the mean.

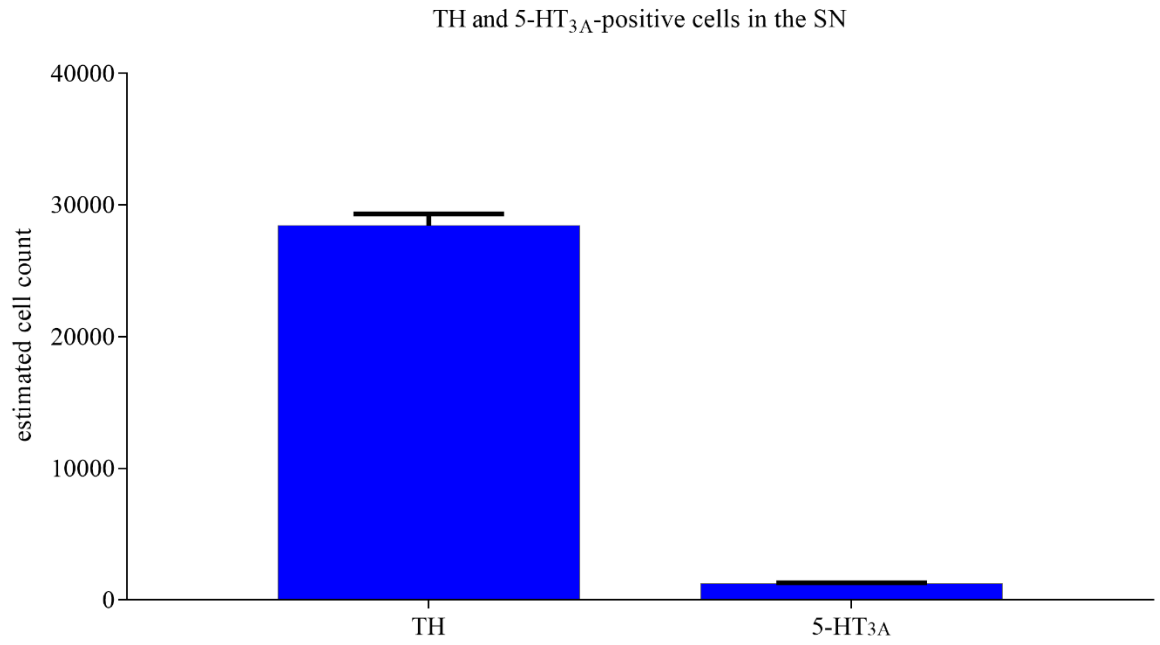

Figure 8. Average estimates of TH- and 5-HT_{3A}-positive cells in the rat substantia nigra. Data are presented as the mean ± SEM.

3.4 Immunohistochemistry in the dorsal raphe nucleus

Figure 9 shows a whole section of the DRN of the rat showing 5-HT neurons stained for SERT. A faint band of SERT-positive cells is visible ventromedially to the central aqueduct. Figure 10 shows the localisation of SERT-positive cells in the DRN. Higher magnification of the DRN after SERT and 5-HT_{3A} IHC are shown in Figure 10A and B, in which SERT-positive cells of the DRN were clearly visible (Figure 10A) whereas 5-HT_{3A}-positive cells were not encountered in the DRN. Figure 10 C-E display higher magnification of SERT-positive and 5-HT_{3A}-negative cells found in the DRN. The differences in intensity of SERT-positive signals compared to background 5-HT_{3A} signalling is apparent. No overlap is seen in the multichannel image (Figure 10E).

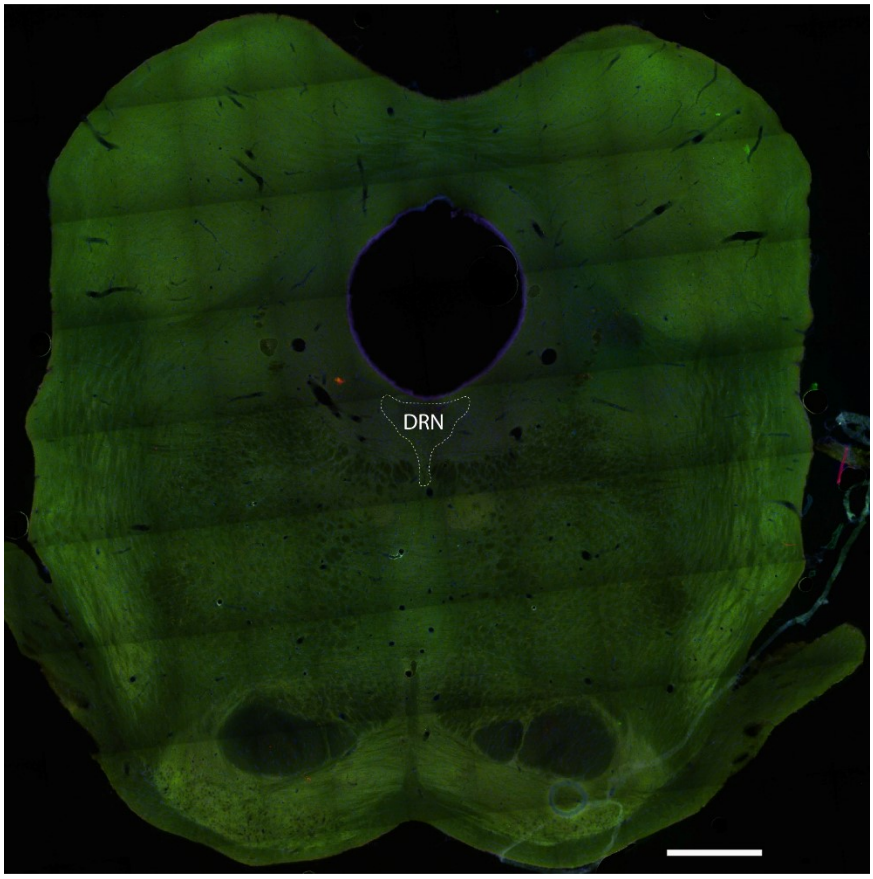

Figure 9. Representative section of the dorsal raphe nucleus of the rats showing 5-HT neurons stained for SERT. Scale bar: 1,000 μm.

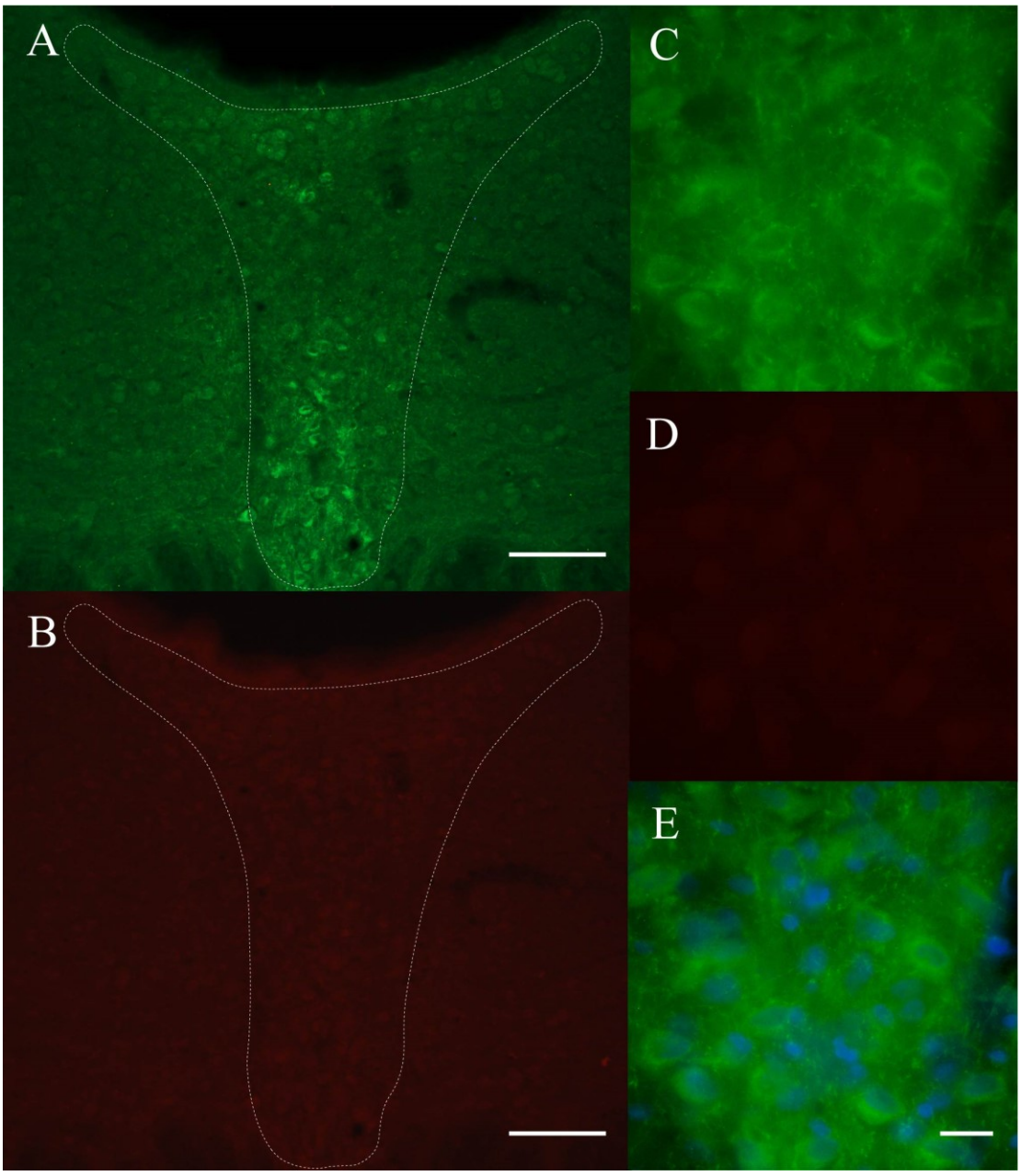

Figure 10. Multichannel positive staining of SERT in the dorsal raphe nucleus. Double-labelled immunofluorescent staining was used to quantify SERT- (green) and 5-HT_{3A}- (red) expressing cells in the DRN of rats (delineated). **A.** 10× magnification in FITC channel, showing robust staining of SERT-positive neurons. **B.** 10× magnification in Texas Red channel, illustrating the absence of clearly discernible positive signals. **C.** 60× magnification in FITC channel. **D.** 60× magnification in Texas Red channel. **E.** 60× magnification multichannel image with FITC, Texas Red and DAPI (blue) staining, with clear SERT staining and lack of yellow staining, which would have indicated signal overlap. Scale bars: 500 µm in A and B, 20 µm in C-E.

3.5 Quantitative distribution of SERT and 5-HT$_{3A}$-positive cells in the dorsal raphe nucleus

The number of 5-HT cell bodies within the DRN of the rats is summarised in Table 2. The average estimate of SERT-positive cells in the DRN was of 12,852 ± 462 (CE = 0.06) (Figure11). 5-HT cell counts per section ranged from 0-183 cells. No 5-HT$_{3A}$-positive cells were identified in the DRN, despite positive staining in the adjacent LDTg (Figure 5C).

animal ID	individual cell count estimate (SERT)	individual cell count estimate (5-HT$_{3A}$)
1	13,131	0
2	8,774	0
3	14,289	0
4	13,741	0
5	17,153	0
6	10,024	0
mean	12,852	0
SEM	462	0

Table 2. Total number of SERT- and 5-HT$_{3A}$-positive cells in the dorsal raphe nucleus of the rats. SEM: Standard error of the mean.

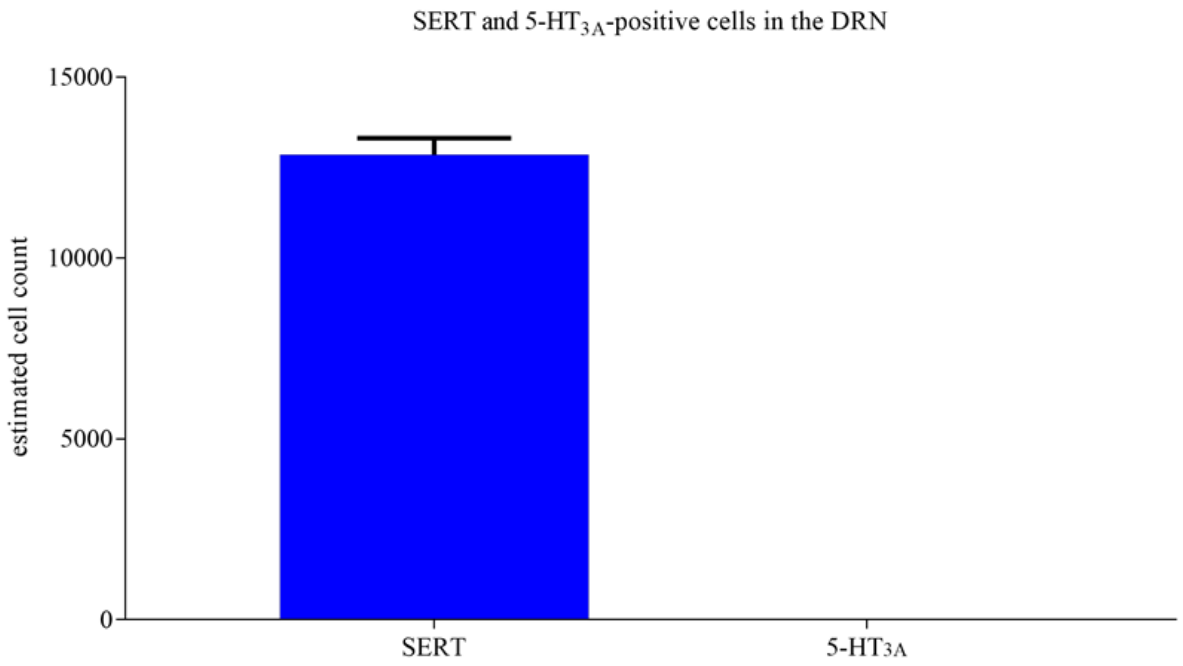

Figure 11. Average estimates of SERT- and 5-HT$_{3A}$-positive cells in the dorsal raphe nucleus. Data are presented as the mean ± S.E.M.

Discussion

The results presented in the previous section report a low expression of 5-HT_3 receptors in the SN and an absence thereof in the DRN. Although we initially hypothesised that 5-HT_3 receptors would be present on monoaminergic neurons in the SN and DRN given the alleviation of dyskinesia in the 6-OHDA-lesioned rat with the 5-HT_3 antagonist ondansetron and improvements in visual hallucinations in humans with PD, it would appear that these effects may not mediated by a direct monoaminergic mechanism with respect to the SN and DRN (Zoldan *et al.*, 1993; Zoldan *et al.*, 1995; Kwan, 2017). Instead, it is conceivable that these effects are due to a decreased post-synaptic effect of serotonergic signaling in key regions involved in L-DOPA-induced psychosis and dyskinesia, notably the striatum, cortex, and other limbic regions in the brain, rather than direct modulation of the SN and the DRN via 5-HT_3 receptors (Zoldan *et al.*, 1995). Similarly, it is plausible that, if ondansetron indeed had an influence on these psychosis- and dyskinesia-associated regions, this may have been due to afferent projections without 5-HT_3 receptor mediation at the synapse within these structures.

4.1 Methodological considerations

The rat was the organism of choice for these experiments. The circuitry of the BG has been widely studied in rodents, and they have been used to model several diseases associated with its dysfunction, such as PD and HD (Cenci *et al.*, 2002; Stathis *et al.*, 2007; Ellenbroek and Youn, 2016). To perform the proposed IHC experiments, brains first needed to be histologically processed. There are numerous techniques which can be employed to preserve neurological tissue, each with their own advantages and disadvantages. Perfusion fixation improves tissue morphology when cryosectioning and more evenly fixes larger tissues in less time compared to immersion fixation (Jung-Hwa *et al.*, 2007; Kasukurthi *et al.*, 2009). Similarly, PFA has empirically been

shown to better preserve gross tissue morphology as well as ultrastructural features such as protein tertiary structure compared to alternatives such as traditional formaldehyde, alcohol or acetone fixation (Cinar *et al.*, 2006). Perfusion-fixation is generally accomplished via transcardial perfusion, first with saline to flush the circulatory system of blood, then followed by a PFA fixing solution (Gage *et al.*, 2012). Tissue thickness must also be judiciously predetermined to preserve tissue morphology in free-floating IHC, while being appropriately thick following shrinkage during processing for stereological analysis, which requires a minimum thickness (Glaser, 2007; MicroBrightfield, 2018). Free-floating IHC was selected over slide-mounted IHC because it permits better antibody penetration into the tissue even with the increased thickness [indeed, slide-based IHC often uses sections which are not sufficiently thick for stereological analysis] (Glaser, 2007; MicroBrightfield, 2018).

In terms of antibodies used for IHC, TH staining has been shown to be an effective indicator of dopaminergic neurons as TH is the rate-limiting step of dopamine synthesis (Hökfelt *et al.*, 1976), as discussed earlier. Similarly, targeting SERT is an effective way of labelling 5-HT-producing neurons, as it is responsible for 5-HT reuptake following its ubiquitous release by 5-HT cells: in axons, dendrites, soma and pre-synaptically (Descarries *et al.*, 1982; Kapadia *et al.*, 1985; Liposits *et al.*, 1985; Chazal and Ralston, 1987; Adell *et al.*, 2002; Kalueff *et al.*, 2010). Antibodies were selected based on prior use in IHC experiments in murine rodents, increasing their likelihood of success. Similarly, antibody titres were based on published concentrations in the literature, while optimal dilutions and staining protocols were determined empirically (Carlsson *et al.*, 2009; Lu *et al.*, 2010; Shin *et al.*, 2012; Tronci *et al.*, 2012; Hitora-Imamura *et al.*, 2015; Schreglmann *et al.*, 2015; Van Rompuy *et al.*, 2015; Zharikov *et al.*, 2015; Chang *et al.*, 2016; Hoffman *et al.*,

2017). For instance, there was a high likelihood of requiring AR to unmask antigens following the PFA-fixation process (Ramos-Vara, 2005; Ivell *et al.*, 2014).

To ensure the validity of the obtained staining, negative controls were run for all antibodies, whereas positive controls were only performed for the experimental rather than anatomical markers (Ivell *et al.*, 2014). The rationale for this is that TH and SERT are both known to be expressed in the SN and DRN, respectively (Zhao *et al.*, 2003; Li *et al.*, 2016). Conversely, the presence of 5-HT_3 receptors in the SN and DRN was, prior to this study, ambiguous owing to different authors reporting different patterns of expression in these ROIs depending on the methods or radiolabels used, for instance (Kilpatrick *et al.*, 1987; Kilpatrick *et al.*, 1988; Waeber *et al.*, 1988; Barnes *et al.*, 1989a; Kilpatrick *et al.*, 1989; Waeber *et al.*, 1989; Barnes *et al.*, 1990; Pratt *et al.*, 1990; Waeber *et al.*, 1990; Gehlert *et al.*, 1991; Jones *et al.*, 1992; Laporte *et al.*, 1992b; Tecott *et al.*, 1993; Morales and Bloom, 1997; Morales *et al.*, 1998; Morales and Wang, 2002; Koyama *et al.*, 2017). The improved resolution, *i.e.* cellular and potentially sub-cellular, as well as improved specificity of immunolabelling compared to radiolabeling renders the present results more compelling. For a comparison of relative differences between various direct and indirect methods of receptor detection, see Table 3. Positive controls in regions with known 5-HT_3 receptor expression were therefore required to validate the antibody. Without these, false-negative results could have been obtained.

Direct Methods		Quantitation	Neuronal Chemical Specificity	Sensitivity	Anatomical Resolution	Ultrastructural Resolution
	IHC	+++	+++	++	+++	+++*
	Radiolabeling	++	+	+	+	-
	In situ hybridisation	+++	++	+++	+++	+
Indirect Methods						
	RT-PCR	+	-	+++	-	-
	Western Blot	+	-	+++	-	-
	Electrophysiology	-	-	-	+	-
	Pharmacological Experiments	-	±	+	±	-
	Microdialysis	-	±	+	+	-

Table 3. **Comparison of different receptor detection techniques.** *In situ* hibridisation and RT-PCR examine RNA expression, not protein expression. *IHC methods appropriate for scanning electron microscopy provide the highest ultrastructural resolution possible. RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

In terms of histological analysis, unbiased stereology is a method for estimating the geometric properties of 3-dimensional biological objects in 2-dimensional space (Burke *et al.*, 2009; West, 2012). Using SRS and an unbiased counting frame for the inclusion or exclusion of said objects, unbiased stereology removes systematic bias from cell counts (Glaser, 2007; Burke *et al.*, 2009; West, 2012). The optical dissector probe (also known as the optical fractionator) examines cells through sequential focal planes, allowing the discrimination of individual cells without repeated measures. This facilitates the estimation of cell counts as it is based on all parts of an ROI having an equal probability of being sampled. Therefore, there is rarely a need for more than 150 sampling events over 10-20 sections in a given individual (West *et al.*, 1991; West, 2012). Moreover, with the use of semi-automated computer-interfaced stereological microscope, sampling time can be reduced. Indeed, unbiased stereology has rapidly become the method of choice for biological quantification at the microscopic level (Saper, 1996).

4.2 Validity of immunostaining

The present study demonstrated robust staining of dopaminergic and 5-HT cells in the SN and DRN, respectively. However, in these ROIs, there was minimal 5-HT_{3A} staining, if any, and its detection was marred by high non-specific background. It is possible that this background was exacerbated by the dense nature of both structures, which would be liable to reflect more light off biological matter than less dense regions. Nonetheless, we believe the methods employed to fluorescently label 5-HT_{3A}-expressing cells were valid, given 5-HT_{3A} subunit detection across multiple positive control ROIs, *e.g*. the OB, VMH, LDTg and NTS. These ROIs were selected over other known regions of expression such as the hippocampus, cortex and spinal cord as they are less diffuse anatomical regions (Morales *et al.*, 1996a; Morales *et al.*, 1998). Moreover, in the case of the spinal cord, there was difficulty in obtaining adequate samples given its small size and fragility. Hence, we opted for control regions with clear, specific anatomical boundaries that could be obtained in a handful of sections, as opposed to requiring staining large quantities of tissue to identify sparse 5-HT_3 receptors. We also saw incidental cerebellar staining on some sections. In such regions, the anti-5-HT_{3A} antibody performed well, with clearly fluorescing cells displaying primarily somatic staining, with projections and dendrites sometimes visible. Critically, despite high background in these regions as well, positive signals were discernible therefrom. Although positive controls were not run for the anti-TH and anti-SERT antibodies, these labelled nuclei outside of the ROIs appropriately (*e.g*. VTA in the case of TH and MnR in the case of SERT). Negative controls, consisting of a lack of primary and/or secondary antibodies, were performed for all antibodies used. The anti-TH and anti-SERT antibodies were the most specific, whereas the anti-5HT_{3A} antibody showed some non-specific staining and high background. Nevertheless, as mentioned above, this non-specific staining was distinguishable from the specific staining seen in

positive controls, and was ignored during analysis. In addition, perivascular cells displayed fluorescence in both FITC and Texas Red channels without the presence of antibodies. Once again, these staining patterns were readily distinguishable from specific staining, and were ignored during analysis. Given these considerations, as well as the reproducibility of 5-HT_{3A} staining across multiple positive control regions, we believe our IHC staining to be appropriately specific for the project at hand.

4.3 5-HT_3 receptors in the substantia nigra

The present results suggest a lack of 5-HT_{3A} expression at the protein level in the SNc of the rat. This finding is equivocal, given the high background staining seen when examining the Texas Red filtre, the channel under which the secondary reporter for 5-HT_{3A} fluoresces. Future studies using a more specific primary antibody with less background staining are needed to validate the present IF-IHC findings, although such specific primary antibodies may not be commercially available yet. An alternative approach would be, rather than relying on positive control ROIs for antibody validation, to validate candidate antibodies with knock-out animals for the protein of interest. Most knock-out animals models have used mice, with genetic toolboxes such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) for rats having been developed in recent years being uncommon (Geurts *et al.*, 2009; Huang *et al.*, 2011; Tesson *et al.*, 2011). Currently, no rat 5-HT_3 knock-out rats exist, however studies in mice demonstrate that the lack of this receptor is not lethal (Zeitz *et al.*, 2002; Kelley *et al.*, 2003; Bhatnagar *et al.*, 2004a; Bhatnagar *et al.*, 2004b; Smit-Rigter *et al.*, 2010). It is noteworthy that the findings reported here are in line with those of others who have attempted to map the distribution of 5-HT_3 receptors using methods such as autoradiographic binding, *in situ*

hybridisation and precipitation-based IHC in the rat and also encountered variable levels of non-specific binding/labelling (Kilpatrick *et al.*, 1988; Barnes *et al.*, 1990; Waeber *et al.*, 1990; Gehlert *et al.*, 1991; Laporte *et al.*, 1992b; Gehlert *et al.*, 1993; Steward *et al.*, 1993b; Morales *et al.*, 1996b; Morales *et al.*, 1998; Spier *et al.*, 1999; Geurts *et al.*, 2002).

In agreement with the findings presented here, data from studies conducted in other rodent species such as the mouse and the Syrian hamster, also indicate a lack of cellular 5-HT_3 receptor expression in the SNc (Carrillo *et al.*, 2010; Koyama *et al.*, 2017). Indeed, the literature review conducted identified only one study which found low-level expression of $5HT_3$ receptors in the rat SN using antibody radiolabelling (Doucet *et al.*, 1999). Homogenate membrane binding assays also demonstrated low-level 5-HT_3 receptor binding in rat SN homogenates when [^{125}I]-iodo-zacopride and [^{3}H]-zacopride were used as radioligands (Laporte *et al.*, 1992b). None of these last 2 studies separated the SNc from the SNr. These rodent data are in agreement with data in humans, in whom low 5-HT_3 receptor levels are encountered within the SN (Bufton *et al.*, 1993b).

Results obtained via non-visual methods of detection such as membrane binding assays, *in vivo* electrophysiology or behavioural assays have both implied and casted doubt on the presence of 5-HT_3 receptors in the SNc (Sorensen *et al.*, 1989; Ashby *et al.*, 1990; Rasmussen *et al.*, 1991; Prisco *et al.*, 1992; Palfreyman *et al.*, 1993; Alex and Pehek, 2007). Thus, it was found that chronic, but not acute, 5-HT_3 blockade with MDL-73,147EF resulted in reduced electrophysiological activity in SNc dopaminergic neurons (Sorensen *et al.*, 1989; Palfreyman *et al.*, 1993). It is noteworthy that, because these last 2 studies employed chronic systemic injections, an effect on 5-HT_3 receptors localised on structures known to provide afferences to the SNc, *e.g.* the cortex, PPN, rostromedial tegmental nucleus, superior colliculus, GPe and thalamus could have underlied the effect encountered here (Fibiger and Miller, 1977; Oertel and Mugnaini, 1984; Sorensen *et al.*,

1989; Weiner *et al.*, 1990; Palfreyman *et al.*, 1993; Gervais and Rouillard, 2000; Chen and Rice, 2002; Forster and Blaha, 2003; Wooltorton *et al.*, 2003; Haber, 2014; Miguelez *et al.*, 2014). In contrast, other 5-HT_3 antagonists such as granisetron, DAU 6215, zatosetron and BRL-43,694 all failed to replicate this effect either acutely or chronically (Ashby *et al.*, 1990; Rasmussen *et al.*, 1991; Prisco *et al.*, 1992). It was suggested that these differences may be due to the different chemical structures of of the different antagonists (Rasmussen *et al.*, 1991). Thus, the herein results are in line with the prevailing conclusion, supported by anatomical, pharmacological and electrophysiological studies, that there is an absence of 5-HT_3 receptors in the rat SNc. Similarly, the lack of expression on dopaminergic neurons is consistent with human data, seeing how 5-HT_3 receptor binding in nigrostriatal dopaminergic terminals did not decrease with the onset of PD (Steward *et al.*, 1993a).

As alluded to in the discussion above, non-pharmacological studies indicating a presence of 5-HT_3 receptors in the rat SN did not differentiate between the SNc and SNr, and therefore do not preclude their presence in the SNr rather than the SNc (Laporte *et al.*, 1992b; Doucet *et al.*, 1999). Because no 5-HT_3 receptor staining was encountered in the rat SNc in the current study, this would suggest that the 5-HT_3 receptors detected in the SN in those previous experiments might be localised in the SNr, which is what was found here.

Although the initial intent of using stereological sampling was to eliminate bias from estimates of 5-HT_3 receptors in the SN, they appear to be too sparse to be adequately quantified by the method, as a fundamental assumption thereof is a reasonably uniform (*i.e.* dense) population (West, 2012). In so far as few 5-HT_{3A}-positive cells were counted (1,250 bilateral estimate, CE = 0.24, Table 1) compared to the total estimate of 52,600 Nissl-stained cells (CE = 0.07) by Oorschot (1996), it is not surprising that the CE obtained is > 0.1. Indeed, because only 0-10 cells were

counted per section, stereological sampling may not have been the most appropriate method for such sparse quantification. Instead, whole sampling, beyond the scope of the present project, would be better suited to quantify the density of 5-HT_3 receptors in the SNr.

Inasmuch as the SNr is a primarily GABAergic structure, the presence of 5-HT_3Rs is intriguing as, for instance, 90% of 5-HT_3 receptors expressed in the neocortex and hippocampus are present on GABAergic neurons (Morales *et al.*, 1996a; Morales and Bloom, 1997; Richards *et al.*, 1997). Hence, further studies investigating whether 5-HT_3 receptor-expressing SNr cells are GABAergic, and if so, to which sub-family they belong to, could be of interest. Hypothetically, the presence of 5-HT_3 receptors in the SNr could play a role in the regulation of seizures, as the SNr may be a modulator thereof (Velíšek *et al.*, 2002). This link between the SNr and seizures appears to relate to GABAergic projections from the SNr, with the anteromedial SNr being anticonvulsant and the posterodorsal SNr being proconvulsant (Moshé *et al.*, 1995). Furthermore, a study in mice found that 5-HT_3 activation with SR-57,227, achieved via systemic injection, was anticonvulsant, with a proposed mechanism of increased GABAergic signalling (Gholipour *et al.*, 2009). While mechanistic insight remains needed, it is plausible that these effects may have been mediated by GABAergic SNr neurons. Outside of this context, studies in primates have implicated the anterolateral segment of the SNr in the development of parkinsonian motor manifestations (Wichmann *et al.*, 2001). Further investigations into the SNr, as well as the potential for 5-HT_3 modulation therein could be of interest for the treatment of neurological disorders.

4.4 5-HT_3 receptors in the dorsal raphe nucleus

The present results suggest a lack of 5-HT_{3A} subunit expression at the protein level in the rat DRN. This finding is equivocal, considering the high background staining seen when examining

the Texas Red filtre, the channel under which the secondary reporter for 5-HT_{3A} signals fluoresces. Nevertheless, this outcome is in agreement with other studies that have attempted to map the distribution of 5-HT_3 receptors using methods such as autoradiographic binding, *in situ* hybridisation and precipitation-based IHC in the rat (Kilpatrick *et al.*, 1987; Kilpatrick *et al.*, 1988, 1989; Barnes *et al.*, 1990; Waeber *et al.*, 1990; Gehlert *et al.*, 1991; Gehlert *et al.*, 1993; Kidd *et al.*, 1993; Steward *et al.*, 1993b; Morales *et al.*, 1996b; Morales *et al.*, 1998). Indeed, only a single study using visual methods found low-level 5-HT_3 receptor binding in the rat DRN (Laporte *et al.*, 1992b). However, there has also been indirect evidence of the presence of 5-HT_3 receptors in the DRN. Thus, microinjections of the 5-HT_3 agonist m-chlorophenylbiguanide (m-CPBG) into the DRN had effects on rapid eye movement (REM) sleep in rats although, through pharmacological experiments, it was later shown that this effect was mediated by glutamatergic, not 5-HT, DRN cells expressing 5-HT_3 receptors (Monti and Jantos, 2008; Monti *et al.*, 2011). Another studyfound that 5-HT_3 antagonists had no effect of 5-HT DRN cells, either *in vivo* or *in vitro* (Adrien *et al.*, 1992). These data collected from previous studies, combined with data collected in the present study, suggest that 5-HT_3 receptors may not be expressed on 5-HT cells in the rat DRN.

The argument presented in the previous paragraph provided indirect pharmacological evidence of the presence of 5-HT_3 receptors on glutamatergic DRN cells, while the current IHC experiments failed to detect them. One possibility to explain this discrepancy is that the density of 5-HT_3 receptors in the DRN may be very low, below the detection threshold of visual methods such as *in situ* hybridisation or IHC. A caveat of this explanation is that it is hard to reconcile with the results of a study that used autoradiographic binding and detected low levels of 5-HT_3 receptors in the rat DRN (Laporte *et al.*, 1992b). In the context of the present study, it is possible that the 5-HT_3 receptors reported by Laporte et al. (1992b) are those associated with glutamatergic activity

in the DRN by Monti et al. (2008; 2011), and that these 5-HT_3 receptors are expressed on the projections of endogenous glutamatergic neurons (Adrien *et al.*, 1992; Jolas and Aghajanian, 1997). Accordingly, it is conceivable that 5-HT_3 receptors in the DRN are expressed on glutamatergic afferents from other nuclei (Soiza-Reilly and Commons, 2011). If this were the case, given the primarily somatic labelling of the anti-5-HT_{3A} antibody used in the present study, such axonal 5-HT_3 receptors would unlikely have been observed. Moreover, from a stereological point of view, such projections would not have been counted as it is virtually impossible to ascertain to which cell a given projection belongs, and that said cell would only be counted once (West, 2012). Nevertheless, experiments to determine whether the tenuous expression of 5-HT_3 receptors by glutamatergic cells are on local (vesicular glutamate transporter type 3 [$VGluT_3$]-positive) cells, or on projections from other loci ($VGluT_{1/2}$-positive) would be of interest (Soiza-Reilly and Commons, 2011). However, owing to a lack of detection of 5-HT_3 receptors in the DRN through visual means other than autoradiographic binding, such experiments would likely have to be pharmacological, and therefore not quantitative. Biochemical experiments such as Western blots could yield quantitative data, at the expense of anatomical resolution (Taylor and Posch, 2014).

4.5 Validity of stereological estimates

The stereological parameters used were determined based on previously published ones in the literature (Oorschot, 1996; Nair-Roberts *et al.*, 2008; Strackx *et al.*, 2008; Maia *et al.*, 2016), as well as empirical determination during preliminary experiments. In turn, these yielded estimates for TH-positive and SERT-positive cells in the SN and DRN with CEs below the minimum Gundersen m = 1 CE threshold of 0.1 and within the appropriate CE boundary of 0.06 for biological accuracy (West *et al.*, 1991). Thus, the sampling parameters employed were deemed

valid for both TH- and SERT-positive cells. Using these parameters, estimations of the number of 5-HT_3 receptors did not achieve appropriate CEs. An alternative method to the one employed would have been to perform more intensive sampling, or even whole-structure sampling. However, this would have defied the rationale of stereology, which aims to produce reliable cell-count estimates with reduced sampling or time spent counting (West *et al.*, 1991; West, 2012). Hence, retrospectively, given the paucity of 5-HT_3 receptors in these ROIs, unbiased stereology may not have been the most appropriate quantitative methodology. Qualitative investigations were carried out following the stereological investigations to determine whether the sampling scheme employed was missing large populations of 5-HT_3 receptors. These assessments did not demonstrate any noticeable groupings of 5-HT_{3A} signals missed by the stereological sampling in experimental ROIs. Despite these caveats, stereological sampling was theoretically the least-biased method of 5-HT_3 receptors quantification.For a more thorough future quantification, assumption-based methods such as direct cell counting may have to be employed.

For TH staining, the number of TH-positive cells estimated bilaterally in the SN were in accordance with previous results published in the literature (Ahmad *et al.*, 2008; Nair-Roberts *et al.*, 2008). For SERT staining, the average estimate of 5-HT cells was less robust. The obtained average estimate of 12,852 (± 462) is similar to a previous estimation of 11,428 (± 207) 5-HT cells (Descarries *et al.*, 1982). However, it is lower than the estimate provided by a later study, which estimated to approximately 33,000 the number of 5-HT cells in the DRN (Strackx *et al.*, 2008). An important distinction between the aforementioned publications are that the former used model-based cell counting methods rather than stereology to estimate the number of serotonergic cells in the DRN, whereas the latter employed stereology. Although differences in cell estimates tend to occur between classical and stereological counting, they tend not to be so marked (Baquet *et al.*,

2009). These authors observed little difference in cell counts of mouse SNc using design-based or method-based counting (Baquet *et al.*, 2009). It would therefore be unlikely for such a vast difference to occur between the studies conducted by Descarries et al. (1982) and Strackx et al. (2008). Moreover, the results of the latter appear to be very close to estimates of total cells in the DRN of 33,008 [± 2345] (King *et al.*, 2002). Raising further questioning on the robustness of the estimates provided by Strackx et al. (2008) is the fact that, as mentioned in section 1.3.1, the DRN is a nucleus consisting primarily of neuropil, has a high density of glial and other cell types, such as GABAergic, dopaminergic and glutamatergic cells distinct from 5-HT cells (Belin *et al.*, 1979; Molliver, 1987; Jacobs and Azmitia, 1992; Jolas and Aghajanian, 1997; Adell *et al.*, 2002; Allers and Sharp, 2003; Commons, 2009; Fu *et al.*, 2010; Hioki *et al.*, 2010; Jahanshahi *et al.*, 2013; Soiza-Reilly and Commons, 2014). The number of 5-HT cells should therefore be lower than total cell counts, and has been estimated to represent approximately two-thirds thereof in the rat (Fu *et al.*, 2010). One other stereological study was identified which reported 5-HT cell count estimates around 18,500, with relatively high CEs ranging from 0.07 to 0.09 for various DRN sub-regions (Maia *et al.*, 2016). While the data generated by the present study does not correspond to the estimate of 20,000 5-HT cells (assuming a whole-cell count of roughly 30,000), the CEs for the estimates herein are more robust than those provided by Maia et al. (2016). Unfortunately, there appears to be little additional literature on stereological estimates of 5-HT cell counts in the rat DRN, as most studies found do not explicitly state these, but rather percent changes or other relative assessments (Díaz-Cabiale *et al.*, 2011; Van den Hove *et al.*, 2014).

Conclusion and Summary

To date, relatively few studies have examined the precise, high resolution anatomical distribution and density of 5-HT_3 receptors in the rat brain. Data on the distribution of this receptor in humans is difficult to obtain owing to limitations of *in vivo* techniques available (*e.g*. positron emission tomography), and the potential confounds of *post-mortem* studies. Limitations in our knowledge pertaining to 5-HT_3 receptors in non-human primates persist for similar reasons. Pharmacological modulation of 5-HT_3 receptors has already been shown to be a clinical tool for the treatment of chemotherapy-induced nausea, and preliminary studies indicate an additional therapeutic potential for the treatment of visual hallucinations in patients with advanced PD (Zoldan *et al.*, 1993; Zoldan *et al.*, 1995; Hesketh *et al.*, 2017). Pre-clinically, activation of these receptors has been shown to be potentially beneficial in the treating of epileptic seizures (Gholipour *et al.*, 2009). Seeing how the role of 5-HT in the CNS as well as PNS is vast, and that 5-HT_3 receptors are the only ionotropic 5-HT receptor identified to date, this receptor is an attractive target for potential direct modulation of fast synaptic transmission in neural circuits (Férézou *et al.*, 2002; Nichols and Nichols, 2008). IF-IHC renders possible the accurate identification of anatomical regions containing 5-HT_3 receptors, in contrast to previous radioligand binding experiments, which offer a lesser cellular resolution (Kilpatrick *et al.*, 1987; Kilpatrick *et al.*, 1988; Barnes *et al.*, 1989a; Barnes *et al.*, 1989b; Kilpatrick *et al.*, 1989; Barnes *et al.*, 1990; Pratt *et al.*, 1990; Jones *et al.*, 1992; Laporte *et al.*, 1992b). The results presented herein indicate a low density of 5-HT_3 receptors in the SN, these being located solely in the SNr, and not the SNc. They also do not indicate the presence of these receptors in the DRN. Therefore, the present results do not suggest a direct role for these receptors in monoaminergic transmission in these nuclei. They rather suggest that the effects of 5-HT_3 antagonism in the context of disorders of the BG are

likely to be due to indirect effects of these receptors with respect to dopaminergic and 5-HT inputs to the BG.

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