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Evolução molecular do receptor serotoninérgico 5-HT3 em primatas do novo mundo e outros cordados

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“Felix qui potuit rerum cognoscere causas”

[Happy is the person who can learn the nature of things].

Virgil

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que no conoce fronteras ni distancias.

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List of abbreviations

5-HT – 5-hydroxytryptamine, serotonin.

5-HTT – 5-hydroxytryptamine transporter, also known as SERT

5-HTTLPR – 5-hydroxytryptamine transporter linked polymorphic region

7-TM – 7-transmembrane receptors, also known as GPCRs

AA – Arachidonic acid

AC – Adenylyl cyclase

AD – Alzheimer's disease

ACh – Acetylcholine

AChBP – Acetylcholine binding protein

ASD – Autism spectrum disorders

ATP – Adenosine triphosphate

BBB – Brain-blood barrier

cAMP – Cyclic adenosine monophosphate

CHO – Chinese hamster ovary cells

CNS – Central nervous system

DAG – Diacylglycerol

EC – Enterochromaffin cells

EC50 – Half maximal effective concentration

ECD – Extracellular domain

ENS – Enteric nervous system

ER – Endoplasmic reticulum

ES – Evolutionary synthesis

ERK – Extracellular signal-regulated kinases

FBPase – Fructose 1,6-Bisphosphatase

G6Pase – Glucose-6-phosphatase

GABA – Gamma-aminobutyric acid

$GABA_AR$ – Gamma-aminobutyric acid receptor

GI – Gastrointestinal

GlyR – Glycine receptor

GPCR – G protein-coupled receptor

GRK – G protein-coupled receptor kinase

GTP – Guanosine triphosphate

HEK-293 – Human embryonic kidney cells.

HPA – Hypothalamic-pituitary-adrenal axis

IBS – Irritable bowel syndrome

ICD – Intracellular domain

IP3 – Inositol triphosphate

IPAN – Intrinsic primary afferent neuron

kDa – Kilodalton

kB – Kilobase

Kg – Kilogram

Ma – Million years ago

MAPK – Mitogen-activated protein kinase

MS – Multiple sclerosis

nAChR – Nicotinic acetylcholine receptor

NAM – Negative allosteric modulator

NTS – Nucleus tractus solitarius

PAM – Positive allosteric modulator

PD – Parkinson’s disease

PIP2 – Phosphatidylinositol 4,5-bisphosphate

PKA – Protein kinase A

PKC – Protein kinase C

PLA2 – Phospholipase A2

PLC-β – Phospholipase C β-isoform

pLGIC – Pentameric ligand-gated ion channel

PNS – Peripheral nervous system

pS – Picosiemen

RIC-3 – Resistance to inhibitors of cholinesterase 3 protein

ROS – Reactive oxygen species

SCFA – Short-chain fatty acid

SERT – Serotonin transporter, also known as 5-HTT

SLC6A4 – Solute carrier family 6 member 4

SSRI – Selective serotonin reuptake inhibitors

SVZ – (Cortical) subventricular zone

TMD – Transmembrane domain

TPH – Tryptophan hydroxylase

VMAT – Vesicle monoamine transporter

ZAC – Zinc-activated channel

Resumo

A serotonina (5-hidroxitriptamina, 5-HT) é uma molécula evolutivamente antiga, ubíqua e multifacetada, profundamente envolvida na regulação de muitos processos fisiológicos e comportamentais em animais, plantas, fungos e outros organismos. Esses processos incluem desenvolvimento, funções cardiovasculares e endócrinas, percepção sensorial, apetite, comportamento sexual, sono, cognição e memória. As funções da 5-HT são mediadas por seus receptores; em humanos, eles compõem um conjunto de 13 receptores ligados a proteína G (GPCR) e um receptor pentamérico de canal iónico (pLGIC): 5-HT3 (codificado por até cinco genes: *HTR3A*, *HTR3B, HTR3C, HTR3D,* e *HTR3E*). O receptor 5-HT3 forma um poro seletivo de íons que abre após ativação do receptor por 5-HT, permitindo a entrada de cátions na célula, e a despolarização neuronal, produzindo uma resposta excitatória rápida.

Estabelecer relações entre variações genéticas e fenotípicas entre organismos durante a evolução é um dos maiores objetivos da biologia atual. Nesta Tese o receptor 5-HT3 é o objeto de estudo, para a melhor compreensão de sua evolução molecular, em pequena (Primatas do novo mundo ou *NWM*, primatas e mamíferos próximos, Capítulo 3) e grande escala (Cordados, Capítulo 4). No Capítulo 3 são apresentadas evidências de seleção positiva com possível importância funcional no gene *HTR3A* nos primatas do novo mundo, dentro do contexto dos primatas e mamíferos. Específicamente, quatro (4) sítios foram identificados (398, 403, 432 e 416) na região desordenada do domínio intracelular M3-M4, usando análises moleculares. Além disso, foram encontradas associações estatísticas desses sítios com traços reprodutivos e de comportamento social (tais como dispersão feminina do grupo natal e monogamia social), usando abordagens estatísticas clássicas e bayesianas, e foram identificados motivos lineares com potencial importância na interação com enzimas regulatórias (por ex. *GSK3*). Esses resultados sugerem que as variáveis achadas podem ter um papel importante na aquisição e manutenção de comportamentos adaptativos nos primatas do novo mundo.

O Capítulo 4 foca na emergência e evolução dos diferentes membros da família gênica do receptor 5-HT3 nos cordados. Nele propomos que todos os membros da família 5-HT3 surgiram a partir dum único gene, que foi duplicado durante os dois grandes eventos de duplicação genômica que aconteceram cedo na evolução dos vertebrados, e que posteriormente experimentou sucessivas duplicações em tandem e perdas nas diferentes linhagens dos cordados, sugerindo um cenário complexo. Assim, *HTR3A* aparece em todas as linhagens investigadas, sob forte seleção purificadora, enquanto outras subunidades (*HTR3C, HTR3D* e *HTR3E*) foram perdidas e/ou duplicadas durante a evolução dos vertebrados, e experimentaram relaxamentos nas pressões seletivas. Esses achados sugerem um cenário no qual subunidades essenciais (como *HTR3A*) foram conservadas nas espécies devido à sua condição essencial para a formação e função do receptor, e somente variantes pontuais (como as descritas no Capítulo 3) foram permitidas e selecionadas em taxa e contextos evolutivos específicos. No entanto, subunidades "não essenciais" puderam acumular mais mutações, chegando inclusive a pseudogenizar, permitindo também a emergência de novas propriedades estruturais, assim como um maior repertório de receptores. Nossos resultados fornecem uma primeira visão da complexa história evolutiva do receptor 5-HT3, e nos permitem especular que a diversidade observada constitui um exemplo de evolvabilidade do sistema, que permitiu a regulação fina de uma ampla quantidade de processos fisiológicos e comportamentais nos vertebrados.

Abstract

Serotonin (5-hydroxytryptamine, 5-HT) is an evolutionary old, widespread, and multifaceted molecule, with profound involvements in the regulation of many behavioral and physiological processes in animals, plants, fungi, and other organisms. These processes include development, cardiovascular and endocrine function, sensory perception, appetite, sexual behaviour, sleep, cognition, and memory. Most serotonergic functions are mediated through its receptors, which in humans comprise a set of 13 G protein-coupled receptors, and a single ligand-gated ion channel: the 5-HT3 receptor. 5HT3 receptor (encoded by up to five genes *HTR3A*, *HTR3B, HTR3C, HTR3D,* and *HTR3E*) forms a pentameric ion-selective pore that opens upon activation by 5-HT, allowing cation influx into the cell and membrane depolarization, and leading to a rapid, excitatory response.

Establishing relationships between genetic and phenotypic variation across organisms along the course of evolution is one of the major goals in biology. In this Thesis, the 5-HT3 receptor is the study subject, in order to better understand its evolution at a molecular level on a small (New World monkeys, primates and related mammals, Chapter 3) and a bigger scale (all major Chordata lineages, Chapter 4). In Chapter 3, I present evidences for positive selection with putative functional importance in *HTR3A* variability in New World Monkeys, within a primate and mammalian context. Specifically, I identified four sites (398, 403, 432 and 416) located at disordered regions of the intracellular (M3-M4) domain, by using molecular analyses. In addition, I found statistical associations with reproductive and social behaviours (such as female-biased dispersal and social monogamy), using two different approaches (classical and Bayesian), and I searched for linear motifs that arose from the variants under positive selection, which that might potentially mediate protein-protein interaction with regulatory enzymes (e.g. *GSK3*). Our results suggest that the variants we found may play a role in mediating the acquisition and maintenance of adaptive behaviours in NWMs.

In Chapter 4, I focused on the emergence and evolution of the members of the 5-HT3 receptor family in Chordata. I postulate that all 5-HT3 members arose from a single gene that underwent subsequent expansions during the two events of whole genome duplications (WGD) that occurred early in vertebrate evolution, followed by multiple events of tandem duplication and loss across lineages. This work shows a striking variability in number of subunits among chordate groups, revealing a more complex scenario than previously expected. Thus, *HTR3A* is found in all species surveyed, and it under strong purifying selection, while other subunits (e.g. *HTR3C, HTR3D* and *HTR3E*) are lost and/or duplicated in many diverse lineages, and experimented a relaxation in selective pressure. These findings are compatible with a scenario in which essential subunits (such as *HTR3A*) are conserved across species due to their essentiality for receptor formation and function, and only punctual variants (such as those we described in Chapter 3) are allowed and selected in specific taxa and contexts. On the other hand, *non-essential* subunits (e.g. CDE subunits) can accumulate more mutations (and even pseudogenize), thus allowing the emergence of novel structural properties, and thus a larger array of possible receptors. Altogether, the results presented in this Thesis provide a first glimpse at the complex evolutionary history of the 5-HT3 receptor family, and allow us to speculate that the observed diversity at the genic level constitute an example of system evolvability. Such flexibility, likely allowed fine-tuned regulation of a broad array of physiological and behavioral functions across vertebrates living under different ecological niches and selective pressures.

Chapter 1. Introduction

1.1. Initial considerations

Nature has the unique ability to both fascinate humans and to prove us wrong, as the true essence of things rarely matches their external appearance. Fortunately, science provides us with a set of tools that, when properly used, constitute an outstanding way to understand the universe.

The concept of evolution is one of the most powerful ideas in the history of science. With the publication of *The origin of species* (Darwin 1859), Charles Darwin ignited an intellectual revolution that quickly spread and had far-reaching effects not only in biology but also in numerous areas of thought (such as economics, sociology and linguistics). In addition, Darwin challenged the prevailing Western philosophical and theological view of a static natural world, created by supernatural powers that put humans in the center. Instead, all living organisms (and their genes) share an evolutionary history, and have common ancestors tracing back to over billions of years. The environmental conditions faced by organisms and their ancestors, through the power of natural selection, shaped the enormous variability of sizes, shapes, colors and behaviors observed in nature. Indeed, as Theodosius Dobzhansky famously stated, "*Nothing in biology makes sense except in the light of evolution*".

Evolution is, simply put, change over time. Biological evolution is fueled by random mutations, and shaped by the combination of different selective forces, such as directional (Darwinian) selection, which favors specific traits over others, and purifying selection, which maintains successful biological strategies over time. Evolutionary theory provides an elegant framework to answer questions about the past, the present, and the future of the natural world in which we live, and its practical and theoretical applications range from development of new drugs against resistant pathogens to the reconstruction of life's history. Darwin's elegant explanation for how living organisms evolve was latter enriched in the first half of the twentieth century when the integration of Mendelian inheritance and population-level thinking came together to form the Modern Synthesis (or more recently referred to as Evolutionary Synthesis, ES). ES, like most ideas, has evolved since its initial postulation, being readily updated with the advent of new discoveries and techniques. Some relevant extensions occurred during the prolific 1960-1980 decades. One of the most remarkable was

the neutral theory (Kimura 1968; King and Jukes 1969; Kimura and Ohta 1971), which provided a different interpretation of nucleotide polymorphisms, and challenged the prevalent view of the time that positive selection is the almost exclusive factor in evolution - a view that was also questioned by other notable researchers, like Stephen Jay Gould and Richard Lewontin (Gould and Lewontin 1979). Nowadays there is a vivid debate among evolutionary biologists about whether ES should be extended to include other processes, such as cultural evolution, niche construction, and non-genetic inheritance (e.g. epigenetics) (Laland et al. 2014; Laland et al. 2015; Futuyma 2017).

One of the fundamental landmarks of molecular and evolutionary biology was the establishment of DNA as a document of evolutionary history by Zuckerkandl and Pauling (1965), which contributed to the birth and development of Phylogenetics and Comparative Genetics (Zuckerkandl and Pauling 1965). Both disciplines emerged as fundamental tools to answer many biological questions, including genome annotation, identification of functional sequences, inference of ancestral phenotypes and population characteristics, and reconstruction of evolutionary history. The 21st century witnessed a revolution in genetics, marked by the publication of the first draft of the human genome in 2001 (International Human Genome Sequencing Consortium 2001; Venter et al. 2001). Thereafter, advances in sequencing technologies (e.g. Next-generation sequencing) allowed large-scale sequencing of a vast number of genomes, and the generation of unprecedented amounts of genomic data, much of it deposited in publicly available databanks (e.g. Ensembl, DDBJ, Genbank). Processing and using this information to learn more about the connections between genetic variability, natural selection, and its phenotypic consequences are main goals of modern evolutionary biology.

1.2. Serotonin: a tiny multifaceted neurotransmitter

Serotonin (or 5-hydroxytryptamine, 5-HT) is a biogenic monoamine that acts as a neurotransmitter, neurohormone, and mitogen in the central (CNS) and in the peripheral nervous system (PNS). Although popularly known as a mood regulator, contributing to happiness and well-being, serotonin is involved in a vast variety of essential physiological functions that include development, cardiovascular and endocrine function, sensory perception, appetite, sexual behaviour, sleep, cognition, and memory (Lucki 1998; Cools et al. 2008; Berger et al. 2009). Some of these functions will be addressed in more detail below.

As a result of these varied and important roles, and especially due to its relevance in mental health, serotonin has been intensely studied for over half a century, and it is still nowadays one of the neurotransmitters that generate more interest among researchers, with over 140,100 indexed publications in the PubMed database as of August 2018, and 3,600+ publications per year in the last decade (**Figure 1**).

The fact that serotonin mediates virtually all human behavioral processes contrasts with the fact that less than one in a million CNS neurons produce serotonin and most part of total body serotonin is found outside the CNS. Indeed, only ~5% of body serotonin is found in the CNS, the vast majority of it stored in the periphery (Lesurtel et al. 2008; Bertrand and Bertrand 2010). Furthermore, the brain-blood barrier (BBB) is fairly impermeable to plasma serotonin, which supports the idea that central and peripheral serotonin pools are independent entities, and may have opposing effect in homeostasis (Namkung et al. 2015; Spohn and Mawe 2017) yet a study reported serotonin efflux through the BBB in rats (Nakatani et al. 2008).

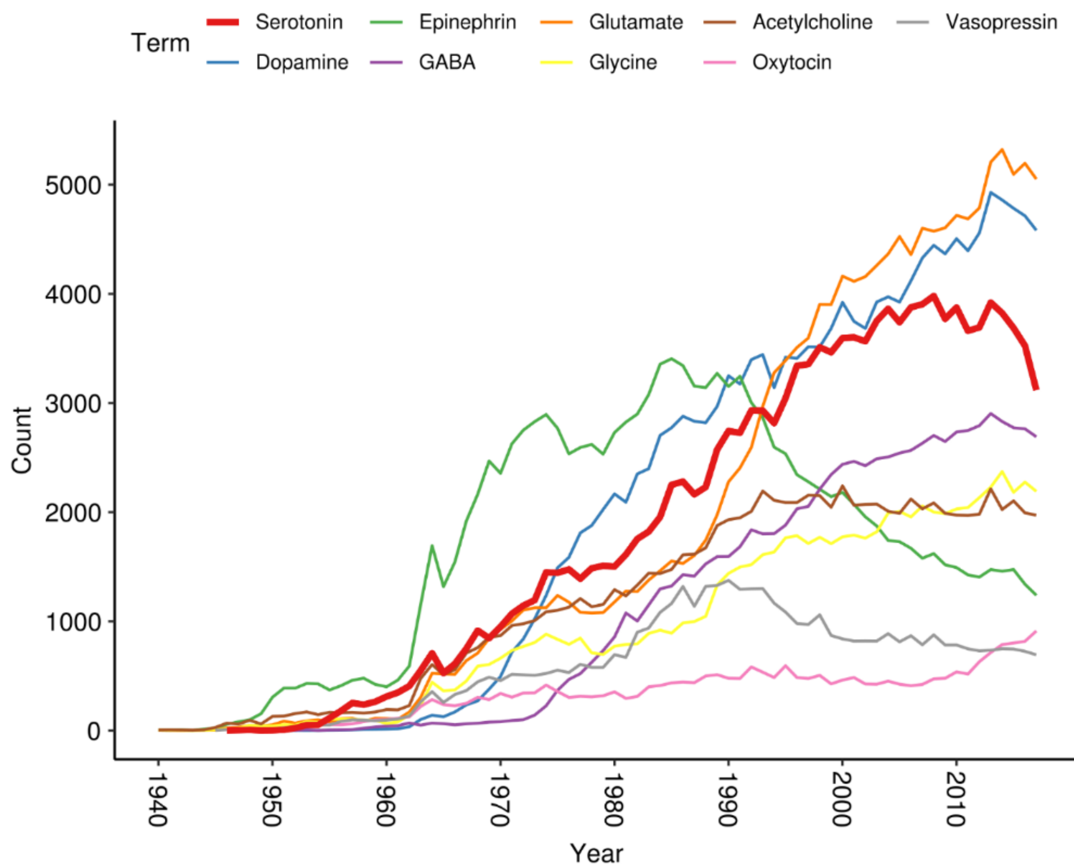


Figure 1. Number of publications indexed in PubMed from 1950 to 2017, using different neurotransmitters as query terms. Serotonin is highlighted by a thicker line.

1.2.1. Discovery

Serotonin was discovered more than sixty years ago and such finding, yet fortuitous, constituted a milestone in the history of Neuroscience. Indeed, it was first isolated independently by the Italian physiologist Vittorio Erspamer who isolated it from enterochromaffin cells (ECs) in the gut epithelium, and named it *enteramine* (Vialli and Erspamer 1937) - and the American biochemists Arda Green and Maurice Rapport at Irvine Page's laboratory at Cleveland Clinic (Rapport et al. 1948). At the time, Page's laboratory studied hypertension-producing factors in blood, but an unidentified serum substance was produced as blood coagulated, and was suspected to interfere in the experiments, so it was Rapport's job to isolate and remove it from blood samples. This substance was isolated, and named *serotonin*, given its presence in the serum (*sero*), and its constrictive activity on the blood vessels tone (*tonin*). In 1952, it was established that serotonin and enteramine were in fact the same substance (Erspamer and Asero 1952).

Serotonin, was first associated to platelets, yet quickly found in other tissues such as the liver, kidney, lung, and gastrointestinal tract. However, it was Dr. Betty Twarog (working at Page's laboratory) who, suspecting that an unknown neurotransmitter that she had previously found in invertebrates could be acting as well in vertebrates, found serotonin in specific areas of the mammalian brain, thus bringing serotonin into the Neuroscience realm, as well as ironically diminishing the likelihood of its involvement in hypertension (Twarog and Page 1953). Another breakthrough was related to the famous discovery of the potent psychoactive lysergic acid diethylamide (LSD-25) by Dr. Albert Hofmann in 1943 (Hoffman 1979), and the observation that a tryptamine fragment embedded in the LSD structure was also the scaffold of the newly discovered serotonin. This finding, together with resemblances of LSD and serotonin effects in some experiments, led to the hypothesis that the mind-altering properties of LSD were directly associated with alterations in the serotonergic function in the brain (Woolley and Shaw 1954) an idea that had profound implications in Neuroscience, and marked the beginning of modern Neuropsychopharmacology (Nichols and Nichols 2008), as well as a dramatic increase in the interest in understanding the role of serotonin in behaviour and psychiatric diseases.

1.2.2. Structure, biosynthesis, and metabolism

Relatively simple in structure, 5-HT is composed of an indole nucleus, a two-carbon aliphatic chain, and a basic amino group (Rapport 1949). Serotonin is biosynthesized from L-tryptophan the largest and the most hydrophobic of amino acids (Aoyagi et al. 2001) in two enzymatic steps: (1) ring hydroxylation of tryptophan to form 5-hydroxytryptophan (5-HTP) by a tryptophan hydroxylase (TPH), and (2) side chain decarboxylation by aromatic amino acid decarboxylase to form 5-HT (Clark et al. 1954; Fitzpatrick 1999) (Figure 2). The first step is a rate-limiting step in animals, since it depends on tryptophan availability, typically low in animals (see *Evolutionary perspectives* section). In mammals, two genes (*TPH1* and *TPH2*) encode for two tryptophan hydroxylases (Walther 2003), and show different expression patterns: TPH1 is expressed in non-neural peripheral tissues, like enterochromaffin cells (ECs) and in the pineal gland, whereas TPH2 is expressed in neurons, fundamentally in the raphe nuclei and the myenteric plexus (Walther 2003; Côté et al. 2003; Patel et al. 2004). After synthesis, serotonin is quickly transported into dense vesicles by vesicular monoamine transporters (VMAT), which appear in two isoforms: VMAT1 in neuroendocrine cells, and VMAT2 in neurons (Erickson et al. 1996). Storage prevents metabolism, and permits serotonin release into the synaptic cleft via exocytosis under tight regulation (See *Serotonergic synapse* section).

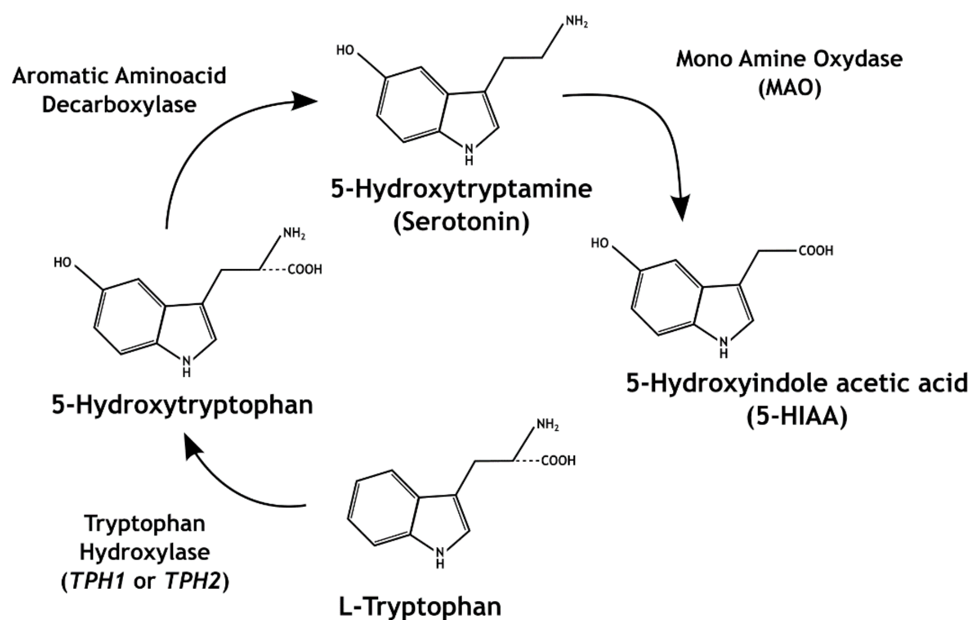


Figure 2. Schematic 5-HT biosynthetic and metabolic pathway.

Serotonin can follow additional enzymatic steps; one of them is metabolism, by monoamine oxidase (MAO)(Sandler et al. 1981), which transforms it into 5-hydroxyindole acetic acid (5-HIAA), a biologically inert compound that is excreted mainly through urine (McIsaac and Page 1959). Just like TPH and VMAT, MAO appears in two major forms (MAO-A and MAO-B), codified by two different genes, and they participate in the metabolic pathways of other amines (e.g. norepinephrine, dopamine, and histidine), being MAO-A the form with highest affinity for serotonin, and its main metabolizer (Tyce 1990).

Alternatively, in the pineal gland of vertebrates, serotonin can be methylated to produce melatonin, another critical compound, used by animals as a biological signal to regulate circadian rhythm, and photoperiodic (i.e. depending on day length) annual functions, such as camouflage colouring, coat growth, and reproduction, and skin colour change in some reptiles and amphibians (Sugden et al. 2004). In addition, plants use melatonin as a powerful antioxidant (Hardeland 2005).

1.2.3. Serotonergic synapse

In the CNS, upon neuronal depolarization, serotonin is released into the synaptic cleft. It can bind to post-synaptic serotonin receptors (5-HT receptors), into the synaptic cleft, where it reaches its receptor and triggers a series of molecular chain reactions of diverse nature that will ultimately produce an effect, or alternatively, to serotonin autoreceptors on the pre-synaptic membrane (Figure 3). The strength and duration of the signalling largely depend on the abundance of serotonin in the synaptic cleft, which is regulated mainly by two factors: (1) serotonin reuptake, and (2) negative feedback. The first, and main factor is carried out by the highly selective serotonin transporter (5-HTT or SERT), which is located on the presynaptic membrane, is responsible for serotonin reuptake (i.e. removing it from the synaptic cleft back into the presynaptic neuron) and in consequence, it is a classic pharmacological target, typically blocked by a group of antidepressant drugs, collectively called selective serotonin reuptake inhibitors (SSRIs). Once transported back into the presynaptic neuron, serotonin is metabolized by MAOA in the cytosol, or recycled back into presynaptic vesicles for re-release, where it is protected from metabolism. The second factor is exerted via serotonin binding to a serotonin autoreceptor in the presynaptic neuron 5-HT1A and 5-HT1B, such activation triggers a negative feedback against further release of serotonin into the synaptic cleft (Cerrito and Raiteri 1979). Serotonergic receptors can also

function as hetero-receptors in non-serotonergic neurons (e.g. GABAergic, adrenergic and cholinergic neurons), thus regulating other neurotransmitters release (Fink and Göthert 2007).

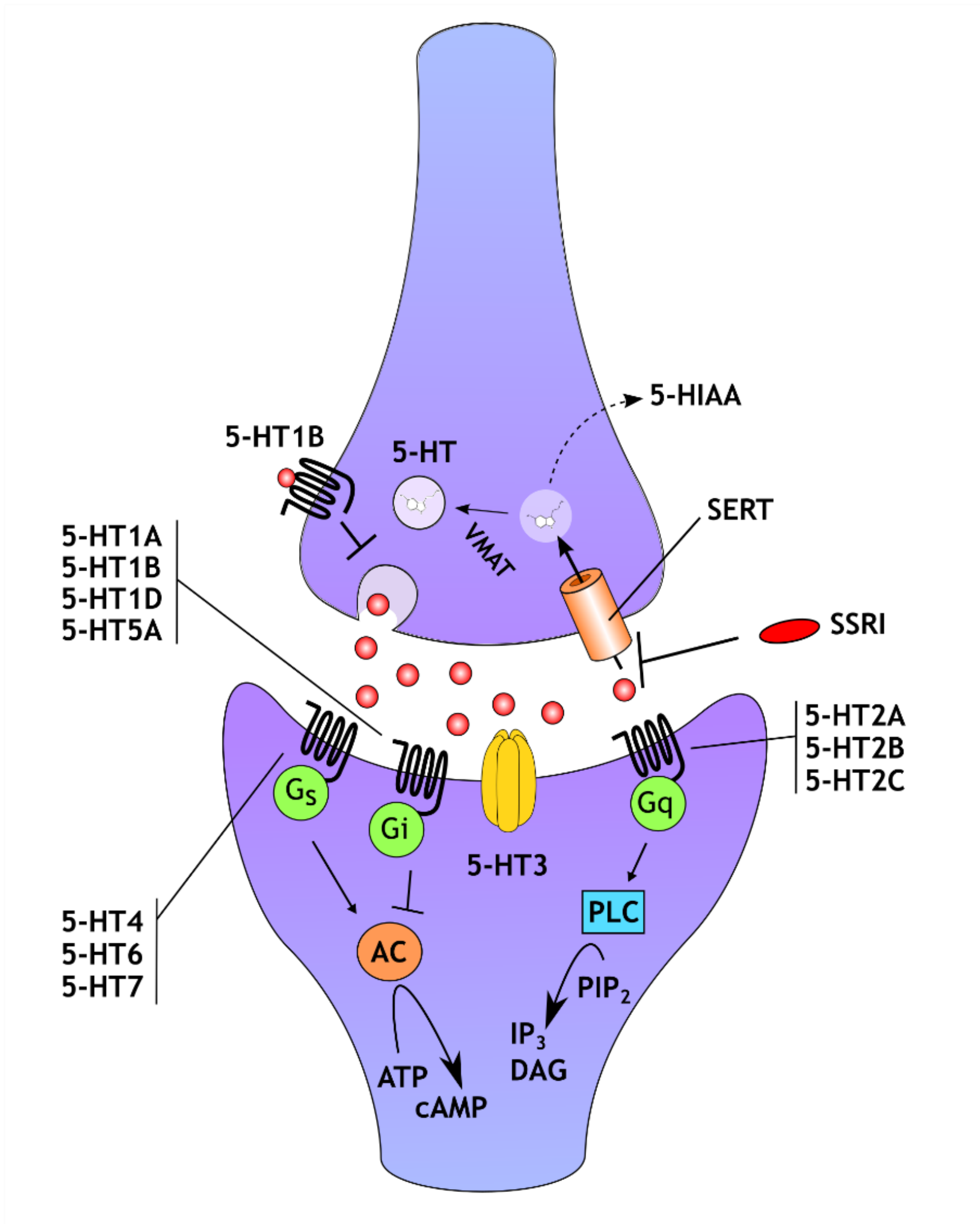
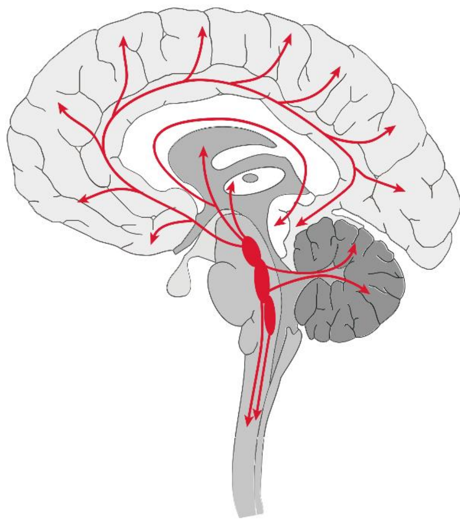


Figure 3. Model of neuronal synapse in which the different 5-HT receptors are depicted. Serotonin is released into the synaptic cleft, where it binds to one of its receptors (each type will trigger different signaling pathways) and can be later reuptaken.

1.2.4. Distribution in the CNS, PNS, and peripheral tissues

Unlike other animal groups (e.g. molluscs and arthropods) in which serotonergic neurons are distributed along the entire CNS (Gillette 2006), central serotonin synthesis in mammals is restricted to nine discrete groups of cell bodies isolated to the pons and midbrain, known as the raphe nuclei. The raphe nuclei represent the major nuclei with both ascending serotonergic fibres projecting to cortical, limbic, midbrain, and hindbrain regions, and descending fibres that extend to the medulla and spinal cord (Dahlström and Fuxe 1964; Törk 1990; Baumgarten and Göthert 1997; Berger et al. 2009) (Figure 4). Such wide distribution of serotonergic fibres, reaching virtually all brain areas, accounts for its involvement in regulation of such wide array of physiological and behavioural processes, and illustrates why deregulations in the serotonergic system have been related to numerous human mental conditions, and thus its critical relevance in medical research.

As mentioned before, ~95% of serotonin is outside the CNS, where is produced and stored mainly produced and stored in enterochromaffin cells (ECs), a type of enteroendrocrine cells (EECs) that localize in the intestinal epithelium, among enterocytes (**Figure 5**). In a lesser extent (~1%) serotonin is synthesized by neurons in the enteric nervous system (ENS), following the same biosynthetic pathway as in CNS neurons. Since the afferent nerves of the gut do not reach into the lumen, EC cells serve as sensory transducers for chemical and mechanical stimuli, by responding to these cues by producing and releasing serotonin into the lamina propria, where it stimulate extrinsic (i.e. vagal and spinal afferent fibres) or intrinsic (e.g. coming from ENS neurons in the myenteric plexus) fibres, and promotes a variety of modulatory effects in the gut (Furness et al. 1998; Raybould et al. 2004; Blackshaw et al. 2007; Mawe and Hoffman 2013). After release, EC-originated serotonin may be recaptured back into EC cells via SERT, or reach portal circulation, where it is quickly uptaken and stored in vesicles by platelets (via SERT and VMAT, respectively), and then metabolized in the liver (Humphrey and Jaques 1954; Launay et al. 1994; Keating et al. 2013). Alternatively, those not uptaken serotonin molecules eventually reach the lung, were they are metabolized (Tyce 1990).



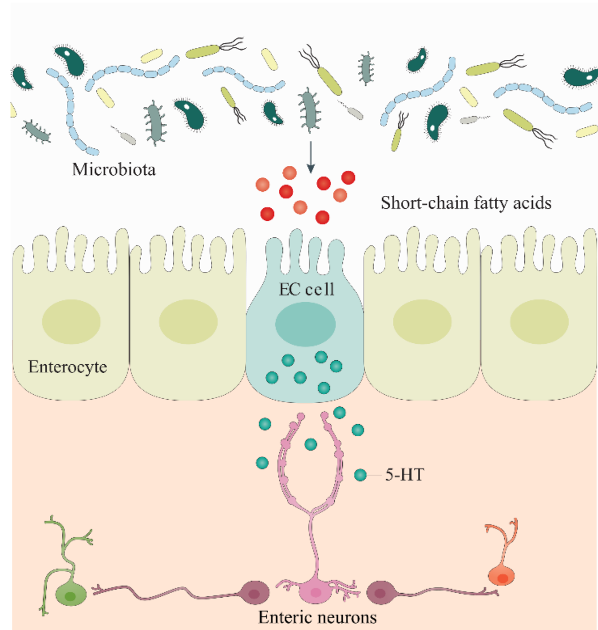


Figure 4. Localization of the raphe nuclei in the human brain (highlighted in red), and neural projections to virtually all brain areas and CNS, based on Berger et al., 2009.

Figure 5. Serotonin in the gut. Gut microbiota produce short-chain fatty acids as fermentation byproduct, which increase TPH1 expression, and thus 5-HT production and release from ECs. 5-HT thus activates enteric neurons that, among other effects, trigger gut mot motility. Adapted from Spohn and Mawe, 2017.

1.2.5. Serotonin functions

Determining the main role and/or the main principle underlying serotonin actions is challenging, given its high amount of receptors, and the fact that its neuronal ramifications reach virtually all brain regions as well as other parts of the organism. Reviewing every involvement of the serotonin systems in the organism is beyond the scope of this Thesis. However, I selected some of them that are of special interest for the focus of the present work.

1.2.5.1 Social behaviour

Behaviour is one of the most complex animal phenotypes, by its variability and its dependence on multiple factors, both genetic and socio-environmental (Robinson et al. 2008). Genetic factors contribute to the structural and functional aspects of the brain, while

socio-environmental factors can shape individual behaviours through modifications in neural circuits and changes in gene expression through epigenetics and other mechanisms. Therefore, like any other phenotype, behaviour is subject to natural selection. Differences in individual social behaviour may be due to the influence of genetic variants in genes involved in the neural circuitry, as well as the influence of the different environments. Likewise, complex social adaptive behavioural relationships do not depend of a single neurotransmitter or cerebral function, and indeed, multiple other neurotransmitters (e.g. oxytocin, vasopressin, dopamine) have been proposed to influence social behaviours. Nonetheless, I will focus on the particular effects associated to serotonin.

In general, serotonin affects sensitivity to both positive and negative environmental stimuli (e.g. reward and stress). Higher central serotonin levels results in increased sensitivity, while low levels are associated with less sensitivity. Thus, elevated serotonin levels are highly context dependent, not good nor detrimental *per se*. In other words, serotonin may have a general effect on attention and perception, in a *for-better-and-for-worse* manner (Belsky et al. 2009; Branchi 2011; Homberg and Lesch 2011). Some authors (see Ellis et al. 2011) have proposed that differential sensitivity in individuals of a population would be advantageous from an evolutionary point of view. For instance, some individuals could detect and change strategies in response to positive and negative environmental changes more readily, thus orienting towards favourable contexts, while being balanced by more stable ("resilient") individuals, that would benefit less from positive environment features, but also would suffer less from the effects of negative experiences (Uher 2009; Branchi 2011; Ellis et al. 2011). Consistent with this is the fact that enhanced sensitivity to threat-related stimuli and punishment is a cardinal feature of several mood and anxiety disorders that implicate central serotonergic systems.

Due to the dramatic social consequences of violence, the role of serotonin in aggression has been widely investigated. Aggression is a manifested behavioural response that targets another individual (conspecific or not) or lifeless thing in a physical or symbolic way with the aim to inflict damage, and plays an important role in evolution. Both defensive aggression and predation are part of the normal behavioural repertoire of most, if not all, species (Vitiello et al. 1990; Kiser et al. 2012). Aggression can take different forms, and is usually subdivided in categories, such as proactive and reactive aggression. Proactive aggression generally has an aim, and physical violence is a mean to achieve it (e.g.

assault/robbery), whereas reactive or hostile aggression is related to affections, and it is driven by perceived hostile intents in others, sometimes provoked by previous aggressive encounters (Craig and Halton 2009). Both forms of aggression have been considered as different strategies to cope with social stimuli (Koolhaas et al. 2007), being reactive aggression the most common in pathological, clinical settings. The relationship between serotonin and aggression vary depending on the type of aggression that we consider. It has been proposed that normal reactive aggressive behaviour, aimed at territorial control and social dominance is positively associated with serotonin neural activity, while proactive aggression has been observed to correlate with low serotonergic levels (de Boer 2009; Kiser et al. 2012).

Serotonin effects depend in a great degree on the proteins involved in its synthesis, metabolism, transport, and signalling. The genes coding for those elements (i.e. TPH1, TPH2, MAOA, SLC6A4, and the serotonin receptors) have been candidates for playing central roles in regulating serotonergic function. Regarding aggression, studies in mice have shown negative correlations between TPH functioning and proactive aggression (Kulikov et al. 2005), as well as an increase in aggression under treatment with TPH inhibitors (Kubala et al. 2008). MAOA function also seems to play a role, and male mice with defective MAOA gene showed increased serotonin (and norepinephrine) levels, decreased 5-HIAA and increased aggressive behaviours (Scott et al. 2008; Bortolato et al. 2018). The serotonin transporter linked polymorphic region (5-HTTLPR) is a common regulatory variant in SERT, which as mentioned above, is essential for regulation of serotonin concentrations in the synaptic cleft. There are two main alleles in 5-HTTLPR: the long (l) and the short (s) form. The short form is associated with SERT down-regulation, thus lowering its availability and function (which in turn lead to increased serotonin levels), and it is widely known for its association with anxiety-related traits and neuroticism. In a study with male Rhesus monkeys, individuals carrying 5-HTTLPR s-allele engaged in high-risk aggressions, but only if they were exposed to adversity early in development (Schwandt et al. 2010).

In addition, 5-HT receptors subtypes appear to have unique, and perhaps opposing, effects on aggression. Aggressive human individuals have been shown to have attenuated response to 5-HT1A receptor agonists (Almeida et al. 2010), and 5-HT1B hetero-receptors in the hypothalamus may be involved in regulating proactive aggression (Olivier and van Oorschot 2005). The relationship between the 5-HT2A receptor and impulsive aggression

has been mixed, with some studies reporting inverse and others positive associations (Meyer et al. 2008; Soloff et al. 2010; Rosell et al. 2010). Finally, the 5-HT2C receptor has been of interest because of its possible anti-aggressive effects when stimulated (Bortolato et al. 2013).

Serotonin also influences social dominance, attachment and hierarchy formation. In male vervet monkeys, individuals with increased serotonin levels achieved dominance by first increasing affiliative behaviours towards group members, thereby creating social support, and then engaging in aggressive encounters with competitors (Raleigh et al. 1991). In contrast, in a different study in Rhesus females, the 5-HTTLPR s-allele (potentially associated with increased serotonin levels) were associated with increased aggression and submission, whereas l/l individuals showed increased dominance and social rank (Jarrell et al. 2008). Another study in Rhesus males showed that 5-HTTLPR genotype was associated with the timing of dispersal from natal groups, being s/s homozygotes the first to leave their group, and l/l the latest (Krawczak et al. 2005). Such effect was not observed in females, since they stay in the natal group throughout life.

Regarding human societies, (Chiao and Blizinsky 2010), suggested that s-allele is associated with increased serotonin levels, increased social sensitivity, and that it would be more frequent in collectivistic cultures. This postulate was later challenged (Bisso-Machado et al. 2013), who compared Native South American populations according their major mode of subsistence, classifying as individualistic (recent history of hunter–gathering) or collectivistic (agriculturalists), and showed that these two groups have an almost identical s-allele frequency (75% and 76%, respectively, Bisso-Machado et al. 2013). More recently, Hünemeier and colleagues (2015) stressed that the Paleo-Indian whose genome is known (named 'Anzick', an individual belonging to the classical North American hunter-gathering Clovis culture, who lived around 13 kya) was an homozygote for the 5-HTTLPR l-allele, a relatively rare genotype in present-day Native Americans (Hünemeier et al. 2015). These results suggest a more heterogeneous and complex demograpic scenario, being challenging to establish an association of one of these alleles with a specific mode of subsistence.

Overall, serotonin levels are positively correlated with sensitivity towards social cues and enhance the perception of dominance of others, as well as social conformity and affiliative behaviour (Edwards and Kravitz 1997; Knutson et al. 1998; aan het Rot et al.

2006; Beacher et al. 2011), which eventually may favour achieving dominance and high social rank (Kiser et al. 2012), condition known to increase fitness in many species.

In conclusion, the serotonergic system affects sensitivity to social and environmental stimuli, and provides the ability to adapt in response to them, thus playing a crucial role in evolution.

1.2.5.2. Sex

In addition to the effects of serotonin on adaptive social behaviour, it also has an effect on sexual behaviour and physiology. The classical view is that serotonin inhibits copulation, although this paradigm has changed in the past years toward a more complex scenario, in which the action of serotonin depends on several factors, like gender, type of sexual behaviour analysed, and type of receptor involved. The strong relationship between the increase in central serotonin during SSRI treatment, and delayed ejaculation and decreased libido in men and rodents in many cases, causing the former to abandon treatment because of these side effects has been well established (de Jong et al. 2006; Chan et al. 2011). On the other hand, SSRIs are successfully used for treating premature ejaculation (Moreland and Makela 2005). In rhesus males, a study showed that individuals with 5-HTTLRP s/s genotype had lower reproductive output compared to their l/l and l/s counterparts, which makes sense if we consider the s/s allele as producing less effective SERT (Krawczak et al. 2005).

In general, global increases in 5-HT levels inhibit male or female sexual behaviour while reductions increase sexual behaviour; yet selectively stimulating specific 5-HT receptors reveal both inhibitory and facilitatory effects.

5-HT1A receptors have been suggested to have an important effect in serotonin-mediated sexual effects, since 5-HT1A desensitization has been observed following SSRI treatment and in knockout rats. Furthermore, treatment with 5-HT1A agonists are helpful in sexual impairments (Hensler 2002), which could be explained by the inhibitory effects of presynaptic 5-HT1A receptors on serotonergic firing by neurons in the raphe nuclei, in situations of high serotonergic levels (like those of prolonged SSRI treatment). Interestingly, these mechanisms seems to depend on the local expression of receptor subtypes and sex (de Jong et al. 2006). Indeed, under conditions of elevated serotonin levels, 5-HT1A receptor agonists seem to facilitate sexual activity in males, but inhibit it in females, but this

discordance seems to be partly dependent on the definitions of different female sexual activities, which have different brain regions involved (Snoeren et al. 2014).

1.2.5.3. Emotions and mood

In adverse life contexts in childhood and adulthood (e.g. physical or sexual abuse, social rejection), 5-HTTLPR s-allele increases the risks for depression and related emotion-related disorders, suggesting a strong s-allele × environment interaction (Lesch et al. 1996; Caspi et al. 2003; Canli and Lesch 2007). This association may seem at odds with the well-known ameliorating effects of long-term treatment with SSRIs like fluoxetine hydrochloride (PROZAC™) in depression and affective disorders, given that SSRIs selectively block SERT, thus increasing serotonin concentration in the synaptic cleft (Wong et al. 2005). However, if we consider the developmental side, some studies have suggested differential effects of altered serotonergic transmission in adult and developing individuals, supporting the idea that reduced expression of 5-HTT during development may increase the odds for depressive behaviour in adulthood, even though blocking serotonin reuptake in adults have antidepressant effects (Ansorge et al. 2004; Pezawas et al. 2005; Nautiyal and Hen 2017).

1.2.5.4. Neural development

The serotonergic system is the first among neurotransmitters to arise during development (i.e. halfway gestation, or E11 phase in rodents, and during the second trimester in humans), when serotonin is released by growing axons even before conventional synapses are established (Levitt and Rakic 1982; Gaspar et al. 2003; Kepser and Homberg 2015). In humans, serotonin levels peak in early brain development, decline during childhood, and become more or less stable during adulthood (Azmitia and Whitaker-Azmitia 2000). The first serotonergic neurons during human development are evident in the brainstem by 5 weeks of gestation, and increase dramatically in the dorsal raphe nuclei by the tenth week (Shen et al. 1989; Sundström et al. 1993). In addition, even before raphe nuclei neurons appear, placenta serves as a transient source of serotonin for the fetal forebrain (Bonnin et al. 2011). These findings highlight the importance of serotonin as a developmental signal, and indicate a role in modulation of different early developmental processes, like synaptogenesis and neurite outgrowth (Mazer et al. 1997; Udo et al. 2005). In consequence, it is not surprising that disruptions of the serotonergic system in early development have

been associated with neurodevelopmental disorders like autism spectrum disorders (ASD) or down syndrome (Whitaker-Azmitia 2001).

During early development, serotonin is involved in cell survival, and later plays a role in cell migration and differentiation. In the adult brain, it contributes to synaptic maintenance and plasticity, in many cases by the same mechanisms used in development (Whitaker-Azmitia 2010).

Serotonin morphogenic effects during development are varied and sometimes contradictory, depending on cell types, species, and developmental stages. For example, increased levels facilitate dendritic differentiation in cerebral cortex, increases neurite elongation of thalamic and hippocampal neurons in rats (Liu and Lauder 1991; Lieske et al. 1999; Norrholm and Ouimet 2000), while depletion impairs dendrite length and spine formation in hippocampus (Alves et al. 2002).

On the other hand, some studies in snails have reported negative effects on neuronal morphology, showing that it also reduced neurite branching and elongation of embryonic neurons, while decreased serotonin facilitates outgrowth of distal neurites and neurite branching in embryonic neurons (Haydon et al. 1984; Goldberg et al. 1991; Diefenbach et al. 1995). In addition, serotonin application induces neurite retraction and growth cone collapse in dorsal ganglion neurons in chicken (Igarashi et al. 1995).

Serotonin seems to be involved also in many other processes, like neuronal network formation and remodelling, as well as behaviour regulation. As showed by Alenina and colleagues (2009), serotonin depletion (by TPH2 knockout in mice) leads to altered behaviour and functions, such as daytime sleepiness, neglect in maternal care and thus poor pup survival, as well as alterations in growth, body temperature and heart rate, without gross alterations of neuron morphology being observed (Alenina et al. 2009). In addition, reduced brain growth and delayed cortex maturation have been associated with partial serotonin reduction in the brain during postnatal development (Migliarini et al. 2013; Narboux-Neîme et al. 2013).

The role of serotonin levels during development may depend on their temporary pattern, and evidence has suggested the existence of a developmentally critical window (during the early postnatal period, in rodents) in which serotonin levels alteration may permanently influence neuronal circuits (Daubert and Condron 2010; Migliarini et al. 2013).

Although our understanding of the mechanisms behind these processes is far from complete, the different outcomes seem to be caused by the diversity of serotonin receptors and/or their downstream effects in different brain areas. Indeed, targeted deletions of serotonin receptors do not cause marked alterations in brain development in mice, supporting the idea that each receptor have a limited set of effects and periods of action during development (Gaspar et al. 2003; Wirth et al. 2017).

Of all serotonin receptors, most attention has been devoted to 5-HT1 and 5-HT2 subfamilies regarding their action in development (Whitaker-Azmitia 2001; Gaspar et al. 2003; Bonnin et al. 2006). However, new evidence suggests an important role of 5-HT3 receptor in neural development. For example, 5-HT3 receptors are expressed on embryonic immature GABAergic interneurons and neuroblasts in early postnatal migratory streams, and in the adult neocortical subventricular zone (SVZ), where they contribute to cortical neurogenesis (Inta et al. 2008; Vucurovic et al. 2010). Therefore, they might be involved in fine regulation of neuronal excitability, migration, maturation, and formation of inhibitory networks during development and in adult stages.

Indeed, two recent studies showed that 5-HT3A receptors are required for interneuron migration when they invade the developing neocortex (Murthy et al. 2014), and for postnatal neuroblast migration, via mediated Ca^{2+} influx (García-González et al. 2017), a mechanism suggested to be evolutionarily conserved, as indicated by the presence of serotonergic axons in migratory pathways in other vertebrates. Furthermore, 5-HT3 receptors are expressed in Cajal–Retzius cells (neocortex) and granule cells (cerebellum), where they regulate the wiring of the local microcircuitry. This opens the possibility that 5-HT3 receptors are involved in the formation of higher-level neuronal structures (Chameau et al. 2009; Oostland and van Hooft 2013).

Some studies have also raised awareness of potential risks that SSRI treatment in pregnant women could pose to fetal brain development, and the effects that disturbing serotonergic circuits at this stage could have, since evidence suggest that disorders like ASD might be caused by disruptions of the serotonergic system during brain development (Anderson et al. 1987; Naffah-Mazzacoratti et al. 1993; Chugani 2002). Indeed, the use of SSRIs by pregnant women, especially during the first trimester, may increase the risk of ASD in the offspring, while early postnatal exposure in mice leads to anxiety-like behaviour (Ansorge et al. 2004; Croen et al. 2011). In addition, a recent study showed that releasing

serotonin in the nucleus accumbens of mouse ASD models reverts social deficits, thus demonstrating a role of serotonin in the nucleus accumbens in social behaviours (Walsh et al. 2018).

1.2.5.5. Gastrointestinal motility and function

Most serotonin in the body is found is found in the gut, where it acts as a paracrine signalling molecule to regulate the major functions of the gut, comprising secretion, vasodilation, and motility, among other functions discussed in this chapter and elsewhere (e.g. Spohn and Mawe 2017). As mentioned before, most serotonin in the gut is produced and stored in EC cells in the GI, where is synthesized by TPH1. ECs serve as sensors in the intestinal lumen, being able to response to neurochemical and mechanical stimuli, as well as nutrients and immune modulators. ECs modulate stimulation of intrinsic and extrinsic afferent fibres that reach the lamina propria (under the mucosal layer) and contain 5-HT receptors, thus triggering downstream effects upon activation (Figure 5). In particular, gut motility is mediated mainly by the activation of 5-HT3 and 5-HT4 receptors in vagal afferent terminals, and 5-HT3 receptors in intrinsic fibres from the myenteric plexus (Bertrand et al. 2000; Hoffman et al. 2012).

EC cells can also detect abnormally high circulating glucose levels in the intestine, which may occur during pathological states like diarrhoea and fast transit, and release 5-HT as a response to it. Also, EC cells can also respond to lack of nutrient availability (Sumara et al. 2012; Zelkas et al. 2015).

The role and of serotonin in gut motility, and whether it is essential or not is still hotly debated, as illustrated by two opposing articles published in 2015 (Smith and Gershon 2015; Spencer et al. 2015). However, it seems clear that serotonin is a potent stimulant of gut motility and stomach emptying, as well as being essential for nutrient and water absorption (Imada-Shirakata et al. 1997; Martin et al. 2017).

1.2.5.6. Gut microbiota

The past few years have witnessed a paradigm shift in Neuroscience, derived from the evidence connecting gut microbiota and the brain in a bidirectional way, through multiple neurocrine and endocrine signalling mechanisms, collectively called the gut-brain axis

(Mayer et al. 2014; Fung et al. 2017). Adult humans carry ~10^{13}-10^{14} microorganisms in their gut, outnumbering all our own cells, and weighing about 1 Kg approximately the same as the brain. The gut microbiota forms a complex community, which has been evidenced to be essential for metabolic and immune health (Lynch and Pedersen 2016). In addition, studies with animal models have evidenced a role of gut microbiota in different disorders, such as constipation and gut motility disorders, depression, anxiety, Multiple sclerosis (MS), Parkinson's disease (PD), Alzheimer's disease (AD) and symptomatologies of ASD (Hsiao et al. 2013; Minter et al. 2016; Kelly et al. 2016; Sampson et al. 2016; De Palma et al. 2017; Berer et al. 2017; Cekanaviciute et al. 2017; Ge et al. 2017).

The specific mechanisms underlying this bidirectional communication have not been fully elucidated yet, although several possible pathways have been proposed. For instance, the hypothalamic-pituitary-adrenal axis (HPA), the core element in stress modulation, which dysregulation caused by psychological or physical stress is likely involved in disorders related to the microbiota-gut-brain axis, such as irritable bowel syndrome (IBS) in which serotonin plays an important role, specifically through 5-HT3 and 5-HT4 receptors (Sikander et al. 2009; Yaakob et al. 2015). Indeed, 5-HT3 receptors colonic motility, secretion, and nociception, so antagonists like ondasetron, alosetron, and granisetron are often used in clinical practice to alleviate IBS-related diarrhoea and abdominal pain (O'Mahony et al. 2015; Yaakob et al. 2015). Another suggested pathway is the stimulation of the vagus nerve the principal component of the parasympathetic system, which is inhibited by stress, and that plays a role in IBS, among other disorders (Bravo et al. 2011; Bonaz et al. 2018). Vagal afferent fibres are found in all layers of the intestinal wall, were they indirectly interact with the microbiota through diffusion of bacterial metabolites or compounds, or through secretion of neurotransmitters and hormones by EECs including serotonin (Li et al. 2000). The microbiota can also produce neurotransmitters, like GABA, dopamine, and norepinephrine (although only the first is known to be produced *in vivo*), thus altering their levels. In contrast, no known serotonin-producing bacteria is found in the gut, which suggests that microbiota-mediated serotonin production is indirect (Strandwitz 2018). Indeed, secretion of short-chain fatty acids (SCFAs) by intestinal microbiota stimulates serotonin synthesis and release in EC cells via increased TPH1 expression. This process in turn activates intrinsic and extrinsic fibres with serotonin receptors, thus promoting intestinal motility, among other effects (Figure 5; Yano et al. 2015; Spohn and Mawe 2017; Bonaz et al. 2018).

In summary, gut microbiota participates in a bidirectional communication with the brain, influencing key brain processes and modulating behaviours by mechanisms that include neurotransmitter production, modulation of host neurotransmitter production, and activation of vagal pathways. However, the complete set of mechanisms acting on the microbiota-gut-brain axis are still being elucidated.

1.2.5.7. Metabolic homeostasis

The known roles of 5-HT obesity and metabolic regulation have been hotly debated for decades. Indeed, 5-HT has been long showed to regulate satiety and feeding behaviour through both peripheral and brain mechanisms (Blundell 1986; Voigt and Fink 2015). High availability of 5-HT (or activation of its receptors) reduce food intake, while low 5-HT levels (or receptor blockade) induce feeding behaviour. Of note, central and peripheral 5-HT storages are independent of each other (i.e. 5-HT does not cross the BBB), so regulation at brain and peripheral levels is exerted through different, independent mechanisms. 5-HT acts in multiple endocrine tissues (e.g. pancreas and liver), where it is actively involved in glucose metabolism, with contrasting effects depending on the tissue. For instance, pancreatic β-cells produce their own 5-HT (as they express both TPH1 and TPH2), and store it along with insulin in secretory granules (Kim et al. 2010). Then, after increase in circulating glucose, 5-HT promotes insulin secretion in a receptor-dependent (by activating 5-HT3 and 5-HT2B receptors), and/or -independent manner, in a process called serotonylation (Paulmann et al. 2009; Kim et al. 2010; Kim et al. 2015). In the liver, serotonin exerts an opposite effect. EC-originated 5-HT activates 5-HT2B receptors, which in turn promote activation of Fructose-1,6-bisphosphatase (FBPase) and Glucose-6-phosphatase (G6Pase), thus promoting gluconeogenesis. In addition, 5-HT2B receptor activation reduces hepatocyte glucose uptake in a Glut2-dependent manner, thus increasing circulating glucose levels (Sumara et al. 2012). In the same way, experiments in mice revealed that 5-HT promotes lipolysis and inhibits lipogenesis in white adipose tissue, while activating thermogenesis in brown adipose tissue (Oh et al. 2015).

1.2.5.8. Hematopoiesis

Serotonin regulates erythropoiesis in the bone marrow, and erythrocyte survival (Yang et al. 2007; Amireault et al. 2011), although it is unclear whether the serotonin source is the

bone marrow or if it originates in the gut and then circulates through platelets. This was observed by experiments with Tph1-knockout mice, which showed anaemia and low red blood cell count in the bone marrow, which was reversed upon exposure to 5-HT (Amireault et al. 2011; Amireault et al. 2013).

1.2.5.9. Bone formation

Bone formation and remodelling is an important process for life, which is regulated by many different molecules, including growth factors, cytokines and other hormones being serotonin one of them (for a review on this topic, see Siddiqui and Partridge 2016). Interestingly, 5-HT role in bone metabolism as it is in other processes can be somewhat contradictory, as it acts both promoting and suppressing bone formation, and the effect seems to depend on where it is synthesized and where it acts. For example, brain 5-HT acts in favour of bone formation, by activating 5-HT2C receptors in the ventromedial hypothalamic nuclei, which increases sympathetic tone, and promote osteoblast proliferation. Such activation is inhibited by leptin (Yadav et al. 2009). In contrast, EC-originated 5-HT decreases osteoblast proliferation (Yadav et al. 2008).

1.2.6. Serotonin receptors

As already seen above, serotonin has a broad and fundamental spectrum of functions mediated by interaction with its receptors. Below I provide more details about each receptor type.

1.2.6.1. Diversity, classification, and function

The first evidence that serotonin could act via specific receptors was provided by Rocha e Silva and colleagues at the *Escola Paulista de Medicina* (São Paulo, Brazil), who discovered that the actions in the pig ileum of that recently identified compound could be inhibited by cocaine at micromolar concentrations (Rocha E Silva et al. 1953). In 1957, Gaddum and Picarelli proposed a dual classification of serotonin receptors, based on their location and pharmacological properties: “M” and “D” receptors (Gaddum and Picarelli 1957). Later, many more serotonin receptors were discovered, conforming the large receptor repertoire of 14 known members encoded by 18 genes that we know today in humans (see **Table 1**). The first international classification of serotonin receptors was published by Hoyer

and colleagues in 1994 (Hoyer et al. 1994) and, on the basis of selective agonist/antagonist ligand affinities, cloned sequence homology, and intracellular transduction mechanisms, serotonin receptors were classified into seven subfamilies (named 5-HT[1-7]R), with differing number of receptors per subfamily.

Serotonin receptors belong to two different, and phylogenetically ancient protein superfamilies: subfamilies 1, 2, 4, 5, 6 and 7 belong to the G protein-coupled receptors (GPCRs) superfamily (more specifically, to "type A" or Rhodopsin-like receptors), while receptor 3 (5-HT3 receptor) belongs to the pentameric ligand-gated ion channels (pLGICs) superfamily. In genetic terms, each serotonin GPCR is encoded by a single gene (N.B. in humans, *HTR5BP* is a pseudogene, and thus produce no 5-HT5B receptor), while there are up to five known genes (*HTR3A-E*) which encode for subunits of the 5-HT3 receptor.

Table 1. Serotonin receptors known as of today in humans, with their chromosomal locations, gene and protein length, exons, primary signalling pathway and potential.

Receptor	Gene	Locus	Gene length (pb)	Protein length (aa)	Exons	Primary signaling system	Potential
5-HT1A	*HTR1A*	5q11.2-q13	2,152	422	1	Gαi/o	Inhibitory
5-HT1B	*HTR1B*	6q13	1,543	390	1	Gαi/o	Inhibitory
5-HT1D	*HTR1D*	1p36.3-p34.3	4,230	377	1	Gαi/o	Inhibitory
5-HT1E	*HTR1E*	6q14-q15	79,378	365	2	Gαi/o	Inhibitory
5-HT1F	*HTR1F*	3p12	11,194	366	1	Gαi/o	Inhibitory
5-HT2A	*HTR2A*	13q14-q21	65,485	471	3	Gαq/11	Excitatory
5-HT2B	*HTR2B*	2q36.3-q37.1	16,889	481	4	Gαq/11	Excitatory
5-HT2C	*HTR2C*	Xq23	326,074	458	6	Gαq/11	Excitatory
5-HT3	*HTR3A*	11q23.1-q23.2	15,433	516	8	Ion channel	Excitatory
	HTR3B	11q23.1	141,889	441	9	Ion channel	Excitatory
	HTR3C	3q27	7,627	447	9	Ion channel	Excitatory
	HTR3D	3q27	7,826	454	8	Ion channel	Excitatory
	HTR3E	3q27	9,932	482	7	Ion channel	Excitatory
5-HT4	*HTR4*	5q31-33	226,204	428	8	Gαs	Excitatory
5-HT5A	*HTR5A*	7q36.1	15,426	357	2	Gαi/o	Inhibitory
5-HT5BP	*HTR5BP*	2q14.1	Pseudo-gene (in humans)				
5-HT6	*HTR6*	1p36-p35	14,276	440	3	Gαs	Excitatory
5-HT7	*HTR7*	10q21-q24	117,097	479	4	Gαs	Excitatory

1.2.6.2. G protein-coupled receptors

GPCRs are the largest family of membrane proteins and mediate most cellular responses to hormones and neurotransmitters, as well as being key for essential sensory functions like vision, olfaction and taste. Structurally, all GPCRs (also known as 7-transmembrane receptors or 7-TMs) are characterized by a common topology, composed seven-transmembrane α-helical segments, separated by three extracellular loops, three intracellular loops, an amino-terminal extracellular domain and a carboxy-terminal intracellular domain (Kroeze et al. 2002; Lefkowitz 2004). GPCRs are subdivided into five families, based on sequence and structural similarity: Rhodopsin (family A, to which serotonin GPCRs belong), secretin (family B), glutamate (family C), adhesion and Frizzled/Taste2. Of them, the rhodopsin family is the largest and most diverse, with unique combinations of signal-transduction activities involving multiple G-protein subtypes, as well as G-protein-independent signalling pathways and complex regulatory processes. Interestingly, despite this diversity, members are characterized by conserved sequence motifs that imply shared structural features and activation mechanisms. The classical (or canonical) role of GPCRs is to couple the binding of agonists to the activation of specific hetero-trimeric G proteins, leading to the modulation of downstream effector proteins.

G-proteins are comprised of a Gα subunit (classified into Gαi/o, Gαq/11, Gα12/13 and Gαs) according to their signalling system) and a dimeric inseparable Gβγ subunit, also known as the βγ complex. (see Table 1 and Figure 6). While the receptor is unstimulated, the Gα subunit binds guanosine diphosphate (GDP) and the βγ complex, and remains dissociated from the receptor. Receptor activation by its ligand at the extracellular GPCR domain, it induces conformational changes of the intracellular GPCR domain, giving rise to GPCR coupling to the G heterotrimer. Consequently, the Gα protein exchanges GDP for guanosine triphosphate (GTP), causing the dissociation of the GTP-bound α-subunit from the βγ complex and their separation from the activated receptor. Thus, Gα and βγ activate a cascade of further signalling events that finally result in a change in cell function. Serotonergic GPCRs bind to all three canonical Gα classes, with differential effects.

Gαi/o proteins (5-HT1 and 5-HT5 subtypes) inhibit several classes of adenylyl cyclases (AC), responsible for cyclic AMP (cAMP) generation. Gαq/11 proteins (5-HT2 subtypes) act regulating phospholipase C β-isoform (PLC-β), which produces diacyl glycerol (DAG) and inositol phosphate (IP), signalling molecules for protein kinase C (PKC), ultimately

leading to increased intracellular calcium. Finally, Gαs (5-HT4, 5-HT6 and 5-HT7 receptors) exert stimulatory regulation of ACs, resulting in the transformation of ATP into cAMP (Offermanns 2003; Nichols and Nichols 2008; McCorvy and Roth 2015).

In addition to the described canonical signalling, intense work in the field has revealed that GPCRs have more complex signalling behaviours, being able to couple to more than one G-protein (e.g. inhibition of cAMP possibly through Gαi activation by 5-HT2A,(Garnovskaya et al. 1995), or trigger non-canonical signalling via phosphorylation by G protein-coupled receptor kinases (GRKs) and subsequent β-arrestin recruitment, a mechanism in GPCR desensitization with clinical importance (Freedman and Lefkowitz 1996; Violin et al. 2014). The GPCR-β-arresting complex can also lead to receptor internalization, which controls both the duration of the response and subsequent downstream signalling of the receptor-ligand complex. On top of that, β-arrestins acting to attenuate G protein-dependent signalling can initiate parallel, G protein-independent signals (Lefkowitz and Shenoy 2005; Bohn and Schmid 2010; Giulietti et al. 2014).

The importance of serotonin receptors diversity comes from the fact that each receptor leads to diverse effects when stimulated. They are also differentially expressed in different areas across the brain and the whole organism, thus connecting serotonin to its wide range of functions (Hoyer et al. 2002; Berger et al. 2009).

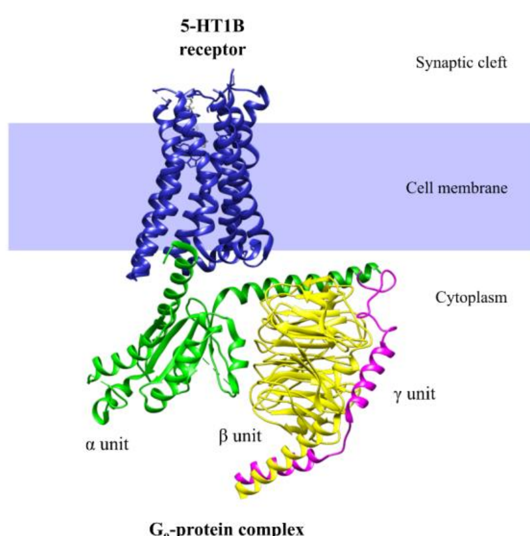


Figure 6. Structural model of the human 5-HT1B receptor (blue), one of the serotonergic GPCRs, bound to a G_o-protein complex, with α (green), β (yellow) and γ (pink) subunits. Built from protein model deposited in PDB (6g79), as published by García-Nafría et al. 2018.

5-HT1 receptors

This intron-less subfamily comprises five different receptors (5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E and 5-HT1F), with a high degree of identity in humans (40-63%, Hoyer and Martin 1997). The initially identified as 5-HT1C receptor was later re-classified as 5-HT2C, and therefore there is no 5-HT1C. Although these subtypes share 40–63% sequence homology, they exhibit different pharmacological properties and diverse expression patterns in the brain (Peroutka and Snyder 1979; Gerhardt and Van Heerikhuizen 1997; Masson et al. 2012; Di Giovanni 2013). The 5-HT1A receptor is the most extensively distributed of all the 5-HT receptors, it was the first 5-HT receptor gene identified, and one of the first of the family to be cloned in human and rat (Kobilka et al. 1987; Albert et al. 1990). In the central nervous system, 5-HT1A receptors are present in high density in the cerebral cortex, hippocampus, septum, amygdala, and raphe nucleus, in small amounts in the basal ganglia and thalamus (el Mestikawy et al. 1991), in myenteric plexus and whole gastrointestinal tract. The 5-HT1A receptor differs from other 5-HT receptors because it couples to different pathways, depending on the targeted cell. Its activity usually promotes a reduction in neuronal excitability and firing, provokes a variation in cAMP and $Ca2^{+}$, levels which may be linked to specific types of behaviour and cognition (Rojas and Fiedler 2016). As mentioned before, 5-HT1A receptors also act as autoreceptors receptors, thus inhibiting neuronal spike activity in the dorsal raphe nucleus and 5-HT release into the synaptic cleft (Barnes and Sharp 1999).

The 5-HT1B receptor is present in many parts of the human brain, specially basal ganglia, striatum and the frontal cortex. In the brain, 5-HT1B receptor are presynaptic, and are localized in axon terminals (Boschert et al. 1994), thus causing serotonin release inhibition and behavioural effects. It has been shown to be involved in several physiological functions, behaviours and psychiatric diseases including locomotor activity, drug abuse reinforcement, migraine, anxiety states and aggressive behaviour, as well as memory and learning processes (Buhot et al. 2003; Sari 2004).

It was originally thought that the 5-HT1B receptor is primarily or exclusively expressed in rodent tissues, whereas the closely related 5-HT1D receptor would be expressed in other species (such as human, cow, and dog), given their similar pharmacology, and the lower very low expression levels (Hannon and Hoyer 2008; Masson et al. 2012). While 5-HT1B is widely expressed in the CNS, 5-HT1D is predominantly expressed in the raphe nuclei,

substantia nigra, dorsal subiculum, and basal ganglia (Hoyer et al. 1990). Two other members of the 5-HT1 receptor family were identified in human as the 5-HT1E and 5-HT1F receptors, with closely related sequences. Interestingly, the 5-HT1E receptor is not found in some rodents, although it is present in the guinea pig genome. 5-HT1E is expressed in the cortical areas, caudate, putamen, and amygdala, while 5-HT1F is found in the dorsal raphe nucleus, hippocampus, cerebral cortex, striatum, thalamus, and hypothalamus (Adham et al. 1993; Bruinvels et al. 1994).

5-HT2 receptors

There are three 5-HT2 receptor subtypes (5-HT2A, 5-HT2B, and 5-HT2C). They share about 50% sequence identity in humans, and show similarities with regard to molecular structure, pharmacology, and signal transduction pathways. They couple preferentially to Gαq/11 proteins, which hydrolyses PIP2 to form IP3 and DAG. IP3 acts as a second messenger to stimulate the release of Ca^{2+} from the endoplasmic reticulum (ER), while DAG activates PKC. In addition, 5-HT2 receptors can activate phospholipase A2 (PLA2) to evoke the release of arachidonic acid (AA) or stimulate mitogen-activated protein kinases (MAPK), specifically extracellular signal–regulated kinases ERK1 and ERK2 (Conn and Sanders-Bush 1984; Masson et al. 2012). 5-HT2 receptors have a known role in muscle contraction and stimulation in the brain. 5-HT2A and 5-HT2C receptors show a broad distribution in the brain, whereas 5-HT2B expression is restricted to a few brain regions (cerebellum, lateral septum, dorsal hypothalamus, hippocampus, and medial amygdala) (Masson et al. 2012). 5-HT2A receptors are involved in a number of psychiatric disorders, including schizophrenia, as 5-HT2A expression is significantly decreased in patients and individuals at risk (Burnet et al. 1996; Hurlemann et al. 2005; Vazquez-Borsetti et al. 2009). In addition, 5-HT2A has been attributed to mediate other functions, such as platelet aggregation and increased capillary permeability after exposure to serotonin.

5-HT2B receptor plays a key role in the differentiation of cranial neural crest cells and heart development (Choi et al. 1997; Nebigil et al. 2001; Nebigil 2003). 5-HT2C receptor may play a

modulatory role in synaptic plasticity regulation (Kojic et al. 2000), depression (Millan 2005; Han et al. 2015) and feeding behaviour (Hurren and Berlie 2011; Zhang et al. 2013), among others.

5-HT4 receptor

The 5-HT4 receptor is encoded by *HTR4*, which spans >200 kB in the human genome, has 8 exons, and up to 11 splice variants (Wirth et al. 2017). 5-HT4 receptor is expressed pre- and post-synaptically in neurons of the CNS in various regions of the limbic system and basal ganglia components (Millan et al. 2008). 5-HT4 receptors have stimulatory effects on cellular activation states through increases in the activity of AC and cAMP, mediated via association with Gαs. It participates in multiple physiological processes, like respiratory rhythm, and in cognitive processes like short and long-term memory formation and learning (Millan et al. 2008; Bockaert et al. 2008). Furthermore, they are found in the gut epithelium and enteric neurons where, together with 5-HT3 receptors, promote motility, secretion, and enhance the development and survival of enteric neurons by exerting anti-inflammatory effects (Hoffman et al. 2012; Mawe and Hoffman 2013; Spohn et al. 2016; Spohn and Mawe 2017).

5-HT5 receptors

There are two types of 5-HT5 receptors: 5-HT5A and 5-HT5B. While mouse and other mammals (such as primates) have two different functional receptors, in humans 5-HT5B gene (HTR5BP) pseudogenized by the appearance of premature stop codons (Grailhe et al. 2001). Although the human 5-HT5A receptor was identified almost 25 years ago (Rees et al. 1994), relatively little is known so far about the signalling pathways and functions of this subfamily. In adult mice, this receptor has been detected in the cerebral cortex, dentate gyrus, hippocampus, cerebellum, astrocytes and olfactory bulb (Plassat et al. 1992; Carson et al. 1996). In addition, it is expressed in the circadian timing system of rodents suggests that 5-HT5 receptor may be involved in circadian rhythm regulation (Duncan et al. 2000).

5-HT6 receptor

The 5-HT6 receptor was first cloned in rat in 1993, and characterized by its high affinity for typical and atypical antipsychotics (Ruat et al. 1993a; Sleight et al. 1998). 5-HT6 receptors are expressed postsynaptically in multiple brain areas (i.e. nucleus accumbens, cortex, olfactory tubercle, cortex, hippocampus, thalamus, amygdala, hypothalamus, and cerebellum) (Ruat et al. 1993a; Yoshioka et al. 1998). Activation of 5-HT6 stimulates AC activity by interacting with Gαs. It has been observed that it subsequently activates the

protein kinase A (PKA) pathway in mouse neuroblastoma cell lines and striatal neurons, and pig caudate membranes, although the mechanisms are still incompletely known (Sleight et al. 1998; Masson et al. 2012). Upon activation, 5-HT6 interacts with the Tyrosine protein-kinase Fyn, a key developmental target of the Src kinase family, prompting Erk1/2 phosphorylation in a Fyn-dependent manner (Yun et al. 2007). In turn, Fyn can increase 5-HT6R surface expression via direct binding to its C-terminus. The 5-HT6R also physically interacts with Jun activation domain-binding protein-1 (Jab1), resulting in Jab1 translocation to the nucleus, consequently increasing phosphorylation of the transcription factor c-Jun (Yun et al. 2010).

5-HT6 has been associated with memory and learning, and emerged as a promising target for treating various mental and neurodegenerative diseases, such as schizophrenia, PD, and especially AD (Da Silva Costa-Aze et al. 2012; Benhamú et al. 2014; Claeysen et al. 2015; Quiedeville et al. 2015; Liu et al. 2016; Ferrero et al. 2017; Khoury et al. 2018). Learning and memory potentiation is correlated with increased excitatory activity in the glutamatergic system and with decreased inhibitory activity in the GABAergic system (Myhrer 2003; Codony et al. 2011). 5-HT6 receptor is located on GABAergic and glutamatergic principal neurons, and on a subset of interneurons that express the 5-HT3A receptor. 5-HT6 receptor may thus regulate the balance between excitatory and inhibitory signalling in the brain (Helboe et al. 2015). Indeed, 5-HT6 antagonists have antiamnesic effects, and the expression of 5-HT6 receptors is diminished in AD patients (Benhamú et al. 2014).

In recent times, 5-HT6 receptor have become a potential candidate to be involved in neurodevelopment, as it regulate a variety of steps and cellular processes required for precise neural circuit assembly (Wang et al. 2014; Dayer et al. 2015).

5-HT7 receptor

The 5-HT7 receptor was discovered independently by several laboratories in 1993 (e.g. (Bard et al. 1993; Plassat et al. 1993; Lovenberg et al. 1993; Ruat et al. 1993b) and identified as associated with AC and cAMP formation, and involved in circadian rhythm regulation in mammals. Indeed, 5-HT7 couples with the Gαs proteins, which leads to AC activation, activating the PKA pathway and a plethora of downstream cell-specific signalling cascades. Interestingly, 5-HT7 can activate AC independently of Gαs, and activate Erk in a PKA-

independent manner, likely involving EPAC exchange protein, which is activated by cAMP (Lin et al. 2003). mRNA products of *HTR7* are spliced, producing variants that differ in their intracellular C-terminal tail. Such variants differ in terms of pharmacological properties, yet they use similar transduction mechanisms (Vanhoenacker et al. 2000).

Today, we know that 5-HT7 is implicated in both central and peripheral endocrine-related processes, such as sleep-wake cycle and body core temperature control, vasomotricity, nociception, as well as in learning, memory and affective processes (Matthys et al. 2011; Masson et al. 2012; Gellynck et al. 2013; Meneses 2017).

1.2.6.3. Ligand-gated ion channels (pLGICs)

Since this thesis focus on the 5-HT3 receptor, more details about its structure and genetics, as well as about the pLGIC family are provided below. The pentameric ligand-gated ion channels (pLGICs), also known as "Cys-loop" receptors, are a very ancient superfamily of ionotropic neurotransmitter receptors, which biological function is to link neurotransmitter release with ion permeability through an ion channel, thus mediating fast excitatory or inhibitory synaptic transmission through cell depolarization or hyperpolarization, respectively (Dent 2006; Thompson et al. 2010). In addition to neuronal communication, some mammal pLGICs are involved in several physiological processes in various tissues, such as epithelial or immune cells (Wessler and Kirkpatrick 2008; Sanders et al. 2015). pLGICs are protein complexes of 150 to 300 kDa, made up of five identical or homologous symmetrically arranged subunits, filling a cylinder at least 12 nm in height and 7 nm in diameter, and whose rotational axis coincides with the central ion pathway (Figure 7). The binding of an extracellular neurotransmitter produces global conformational changes that stabilize an open channel conformation, and prolonged or repetitive application of neurotransmitters promotes a closed/desensitized state, refractory to activation (Katz and Thesleff 1957; Papke et al. 2011). Furthermore, pLGIC can adopt many different conformational states in order to meet the needs for regulation (Corringer et al. 2012; Nemecz et al. 2016).

In vertebrates, members of this family (with more than 40 genes identified so far) are classified according to their pharmacology, and include nicotinic acetylcholine receptors (nAChRs), gamma-aminobutyric acid type A receptors ($GABA_A$Rs), Glycine receptors (GlyRs), zinc-activated receptors (ZAC) and 5-HT type 3 receptors (5-HT3Rs), being the

latter the focus of the present Thesis. They are also classified in terms of their synaptic effect, dictated by the charge of the ions that flow through the channels, and they are consequently divided into excitatory cationic (nAChRs, ZAC, and 5-HT3Rs) which are permeable to sodium (Na^+), calcium (Ca^{2+}) and potassium (K^+) ions; and inhibitory anionic Cys-loop receptors ($GABA_A$Rs and GlyRs), permeable to chloride ions (Cl^-). Each of these receptor types have different subunits, that are codified by different genes (over 40 genes in vertebrates, Dent, 2010): nAChRs have 17 known subunits (α1-α10, β1-β4, γ, δ, and ε), $GABA_A$Rs have 19 (α1-α6, β1-β3, γ1-γ3, δ, ε, θ, π, and ρ1-ρ3), GlyRs have five (α1-α4, and β), ZAC have only one, and 5HTR3 have five (named A-E). The majority of the subunits, predominantly in neuronal nAChRs and $GABA_A$Rs, assembles in obligatory heteropentamers, while 5-HT3 may form 3A homopentamers, or heteropentamers, combining at least one 3A subunit with one or more of the other types (more on this below). The combinatorial association of subunits generates a large number of receptors with different subunit compositions, stoichiometries, and functional properties, which, together with splice variants, adds complexity to the pLGIC family (Nemecz et al. 2016). With only one known subunit, the ZAC receptor was the last of the family to be discovered, and its function is still unknown (Davies et al. 2003). Although it received its name due to its activation by zinc ions (Zn^{2+}), latter studies showed that it can be activated by hydrogen (H^+) and copper (Cu^{2+}) ions too (Trattnig et al. 2016). ZAC displays very little amino acid sequence similarity with other members in the superfamily, being the human 5-HT3A, 5-HT3B and nACh α7 subunits (~15% amino acid sequence identity) the closest matches to ZAC (Houtani et al. 2005). Interestingly, the pLGIC superfamily combines a seemingly paradoxical lack of conservation at the level of sequence with a highly conserved underlying structure (Dent 2010).

1.2.7. The 5-HT3 receptor

The 5-HT3 receptor (or 5-HT3R) is a typical Cys-loop receptor. It is cationic, closely related to nAChRs, and the only serotonergic pLGIC known to date. In the past few years, the 3D structure of the 5-HT3 receptors was revealed by X-ray crystallography (Hassaine et al. 2014, Figure 7), cryo electron tomography (Kudryashev et al. 2016) and cryo-electromicroscopy (Basak et al. 2018), which have brought useful insights into the receptor physiology and function. Like all other Cys-loop receptors, 5-HT3 is bullet-shaped, with five

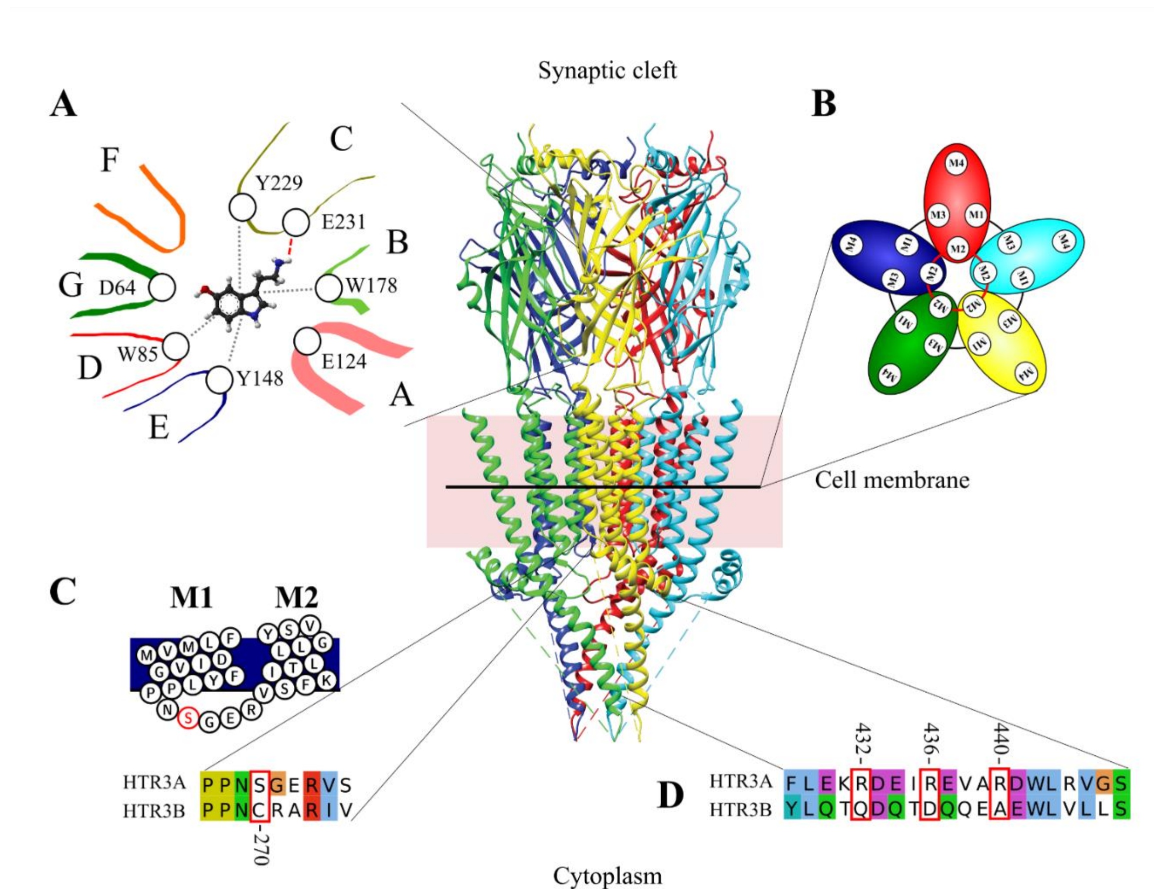


Figure 7. Important features of the 5-HT3 receptor. Each color in the receptor represents a different subunit. **A.** The serotonin binding pocket in the 5-HT3 ECD, formed by 7 loops from the principal (Loops A – C) and the complementary (Loops D – G) subunits. Dashed lines show the residue-serotonin interaction, with π-π interactions in black and ion bridges in red. Adapted from Yuan et al., 2016. **B.** Schematic view of the TMD from the outside, highlighting M2 domain disposition close to the pore, forming an inner ring, and M1-M3 forming an outer ring. Adapted from Wu et al., 2015. **C.** M1-M2 short intracellular loop, suggested to be responsible for differential 3A-3B trafficking to the membrane (Boyd et al., 2003). Key site is highlighted in a red box. **D.** MA-helix in the ICD, highlighting the key sites responsible for differences in receptor conductance between 3A homomers and 3AB heteromers, as described by Kelley et al., 2003. All sequences shown correspond to human genes. All site positions are referent to the canonical human 5-HT3A protein (UniprotID: P46098). Receptor structure built from mouse 5-HT3A X-ray crystal structure deposited in PDB (4pir), published in Hassaine et al., 2014.

subunits forming the channel around the central pathway. The essential ECD and TMD are structurally similar to other pLGICs, but it has a long ICD, with 70-150 residues, which is absent in many pLGICS (e.g. the prokaryotic ELIC and GLIC, and eukaryotic GlyR and $GABA_AR$, but present in nAChRs), and though not being essential for receptor function (Bocquet et al. 2007; Jansen et al. 2008), it is critical for receptor clustering at the synapse (Thompson et al. 2010; Zuber and Unwin 2013), gating kinetics (Bouzat et al. 1994), and conductance (Kelley et al. 2003; Peters et al. 2010).

As mentioned above, five different subunits forming 5-HT3 receptors have been discovered thus far, named 5-HT3A, -B, -C, -D, and -E subunits (see sections below). Of these subunits, only 5-HT3A can form functional homomeric 5-HT3 receptors, although other subunits can form functional heteromeric receptors in co-expression with 3A subunits (Niesler et al. 2007; Holbrook et al. 2009). More details on the different subunits will be provided in the next sections.

1.2.7.1. Discovery and history

The 5-HT3 receptor was first identified by Gaddum and Picarelli as "M" receptor, which was located mainly in the nervous system, and were inhibited by cocaine, morphine and atropine. In addition, they identified a different receptor in the muscle, which they called the "D" receptor, which was later classified as a 5-HT2 receptor (Gaddum and Picarelli 1957; Hoyer et al. 1994). In the 1980s, the first selective antagonists were developed, and their effects as potent antiemetics was evidenced (Miner and Sanger 1986; Costall et al. 1986), which led to the development of other antagonists, such as ondansetron and granisetron. In 1989, the identification of 5-HT3 receptors as ion channels was established (Derkach et al. 1989), and two years later, in 1991, the first subunit (5-HT3A) was cloned (Maricq et al. 1991).

A particularity of 5-HT3 among the pLGIC superfamily (at least until ZAC was discovered in 2003) was that it was the only to form form functional homomeric receptors, with five 3A subunits forming it. However, something was intriguing: 5-HT3 receptors expressed in HEK-293 cells had very different single-channel conductance than those observed in rabbit nodose ganglion (Gill et al. 1995). The mystery lingered for some years, until a new subunit, 5-HT3B was identified as responsible for the differences (Davies et al. 1999; Dubin et al. 1999). Indeed, homomeric 5-HT3A receptors have single-channel

conductance (i.e. the measure of the ease the receptor offers for an ion current to pass from it, inverse to electrical resistance) <1 pS, while heteromeric receptors can reach ~16-30 pS (Davies et al. 1999; Kelley et al. 2003; Peters et al. 2010). In 2003, three additional sequences were discovered in humans by Niesler and colleagues (Niesler et al. 2003), which they called 5-HT3C, 5-HT3D and 5-HTR3E, adding more complexity to the 5-HT3 system. Initially, their absence in the mouse genome (Karnovsky et al. 2003) led researchers to think that they were exclusively human, yet later they were found in other primate and non-primate mammals, thus confirming that they were lost in the rodent lineage (Holbrook et al. 2009).

I will explore the evolution of these genes in more detail in Chapter 4.

1.2.7.2. Distribution

5-HT3 receptors are widely expressed in both PNS and CNS. In the CNS, the highest levels of 5-HT3 binding sites were found in the dorsal vagal complex, in the brainstem. This region comprises the nucleus tractus solitarius (NTS), area postrema and dorsal motor nucleus of the vagus nerve, which are key to the initiation and coordination of the vomiting reflex (Doucet et al. 2000; Chameau and Van Hooft 2006). This may explain the effectiveness of 5-HT3 selective antagonist in treating radiation- and chemotherapy-induced emesis. In addition, a common adverse effect of 5-HT3 receptor antagonists for antinausea and antiemetic purposes is constipation, an action that has been exploited for the treatment of IBS-associated diarrhoea (Talley 1992).

5-HT3 receptors are also expressed in some areas of the forebrain, with highest levels of expression within the amygdala and hippocampus, as evidenced by studies in rat, human, and marmoset brains (Barnes et al. 1990; Tecott et al. 1993; Parker et al. 1996; Chameau and Van Hooft 2006; Shukla et al. 2014). Studies analysing 5-HT3 expression in human brain established the highest density of 5HT3A subunit in hippocampus, striatum, amygdala and thalamus (Houtani et al. 2005). The highest density of 5HT3B receptors was detected in brain stem including hippocampus, amygdala, thalamus and frontal cortex (Hammer et al. 2012). Such findings made 5-HT3 receptor to be subject of growing interest as a potential target involved in emotional and affective disorders, like depression (Gupta et al. 2016). Recently, involvement of the 5-HT3Rs was found in a number of medically relevant processes such as drug addiction, cognitive function, schizophrenia, satiety control, and

immune regulation (Thompson and Lummis 2007; Walstab et al. 2010b; Kato 2013; Browning 2015; Li et al. 2015).

Interestingly, there are marked differences in receptor expression at the regional and cellular level among species. For example, in human forebrain the highest levels are found in the striatum (putamen and caudate nucleus), while lower levels are found in cortical regions (Parker et al. 1996), while this pattern is reversed in rodents (Steward et al. 1993). In contrast, a recent study found 5-HT3A to be restricted to the hippocampus in marmoset and no 5-HT3B expression (Shukla et al. 2014), although Jakab and Goldman-Rakic found 5-HT3A receptors at the cell body of cortical neurons in macaques (Jakab and Goldman-Rakic 2000). Altogether, this evidence support the idea of differences in the expression pattern of 5HT3A in the cortex between species.

In the GI tract, 5-HT3 receptors are found primarily on vagal afferent neurons and fibres (e.g., Lacolley et al. 2006; Babic et al. 2012), and in intrinsic primary afferent neurons (IPAN; Bertrand et al. 2000).

5-HT3 receptors can be localised either pre- or postsynaptically and their activation can modulate the release of a range of neurotransmitters, including dopamine, GABA, cholecystokinin, substance P, and ACh (Miquel et al. 2002; Thompson and Lummis 2006). For example, 5-HT3 receptors have been found presynaptically in varicosities of GABA interneurons in the rat hippocampus and human frontal cortex (Fink and Göthert 2007; Barnes et al. 2009; Leiser et al. 2015), suggesting that the release of GABA is, at least in part, mediated by Ca^{2+} influx into presynaptic terminals (Koyama et al. 2000; Katsurabayashi et al. 2003; Turner et al. 2004). Post-synaptically, 5-HT3 receptors are involved in regulating neural network excitability, derived from its fast synaptic transmission properties (Chameau and Van Hooft 2006).

1.2.7.3. Subunits and receptor properties

The genes coding for 3A and 3B subunits (*HTR3A* and *HTR3B*) are located next to each other in the long arm of human chromosome 11 (11q23). They share 41% amino acid sequence identity, and probably arose by local duplication event (Davies et al. 1999). Genes encoding 3C, 3D and 3E (*HTR3C*, *HTR3D* and *HTR3E*) are also located next to each other in the long branch of chromosome 3 (3q27, Karnovsky et al. 2003).

Subunits 5-HT3C, 5-HT3D and 5-HT3E were more recently discovered, and less well explored than 5-HT3A and 5-HT3B, although 5-HT3C and 5-HT3E subunits are implicated in a range of neurological disorders including pregnancy-related nausea, irritable bowel disease, schizophrenia and autism (Niesler et al. 2007; Holbrook et al. 2009; Schuhmacher et al. 2009; Rehnström et al. 2009; Walstab et al. 2010b; Yaakob et al. 2011). The 5-HT3D subunit was initially considered to be non-functional, as it lacks the signal peptide and a large region of the N-terminal domain, including the Cys loop and loop D (Niesler et al. 2003), although it was later proved that a longer form of this subunit exists, with most of the expected Cys-loop features (Holbrook et al. 2009). Interestingly, comparative expression analyses in humans revealed different expression patterns among subunits, with 3A and 3B expressed both in the CNS and gut (yet preferentially in the CNS) while C-E subunits show a peripheral restricted pattern, with high levels in the GI tract particularly in myenteric neurons and submucosal plexus of the large intestine), kidney and liver (Niesler et al. 2003; Tzvetkov et al. 2007; Barnes et al. 2009; Kapeller et al. 2011). This evidence points to a specific role of CDE heteroreceptors in the PNS of some species, distinct from the more general functions of 5-HT3A homo- and 5-HT3AB heteroreceptors.

Molecular and functional characterization indicated that no subunit other than 5-HT3A can form functional homomeric 5-HT3, but other subunits can form functional heteromeric receptors, with different physiological properties, in co-expression with 3A. One of the reasons for the inability for other subunits to form homomeric receptors was suggested to be that they cannot reach the cell membrane on their own. Boyd and colleagues (2003) showed that 5-HT3B subunit fails to exit the endoplasmic reticulum (ER) due, at least in part, to a retention motif (CRAR, SGER in 5-HT3A) within the short first intracellular loop between M1 and M2 (Boyd et al. 2002; Boyd et al. 2003, Figure 7C). When co-expressed with 3A, 3A subunit may shield this motif, thus allowing the heteromeric receptor reach the cell membrane. The same conclusion was reached for 3C, 3D, and 3E subunits, thus indicating that 5-HT3A plays a crucial role in receptor trafficking (Niesler et al. 2007). However, other researchers found that B, C, D and E subunits can independently reach the membrane although not form functional A-less receptors (Holbrook et al. 2009). The reasons for these discrepancies are yet unclear, but it may lay in the differences in the expression systems used (e.g. HEK and CHO cells) and/or their intracellular machinery (e.g. glycosylation machinery, chaperones), which are important for trafficking and expression (Price et al.

2017). The assembly and trafficking of newly synthesized proteins is achieved by the action of chaperones, and pentameric ligand-gated ion channels (pLGICs) are no exception, with many interacting proteins have been identified so far (Collingridge et al. 2004). Specifically, the resistance to inhibitors of cholinesterase type 3 (RIC-3) protein is crucial for maturation and folding of some pLGICs, including nAChRs and 5-HT3R (Halevi et al. 2002; Castillo et al. 2005; Walstab et al. 2010a; Alexander et al. 2010), and it was suggested to be essential for homomeric (but not heteromeric) 5-HT3A receptor trafficking and expression on the cell membrane (Cheng et al. 2005; Cheng et al. 2007; Walstab et al. 2010a). In addition, post-translational modifications, like N-glycosylation promote subunit recruitment and expression in the membrane (Monk et al. 2004; Massoura et al. 2011).

1.2.7.4. Receptor Structure

The 5-HT3 receptor is structurally a typical pLGIC. In this section I will provide further details on the receptor structure and properties, many of which are shared with other pLGICs.

The Extracellular domain

The extracellular domain (ECD) comprises the most external part of the receptor, and contains the agonist binding site (Figure 7A). It consists of an N-terminal α-helix and a core of ten β-strands that form an αβ-sandwich structure. The inner β-sheet is formed by β1, β2, β3, β5, β6 and β8 and the outer β-sheet by β4, β7, β9 and β10. The N- and C-termini are located at the top and bottom of the pentamer fold, respectively. The C-terminus of β10 is connected to the N-terminus of TM1. The linker between strands β6 and β7 forms the signature Cys-loop (a couple disulphide-bonded Cys residues forming a structure loop), which is close to the transmembrane domain and may play a role in the propagation of conformational changes from the ECD to the transmembrane domain (TMD) (Yakel 2010). The degree of conservation of the Cys-loop made this family be also called Cys-loop receptors. Once considered to be universal, functional Cys-less prokaryotic receptors were discovered in the cyanobacteria *Gloeobacter violaceus* (GLIC, Bocquet et al. 2007) and in the enterobacteria *Dakeya chrysanthemi* – formerly known as *Erwinia chrysanthemi* (ELIC, Zimmermann and Dutzler 2011). Posteriorly, evolutionary studies searching for deep pLGIC homologues across the Tree of Life found the Cys-loop not to be present in many domains of life, such as invertebrate Metazoa, Prokaryota and Archaea (Jaiteh et al. 2016). Instead,

Jaiteh and colleagues found a “universally” conserved proline residue, proximate to the Cys-loop tips, and proposed “Pro-loop receptors” as a more appropriate name.
At rest, pLGICs are in closed conformation. Activation of pLGICs involves a cascade of structural events that occur in the μs to ms timescale (Chakrapani and Auerbach 2005). Ligand binding induces the C-loop to move towards the bound ligand, and then a wave of structural change occurs across the ECD, including β1 and β2 strands, which are in close proximity to the TMD. These changes promote conformational change in the TMD and eventual channel opening (Bouzat 2012; Althoff et al. 2014).
An interesting pattern in pLGIC ECD is the over-representation of aromatic residues, especially Tyr and Trp, if we compare with other proteins. This is probably due to the appropriate characteristics of these side chains for molecular recognition, since they can form non-polar, H-binding, and cation-π interactions (Koide and Sidhu 2009). Given that Cys-loop receptors are gated by chemically different compounds, there must naturally be a substantial degree of divergence at critical agonist-binding side chains in the agonist-binding site. Indeed, when considering the most conserved residues in the pLGIC ECD, they lie outside the agonist-binding loops (Hibbs and Gouaux 2011). However, two motifs within agonist-binding loops are conserved across all Cys-loop receptors: a tryptophan in Loop D (W85 in 5-HT3A), and a Trp-X-Pro (or WxP, “x” meaning any amino acid) motif in Loop A (W116-P118 in 5-HT3A), which is known to contribute to structural integrity, at least in 5-HT3 receptors (Deane and Lummis 2001).

The ligand binding site comprises a hydrophobic box formed between three peptide loops (termed Loop A C) from one subunit and three β-sheets (termed Loop D–G) from another subunit (Sine 2002; Yuan et al. 2016, see Figure 7A). These subunits are called principal and complementary subunits, respectively. Noteworthy, this loop nomenclature was proposed before the receptor secondary structure was unravelled by crystallography, and hence the counter-intuitive denomination, since they are not all loops. Some proposed key residues in serotonin binding are E124 (although N123 has also been proposed to interact with serotonin, see Lynagh and Pless 2014) in Loop A, W178 in Loop B, Y229 and E231 in Loop C, and W85 in Loop D (Figure 7A; Lummis 2012; Lynagh and Pless 2014; Yuan et al. 2016). Some other sites have been proposed to have relevance (e.g. sites in Loop E and Loop F, Venkataraman et al. 2002; Thompson et al. 2006), but their specific role less clear. Single-point mutations in nAChR and 5-HT3 receptor ECD are sufficient to switch agonist actions

of serotonin and acetylcholine, respectively, evidencing that share a broadly similar binding site architecture with overlapping ligand recognition characteristics (Palma et al. 1996; Steward et al. 2000; Thompson and Lummis 2006).

The 5-HT3 serotonin binding pocket is similar to that of acetylcholine in the nAChR, in that the amine nitrogen of serotonin forms a cation-π interaction with a W in the Loop B (W178, Beene et al. 2002) and the polar 5-hydroxyl is likely oriented toward the complementary face, according to mutagenesis and homology modelling (Beene 2004). Such arrangement is supported by the crystal structure of a serotonin-bound sea slug (*Aplysia californica*) AChBP, with substitutions equivalent to those of 5-HT3 receptor that enhance serotonin binding (Kesters et al. 2013). Thus, it is only a few differences that confer on 5-HT3 receptor its selective recognition of serotonin, including a Loop A E124 (N in nAChRs) and a pre-Loop B 174T (K in most nAChRs and AChBPs), perhaps in combination with a longer Loop C (Kesters et al. 2013; Lynagh and Pless 2014).

It has been suggested that in 5-HT3 the binding pocket lies in an A-A interface (i.e. between two 3A subunits, Brady et al. 2001; Lochner and Lummis 2010), an idea supported by the finding that homomeric and 3AB receptors are similar in their pharmacology. However, Barrera and colleagues determined, using atomic force microscopy, a 5-HT3AB receptor with B-A-B-B-A arrangement (Barrera et al. 2005). Currently, there is no consensus on how many serotonin molecules are required to activate the 5-HT3 receptor although Rayes and colleagues reported between two and five serotonin molecules (Rayes et al. 2009).

The Transmembrane domain

The transmembrane domain (TMD) is composed of four α-helices, named TM1-TM4, which perpendicularly span through the membrane bilayer. They are distributed in a similar way across the five subunits, with TM2 helices forming the ion channel pore, forming a closely packed bundle with TM1 and TM3 (which form a concentric outer ring), and TM4 in the most peripheral part of the receptor (Figure 7B).

The TMD is known to contain several allosteric sites, to which a wide range of both organic and inorganic substances can bind for regulation (Changeux et al. 1984). For example, ions, anaesthetic drugs like benzodiazepines, as well as alcohol and other therapeutics like Ivermectin can modulate pLGICs allosterically, adding up for their pharmacological importance (Thompson et al. 2010). These modulators can act stimulating or inhibiting the

channel, and are consequently named positive or negative allosteric modulators” (PAMs/NAMs, respectively). PAMs/NAMs typically exhibit little intrinsic activity but provide selective potentiation/inhibition of physiological activity without directly interfering with the ongoing signalling processes (Wu et al. 2015). The extracellular M2–M3 loop forms part of the interface that links the ECD with the TMD. It has a critical role in transmitting the energy of binding into channel opening, and mutations in this region disrupt activation in nACh, 5-HT3, GABA and Gly receptors (Grosman et al. 2000; Deane and Lummis 2001).

The Intracellular domain

The intracellular domain (ICD) comprises the cytoplasm-facing region of the receptor, which despite not being universal for every pLGIC nor indispensable for receptor function, it plays important roles in trafficking and clustering at the synapse plasma membrane, gating, post-translational modification, single-channel channel conductance, rectification, desensitization kinetics and intracellular regulation of channel function (Peters et al. 2004; Connolly 2008; Baptista-Hon et al. 2013). In 5-HT3A it comprises a 70-150 amino-acid chain which sequence is the least conserved among pLGICs, and which structure has been only partially resolved. Indeed, structural models from X-ray crystallography (Hassaine et al. 2014) and Cryo-EM (Basak et al. 2018) described two α-helical portions in the post-M3 and pre-M4 section, named MX-helix and MA-helix respectively. MX-helix could have only its firsts 20 residues structure determined as an α-helix that extends away from the pore, forming a belt around the MA helical bundle. The structure of the rest of the MX-helix could not be modelled, due to the flexible and disordered nature of this region. The MA-helix is structurally a continuous extension of M4, projecting interiorly into the cytoplasm. The ICDs of the five receptor subunits converge in the central axis forming an inverted tepee shape, thus forming a vestibule perforated by five narrow fenestrations (‘portals’) that form an obligate pathway through which ions must flux (Peters et al. 2010; Carland et al. 2013).

As mentioned above, 5-HT3AB receptors differ from 5-HT3A homoreceptors in conductance (< 1pS vs. 16-30 pS). Yet they also in other biophysical properties, like their EC50 (i.e. half maximal effective concentration, or the concentration of a drug to elicit a response halfway between baseline and maximum; used for measuring drug efficiency), Hill slope (i.e. the slope of the dose-response curve), desensitization kinetics, and the shape of current-voltage relationship (Dubin et al. 1999; Davies et al. 2003; Livesey et al. 2008; Peters

et al. 2010), although these differences seem to change depending on the species studied (Thompson and Lummis 2013).

Apparently, the reason for the differences in single-channel conductance lies in the ICD, particularly in the MA-helix (Kelley et al. 2003; Peters et al. 2004; Peters et al. 2005; Hales et al. 2006). Indeed, Kelley and colleagues identified three amino-acid positions in the MA-helix that were responsible for these differences. Human 5-HTR3A has three arginines (RRR) at positions 432, 436 and 440 (according to canonical protein co-ordinates, UniprotID: P46098), while 5-HT3B has glutamine, aspartate, and alanine (QDA) at these positions (Figure 7D). Replacing amino-acids at those specific positions in 5-HT3A by those of 5-HT3B had a dramatic effect on conductance, being position 436 especially relevant. These findings were further supported by Hales and colleagues (Hales et al. 2006), who extended the experiment to nAChRs, confirming the role of these sites in conductance. In addition, the ICD has been suggested to be the driver for receptor oligomerization and for pentamer formation, as well as being able and sufficient for interaction with RIC-3 (Pandhare et al. 2016).

1.3. Evolutionary perspectives

One of the biggest enigmas surrounding serotonin is how such a simple neurotransmitter can be in charge of such diverse and essential functions in the organism. This enigma is further complicated if we consider that serotonin exist in every organ, and in nearly all eukaryote organisms (Salmoun et al. 2002; Gillette 2006; Azmitia 2007; Azmitia 2010; Erland et al. 2016).

The widespread distribution, closely reaching the root of the Tree of Life, together with its multiple functions supports the idea that serotonin is phylogenetically very ancient, and that it evolved prior to the emergence of neurons. Serotonin evolutionary history is inevitably linked to that of its precursor tryptophan and its metabolites, a history that probably began in unicellular organism around 3 billions years ago (Azmitia 2010). Serotonin synthesis from tryptophan requires oxygen at the first enzymatic step, carried out by tryptophan hydroxylases (TPH1 and TPH2 in humans, as reviewed in *Biosynthesis and metabolism* section of the present Thesis), and due to the low levels of oxygen in the early stages of life on Earth, only organisms capable of producing oxygen via photosynthesis would be able to produce serotonin. In fact, tryptophan structure contains an indole ring that absorbs light,

with peak fluorescence absorption at UVB, and emission in the range of UVA-blue light, making it an essential amino acid for light absorption by chloroplasts, and consequently for photosynthesis (Lin and Sakmar 1996; Angiolillo and Vanderkooi 1996; Vavilin et al. 1999).

Interestingly, some of the tryptophan derivatives (e.g. melatonin and auxin), present in plants and algae are powerful antioxidants, and act as protectors against oxidative photosynthetic by-products, such as reactive oxygen species (ROS) (Grossweiner 1984; Asada 2006). Also, auxin and other serotonin-like tryptophan derivatives regulate the movement of roots, and guide leaves towards light sources (Ivanchenko et al. 2006).

The enzymatic machinery needed for tryptophan synthesis is widely conserved in plants and prokaryotes, which together with high oxygen availability, allows plants to produce serotonin in high concentrations (Radwanski and Last 1995). On the other hand, animals lack chloroplasts and tryptophan biosynthetic enzymes, thus depending on food intake for tryptophan supply. In consequence, serotonin production and concentrations in animals are very low compared to plants, which may explain why serotonin-producing cells are relatively few in animals, and why they concentrate in discrete, specialized areas of the body, as animal neural systems increase in complexity.

Neurons and neural systems are among the most astonishing products of evolution, which allowed animals to develop and maintain increasingly complex behaviours while evolving large multicellular bodies. The emergence of neurons along animal evolution has been a hotly debated topic for decades, and despite the advent of new techniques and genomic data, we have not yet been able to elucidate their origin. Such difficulties stem from the fact that neurons are complex entities, composed of varied molecular machineries with different evolutionary histories, and establishing connections between them and their function in ancient organisms from genomic data only is not feasible (Liebeskind et al., 2017). Still, the advent of genomics and the consequent improvement of our knowledge about the tree of life has provided some useful insights into the evolutionary scenarios in which neural systems emerged. The sequencing of genomes from Ctenophora phylum, and its placement as a sister group to all other Metazoa (Moroz, et al., 2014; Moroz, 2015, Wheelan et al., 2015) implied a changed the paradigm, since this basal animal lineage has complex neuron systems and behaviours, while sponges and placozoans do not. This led to two contending hypotheses: (1) neural systems have a single origin, and were subsequently

lost in sponges and placozoans (Rokas, 2013), or (2) neural systems arose independently in ctenophores and cnidarian/bilaterian animals (Moroz 2015).

A hallmark of all neural systems is signal transduction from neuron to neuron, which is mediated by a varied array of receptors (some of them have been reviewed above) present in most organisms, that experienced independent expansions and losses across animal lineages. All major neurotransmitters use ionotropic (e.g. pLGICs) and metabotropic (e.g. GPCRs) receptors that are distant phylogenetically, and show substantial differences between vertebrates and invertebrates, suggesting a role of convergent evolution in receptor specificity. Interestingly, ctenophores do not use serotonin as a neurotransmitter, nor have receptors for it – nor pLGICs altogether. Instead, they seem to rely on glutamate, GABA, and other specific signal molecules without recognized analogues in metazoans (Moroz et al. 2014; Moroz 2015).

Strikingly, the evolution of neural systems is very slow, when compared with behavioural evolution, and neural-related genes show among the lowest evolutionary rates in vertebrates (Wang et al., 2007; Roux et al., 2017), which indicates a possible role of mechanisms like compartmentalization and subfunctionalization in the evolution of neuromodulation and behaviour (Katz & Lillvis, 2014).

1.4. New World Monkeys

The Platyrrhini, or New World Monkeys (NWM) are a parvorder of arboreal primates that live exclusively in the tropical regions of Central and South America. NWMs have an evolutionary history that dates back to ~30-43.5 Mya since they split from their African relatives (Perelman et al., 2011; Pérez et al., 2013; Fleagle et al., 2013). They are the only primates besides humans to occupy the continent, and they experienced an adaptive radiation that turn them into a diverse group in terms of morphology, ecological niches, and behaviours (Aristide et al., 2015). Some morphological characteristics of this group include their small to medium size (~100g-10Kg), their flat nose with often sideways-directed nostrils (hence the taxon Latin name *platy-* = flat, *-rrhini* = nose), and the prehensile tail present in some species, that it used as a fifth limb. In addition, NWMs show some reproductive and behavioural characteristics that are rare among primates, such as twinning (Harris et al., 2014), direct paternal care (Fernandez-Duque et al., 2009), complex (and diverse) social

organization (Fleagle et al., 2013) and social monogamy (Leutenegger, 1980), which make them animal models of outstanding interest for behavioural genetics research. Chapter 3 includes a study on the genetic diversity of *HTR3A* in 20 different NWM species, and explores the relationship between this diversity and behavioural and life-history traits.

Chapter 2. Justificative and Objectives

Justificative and general objective

Since the discovery of 5-HT, great advances have been made in the fields of Neuroscience, Genetics, and Medicine towards understanding serotonin functions and its mechanisms. However, few works to date have aimed to investigate the serotonergic system in an evolutionary context. Such knowledge would provide useful insights on how serotonergic functions emerged and change over time, and thus the present Thesis will focus on the molecular evolution of the 5-HT3 receptor gene family (*HTR3A, HTR3B, HTR3C, HTR3D, HTR3E*) in chordates, with focus on New World monkeys (Platyrrhini), in order to understand the evolutionary patterns that arose during the evolutionary history of the receptor, and how such patterns are connected with behavioural traits.

Specific objectives

(1) To analyse taxon-specific variants in *HTR3A* in New World Monkeys that could potentially be subject of positive selection, and connected with taxon-specific behaviours. Results in Chapter 3.

(2) To reconstruct the evolutionary history of 5-HT3 receptor in Chordata, in order to understand how the current genetic landscape in vertebrates came to be, and its evolutionary meaning. Results in Chapter 4.

Chapter 3. Results - Serotonin, selection, and New World Monkeys

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Serotonin, behavior, and natural selection in New World monkeys

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5-HT3 receptor;
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Platyrrhini.

Abstract

Traits that undergo massive natural selection pressure, with multiple events of positive selection, are hard to find. Social behaviour, in social animals, is crucial for survival, and genetic networks involved in behaviour, such as those of serotonin (5-HT) and other neurotransmitters, must be the target of natural selection. Here, we used molecular analyses to search for signals of positive selection in the 5-HT system and found such signals in the M3-M4 intracellular domain of the 5-HT3A serotonin receptor subunit (HTR3A) in primates. We detected four amino acid sites with signs of putatively positive selection (398, 403, 432 and 416); the first three showed indications of being selected in New World monkeys (NWM, Platyrrhini), specifically in the Callitrichinae branch. Additionally, we searched for associations of these amino acid variants with social behavioural traits (i.e. sex-biased dispersal, dominance and social monogamy) using classical and Bayesian methods, and found statistically significant associations for unbiased sex dispersal (398L and 416S), unbiased sex dominance (416S) and social monogamy (416S), as well as significant positive correlation between female dispersal and 403G. Furthermore, we found putatively functional protein motifs determined by three selected sites, of which we highlight a ligand motif to GSK3 in the 416S variant, appearing only in Platyrrhini. 5-HT, 5-HT3A receptor and GSK3 are part of a network that participates in neurodevelopment and regulates behaviour, among other functions. We suggest that these genetic variations, together with those found in other neurotransmitter systems, must contribute to adaptive behaviours and consequently to fitness in NWMs.

Introduction

Finding scientific evidence of positive selection is a rare event and an especially challenging goal if genes related to complex phenotypes are considered. Any attempt to establish connections between genes and multifactorial inheritance traits (e.g. behaviour) must take into account the different levels at which the condition is regulated, as well as the complex interactions between genetics and environmental elements (Bradley & Lawler, 2011; Plomin *et al.*, 2013). Regulation of behaviour begins during brain and neural development, and it is shaped by chemical signals (transcription and growth factors, neurotransmitters and their receptors) that modulate the growth and the activity of neural circuits. This process extends to adulthood by the action of different molecules, including neurotransmitters that act during development (Baran, 2017). In addition, behaviour is extraordinarily plastic in response to environmental demands, which requires many regulatory

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Chapter 4. Results - The making of a receptor: An evolutionary scenario for 5-HT3 receptor in Chordata

Manuscript in preparation.

The making of a receptor: an evolutionary scenario for 5-HT3 receptor in Chordata

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Abstract

Background: The serotonin receptor type 3 (5-HT3) is the only serotonergic ionotropic receptor, belonging to the pentameric ligand-gated ion channel superfamily. The 5-HT3 receptor is involved in a variety of essential functions in the central and peripheral nervous systems of vertebrates. However, we lack information about the evolutionary processes that shaped 5-HT3 receptors. Here, using phylogenetic and molecular evolution approaches, we attempt to elucidate the evolutionary history of 5-HT3 receptors in Chordata.

Methods: We searched for members of the 5-HT3 family in 213 available chordate genomes, reconstructed the gene family and inferred the main events that occurred during the gene family evolution, using a phylogenetic framework.

Results and discussion: *HTR3A* appears in all species surveyed, coherently with its essentiality to form 5-HT3 receptors. On the other hand, *HTR3B* and *HTR3C, HTR3D* and *HTR3E* have a more complex history, involving multiple duplication and loss events during vertebrate evolution. Surprisingly birds, lizards, and serpents seem to lack *HTR3B* and *CDE-like* genes. We found an additional putative 5-HT3 subunit, which we called HTR3F. Finally, we propose two alternative scenarios for the origin and evolution of the complex and dynamic evolution of the 5-HT3 receptor family.

Introduction

The development and maintenance of adaptive complex behaviors and motor functions in mammals and other animal groups were achieved by the emergence and evolution of fine-tuned, tightly regulated central and peripheral nervous systems (Liebeskind et al. 2017). Neural networks throughout nervous systems rely upon a diversity of neurotransmitter systems for both their initial formation and mature function.

Serotonin (5-hydroxytryptamine, 5-HT) is one of the most important neurotransmitters, involved in many different and essential biological functions, such as

development, cardiovascular and endocrine function, body temperature, digestion, gut motility, blood clotting, sensory perception, appetite, sexual behaviour, sleep, cognition and memory, among many other functions (Lucki 1998; Barnes et al. 2009; Berger et al. 2009; Švob Štrac et al. 2016). It appears early in evolution, which is reflected by its broad distribution across life (e.g. animals, plant, fungi, unicellular eukaryotes, and bacteria) and in development, suggesting that it played a crucial role in the evolution of living organisms (Azmitia 2010).

5-HT3 is the only pentameric ligand-gated ion channel serotonergic receptor (pLGIC, traditionally called "Cys-loop") in vertebrates - other thirteen receptors (organized into six families, 5-HT$_{1,2,4\text{-}7}$) belong to the GPCR rhodopsin-like superfamily, and participate in slow neurotransmission, using G proteins as second messengers(Hoyer et al. 1994; Nichols and Nichols 2008; McCorvy and Roth 2015). pLGICscomprise one of the most crucial and evolutionary old neurotransmitter receptor superfamily that originated from a common ancestor that existed prior to the eukaryote-prokaryote split, and has extant representatives in all kingdoms of life (Cockcroft et al. 1990; Ortells and Lunt 1995; Tasneem et al. 2005; Jaiteh et al. 2016). In bilateral animals, pLGICs are essential for fast signal transmission between neurons, by allowing cation or anion flux into the postsynaptic neuron upon channel activation by neurotransmitters (Yakel 2010; Thompson et al. 2010; Corringer et al. 2012). pLGIC receptor superfamily includes cation-selective excitatory channels, namely nicotinic acetylcholine receptors (nAChRs), zinc-activated (ZAC) receptors, and serotonin type 3 (5-HT3) receptors, and anion-selective inhibitory γ-aminobutyric acid (GABA$_A$R) and glycine (GlyR) receptors. All pLGICs share a common structure consisting of five identical or homologous subunits consisting of an amino-terminal ligand-binding domain and four transmembrane domains that assemble to form a pentameric channel with identical or related subunits around an ion-selective pore. More than 40 genes encoding pLGIC subunits have been identified in vertebrates, and receptor composition determines ion selectivity, neurotransmitter affinity, subcellular localization, gating kinetics, and pharmacology (Dent 2006; Dent 2010). Despite the high divergence at the sequence level, the overall structure of pLGICs is highly conserved (Thompson et al. 2010). In the past few years, the 3D structure of the 5-HT3 receptors was revealed by X-ray crystallography (Hassaine et al. 2014), cryo-ET (Kudryashev et al. 2016) and cryo-EM (Basak et al. 2018), which have brought useful insights into the receptor physiology and

function. Structurally, 5-HT3 is a typical pLGIC with an extracellular domain responsible for ligand binding, a transmembrane domain formed by four transmembrane α-helices, and an intracellular domain, with an inverted tepee shape towards inside the cell that forms a portal through which ion current flows (Peters et al. 2010; Carland et al. 2013). 5-HT3 receptors are widely expressed in both PNS and CNS. In the CNS, the highest levels of 5-HT3 binding sites were found in the dorsal vagal complex, in the brainstem. This region comprises the nucleus tractus solitarius (NTS), area postrema and dorsal motor nucleus of the vagus nerve, which are key to the initiation and coordination of the vomiting reflex (Doucet et al. 2000; Chameau and Van Hooft 2006). In consequence, 5-HT3 selective antagonists (e.g. ondansetron, granisetron, and palonosetron) and are widely used in treating radiation- and chemotherapy-induced emesis (Thompson and Lummis 2007).

5-HT3 receptors can be localized either pre- or postsynaptically and their activation can modulate the release of a range of neurotransmitters, including dopamine, GABA, cholecystokinin, substance P, and acetylcholine (Miquel et al. 2002; Thompson and Lummis 2006; Fink and Göthert 2007; Barnes et al. 2009; Leiser et al. 2015). For example, 5-HT3 receptors have been found presynaptically in varicosities of GABA interneurons in the rat hippocampus and human frontal cortex, suggesting that the release of GABA is, at least in part, mediated by Ca2+ influx into presynaptic terminals (Koyama et al. 2000; Katsurabayashi et al. 2003; Turner et al. 2004). Post-synaptically, 5-HT3 receptors are involved in regulating neural network excitability, derived from its fast synaptic transmission properties (Chameau and Van Hooft 2006).

In the gastrointestinal tract (GI), 5-HT3 receptors are found primarily on vagal afferent neurons and fibers (Lacolley et al. 2006; Babic et al. 2012), and in intrinsic primary afferent neurons (IPAN; Bertrand et al. 2000; Mawe and Hoffman 2013; Browning 2015), where they are involved in mediating pain and motility reflexes, and in the microbiome-gut-brain axis (Browning 2015; O'Mahony et al. 2015; Bonaz et al. 2018).

In humans, five genes encoding 5-HT3 receptor subunits have been described so far. *HTR3A* and *HTR3B* are located next to each other in 11q23, whereas *HTR3C, HTR3D,* and *HTR3E* form a tight cluster in chromosome 3 (3q27)(Davies et al. 1999; Karnovsky et al. 2003). Molecular and functional studies showed that only 5-HT3A subunit can form functional homomeric 5-HT3 receptors, although other subunits can form functional heteromeric receptors in co-expression with 3A subunits (Niesler et al. 2007; Holbrook et al.

2009). Several reasons for this have been proposed, one of them being the location of the binding site between two 3A subunits, an idea supported by the fact that 3A homomeric and 3AB heteromeric receptors show no differences in pharmacology (Brady et al. 2001; Lochner and Lummis 2010; Thompson and Lummis 2013). Another proposed explanation is that 3B subunit fails to exit the endoplasmic reticulum (ER) due, at least in part, to a retention motif (CRAR, SGER in 5-HT3A) within the short first intracellular loop between M1 and M2 (Boyd et al. 2002; Boyd et al. 2003). When co-expressed with *HTR3A*, 3A subunit may shield this motif, thus allowing the heteromeric receptor to reach the cell membrane. The same conclusion was reached for 3C, 3D, and 3E subunits, thus indicating that 5-HT3A plays a crucial role in receptor trafficking (Niesler et al. 2007).CDEsubunits were more recently discovered, and less well explored than 5-HT3A and 5-HT3B, although 5-HT3C and 5-HT3E subunits are implicated in a range of neurological disorders including pregnancy-related nausea, irritable bowel disease, schizophrenia and autism (Niesler et al. 2007; Holbrook et al. 2009; Schuhmacher et al. 2009; Rehnström et al. 2009; Walstab et al. 2010; Yaakob et al. 2011).

Comparative expression analyses in humans revealed different expression patterns among subunits, with 3A and 3B expressed both in the CNS and gut (yet preferentially in the CNS) while C-E subunits show a more peripherally-restricted expression pattern, with high levels in the GI tract, particularly in myenteric neurons and submucosal plexus of the large intestine, kidney, and liver (Niesler et al. 2003; Tzvetkov et al. 2007; Barnes et al. 2009; Kapeller et al. 2011). This evidence points to a specific role of CDE heteroreceptors in the PNS of some species, distinct from the more general functions of 5-HT3A homo- and 5-HT3AB heteroreceptors.

5-HT3 receptors are also expressed in the forebrain, with highest levels of expression within the amygdala and hippocampus, as evidenced by studies in rat, human, and marmoset brains (Barnes et al. 1990; Tecott et al. 1993; Parker et al. 1996; Chameau and Van Hooft 2006; Shukla et al. 2014). Studies utilizing expression analysis in human brain established the highest density of 5-HT3A subunit in the hippocampus, striatum, amygdala, and thalamus (Houtani et al. 2005). The highest density of 5-HT3B-containing receptors was detected in the brainstem including the hippocampus, amygdala, thalamus and frontal cortex (Hammer et al. 2012). Such findings made 5-HT3 receptor to be subject of growing interest as a potential target involved in emotional and affective disorders(Gupta et al. 2016).

Interestingly, there are marked differences in receptor expression at the regional and cellular level among species. For example, in human forebrain, the highest levels are found in the striatum (putamen and caudate nucleus), while lower levels are found in cortical regions (Parker et al. 1996), while this pattern is reversed in rodents (Steward et al. 1993). In contrast, a recent study found 5-HT3A to be restricted to the hippocampus in marmoset and no 5-HT3B expression (Shukla et al. 2014), although Jakab and Goldman-Rakic found 5-HT3A receptors at the cell body of cortical neurons in macaques (Jakab and Goldman-Rakic 2000), support the idea of differences in the expression pattern of 5-HT3A in the cortex between species.

Recently, 5-HT3 receptor was shown to be involved in a number of medically relevant processes such as drug addiction, cognitive function, schizophrenia, satiety control, and immune regulation (Thompson and Lummis 2007; Walstab et al. 2010; Kato 2013; Browning 2015; Li et al. 2015).

Despite the enormous interest devoted to understanding the functional and pharmacological aspects of serotonin and its receptors, to date, few studies have put these findings into an evolutionary context. Here we use a phylogenetic approach to explore the evolutionary history of 5-HT3 receptor gene family in Chordata, where it experienced an expansion from a singleton ancestral gene, followed by multiple processes of lineage-specific duplication and loss, configuring the genomic landscape that we see today.

Materials & Methods

Ortholog search and sequence retrieval

For this study, we searched for HTR3A-E homologs in publicly available databases and collected 671 sequences from 213 chordate species covering all major chordate groups. In order to detect the maximum possible amount of orthologs/paralogs, we mined six different data repositories: Ensembl v93 (Zerbino et al. 2018), OMA Sep. 2017 release (Altenhoff et al. 2018) and EggNOG v4.5.1 (Huerta-Cepas et al. 2016), UniProtKB 2018_05 release (Bateman et al. 2017), NCBI nr/nt collection, and UCSC Genome Browser. For Ensembl, OMA, and EggNOG, we used human HTR3A-E Ensembl gene identifiers (ENSG00000166736, ENSG00000149305, ENSG00000178084, ENSG00000186090, and ENSG00000186038) as query. For Ensembl, we used custom biomaRt-based functions in R (Durinck et al. 2005; R Core Team 2018) to automatically download ortholog IDs and

coding/protein sequences, keeping the longest transcript in genes with multiple transcripts available. In some cases (e.g. *Microcebusmurinus*), sequences for some genes were not available in the v92 release, so we manually retrieved them from previous releases (e.g. Ensembl 85 and 87).

For UniProtKB database mining, we used Interpro family identifiers (IPR008133, IPR008134, respectively, Finn et al. 2017)in order to retrieve putative HTR3A and HTR3B protein sequences. HTR3C, -3D and -3E lack specific Interpro identifiers, so additional sequences for them could not be obtained by this method. Also, non-chordate homologs were excluded from the analysis.

In order to increase the diversity of species represented in our dataset, we used the tblastn algorithm to query the NCBI nr/nt collection for every major taxonomic group in Chordata, using protein sequences from species belonging or close to each group. We kept the best hits for each new species and included them in our alignment. Taxonomic groups with no match to our query protein were double-checked using alternative sequences as query. We used NCBI Genome Data Viewer v4.4 (Available at https://www.ncbi.nlm.nih.gov/genome/gdv/) and UCSC Genome browser (Kent et al. 2002) for several purposes: (1) To confirm gene losses in species in which homologs could not be found, (2) to confirm premature stop codons in unusually short sequences (3) to confirm gene duplication at specific loci, (4) to retrieve sequences based on GENSCAN/Augustus (Burge and Karlin 1997; Stanke et al. 2008) gene predictions for genes and species with no or poorly annotated homologues, and (4) to verify the synteny block of potential homologues.

All sequences were visually inspected using Aliview v1.20 (Larsson 2014). Misidentified sequences in data repositories were reclassified (e.g. HTR3A with an associated IPR008134 identifier), and poor quality sequences were removed, being replaced by alternative sequences in different repositories when possible.

For all protein sequences used in the final dataset, we obtained corresponding nucleotide sequences from Ensembl, NCBI Genbank, or the European Nucleotide Archive (ENA). See Table S1 for a full list of the sequences used in this study.

Presence/absence trees

We tabulated ortholog presence or absence according to our extensive search and constructed trees based on the best-known species tree at NCBI taxonomy. Illustrations were made using iTol v4 server (Letunic and Bork 2016).

Multiple sequence alignments

Protein sequences were aligned using the L-INS-i algorithm in MAFFT v7.394 (Katoh and Standley 2013) with default parameters. For nucleotide sequences, we used PAL2NAL (Suyama et al. 2006) in order to align while keeping codons intact, using protein alignment as a guide. Sequences were trimmed using the gappyout algorithm in trimAl v1.2rev59 (Capella-Gutiérrez et al. 2009).

Phylogenetic analyses

In order to analyze phylogenetic relationships within the 5-HT3 gene family, we built phylogenetic trees by Maximum Likelihood (ML) and Bayesian methods, using RAxML v8.2.4 (Stamatakis 2014) and BEAST v2.5.0 (Bouckaert et al. 2014), respectively. For it, we considered a subset of representative species in our dataset, including 335 sequences and 76 species (Figure S1, Table S1). Prior to ML tree estimation, we identified the GTR+Γ+I model as the best substitution model, using jModeltest v2.1.10 (Guindon and Gascuel 2003; Darriba et al. 2012). We then performed 300 bootstraps prior to ML tree search, using autoMRE criterion.

In BEAST2, we used bModelTest package 1.1.0 (Bouckaert and Drummond 2017) for co-estimation of the best site model during the phylogenetic analysis. The model most frequently used during the analysis (91.12%) was 123345, followed by GTR (123456, 8.05%). We used a Relaxed Log Normal clock model, a Birth-Death tree prior, and a chain of 70,000,000 iterations, sampling every 4,000 iterations.

Domain analysis

We inspected our protein sequences for predicted functional domains using Interproscan v5.30 (Jones et al. 2014) with default settings on the Interpro 69 release. Specifically, we aimed to confirm the presence of two functional domains common to pLGICs: Neur_chan_LBD (PF02931) and Neur_chan_membr (PF02932). These domains correspond

to the extracellular ligand-binding ion-channel domain, and the transmembrane domain, (corresponding to the four transmembrane helices and the intracellular domains), respectively (Table S2).

Microsynteny analysis

We used Genomicus server v92.01 (Nguyen et al. 2018) and NCBI Genome Data Viewer to analyze synteny blocks surrounding our genes of interest, as well as to detect additional putative homologs, not detected by other methods. In both servers, we searched in 15-upstream and 15-downstream windows with respect to our genes of interest. In genomes in scaffolds, contigs with fewer genes were considered in whole.

Positive selection analyses

Given that 5-HT3 gene family is characterized by multiple duplications, one of our goals in this study was to analyze and compare evolutionary rates of members after gene duplication and to search for signals of positive selection. Specifically, we wanted to explore the differences in selective pressures in two clades with a different number of family members: placental mammals (Eutheria) and Sauropsida, a group that comprises reptiles and birds. For it, we compared neutral M8a *vs.* positive selection M8 site models in codeml program of PAML v4.9 software (Yang 2007). We separately tested *HTR3A* in Eutheria and Sauropsida, and for *CDE* genes, we split them according to the groups formed in our phylogenetic analysis. In addition, we tested *HTR3F* in Sauropsida and *ZACN* in Eutheria. Species with more than one *CDE* copy in the same group, or more than one *HTR3F (*e.g. *Pelodiscus sinensis*) were excluded from the analyses.

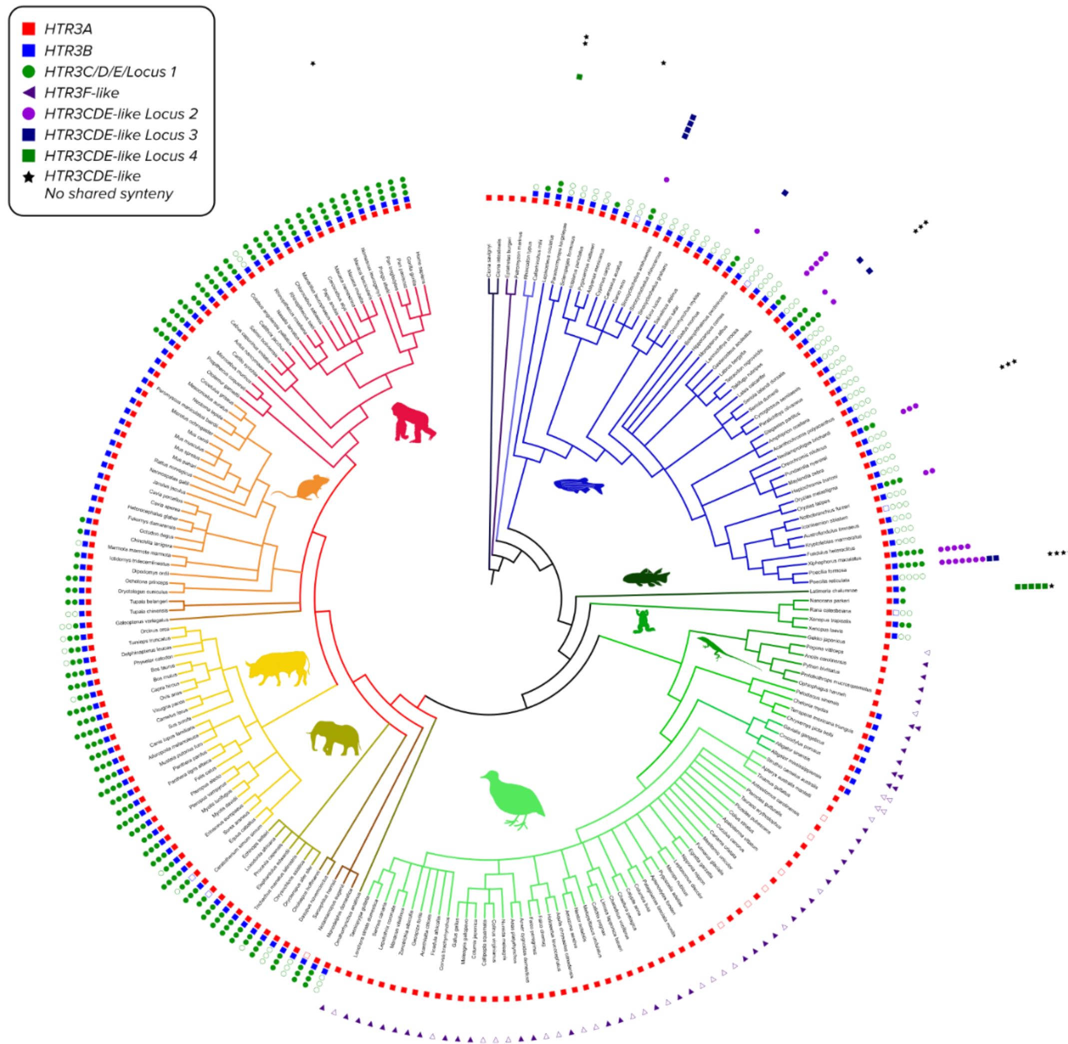

Figure 1. General distribution of 5-HT3 genes in 213 chordates. Branches are coloured to highlight the major Chordata groups. *Deep purple:* Agnatha or lampreys and hagfish, *light purple*: Chondrichthyes, or cartilaginous fishes, *blue*: Actinopterygii, or ray-finned bony fishes, *dark green*: Coelacanth, *medium dark to light green*: Amphibians, Squamata, Testudines, Crocodylia and Aves, *dark brown:* Monotremata, *brown:* Marsupials, *lighter brown:* Xenarthra, *golden:* Afrotheria, *yellow:* Laurasiatheria, *dark orange:* colugos, *orange:* tupaias, *light orange:* Glires, *magenta:* Primates. Empty shapes corresponding to genes that we could not find due to low genome quality and other technical factors, but we assume their existence based on their phylogenetic position.

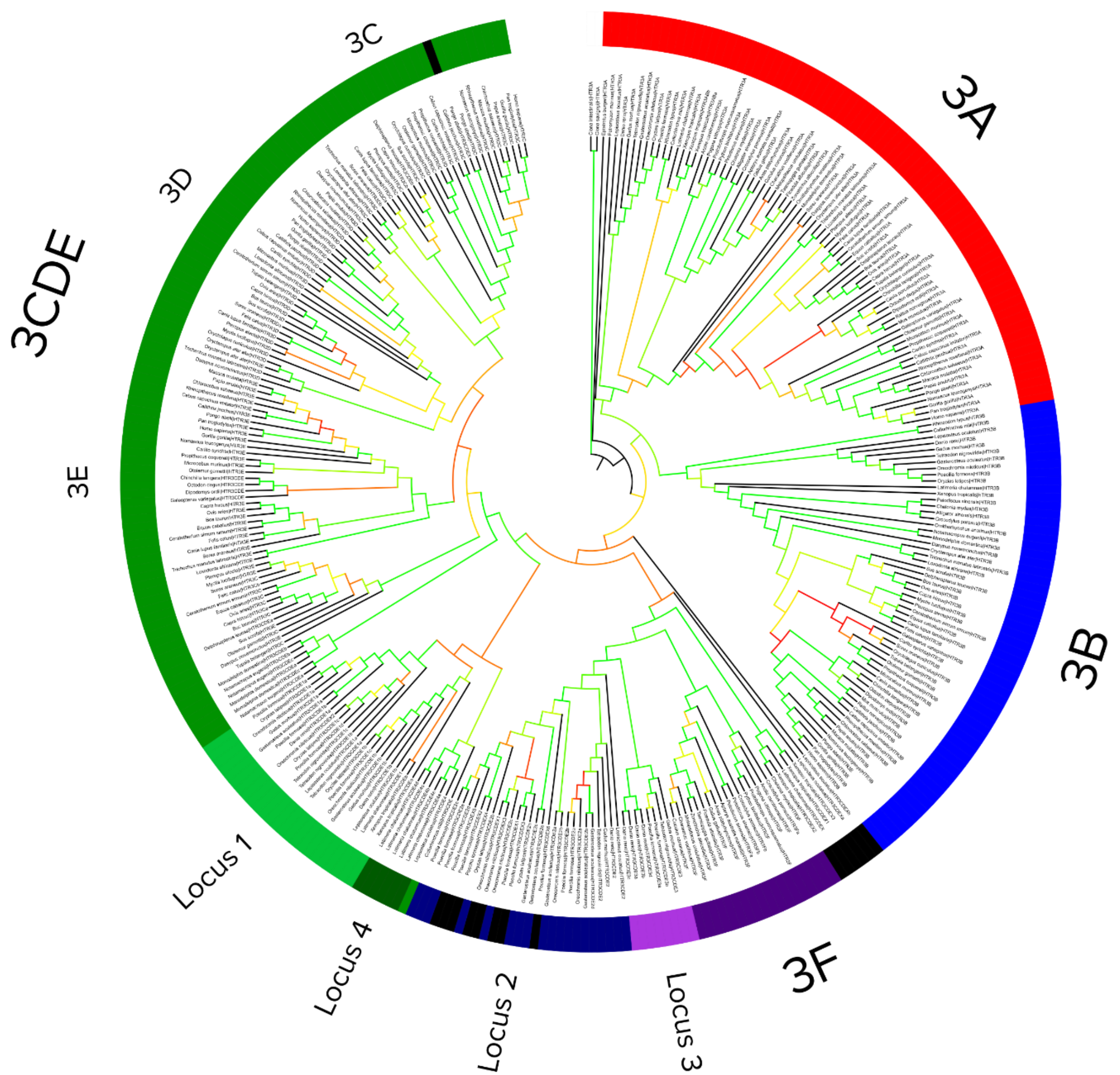

Figure 2. Gene tree using Maximum Likelihood. Branch colours indicate bootstrap support (green = high, red = low). Colored ring represents each gene or gene groups of the family (See Results and discussion).

Results and Discussion

The evolutionary history of 5-HT3 receptor gene family in chordates is complex and characterized by multiple duplications and gene losses in most chordate lineages (Figures 1 and 2). Here we performed exhaustive searches for 5-HT3 homologs in the genomes 213 chordate species, in order to characterize every member of the family, and study their evolutionary relationships using phylogenetic and molecular evolution approaches.

HTR3A and *HTR3B* appear next to each other in most chordates surveyed in this study, sharing a remarkable degree of synteny across different groups (Figures 3). In turn, *HTR3C*, *HTR3D,* and *HTR3E* show a more complicated pattern, with multiple expansions and losses across chordates (Figure 1 and Figure 4). The latter genes were first described in humans, and given a letter per each gene. However, our analyses revealed multiple duplication and loss events in different vertebrate lineages, which renders this nomenclature insufficient to describe the diversity of *CDE*-like genes in vertebrates. Therefore, for a better understanding, here we denominate non-mammalian *CDE*-like genes, homologous to all three mammalian *HTR3C, HTR3D,* and *HTR3E*, as *HTR3CDE*. In addition, we found a putative extra subunit in several non-mammalian lineages (e.g. birds, turtles and reptiles), with high degree of identity among lineages, which we refer here to as *HTR3F,* and we assume that the pLGIC *ZACN* share its evolutionary history with the 5-HT3 receptors based on their synteny (Figure 5) and phylogenetic analyses (data not shown).

Basal chordates: lancelets and sea squirts

Our search included members of the most basal Chordata subphyla: Cephalochordata (lancelet, *Branchiostoma floridae* and *Branchiostoma belcheri*) and the Urochordata (sea squirts*, Ciona intestinalis,* and *Ciona savignyi*). No ortholog was found in lancelets (braFlo1 assembly and B.belcheri v7h2). However, it is known that lancelets have at least one ortholog of the serotonergic biosynthetic enzyme tryptophan hydroxylase (Tph), the serotonin transporter (5-HTT), and at least three serotonergic GPCR orthologs (Nordström et al. 2008; Candiani et al. 2012). This indicates that lancelets use serotonergic signaling through GPCRs, having probably lost the ionotropic pathway (Figure 5).

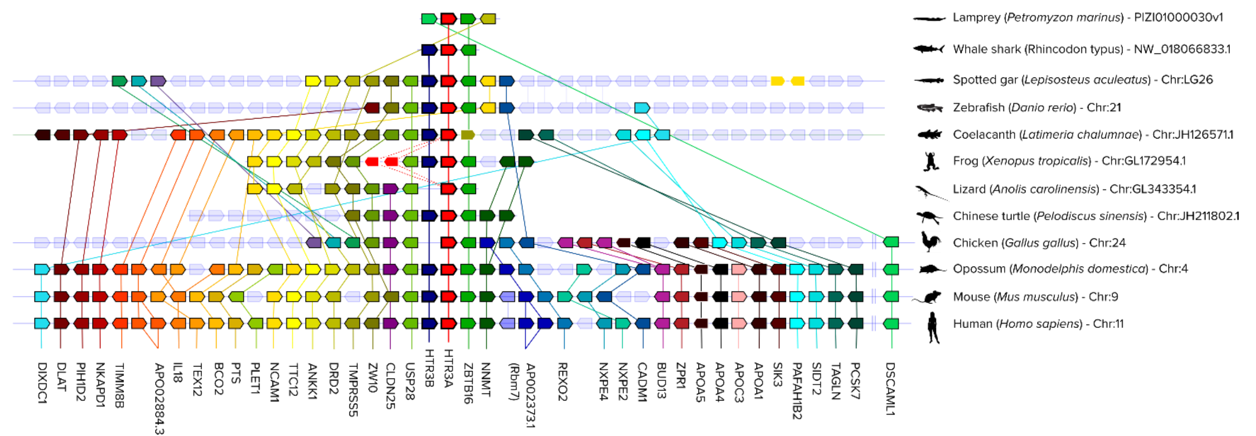

Figure 3. Microsynteny analysis of the *HTR3A* and *HTR3B* locus. Orthologous and paralogous genes are represented by pentagons indicating gene direction share the same colours and are connected with lines. Empty pentagons represent genes with no relevant shared synteny. Numbers after species names indicate the chromosomal location or contig of the syntenic block.

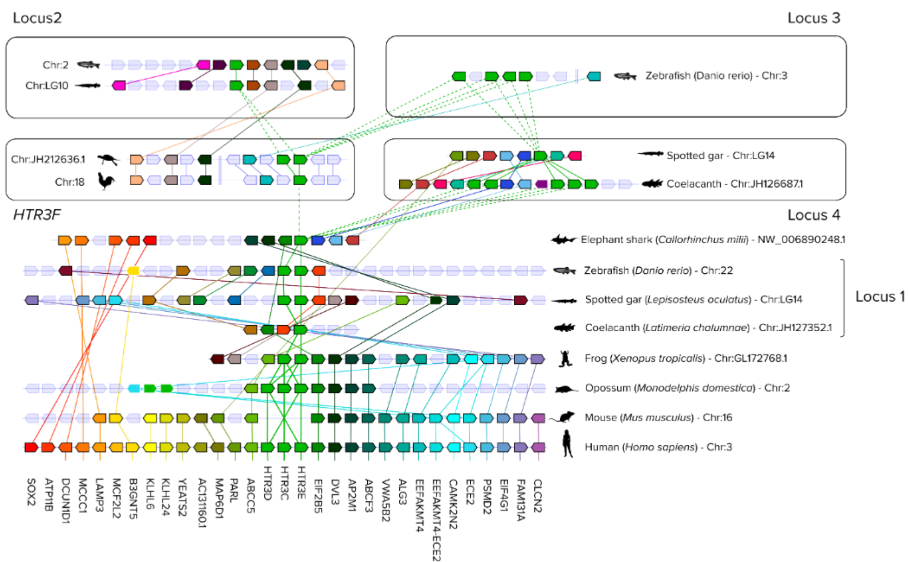

Figure 4. Microsynteny analysis of *HTR3CDE, HTR3F,* and *CDE-like* Locus 1-4 loci, using representative species of each major lineage. Solid lines between chromosomes represent more likely orthologs than dashed lines.

On the other hand, we retrieved one putative 5-HT3 gene in each of the two sea squirts (genome assemblies KH/Ci3 and CSAV2.0, respectively), with no shared synteny.

Phylogenetic analyses showed that sea squirts genes group together and form a sister branch to vertebrate 5-HT3 receptors (Figure 2), suggesting that these single genes may represent the putative ancestral state of a singleton gene in the origin of the 5-HT3 gene family. Alternatively, independent losses may have occurred in Ciona genomes, which are known to have undergone extensive structural rearrangements and are characterized by high evolutionary rates (Delsuc et al. 2018).

Jawless vertebrates: marine lamprey and the inshore hagfish

The extant jawless vertebrates (Agnatha), a vertebrate group sister to jawed vertebrates (Gnathostomata) that diverged ~550 Ma (Smith and Keinath 2015) is represented in our sample by two species: the sea lamprey (*Petromyzon marinus*) and the inshore hagfish (*Eptatretus burgeri*). In both species, we recovered single putative orthologs, containing the two typical pLGIC domains. Lamprey gene (ENSPMAG00000007406) shares a reasonable degree of synteny with other vertebrates (Figure 3), although the scaffold status of the most recently published genome (PetMar3) restricted our search to the length of the contig (PIZI01000030v1). The putative hagfish ortholog (ENSEBUG00000009656) has no recognizable shared synteny with other vertebrates. However, we found a predicted short protein (192 amino acids, ENSEBUG00000010488) within ~300 kb, with a predicted ligand-gated ion channel transmembrane domain. The finding of singleton 5-HT3 genes in both jawless vertebrates and Ciona would support the idea that the Chordata 5-HT3 receptor gene family originated from a single gene. On the other hand, there are controversies about whether these animals underwent a second round of whole genome duplication (WGD, 2RWGD) that occurred early in vertebrate evolution (Dehal and Boore 2005). In addition, the lamprey genome underwent somatic rearrangements, losing portions that might include protein-coding genes (Pascual-Anaya et al. 2018), and therefore extant jawless vertebrates may have independently lost members of the family.

Jawed vertebrates: sharks, fishes, frogs, reptiles, birds, and mammals

Jawed vertebrates show a complex pattern, with multiple expansions and losses across lineages. We found the first instance of *HTR3A* and *HTR3B* and *HTR3CDE* genes in the cartilaginous fishes. Thus, we found three genes in the elephant shark (*Callorhincus milii*) but only *HTR3A* and *HTR3B* in the whale shark (*Rhincodon typus*). *HTR3A* and *HTR3B* were found together in the same contig in the whale shark (Figure 3), as they are found in most vertebrates. In the elephant shark, both genes were found in different contigs so shared

synteny could not be asserted. However, we found the putative *HTR3CDE* gene to share synteny with *HTR3CDE* of mammals and other vertebrates (Figure 4). Since both genomes are currently in scaffolds, it is a possibility that *HTR3A* and *HTR3B* are together in the elephant shark genome, and that the whale shark has an *HTR3CDE* gene. The presence of the duplicated tandem genes on early vertebrates support the hypotheses that these events occurred early in vertebrate evolution but it is unclear whether it happened before or after the 2RWGD.

Our search included 48 species of ray-finned bony fishes, belonging to the Neopterygii subclass, including the spotted gar (*Lepisosteus oculatus*), and 47 Teleostei. We found *HTR3A* and *HTR3B* in all of them, and a variable number of *HTR3CDE* homologs, representing the greatest expansion of the 5-HT3 receptor family in Chordates. We restricted our analyses to 8 representative species with good genome quality (Amazon molly/*Poecilia formosa*, medaka/*Oryzias latipes*, tilapia/*Oreochromis niloticus*, pufferfish/*Takufugu rubripes*, stickleback/*Gasterosteus aculeatus*, Atlantic cod/*Gadus morhua*, zebrafish/*Danio rerio*, and spotted gar/*Lepisosteus oculatus*). We found up to 4 loci containing 1-5 putative homologs each (Figure 4). Interestingly, locus 1 and locus 4 genes in spotted gar are located on the same chromosome (LG14), within <3Mb, which suggests that they have a common origin by tandem duplication. Locus 2 and locus 3 in Teleostei showed a variable number of genes, and are located in different chromosomes, with a certain degree of shared synteny, suggesting that these genes may have arisen as a direct consequence of the third round of whole genome duplication (3RWGD), and subsequent asymmetric losses (Amores et al. 1998; Jaillon et al. 2004; Kasahara et al. 2007; Moriyama and Koshiba-Takeuchi 2018). This idea is further supported by the finding of only locus 2 genes in spotted gar, that did not undergo a 3RWGD (Parichy 2016). In addition, we found a variable number of genes with no apparent shared synteny with other fish or vertebrate 5-*HT3* genes (Figure 1).

Our phylogenetic analyses (Figure 2 and S2) revealed also that genes in locus 1 and 4 form groups sister to mammalian *CDE*, while loci 2 and 3 group together with *HTR3F*. In concordance, we observed more genes with shared synteny among ray-finned fish locus 1 and mammalian *CDE*, and among locus 2 and 3 and *HTR3F*. Interestingly, only a few teleost species analyzed have genes in locus 3 (Amazon molly, Atlantic cod, zebrafish, fugu, and pufferfish). Given this restricted distribution, as well as their phylogenetic position grouping with locus 2 and the fact that they share some genes in their synteny block with *HTR3F* in

Tetrapoda (e.g. *CRD2L* in chicken, *CDR2A* in zebrafish, Figure 4), it is a possibility that locus 3 arose from the third round of genome duplication (3RWGD).

The Western Indian coelacanth (*Latimeria chalumnae*), a lobe-finned fish closely related to tetrapods, has both *HTR3A* and *HTR3B*, and 6 putative *CDE* homologs: one in locus 1 and five in locus 4. Genes in locus 4 are concentrated in a 300 kb cluster and are found next to genes with orthologs in close synteny with elephant shark *CDE* such as *GMNC, IL1RAP,* and *FGF12*, as well as locus 4 in spotted gar. Interestingly, locus 1 and locus 4 genes in spotted gar are located in the same chromosome (LG14), within <3Mb, which suggests that they have a common origin by tandem duplication, although we could not confirm this in coelacanth since both loci were in different contigs (Figure 5).

Our search in Sauropsida (birds, crocodiles, lizards, serpents, and turtles) yielded a surprising finding: most groups (except for turtles and crocodiles) seem to have lost *HTR3B* (Figures 1 and 3). Such phylogenetic pattern suggests that *HTR3B* was independently lost at least twice in the Sauropsida lineage: one in squamates (lizards and snakes), and another after the birds and crocodiles split. In addition, all members of Sauropsida seem to have lost all *CDE* genes. Instead, we found a putative new ortholog, which here we refer to as *HTR3F*. The protein product shows relatively low identity with mammalian *CDE* and *HTR3A* (~30-33%), although it is relatively conserved among Sauropsida (~50-90% identity), and has both ligand-binding and transmembrane domains predicted for a functional pLGIC.

We surveyed 90 mammal species, including one Monotremata (platypus, *Ornithorhynchus anatinus*), three marsupials (opossum/*Monodelphis domestica*, wallaby/*Notamacropus eugenii,* and Tasmanian devil/*Sarcophilus harrisii*), and 84 placental mammals, covering all major orders (Figure 1). All species surveyed with good genome quality had *HTR3A* and *HTR3B*, with conserved shared synteny across species.

Interestingly, we found a predicted premature stop codon in the horse (*Equus caballus*) *HTR3B*, which yields a predicted shorter (163 aa) protein, with only one of the predicted functional pLGIC domains (PF02931, ligand-binding domain; see Material and Methods section) covering 137 aa, and thus likely nonfunctional. We also found the same stop codon in the genome of the close relative Przewalski horse (*Equus przewalskii,* not included in our analysis).

Regarding *HTR3CDE* genes, we could not find any in platypus, although their loss could not be confirmed due to low genome quality. In marsupial and most placental mammals,

CDE genes form a small cluster of three genes, that were named in humans as *HTR3D*, *HTR3C,* and *HTR3E* (following chromosomal order). Interestingly, our phylogenetic analyses place marsupial *CDE* as an outgroup to placental mammal genes (Figure 2).

Placental mammal *CDE* genes also show some degree of variability in copy number among groups. Indeed, some members of the Rodentia order lost all *CDE* genes, as showed by earlier studies (Karnovsky et al. 2003; Holbrook et al., 2009). However, we found *CDE*-like singletons in some rodent species, namely degu (*Octodon degus*), chinchilla (*Chinchilla lanigera*), squirrel (*Ictiodomys tridecemlineatus*) and kangaroo rat (*Dypodomis ordii*). Thus, similar to other situation described above (*HTR3B* lost in some Sauropsida lineages), independent loss of *CDE* genes occurred in Rodentia.

Laurasiatherians (a morphologically diverse group of mammals that originated in the northern supercontinent Laurasia; e.g. bats, carnivores, ruminants, whales, and shrews) pose perhaps the best example of *CDE* variability in mammals since we find species with 2 to 4 *CDE* orthologs. For example, megabat (*Pteropus vampyrus*) seems to have only two copies, as confirmed by the most recent genome assembly (Pvam_2.0, coverage 188x), although closely related *Pteropus alecto* seem to have 3 functional copies. Another example is the beluga whale (*Delphinapterus leucas*) that appears to have only two copies. An exploration of its genome (ASM228892v2, coverage 117x) revealed a third, pseudogenized copy. In addition, all putative beluga *CDE* genes seem to be contained within an intron of its neighboring gene, *ABCC5* (Figures 4 and S3), and contain genomic stop codons (Figure S3). On the other hand, species like cat, ferret, and goat seem to have four *CDE* copies – although one of the goat genes (ENSCHIG00000019914, here named *HTR3Cb*) is predicted by NCBI to be a pseudogene.

Most primates, just like humans, have five genes (*HTR3A-E*). However, New World's Nancy Ma's night monkey (*Aotus nancymaae*) seems to have lost *HTR3D*, according to the most recent genome assembly (Anan_2.0, coverage 132x). Although the genome is in scaffolds, *HTR3C* and *HTR3E,* and neighbor genes are in the same unplaced contig (NW_018503256.1), which supports the idea that *HTR3D* was truly lost in the species.

In placental mammals, our phylogenetic analyses divided *CDE* genes into 4 different groups, three of them mostly matching *HTR3C, HTR3D* and *HTR3E* cluster. However, a fourth cluster, sister of the former three, groups a combination of different *CDE* genes of several different orders (Figure 2 and Figure S2). The most likely scenario for this pattern is

that ancestral mammals had at least four *CDE-like* copies, most taxa differentially lost one (or more) copies, and few species (e.g cat, goat) retained all four.

The *HTR3F/ZACN* conundrum

The specific identity and evolutionary history of *HTR3F* are intriguing. ML phylogenetic analyses place it as a sister group to *CDE* (including mammalian and elephant shark CDE, and locus 1 and 4), and groups with fish locus 2 and 3 genes (Figure 2). In addition, its relatively higher (~30%) identity with *HTR3A*, compared to any other known pLGIC probably made it be annotated as "*HTR3A-like*" and "*HTR3C-like*" in NCBI Genbank NR collection and classified by Ensembl (but not by EggNOG) as an ortholog of mammalian *CDE* genes. This evidence made us name it *HTR3F* in this work. On the other hand, *HTR3F* locates in a region with well-conserved synteny in other vertebrates, and which in mammals contain another close pLGIC relative: *ZACN*.

ZACN is a recently discovered cationic pLGIC (Davies et al. 2003), which function is still unknown. It received its name due to its activation by zinc ions (Zn^{2+}), later studies showed that it can be activated by hydrogen (H^{+}) and copper (Cu^{2+}) ions too (Trattnig et al. 2016). To the best of our knowledge, the particular evolutionary history of *ZACN* has not been elucidated so far, but some phylogenetic analyses in the pLGIC superfamily place it as a close relative of the 5-HT3 receptor family (Ortells and Lunt 1995; Tasneem et al. 2005; Nemecz et al. 2016). Furthermore, *ZACN* shares with 5-HT3A receptors its permeability to monovalent cations (Na^{+} and K^{+}), yet unlike 5-HT3A and nAChRs, it is impermeable to divalent cations Ca^{2+} and Mg^{2+} (Trattnig et al. 2016). Interestingly, *ZACN* shows an alternative presence/absence with *HTR3F* in Tetrapoda: *ZACN* is found in most mammals (it was lost in rodents) and seems to be absent in the majority of the reptiles and birds, while *HTR3F* is absent in mammals, although present in most Sauropsida. Exceptions to this are species like the green anole lizard (*Anolis carolinensis*), that retain both genes, which makes us suppose that *HTR3F* and *ZACN* arise from a tandem duplication, regardless of the sequence identities (Figure 5). In concordance with this hypothesis, the synteny in this block is well conserved across most mammals and reptiles and birds lineages. Furthermore, our Bayesian analyses (Figure S2) place *HTR3F,* together with locus 2 and 3 genes as an outgroup to the rest of the 5-HT3 family, with good posterior probability (pp = 0.91). These findings open two great questions: (a) Is *HTR3F*a true 5-HT3 receptor subunit, with the ability to form heteroreceptors with 3A subunit? (b) Is *HTR3F* an independent pLGIC,

closely related to 5-HT3 and *ZACN*? *HTR3F* could have appeared early in vertebrate evolution, and its position as the only 5-HT3 subunit other than 3A in birds and squamates (lizards and serpents in our analyses) might imply functional differences among 5-HT3 heteroreceptors in these groups. On the other hand, if *HTR3F* is an independent pLGIC it would mean that birds, lizards, and serpents have the genetic ability to form exclusively homomeric 5-HT3A receptors, and *HTR3F* could be an independent receptor, perhaps fulfilling the same function as ZACN in mammals, or a completely different one. Future structural and experimental studies to determine *HTR3F* properties will provide answers to these questions.

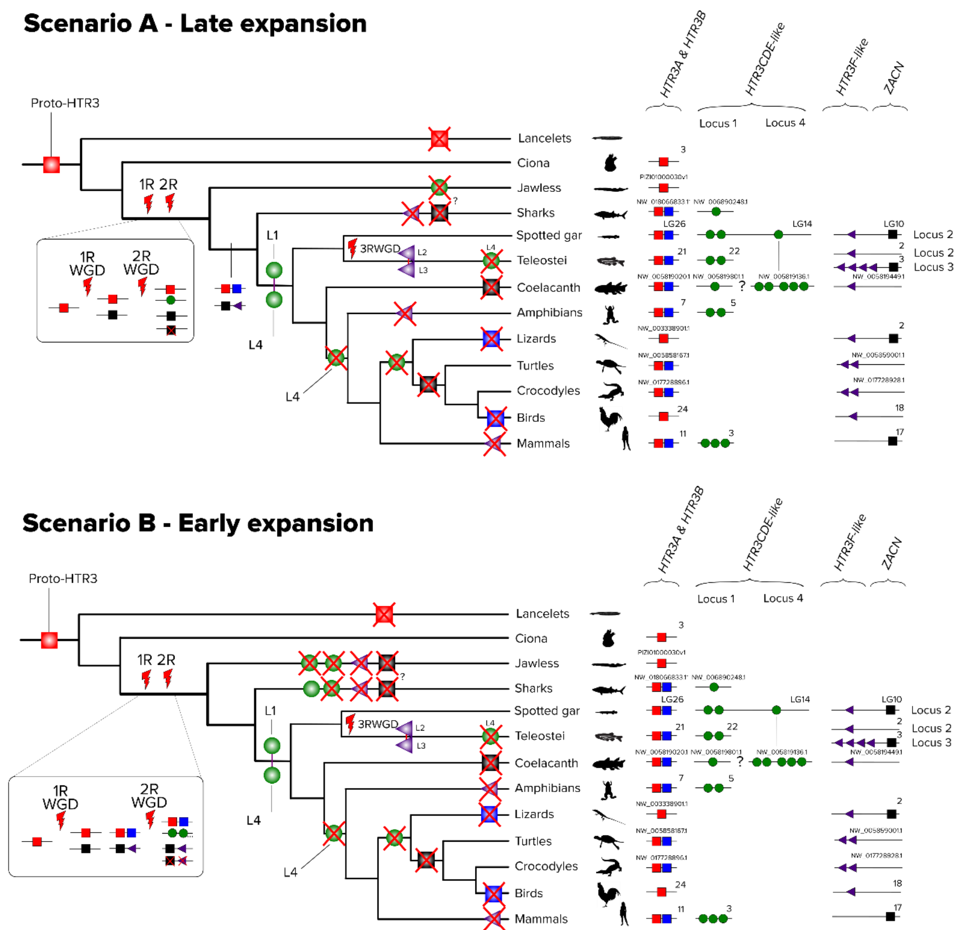

Figure 5. Two proposed 5-HT3 evolutionary scenarios for the origin of the 5-HT3 gene family, highlighting the major events during the family evolution in Chordata. In branches only lost are and duplications events are represented. For simplicity, gene number variability within each branch is not represented. Trees are not time-scaled. A) Late expansion. Proto-

3A and proto-ZACN genes arose after 1RWGD from a common ancestral gene, and proto-3C after 2RWGD. Later on, HTR3B and HTR3F arose by tandem duplication of proto-3A and proto-ZACN, respectively. B) Early expansion. Proto-3A and proto-ZACN arose from 1RWGD, then duplicated in tandem to originate HTR3B and HTR3F, respectively. Afterwards, 2RWGD originated two proto-3CDE-like genes from HTR3A and HTR3B, and an extra proto-ZACN-3F pair. On the right, chromosome regions or contigs harboring the genes are depicted, with the corresponding numbers. For each group, we selected a representative: Mammals: *Homo sapiens,* Birds: *Gallus gallus*, Crocodiles: *Crocodylus porosus,* Turtles: *Pelodiscus sinensis,* Amphibians: *Xenopus tropicalis,* Coelacanth: *Latimeria chalumnae,* Teleostei: *Danio rerio,* Spotted gar: *Lepisosteus oculatus,* Sharks: *Rhincodon typus* (*HTR3A* and *HTR3B*) and *Callorhinchus milii* (*HTR3CDE*), Jawless vertebrates: *Petromyzon marinus,* Ciona: *Ciona intestinalis*, and Lancelet: *Branchiostoma floridae.*

One, two, three, four...? Does it matter to have less or more?

In order to explain the possible origins of the family, we propose two possible scenarios (Figure 5) taking into account the two rounds of whole genome duplication (1RWGD and 2RWGD) that occurred early in vertebrate evolution (Dehal and Boore 2005) and the third round (3RWGD) that occurred in the Teleostei lineage.

The first scenario (Scenario A - the late tandem duplication, after 2RWGD, Figure 5A) corresponds to a *late* expansion, in which a common ancestral gene (here called *proto-HTR3*) underwent the first round of whole genome duplication (1RWGD) that originated two genes (*proto-HTR3A* and *proto-ZACN-HTR3F*) and then the second round (2RWGD) originated *proto-3CDE* and a *proto-ZACN-HTR3F-like* genes. Later, *HTR3B,* CDE-like, and other *proto-ZACN-HTR3F* genes arose by tandem duplication. Thereafter, successive tandem duplications and losses shaped the gene distribution that we see today in chordates. This scenario is supported by the observed gene distribution in the phylogenetic analyses, which suggest that the *AB* duplication and *CDE* expansion occurred at different times.

In a second possible scenario corresponding to an *early* expansion (Scenario B - early tandem duplication, before 2RWGD Figure 5B), tandem duplications that gave rise to *HTR3B*, *proto-CDE,* and *proto-ZACN-HTR3F* occurred before 2RWGD or even before the

1RWGD. This originated two CDE-*like* ancestors that gave rise to all CDE-*like* genes by subsequent tandem duplications, as well as an extra *ZACN-HTR3F* pair that was lost in jawless vertebrates. Noteworthy, the timing of the 2RWGD is a still controversial, and it is unclear whether the 2RWGD occurred before or after the Agnatha-Gnathostomata split (Kuraku et al. 2009; Pascual-Anaya et al. 2018).

In both scenarios described above, we observe that *CDE* genes may have a more complicated evolutionary history than previously expected. With few exceptions, grouping in both ML and Bayesian trees match *HTR3C, HTR3D* and *HTR3E* genes in mammals, but an extra cluster comprising CDE genes from diverse mammalian species arise as a sister group. One of the possible explanations for such observation would be that the ancestral mammalian *CDE* duplicated in tandem twice, giving rise to four genes that were differentially lost in most mammalian species. Alternatively, the observed scenario could have arisen from gene conversion occurring differentially soon after *CDE* expansion in the mammalian lineage (Carson and Scherer 2009; Fawcett and Innan 2011). The latter explanation could also explain the finding that marsupial *CDE* genes group together in the tree, thus preventing the establishment of one-to-one orthology relationships with mammalian CDE (Figure 2).

Positive selection in the 5-HT3 family

Our molecular evolution analyses revealed differences in evolutionary rates and natural selection pressure among genes in placental mammals (Eutheria), and birds and reptiles (Sauropsida) (Table 1). *HTR3A* showed to be under strong purifying selection in both groups, a coherent finding given the essentiality of 3A subunit for receptor functionality. Notwithstanding, previous work from our group found signals of positive selection in several sites of *HTR3A* intracellular domain in New World monkeys, suggesting possible room for adaptive novelties in this gene (Reales et al. 2018), illustrating how an extremely conserved gene may admit changes in some branches of a phylogeny. *HTR3B* shows moderate signals of positive selection, with two sites (332R and 386Q in the human canonical sequence, O95264) located in the intracellular domain. *HTR3C, HTR3D, HTR3E, HTR3F,* and *ZACN* all showed significant signatures of positive selection, being mammalian *CDE*s the ones with highest evolutionary rates, and sites under putative positive selection. However, most sites under putative positive selection are located in the extracellular domain, with few exceptions, and no site was found in the four transmembrane domains, which are expected to be under strong purifying selection, given the essentiality for receptor structure. Thus,

changes in the intracellular and extracellular domains may provide a fundamental window of evolvability for the serotoninergic system, preserving its primordial basal functions.

Table 1. Molecular evolution analyses using site models M8a and M8 reveal different selective pressures and evolutionary rates across genes in placental mammals (Eutheria) and birds and reptiles (Sauropsida).

Gene set	N	Mean *dN/dS* (ω)	M8a (lnL)	M8 (lnL)	LRT P-value (df = 1)	Positive selected sites (pp >0.95)	*dN/dS* (ω10)
HTR3A (Eutheria)	40	0.1375	-14794.62132	-14794.42039	0.526		
HTR3A (Sauropsida)	14	0.1156	-8362.89845	-8361.151554	0.062		
HTR3B (Eutheria)	40	0.4138	-15032.73476	-15026.84531	6×10^{-4}*	327, 381	1.53
HTR3C (Eutheria)	27	0.4426	-11802.14766	-11793.11091	2.13×10^{-5}*	7, 65, 115, **127**, **129**	1.60
HTR3D (Eutheria)	29	0.6050	-15185.78801	-15163.24352	1.88×10^{-11}*	**2**, **3**, 5, 9, 117, **138**, 183, 282	2.18
HTR3E (Eutheria)	29	0.5640	-14436.32978	-14306.71626	2.53×10^{-58}*	**2**, **4**, **5**, **10**, **11**, **12**, **16**, **18**, **21**, **22**, **65**, **128**, **386**	5.00
HTR3CDE (Extra group, Eutheria)	14	0.4268	-7785.73265	-7777.20022	3.61×10^{-5}*	7, 345, **366**, **370**	2.15
HTR3F (Sauropsida)	14	0.5192	-11095.78805	-11093.30418	0.025*	219	1.49
ZACN (Eutheria)	24	0.3414	-10231.83033	-10227.76135	0.004*	204	1.50

Sites in bold indicate posterior probability (pp) > 0.99.

Conclusions

Our study provided the first glimpse of complex and dynamic evolutionary history of 5-HT3 receptors in Chordates, characterized by multiple duplications, losses in all major chordate taxa. We found and characterized a potential additional 5-HT3 homolog, that is found in most birds, reptiles, and fishes, but that seems to be lost in mammals. We named this gene *HTR3F*, and it is located next to *ZACN*, a relatively newly discovered pLGIC, which was lost in most species in which we found *HTR3F*. Further structural studies would provide more insights into the *HTR3F* functional and structural characteristics. We also proposed two alternative scenarios for the origin of the 5-HT3 family in chordates, using a phylogenetic approach. Both scenarios take into account the possible role of WGD in the evolution of the family, including the emergence of the closely related *ZACN*. The study of

the 5-HT3 receptor would benefit from incorporating transcriptomic data, in order to determine if genes not found in scaffold genomes were lost indeed, and if genes that we found are actually expressed. In addition, distribution of subunit expression would shed light on receptor function, as compartmentalization has been shown to play also an important role in the variability of function in neuromodulatory genes.

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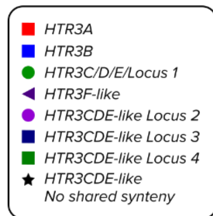

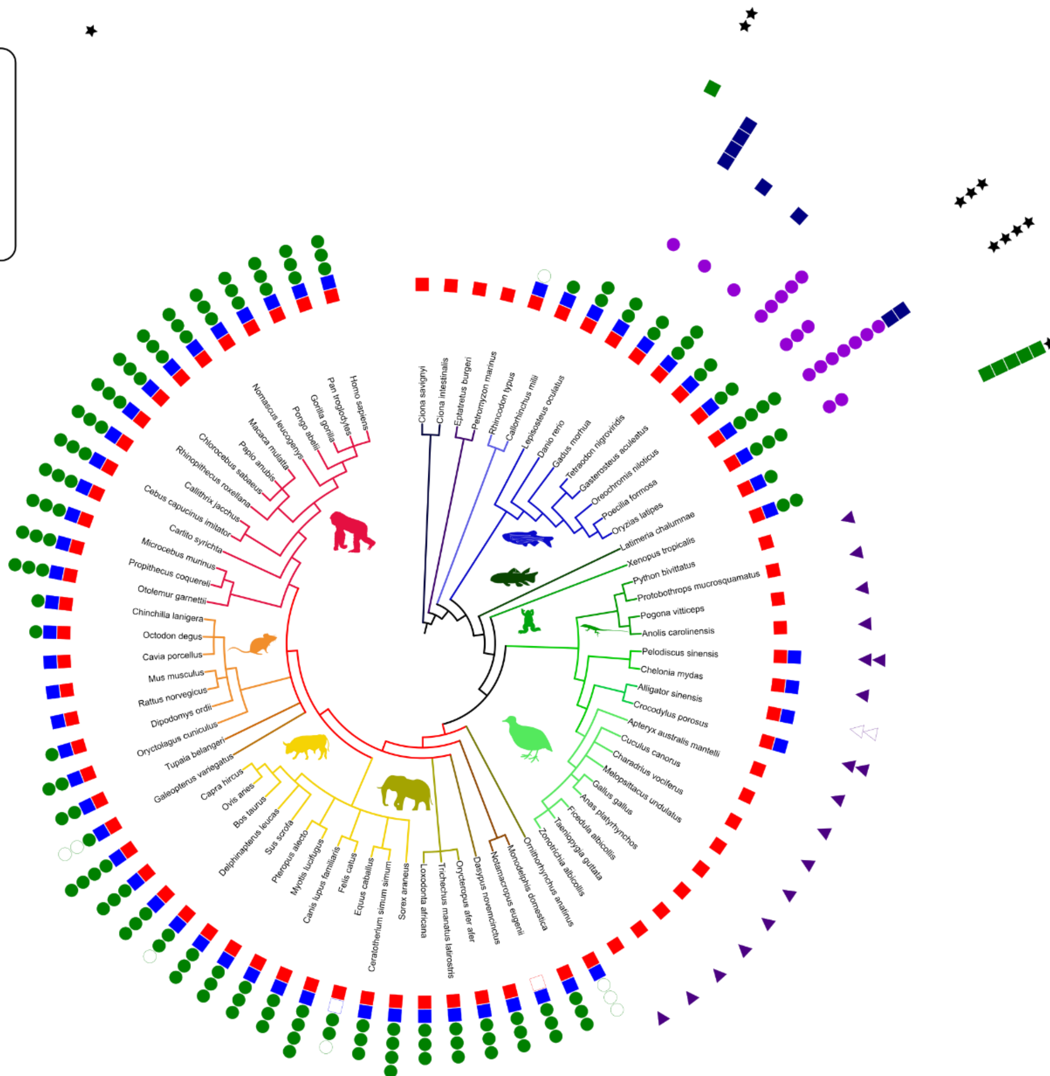

Figure S1. Distribution of 5-HT3 genes in the 76 species subset used for phylogenetic analyses.

Figure S2. Bayesian tree generated by phylogenetic analysis in BEAST2. Branch colours indicate posterior probabilities (green = high, red = low).

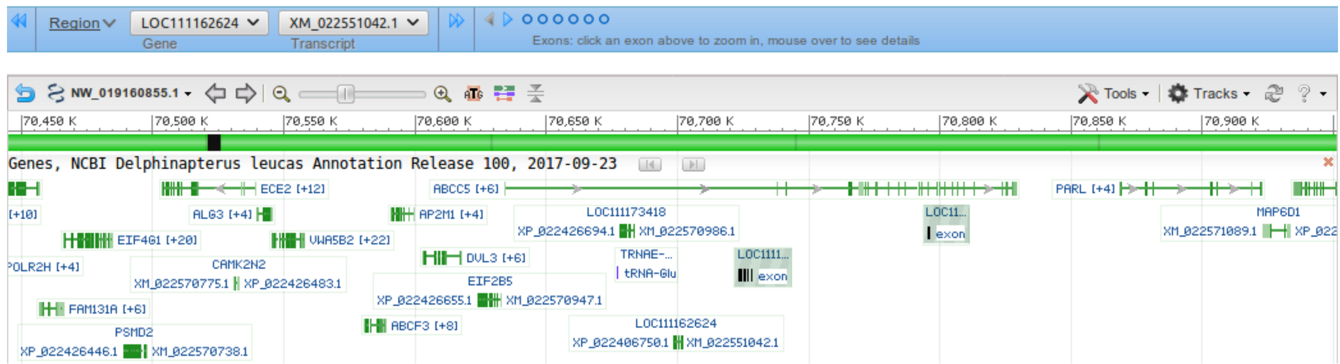

Figure S3. Segment of the beluga whale (*Delphinapterus leucas*) genome as visualised in NCBI Genome Data Viewer. XM_022551042.1 and XM_022570986.1 identifiers correspond to the two putative CDE orthologs, which are contained in a predicted *ABCC5* gene intron.

NOTE: Given the size of the supplementary tables, only the first page is presented here. Full Supplementary Tables are available online in the following link: https://bit.ly/2xjaodL

Table S1. Identifiers and additional information of all sequences used in this study.

Species	CDS ID	Protein ID	Gene denomination	Source
Acanthisitta chloris	XM_009071927.1/XM_009075488.1 Hybrid	XP_009070175.1/XP_009073736.1 Hybrid	HTR3A	ENA/Uniprot KB
Acanthisitta chloris	XM_009084966.1	XP_009083214.1	HTR3F	ENA/Uniprot KB
Acanthochromis polyacanthus	XM_022204845.1	XP_022060537.1	HTR3B	ENA/Uniprot KB
Acanthochromis polyacanthus	XM_022204846.1	XP_022060538.1	HTR3A	Genscan/UCSC Genome Browser
Ailuropoda melanoleuca	ENSAMEG00000005986	ENSAMEG00000005986	HTR3B	Genscan/UCSC Genome Browser
Ailuropoda melanoleuca	ENSAMEG00000013316	ENSAMEG00000013316	HTR3A	ENA/Uniprot KB
Ailuropoda melanoleuca	ENSAMEG00000017308	ENSAMEG00000017308	HTR3E	ENA/Uniprot KB
Ailuropoda melanoleuca	ENSAMEG00000017320	ENSAMEG00000017320	HTR3C	ENA/Uniprot KB
Ailuropoda melanoleuca	ENSAMEG00000017325	ENSAMEG00000017325	HTR3D	Ensembl
Alligator mississippiensis	XM_006269182.2	XP_006269244.1	HTR3B	Ensembl
Alligator mississippiensis	XM_019483989.1	A0A151P163	HTR3A	Ensembl
Alligator sinensis	XM_006026681.2	A0A1U7RVQ5	HTR3B	Ensembl
Alligator sinensis	XM_006026682.1	A0A1U7S741	HTR3A	Ensembl
Amazona aestiva	KQK78711.1	A0A0Q3M7K3	HTR3A	Ensembl
Amphiprion ocellaris	XM_023294816.1	XP_023150584.1	HTR3A	Ensembl
Amphiprion ocellaris	XM_023294817.1	XP_023150585.1	HTR3B	Ensembl
Anas platyrhynchos	ENSAPLG00000002673	ENSAPLG00000002673	HTR3A	Ensembl
Anas platyrhynchos	ENSAPLG00000006297	ENSAPLG00000006297	HTR3F	Ensembl
Anolis carolinensis	ENSACAG00000009214	ENSACAG00000009214	HTR3A	Ensembl
Anolis carolinensis	XM_016991353.1	XP_016846842.1	HTR3F	Ensembl
Anser cygnoides domesticus	XM_013179228.1	XP_013034682.1	HTR3F	Ensembl
Anser cygnoides domesticus	XM_013187098.1	XP_013042552.1	HTR3A	Ensembl
Antrostomus carolinensis	XM_010171342.1	XP_010169644.1	HTR3F	Ensembl
Antrostomus carolinensis	XM_010174154.1	XP_010172456.1	HTR3A	Ensembl

Aotus nancymaae	ENSANAG00000016907	ENSANAG00000016907	HTR3A	Ensembl
Aotus nancymaae	ENSANAG00000025924	ENSANAG00000025924	HTR3B	Ensembl
Aotus nancymaae	ENSANAG00000037618	ENSANAG00000037618	HTR3E	Ensembl
Aotus nancymaae	ENSANAG00000038263	ENSANAG00000038263	HTR3C	Ensembl

Table S2. Protein domain analysis using PFAM domains and InterproScan

ProteinID	Prot Length	Domain ID	Domain name	Start	End	E-value	InterproID	InterproName	Domain Length
Acanthisitta_chloris|XP_009070175.1_XP_009073736.1|HTR3A	387	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	40	246	2.1E-53	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	206
Acanthisitta_chloris|XP_009070175.1_XP_009073736.1|HTR3A	387	PF02932	Neurotransmitter-gated ion-channel transmembrane region	253	373	9.1E-19	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	120
Acanthisitta_chloris|XP_009083214.1|HTR3F	434	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	44	237	4E-30	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	193
Acanthochromis_polyacanthus|XP_022060537.1|HTR3B	459	PF02932	Neurotransmitter-gated ion-channel transmembrane region	245	337	1.2E-21	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	92
Acanthochromis_polyacanthus|XP_022060537.1|HTR3B	459	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	33	238	2.9E-50	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	205
Acanthochromis_polyacanthus|XP_022060538.1|HTR3A	494	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	42	248	5.2E-50	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	206
Acanthochromis_polyacanthus|XP_022060538.1|HTR3A	494	PF02932	Neurotransmitter-gated ion-channel transmembrane region	255	483	2.3E-38	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	228
Ailuropoda_melanoleuca|ENSAMEG00000005986|HTR3B	444	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	4	210	2.5E-48	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	206
Ailuropoda_melanoleuca|ENSAMEG00000005986|HTR3B	444	PF02932	Neurotransmitter-gated ion-channel transmembrane region	217	298	5.3E-13	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	81
Ailuropoda_melanoleuca|ENSAMEG00000013316|HTR3A	512	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	35	242	1.9E-55	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	207
Ailuropoda_melanoleuca|ENSAMEG00000013316|HTR3A	512	PF02932	Neurotransmitter-gated ion-channel transmembrane region	249	307	3.4E-15	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	58
Ailuropoda_melanoleuca|ENSAMEG00000013316|HTR3A	512	PF02932	Neurotransmitter-gated ion-channel transmembrane region	340	502	4.6E-16	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	162
Ailuropoda_melanoleuca|ENSAMEG00000017308|HTR3E	444	PF02932	Neurotransmitter-gated ion-channel transmembrane region	236	337	3.2E-14	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	101
Ailuropoda_melanoleuca|ENSAMEG00000017308|HTR3E	444	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	24	226	2.5E-37	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	202
Ailuropoda_melanoleuca|ENSAMEG00000017320|HTR3C	446	PF02932	Neurotransmitter-gated ion-channel transmembrane region	254	354	1.3E-15	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	100
Ailuropoda_melanoleuca|ENSAMEG00000017320|HTR3C	446	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	52	247	2.8E-39	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	195

Ailuropoda_melanoleuca|ENSAMEG00000017325|HTR3D	457	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	52	247	2E-41	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	195
Ailuropoda_melanoleuca|ENSAMEG00000017325|HTR3D	457	PF02932	Neurotransmitter-gated ion-channel transmembrane region	254	365	2E-16	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	111
Alligator_mississippiensis|A0A151P163|HTR3A	487	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	41	247	9.5E-56	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	206
Alligator_mississippiensis|A0A151P163|HTR3A	487	PF02932	Neurotransmitter-gated ion-channel transmembrane region	254	476	1.6E-36	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	222
Alligator_mississippiensis|XP_006269244.1|HTR3B	462	PF02932	Neurotransmitter-gated ion-channel transmembrane region	242	321	1.1E-12	IPR006029	Neurotransmitter-gated ion-channel transmembrane domain	79
Alligator_mississippiensis|XP_006269244.1|HTR3B	462	PF02931	Neurotransmitter-gated ion-channel ligand binding domain	34	235	4.6E-45	IPR006202	Neurotransmitter-gated ion-channel ligand-binding domain	201

Chapter 5. Final conclusions

The study of evolution in genetics can be undertaken at different levels and time scales, from the analysis of the genetic variability among populations of the same species across few generations (microevolution) to the study of change across different species and taxonomic groups (macroevolution). Both levels of analysis share an underlying framework, but require different approaches and techniques.

Other studies conducted and/or published during this PhD contributed to enhance our understanding of genotype/phenotype connections at a microevolutionary level, using human Native South American (Reales et al. 2017, Annex I) and general Latin American populations (Adhikari et al. 2016, Annex II) as a study subject.

The results obtained in this Thesis, however, contribute to the body of research that aim to understand the evolution of neuroreceptor systems, and their implication in behavior and other phenotypes at a macroevolutionary level. Specific discussions on the results of each objective are contained in their corresponding chapters (chapter 3 and 4), and this section will be devoted to highlight the main insights obtained in this Thesis.

Since its discovery, 5-HT has fascinated researchers due to the dramatic consequences of serotonergic disestabilization on human health, and the variety of functions and processes in which it is involved. In the words of professor Irvine Page in his 1968 book: "*If I had to select a single effect resulting from the discovery of serotonin, I would unhesitatingly suggest its influence in shaping investigators' ideas on cerebral activity"* – and it has not stopped surprising us ever since. Indeed, despite the huge progress, we are still far from fully elucidating all the mechanisms through which 5-HT influences our life and our health. To date, relatively few studies have been directed towards understanding the serotonergic system within its evolutionary context (e.g. Peroutka 1994; Andrés et al. 2007; Azmitia 2010), if we consider serotonin relevance across the Tree of life. Interestingly, serotonin receptors are quite numerous with different affinities and transduction pathways, allowing serotonin to have such diverse effects inside and outside the central nervous system. However, these receptors do not keep a one-to-one receptor-function relationship, and their actions are dependent of where they are expressed, and their interactions with other neurotransmitters. These facts are consistent with the early serotonin appearance as a regulatory molecule of life.

The 5-HT3 receptor is a very special receptor, in that it is the only known vertebrate ion channel activated primarily by serotonin, and that it forms pentamers encoded by several different genes, that diverged greatly in number and sequence during vertebrate evolution, as showed in Chapter 4 of this Thesis.

The observed diversity of 5-HT3 receptor subunits across different taxa provide the raw materials for the formation of a varied repertoire of 5-HT3 receptors, and supports a scenario of extraordinary evolutionary plasticity among taxa. Interestingly, this plasticity does not seem to be equal for all subunits. As previously stated, *HTR3A* is required for receptor formation and function, derived from the fact that all functional 5-HT3 receptors identified *in vivo* have at least one 5-HT3A subunit, and it is the only subunit known to form homomeric receptors. Our results are compatible with this, as *HTR3A* was the only gene of the family that we found in all chordates surveyed, which also supports an ancestral position of *HTR3A*. Noteworthy, when we evaluated the evolutionary rates at the molecular level (*dN/*dS) of 5-HT3 genes in two animal groups with different 5-HT3 repertoires (*Eutheria,* placental mammals and *Sauropsida*, reptiles and birds), we observed that in both groups *HTR3A* had strikingly low evolutionary rates compared to other subunits (e.g. *HTR3C, HTR3D,* and *HTR3E*). These results support the idea that the system evolves differentially, allowing certain subunits to evolve at a faster pace while preserving the core receptor structure and functionality. Pharmacological and structural studies indicated that human heteroreceptors containing 5-HT3C, 5-HT3D, and/or 5-HT3E subunits have subtle yet significant pharmacological and electrical differences (Holbrook et al. 2009; Price et al. 2017), which together with their distinct expression patterns (Kapeller et al. 2011) support the idea that they play specialized physiological roles. On the other hand, *HTR3B* subunit has been widely shown to produce major differences in receptor physicochemical properties (see Chapter 1), which is coherent with the low evolutionary rates that we observed.

Despite high levels of conservation of *HTR3A* at the sequence level, we showed in Chapter 3 that mutations with potential functional implications may arise and be selected within specific taxa, as illustrated by the study of the M3-M4 intracellular domain of 20 New World monkey species. 5-HT3 receptor is a ligand-gated ion channel, which canonical signaling pathway does not include second messengers (as it is the case for its fellow serotonergic receptors); however, it might interact with cytosolic proteins through its disordered region in M3-M4 domain. Interestingly, we found a linear motif for *GSK3* at a

selected site in NWMs. *GSK3* is a multifaceted enzyme that participates in numerous regulatory processes, and has been previously shown to interact with other ligand-gated and voltage-gated ion channels (e.g. Na_v, NMDA and AMPA receptors), as well as with 5-HT1B receptors (see Chapter 3 and references therein). In addition, it has a fundamental role in synaptic plasticity, among others (Bradley et al. 2012).

The establishment of functional connections between molecular changes and behavioral phenotypes will require further experimental evidence, in order to take into account the multiple factors (e.g. molecular dynamics, synaptic plasticity, and environmental interaction) that pave the way from molecules to complex behaviors.

Altogether, the results presented in this Thesis provide a first glimpse at the complex evolutionary history of the 5-HT3 receptor family, and allow us to speculate that the observed diversity at the genic level constitute an example of evolutionary flexibility. Such flexibility, likely allowed fine-tuned regulation of a broad array of physiological and behavioral functions across vertebrates living under different ecological niches and selective pressures.

The 5-HT3 receptor was discovered more than 60 years ago, although most of their human subunits (namely *HTR3C, HTR3D,* and *HTR3E*) had to wait until the *genomics* revolution in the 21st century to be discovered and characterized. Although some remarkable advances have been made in a relatively short time, there are many questions to be answered, such as the specific involvements of gut 5-HT3 receptors in the microbiome-gut-brain axis, and the effects of such interactions on behavior. The present Thesis contributes to the understanding of the evolutionary aspects of the 5-HT3 receptor in a broad range of vertebrates, and also opens the way to further questions, such as the specific role of the novel gene, here referred to as *HTR3F* present in birds, reptiles and some fishes.

Our data and results, together with those by other authors here discussed, allow us to anticipate many surprises in the coming years regarding this instigating genetic system. I hope that I can further contribute to this fascinating field!

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Annex I. A tale of agriculturalists and hunter-gatherers: Exploring the thrifty genotype hypothesis in Native South Americans

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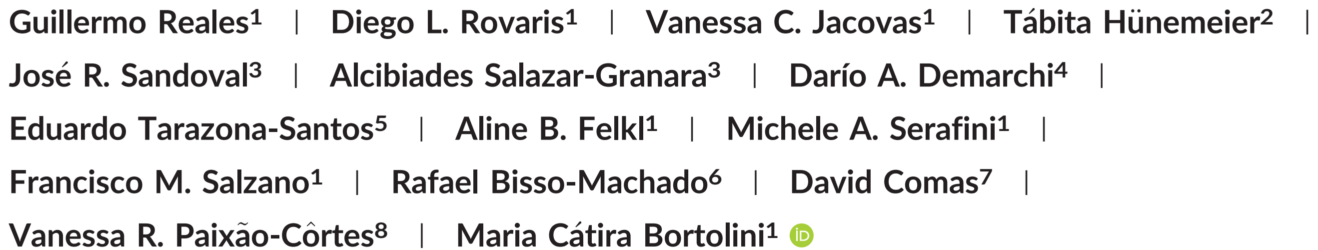
A tale of agriculturalists and hunter-gatherers: Exploring the thrifty genotype hypothesis in native South Americans



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Abstract

Objectives: To determine genetic differences between agriculturalist and hunter-gatherer southern Native American populations for selected metabolism-related markers and to test whether Neel's thrifty genotype hypothesis (TGH) could explain the genetic patterns observed in these populations.

Materials and Methods: 375 Native South American individuals from 17 populations were genotyped using six markers (*APOE* rs429358 and rs7412; *APOA2* rs5082; *CD36* rs3211883; *TCF7L2* rs11196205; and *IGF2BP2* rs11705701). Additionally, *APOE* genotypes from 39 individuals were obtained from the literature. AMOVA, main effects, and gene-gene interaction tests were performed.

Results: We observed differences in allele distribution patterns between agriculturalists and hunter-gatherers for some markers. For instance, between-groups component of genetic variance (F_{CT}) for *APOE* rs429358 showed strong differences in allelic distributions between hunter-gatherers and agriculturalists ($p = 0.00196$). Gene-gene interaction analysis indicated that the *APOE* E4/*CD36* TT and *APOE* E4/*IGF2BP2* A carrier combinations occur at a higher frequency in hunter-gatherers, but this combination is not replicated in archaic (Neanderthal and Denisovan) and ancient (Anzick, Saqqaq, Ust-Ishim, Malta) hunter-gatherer individuals.

Discussion: A complex scenario explains the observed frequencies of the tested markers in hunter-gatherers. Different factors, such as pleotropic alleles, rainforest selective pressures, and population dynamics, may be collectively shaping the observed genetic patterns. We conclude that although TGH seems a plausible hypothesis to explain part of the data, other factors may be important in our tested populations.

KEYWORDS
adaptation, APOE, mode of subsistence, pleiotropy

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Annex II. A genome-wide association study identifies multiple loci for variation in human ear morphology

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A genome-wide association study identifies multiple loci for variation in human ear morphology

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Here we report a genome-wide association study for non-pathological pinna morphology in over 5,000 Latin Americans. We find genome-wide significant association at seven genomic regions affecting: lobe size and attachment, folding of antihelix, helix rolling, ear protrusion and antitragus size (linear regression *P* values 2×10^{-8} to 3×10^{-14}). Four traits are associated with a functional variant in the Ectodysplasin A receptor (*EDAR*) gene, a key regulator of embryonic skin appendage development. We confirm expression of *Edar* in the developing mouse ear and that *Edar*-deficient mice have an abnormally shaped pinna. Two traits are associated with SNPs in a region overlapping the T-Box Protein 15 (*TBX15*) gene, a major determinant of mouse skeletal development. Strongest association in this region is observed for SNP rs17023457 located in an evolutionarily conserved binding site for the transcription factor Cartilage paired-class homeoprotein 1 (*CART1*), and we confirm that rs17023457 alters *in vitro* binding of *CART1*.

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