Universidade de Aveiro Departamento de Ciências Médicas
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Filipa de Jesus Costa

Efeito do desequilíbrio de aminoácidos no desenvolvimento neuronal

The effect of amino acid imbalance in neuronal development

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica do Doutor Ramiro Daniel Carvalho de Almeida, Professor Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro e da Doutora Ana Margarida Sousa, Investigadora e Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro.

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palavras-chave

Triptamina, triptofano, triptofanil-tRNA sintetase, sistema nervoso central, hipocampo, agregação proteica

resumo

A triptamina é um aminoácido não-canónico que pode ser obtido através da dieta, pela descarboxilação do triptofano ou sintetizado pela microbiota intestinal. A triptamina está envolvida na regulação do sistema nervoso entérico e do sistema nervoso central, permitindo uma comunicação bidirecional entre estes dois sistemas (eixo intestino-cérebro). Em concentrações fisiológicas, no sistema nervoso central atua como um modulador. No entanto, em concentrações não fisiológicas tem um efeito neurotóxico e induz agregação proteica. Esta agregação proteica resulta do desequilíbrio entre as concentrações de triptamina e triptofano, que levam à inibição da enzima triptofanil-tRNA sintetase (TrpRS).

O objetivo desta dissertação é estudar o efeito da triptamina em neurónios do sistema nervoso central. Para isso, estimularam-se com diferentes concentrações de triptamina células SH-SY5Y e neurónios do hipocampo de embrião de rato. Verificámos que a triptamina induziu a acumulação de agregados proteicos sem comprometer a viabilidade dos neurónios. Resultados semelhantes foram observados em células SH-SY5Y. No entanto, nesta linha celular o efeito foi dependente da concentração de triptamina. Além disto, a triptamina reduziu o número de sinapses.

Todos estes resultados indicam que o desequilíbrio prolongado da proporção triptamina/ triptofano induz a formação e acumulação de agregados proteicos em neurónios do sistema nervoso central alterando assim as funções neuronais.

keywords

Tryptamine, tryptophan, tryptophanyl-tRNA synthetase, central nervous system, hippocampi, protein aggregates

abstract

Tryptamine is a non-canonical amino acid that can be obtained in the diet, by tryptophan decarboxylation or synthesized by the gut microbiota. The tryptamine acts at the level of enteric and central nervous systems, enabling bidirectional communication between the two systems (gut-brain axis). In the central nervous system, at physiological concentrations, acts as a modulator. However, at non-physiological concentrations it has a neurotoxic effect and induces protein aggregation. This protein aggregation results from the imbalance between tryptamine and tryptophan concentrations, which leads to inhibition of the enzyme tryptophanyl-tRNA synthetase (TrpRS).

The objective of this thesis project is to study the effect of tryptamine in central nervous system neurons. For this, SH-SY5Y cells and rat embryonic hippocampal neurons were stimulated with different tryptamine concentrations. Tryptamine induced an accumulation of protein aggregates without compromising the viability of hippocampal neurons. Similar results were observed in SH-SY5Y cells, however in this case a dose-dependent effect was observed. In addition, tryptamine reduced the number of synaptic clusters.

Together these results indicate that a prolonged imbalance of the tryptamine/ tryptophan ratio induces the formation and accumulation of protein aggregates in central nervous system neurons, altering neuronal function.

Contents

List of Abbreviations

AAAD	Aromatic amino acid decarboxylase
AD	Alzheimer's disease
AhR	Aryl hydrocarbon receptor
ARS	Aminoacyl-tRNA synthetases
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
DIV	Days *in vitro*
DMSO	Dimethyl sulfoxide
ECs	Enterochromaffin cells
EEs	Enteroendocrine cells
ENS	Enteric nervous system
FBS	Fetal bovine serum
F12	L-glutamine
GPCRs	G protein-coupled receptors
HBSS	Hank's balanced salt solution
HeLa	Human cervical carcinoma cell line
HSP27	Heat-shock protein 27
IDO	Indoleamine 2,3-dioxygenase
GFP	Green fluorescent protein
KA	Kynurenic acid
KP	Kynurenine pathway
MAO	Monoamine oxidase enzyme
MAP2	Microtubule-associated protein 2
MDBK	Madin-Darby bovine kidney

MEM	Minimum essential medium
NAD	Nicotinamide adenine dinucleotide
NFT	Neurofibrillary tangles
ns	Non-significant
PKA	Protein kinase A
PKC	Protein kinase C
PSD95	Postsynaptic density protein 95
TAARs	Trace amine-associated receptor
TDO	Tryptophan 2,3-dioxygenase
TPH	Tryptophan hydroxylase
Trp	Tryptophan
TrpRS	Tryptophanyl-tRNA synthetase
TUBB 3	Anti-Tubulin β 3
PD	Parkinson's disease
PDL	Poly-D- Lysine
PFA	Paraformaldehyde
SCFAs	Short-chain fatty acids
SEM	Standard error of the mean
SH-SY5Y	Human neuroblastoma cell line
ROI	Region of interest
ROS	Reactive oxygen species
RT	Room temperature
VAN	Vagal afferent neurons
VEN	Vagal efferent neurons
VGluT	Vesicular glutamate transporter
VMAT	Vesicular monoamine transporter
5-FDU	5-Fluoro-2'-deoxyridine
5-HT	Serotonin
5-HTP	5-hydroxytryptophan

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1 Introduction

1.1 Gut-Brain Axis

The gut-brain axis consists of a communication "path" between the enteric nervous system (ENS) and central nervous system (CNS). This communication is bidirectional (Mayer, 2011) and occurs through the vagal nerve (**Fig. 1**) (O'Mahony *et al.*, 2015). Vagal innervation may occur by vagal efferent neurons (VEN) or vagal afferent neurons (VAN). VEN are responsible for sending motor information from the brain to the peripheral organs. While VAN transmit sensory information from the periphery to the CNS (Cawthon and de La Serre, 2018). VAN cell bodies are located in the nodose ganglion (Sutton, Patterson and Berthoud, 2004). However, the nerve terminals of the VAN are located on the lamina propria of the gastrointestinal tract and can thus interact with the gut microbiota (Patterson, Zheng and Berthoud, 2002).

The microbiome corresponds to the set of all microbes (viruses, bacteria, fungi and protozoa), their genes and genomes. However, the microbiota is defined as a set of microorganisms (bacteria, archaea, lower eukaryotes) present in a given environment. These two terms have different meanings but are frequently used interchangeably (Quigley, 2017). The human body hosts complex microbial communities (~100 trillion microbial symbionts) whose combined association exceeds by about ten times the cells of our body (Savage, 1977). A large number of microbial species reside in the distal gastrointestinal tract and, to the level of bacterial strains, the gut microbiota demonstrates enormous diversity and variation between individuals (Lynch and Pedersen, 2016).

The gut-brain axis is established by metabolic and endocrine messengers as well as immune mediators sensitive to nutrients and components of the intestinal microbiota (microbiota-gut-brain axis) (Mayer, 2011). Ivan Petrovich Pavlov was the first to demonstrate that the brain directly influences gut function (Tansey, 2006).

In recent years it has been noted that the intestinal microbiota encompasses several functions such as: (a) maintenance of the epithelial barrier, (b) inhibition of pathogen adhesion to

intestinal surface, (c) modulation and adequate maturation of the immune system, (d) degradation of otherwise nondigestible carbon sources, and (e) production of different metabolites such as vitamins and short-chain fatty acids (SCFAs) (Sánchez *et al.*, 2017). Some of these functions require a delicate degree of regulation and coordination provided by the ENS (Rao and Gershon, 2016).

The ENS is one division of the autonomic nervous system (composed by the sympathetic, parasympathetic and enteric components) (Langley, 1916). The ENS can function independently of the CNS and it is a complex network containing more than 100 million neurons. The number of efferent fibres that enervate the gut in the vagus nerve is less than the number of afferent fibres (Furness, 2006). The ENS is composed of epithelial sensors such as enteroendocrine cells (EEs), enteric glia and neurons of peripheral ganglia, which constitute the gut connectome (Bohórquez and Liddle, 2015). This is responsible for modulating gut motility, endocrine function, transmucosal fluid movement, and local blood flow (Goyal and Hirano, 1996). Thus, the signals originating in one of these organ systems affect the function of the other (Powell, Walker and Talley, 2017).

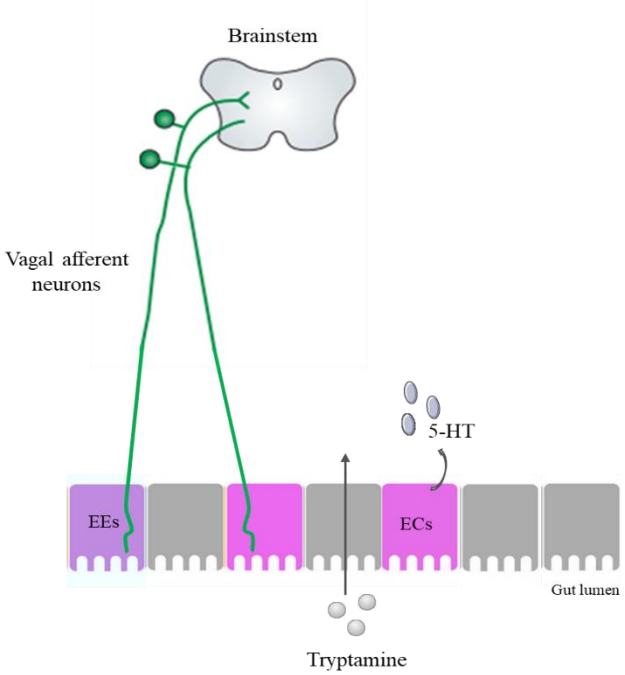

Figure 1. Communication between the gut and the brain.
Serotonin (5-HT) is released by enterochromaffin cells (ECs) and activates specific receptors in the vagus nerve. Serotonin release is induced by tryptamine. Adapted from (Mayer, 2011; Roager and Licht, 2018).

1.1.1 Perturbation of the Gut-Brain Axis and its Implication in Disease

Alterations in brain-gut microbiota interactions are thought to be involved in the pathogenesis of well-known disorders. One such example are functional gastrointestinal disorders (Rhee, Pothoulakis and Mayer, 2009; Mayer, 2011), and more recently have been implicated as a possible mechanism in the pathophysiology of brain disorders (Mayer *et al.*, 2015). It is known that the ENS and CNS have many morphological, physiological and pharmacological characteristics in common, so if bacteria can influence the ENS, they could similarly impact CNS function (Mayer *et al.*, 2015).

The gut microbiome has been associated with neurological disorders. There is some evidence that microbial populations characteristic of the gastrointestinal tract (periodontal, oral and nasal communities) are altered in patients with Alzheimer's disease (AD) and Parkinson's disease (PD) (Westfall *et al.*, 2017) when compared with healthy individuals. *Heliobacteria pylori* infection has been associated with risk of developing PD (Shen *et al.*, 2017) and *Escherichia* and *Shigella* were associate with pro-inflammatory cytokines and amyloid deposition in the brain (Cattaneo *et al.*, 2017). There are many factors that contribute to these microbiota alterations such as aging (Shoemark and Allen, 2015) and diet (Claesson *et al.*, 2012). During aging changes occur in the gut microbiota, as well as a decrease in immune function and a consequent increase in the probability of infection. On the other hand, the diet influences the composition of the gut microbiota (Claesson *et al.*, 2012) and in older people this composition varies considerably between individuals (Claesson *et al.*, 2011). Furthermore, the lower diversity of microbiota, inflammation and disability was associated with poor diets of the elderly (Claesson *et al.*, 2012).

In addition to AD and PD, other neurological and psychiatric disorders have been associated with gut microbiota dysregulation such as Autism, Depression, Multiple Sclerosis and Anxiety (Fung, Olson and Hsiao, 2017). **Table 1** summarizes the changes that occur in the gut microbiota and the neurological diseases associated with this dysregulation.

Table 1. Gut microbiota changes associated with neurological diseases

Neurological diseases	**Alterations in the gut microbiota**	**Refs.**
Parkinson	↓ *Faecalibacterium, Prevotellaceae* ↑ *Blautia, Coprococcus, Roseburia, Proteobacteria*	(Keshavarzian *et al.*, 2015; Scheperjans *et al.*, 2015)
Autism	↓ *Bacteroides / Firmicutes* ratio, *Enterococcus, Streptococcus, Lactococcus, Staphylococcus, Bifidobacteria, Coprococcus, Veillonellaceae, Actinobacterium, Bifidobacterium* ↑ *Lactobacillus, Desulfovibrio, Sutterella, Clostridium, Bacteroides, Porphyromonas, Pseudomonas, Aeromonas, Enterobacteriaceae, Bacteroidetes, Proteobacterium*	(Finegold *et al.*, 2010; De Angelis *et al.*, 2013; Kang *et al.*, 2013; Wang *et al.*, 2013; Tomova *et al.*, 2015)
Depression	↓ *Bifidobacterium, Lactobacillus, Bacteroidaceae, Rikenellaceae, Veillonellaceae, Sutterellaceae, Erysipelotrichaceae, Lachnospiraceae, Prevotellaceae* ↑ *Actinomycineae, Coriobacterineae, Lactobacillaceae, Streptococcaceae, Clostridiales, Eubacteriaceae, Erysipelotrichaceae, Enterobacteriaceae, Alistipes, Fusobacteriaceae, Porphyromonadaceae, Rikenellaceae*	(Jiang *et al.*, 2015; Aizawa *et al.*, 2016; Zheng *et al.*, 2016)
Multiple Sclerosis	↓ *Clostridia clusters XIV* and *IV, Bacteroidetes, Bacteroidaceae, Faecalibacterium, Parabacteroides, Adlercreutzia, Prevotella, Lachnospiraceae, Butyricimonas* ↑ *Streptococcus, Eggerthella, Pseudomonas, Mycoplana, Haemophilus, Blautia, Dorea, Desulfovibroneceae, Methanobrevibacter, Akkermansia*	(Cantarel *et al.*, 2015; Miyake *et al.*, 2015; Chen *et al.*, 2016; Jangi *et al.*, 2016)
Anxiety	Bacterial species that decrease anxiety: *Lactobacillus bulgaricus*, *Bifidobacterium breve*, *Streptococcus thermophilus* and *Bifidobacterium lactis*	(Mohammadi *et al.*, 2016)

1.2 Relevance of Tryptophan metabolism

Tryptophan (Trp) is an essential amino acid for humans (Roager and Licht, 2018). Trp is a precursor of various pathways (**Fig. 2**) and its catabolism gives rise to important molecules such as indole, tryptamine and indole acid derivatives. These catabolites are formed by degradation of Trp mainly by the gut bacterial microbiota (Roager and Licht, 2018).

A major part of ingested Trp (approximately 95%) is converted to quinolinic acid, picolinic acid, kynurenine, kynurenic acid (KA) and nicotinamide adenine dinucleotide (NAD) by kynurenine pathway (KP). Limiting steps of this pathway are the reactions regulated by the enzymes tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) (Peters, 1991).

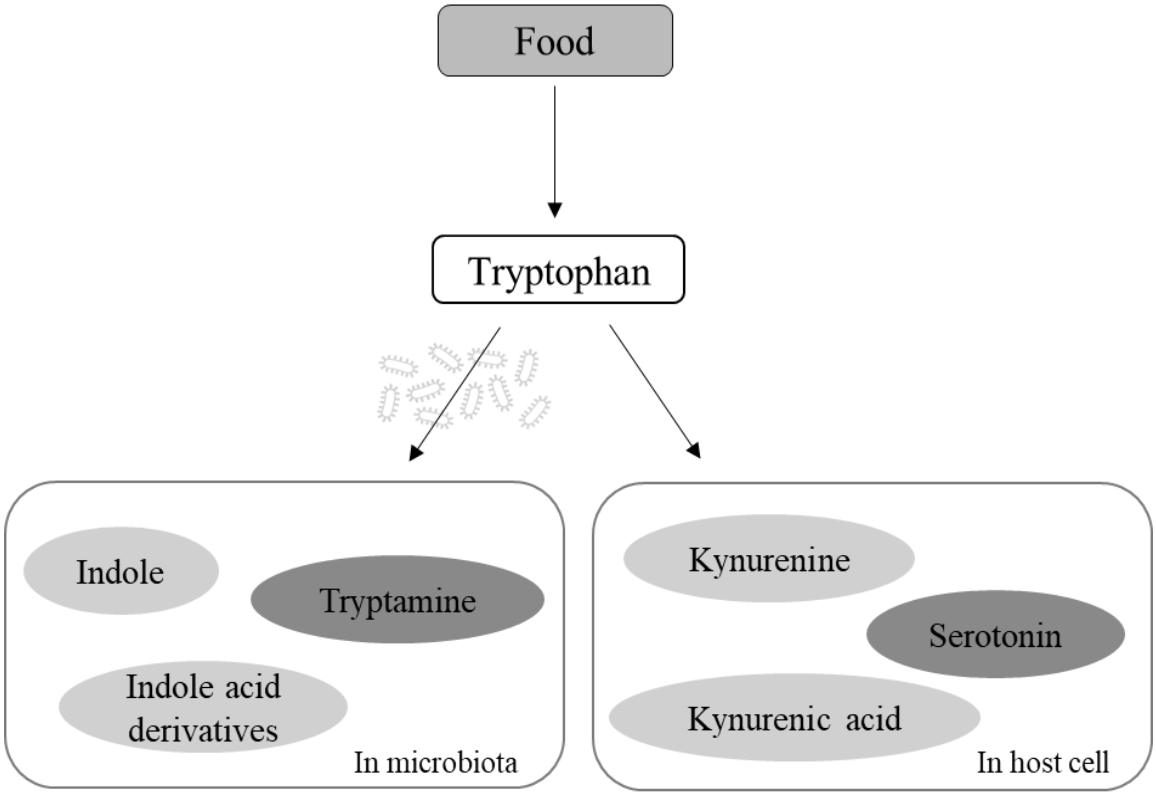

Figure 2. Tryptophan metabolism in gut microbiota and host cell.

Trp is absorbed by the intestine and is available in the circulation in free form or bound to albumin (McMenamy, 1965). Most of the total tryptophan is bound to albumin, whereas only 10% circulates in a free form and can be transported through the blood brain barrier (BBB) (Madras, 1974). Tryptophan crosses the BBB by large amino acid transporter

pathway (O'Mahony *et al.*, 2015). The levels of tryptophan in the extracellular brain fluid are very low comparatively to other neutral amino acids (Valine, Isoleucine and Leucine) since tryptophan competes with them for the same transporters to cross the BBB (O'Kane and Hawkins, 2003).

In the central nervous system (CNS), around 1-2% tryptophan participates in the synthesis of the neurotransmitter serotonin (5-hydroxytryptamin, 5-HT) (Fernstrom, 1985). However, the majority of serotonin is synthesized in the gut from tryptophan in the enterochromaffin cells (ECs) (Spiller, 2008). Serotonin synthesis occurs in two steps, regardless of where it is synthesized (gut or brain). First, tryptophan is converted to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH) and this reaction is the limiting step in serotonin synthesis (Aghajanian and Asher, 1971; Fernstrom, 1985). Consequently, 5-HTP is rapidly converted to 5-HT by aromatic amino acid decarboxylase (AAAD) (Lovenberg, Weissbach and Udenfriend, 1962).

Serotonin is therefore a molecule that intervenes in the signalling of the enteric nervous system (ENS) and central nervous system, enabling bidirectional communication of the brain and gut – gut-brain axis (O'Mahony *et al.*, 2015). Some of the serotonin effects on CNS are motor control, circadian rhythm regulation and vascular tone (O'Mahony *et al.*, 2015). Serotonin released by ECs activates 5-HT family receptors on intrinsic and extrinsic afferent nerves fibers and is responsible for different functions, such as peristaltic movements, vasodilation, sense of pain or nausea, gastric, pancreatic and intestinal secretions (Mawe and Hoffman, 2013).

1.3 Importance of Tryptamine in Biological Systems

Tryptamine is considered a "trace amine", due to the fact that it is physiologically present at low concentrations (Zucchi *et al.*, 2006).

In recent years, it has been found that tryptamine was not only a by-product of serotonin synthesis and possibly played a role in modulating 5-HT transmission (Jones, 1982). Until recently, it was considered that the only way to obtain tryptamine was by tryptophan decarboxylation by AAAD (**Fig. 3**) (Jones, 1982; Facchini, Huber-Allanach and Tari, 2000). This decarboxylase is found in the brain and peripheral tissues (Zucchi *et al.*, 2006). As mentioned earlier, AAAD is also responsible for decarboxylating 5-HTP and synthesizing 5-HT. The optimal pH for this reaction to occur is 7.8-8.0 (Bender and Coulson, 1972). Tryptamine brain concentrations are within the nanogram range, while serotonin is present in the microgram range. Tryptamine is found in low concentrations in CNS (Jones, 1982). Mass spectrometry and mass fragmentography methods have demonstrated in rat that brain tryptamine concentration is 0.5 – 1 ng/g brain tissue (Jones, 1982). These low tryptamine concentrations in the brain may be due, first, to the low affinity of AAAD for tryptophan substrate and, secondly, to the optimal pH for decarboxylation of tryptophan to be 9.0 (Jones, 1982).

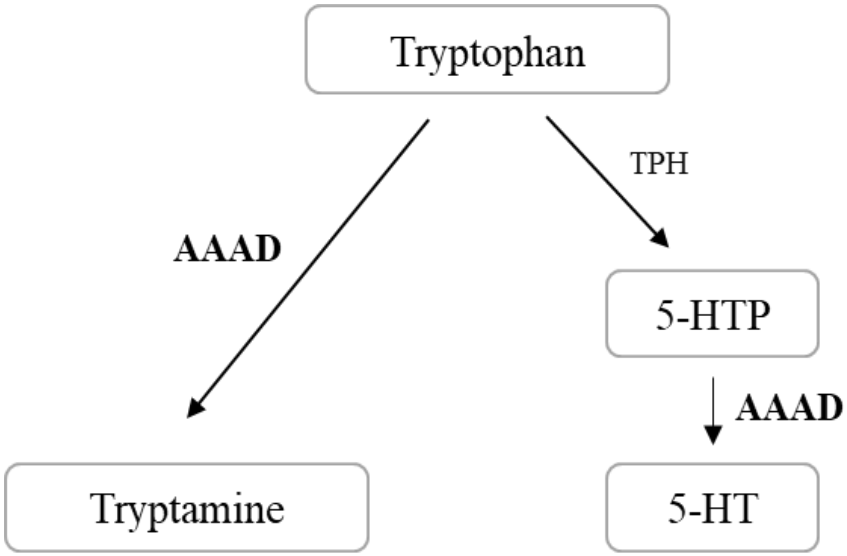

Figure 3. Pathways in which the AAAD enzyme participates.
Tryptophan is decarboxylated by AAAD and converted to tryptamine. AAAD also participates in biosynthesis of serotonin (5-HT), converting the 5-HTP into 5-HT. Adapted from (Jones, 1982).

Recently, some gut microbiota bacteria have been found to be able to synthesize tryptamine by conversion of tryptophan. Decarboxylation process of tryptophan is extremely rare in bacteria (Williams *et al.*, 2014). Williams and colleagues provided evidence showing that the tryptophan decarboxylation process can be performed by gut microbiota. Chander previously discovered decarboxylases of microbial origin that catabolize amines present in food into amino acids (Chander *et al.*, 1989). Recently, the authors demonstrated *in vitro* that *Ruminococcus gnavus* and *Clostridium sporogenes* produce tryptamine by the action of a tryptophan decarboxylase, suggesting that these strains have the potential to excrete tryptamine in the gut lumen (Williams *et al.*, 2014).

The conversion of tryptophan to tryptamine by the gut microbiota corresponds to only a small part (Bergander *et al.*, 2012). However, when the diet is supplemented with tryptophan, tryptamine levels increase considerably (Islam *et al.*, 2017).

Tryptamine can also be directly obtained by diet, because it is present in different foods and drinks such as chick pea, lupine seed following germination (Shalaby, 2000), goat cheese (Novella-Rodríguez *et al.*, 2002), canned fish (Valls, Bell and Kodaira, 2000), beer (Kalač and Křížek, 2003), frozen spinach puree, tomato pasta, ketchup, frozen green pea, tomato, kiwi (Tsuchiya *et al.*, 1995), pomegranate, strawberry (Badria, 2002) and fermented sausages (Martuscelli *et al.*, 2000; Bover-Cid *et al.*, 2001).

Tryptamine is a neuromodulator that acts on ENS and has an effect on the modulation of intestinal homeostasis (Wlodarska, Kostic and Xavier, 2015). In the gut, tryptamine at physiological concentrations promotes the release of 5-HT by ECs (Mawe and Hoffman, 2013). In addition, it can also induce ion release by epithelial cells of intestine (Williams *et al.*, 2014). On the other hand, tryptamine is an inhibitor of the enzyme IDO1 (Gao *et al.*, 2018). This enzyme participates in kynurenine pathway and in physiological conditions is responsible for the regulation of adaptive immunity (Zelante *et al.*, 2013). It has been reported that when IDO1 is overexpressed, cancer cells can escape the immune system (Katz, Muller and Prendergast, 2008). In contrast, when IDO1 is inhibited, immune cells can respond more efficiently to cancer cells (Tourino *et al.*, 2013).

Additionally, tryptamine is the substrate of the enzyme monoamine oxidase (MAO). MAO has two isoforms (A and B) that are localized in mitochondrial outer membrane and is responsible for tryptamine oxidation (Houslay and Tipton, 1974; Paley, Perry and Sokolova, 2013). Generally, MAOs are responsible for neurotransmitters degradation. This allows the regulation of dopamine, serotonin and noradrenaline levels in the brain (Ramsay, 2016). MAO inhibition is responsible for significatively increasing tryptamine concentration in the brain (Houslay and Tipton, 1974).

1.3.1 Tryptamine Receptors and Transporters

Tryptamine receptors are heterogeneously distributed in the brain. The hippocampus is one of the brain regions where there is a high density of tryptamine binding sites (Mousseau and Butterworth, 1994).

Tryptamine is a ligand for trace amine-associated receptor (TAARs). TAARs belong to the family of G protein-coupled receptors (GPCRs) (Zucchi *et al.*, 2006). TAARs are receptors for serotonin but can also bind tryptamine, which potentiates the inhibitory response of cells to this neurotransmitter (Zucchi *et al.*, 2006). These receptors are intracellular and are distributed in the limbic and aminergic systems (Bunzow *et al.*, 2001; Revel *et al.*, 2011). Briefly, when the ligand binds to the TAARs, adenylyl cyclase converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Consequently, cAMP accumulation induces phosphorylation of protein kinase A (PKA) and protein kinase C (PKC) (Bunzow *et al.*, 2001).

Tryptamine also binds to the aryl hydrocarbon receptor (AhR) (Heath-Pagliuso *et al.*, 1998). AhR is highly expressed in cells located at the body's barrier sites (skin, lung, gut) and its primary function is to provide protection against pathogens (Stockinger *et al.*, 2014).

AhR when activated by tryptamine binding initiate an intestinal immunity regulatory response (Islam *et al.*, 2017). In turn, when an intestinal imbalance occurs, AhR is activated in order to produce tryptamine (Gao *et al.*, 2018).

Tryptamine can use the same transporters as serotonin, dopamine and norepinephrine (Finn and Edwards, 1998). There are two vesicular monoamine transport proteins, VMAT1 and VMAT2 (Finn and Edwards, 1998), which differ in ligand distribution and affinity. VMAT2 has a higher affinity for dopamine, norepinephrine and serotonin compared to VMAT1 (Peter *et al.*, 1994; Erickson *et al.*, 1996). In addition, tryptamine is also one of the VMAT2 ligands (Finn and Edwards, 1998).

1.4 Tryptamine is a Competitive Inhibitor to TrpRS

In the translation process, the essential enzyme responsible for introducing tryptophan into proteins is a tryptophanyl-tRNA synthetase (TrpRS). TrpRS belongs to aminoacyl-tRNA synthetases (ARS) family, and its biosynthesis depends on ATP (Paley, 1999). In mammals, TrpRS is formed by two 54-60 kDa subunits (Penneys and Muenchi, 1974). Recent studies showed that TrpRS performed the aminoacylation function of tRNA in the cytoplasm, but this protein is also found in the nuclei and cytoplasm (Paley *et al.*, 1991). TrpRS is also a phosphoprotein and has kinase activity. TrpRS belongs to the class of protein kinases that phosphorylates the serine, tyrosine and threonine residues since TrpRS has a consensus sequence in subdomain II (residues 44-48), characteristic of serine/ threonine/ tyrosine kinases (Paley, 1997).

Although tryptophan is the TrpRS substrate, high concentrations of tryptophan (500 µM) induce its inhibition (Paley, Denisova, *et al.*, 2007). The activity of this enzyme, unlike in most cases, is decreased by an increase in substrate (Bardsley *et al.*, 1983).

Several studies have been conducted in recent years to understand the impact of tryptamine on the TrpRS enzyme. A study, using the Madin-Darby Bovine Kidney cell line (MDBK) as a model, found that tryptamine treatment of cells induced formation of tangles-like filaments formation and increased TrpRS expression (Paley, 1997). A subsequent using a human cervical carcinoma cell line (HeLa), demonstrated that tryptamine prolongs the half-life of TrpRS (from less than one hour to six hours) (Paley, 1999). It also decreases the proliferative capacity of cells of this tumoral cell line (Paley, 1999). In addition, the enzyme TrpRS is known to be involved in cell proliferation (Fukushima *et al.*, 1996). These studies

suggest that drug resistance of cancer cells is with the metabolic stability of this enzyme (Paley, 1999).

Tryptamine may act as a competitive inhibitor of TrpRS (Paley, Denisova, *et al.*, 2007), a process that is dependent on tryptophan levels. Using the human neuroblastoma cell line (SH-SY5Y), Paley and colleagues found increased ratios of tryptamine to tryptophan may lead to TrpRS inactivation and prevent the incorporation of tryptophan into proteins (Paley, Denisova, *et al.*, 2007). Although the half-life of tryptamine in the brain is shorter (54 seconds) when compared with tryptophan (30 seconds - 60 minutes) (Juorio and Durden, 1984; Chanut *et al.*, 1993), it may be sufficient to inhibit TrpRS (Paley, Smelyanski, *et al.*, 2007).

1.4.1 Tryptamine/ Tryptophan Ratio and TrpRS aggregates

Tryptophan concentration and tryptamine/tryptophan ratio is crucial for cell survival and protein biosynthesis. Inhibition of TrpRS by tryptamine depends on tryptophan concentration (Paley, Denisova, *et al.*, 2007).

The ratio of tryptamine to tryptophan may increase in several ways: (a) altered consumption of tryptamine-containing foods; (b) decrease in the consumption of foods from which tryptophan is obtained (Paley, Denisova, *et al.*, 2007) or (c) tryptamine produced by the gut microbiota (Williams *et al.*, 2014). This imbalance between tryptamine and tryptophan concentrations leads to an inhibition of TrpRS by tryptamine and consequent aggregation of the enzyme (Paley, Denisova, *et al.*, 2007). Protein biosynthesis is halted in the codon correspondents to tryptophan via ribosome frameshifting, and as a consequence of the formation of a non-functional protein (Farabaugh, 1996). The accumulation of TrpRS aggregates induces cell death (Paley, Denisova, *et al.*, 2007). Supposedly the deleterious effects of TrpRS are due to aggregation of the enzyme and have been described as morphologically similar to the neurofibrillary tangles (NFT) that characterize AD (Spillantini and Goedert, 1998; Paley, Denisova, *et al.*, 2007).

1.5 Objectives

The objective of this thesis project is to understand the effect of tryptophan metabolism imbalance on the development of hippocampal neurons.

Thus, the specific objectives of this project are to:

i. Determine the effects of tryptamine in SH-SY5Y.
ii. Evaluate the potential neurotoxic effects of tryptamine in hippocampal neurons.
iii. Determine if tryptamine promotes protein aggregation.
iv. Investigate the impact of tryptamine in neuronal development.

2 Materials and Methods

2.1 Cell Culture

2.1.1 Primary Hippocampal Cultures

Primary hippocampal cultures were prepared from Wistar rat embryos (E17-E18). Hippocampal tissues were isolated and digested with 0.15% trypsin (GIBCO – Invitrogen) and 0.01% v/v DNase diluted in Ca2+- and Mg2+- free Hank's balanced salt solution (HBSS; 5.36 mM KCl, 0.44 mM KH2PO4, 137 mM NaCl, 4.16 mM NaHCO3, 0.34 mM Na2HPO4.2H2O, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red) for 15 min at 37ºC. After that, to stop trypsin activity, the hippocampal neurons were washed with HBSS containing 10% fetal bovine serum (FBS; GIBCO – Invitrogen). Cells were dissociated in neurobasal medium (GIBCO - Invitrogen) supplemented with B27 supplement (1:50 dilution; GIBCO – Invitrogen), 25 µM glutamate, 0.5 mM glutamine and 10 000 U/mL penicillin and 10 000 µg/mL streptomycin. Then, cells were plated in 24-well plates at a density of 90 000 cells per well precoated overnight with 0.1 mg/ml poly-D-lysine (PDL). At 4 days *in vitro* (DIV-4), 5-Fluoro-2'-deoxyridine (5-FDU; Sigma-Aldrich) was added to the cell culture to inhibit glial cell growth. The cultures (**Fig. 4**) were maintained in an incubator with 5% CO2/95% humidity at 37 °C.

After DIV-7/8, hippocampal neurons were stimulated with different concentration of tryptamine, for 7 days.

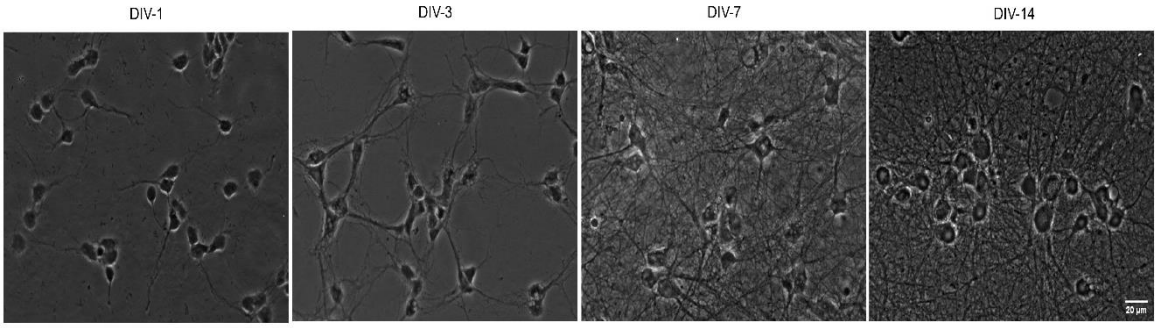

Figure 4. Rat embryonic hippocampal neurons development *in vitro*.
Neurons were plated at DIV-0 and after 1 day *in vitro* (DIV-1) initiate neurite outgrowth. At DIV-3 polarization of neurites occurs and the axons is formed. At DIV-7, the axonal network increases and by DIV-14 is very dense, at this stage neurites are completely differentiated into dendrites and axons. Phase contrast images were acquired using a Zeiss LSM 880 confocal with a plan-Apochromat 20x ph2 objective. Scale bar: 20 µm.

2.1.2 Culture of SH-SY5Y Cell Line

SH-SY5Y was obtained from the Institute of Biomedicine (iBiMED, University of Aveiro) cell culture bank. SH-SY5Y cells were dissociated in 500 µL of minimum essential medium (MEM) supplemented with 10% FBS, L-glutamine (F12) and antibiotic-antimycotic (solution contained 10 000 U/mL of penicillin, 10 000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B). SH-SY5Y cells were plated at a concentration of 70 000 cells in 24-well plates precoated with 0.1 mg/mL PDL. Cells were incubated in a 5% CO2/ 95% humidity at 37 °C.

The cells, after adhered to coverslips, were treated for 3 days with different concentrations of tryptamine.

2.2 Treatment with Tryptamine

2.2.1 Tryptamine Preparation

Tryptamine (#76706, Sigma-Aldrich) was reconstituted in 0.27 M HCl to a stock concentration of 50 mM. Intermediate stock dilutions were prepared at 1 mM and 10 mM.

2.2.2 Cell Treatment with Tryptamine

SH-SY5Y cells were stimulated with tryptamine for18 hours after plating. Culture medium was removed and tryptamine was diluted in MEM: F12 with 0.5% FBS and added to the culture. SH-SY5Y cell line, was treated for 3 days.

Hippocampal neurons were stimulated with tryptamine at DIV-7/8. Tryptamine was diluted in complete medium (neurobasal medium supplemented with B27 supplement, glutamate, glutamine and penicillin and streptomycin). Hippocampal neurons were treated for 7 days.

The tryptamine concentrations used in these experiments were 10 µM, 100 µM and 200 µM. Positive control cells were stimulated with 10 µM MG-132 for 6 hours. MG-132 was reconstituted in dimethyl sulfoxide (DMSO).

2.3 Detection and Quantification of Protein Aggregation

2.3.1 Immunocytochemistry

Hippocampal neurons and SH-SY5Y cells were fixed for 10 minutes in 4% paraformaldehyde (PFA) and 4% sucrose, at room temperature (RT). Cells were washed three times with PBS 1x (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4), permeabilized for 5 min with 0.25% Triton X-100 diluted in PBS, washed three times with PBS, and then blocked for 40 min in 3% bovine serum albumin (BSA) diluted in PBS. After that, cells were incubated with Alexa Flour 647 anti-Tubulin β 3 (TUBB3) antibody, clone Tuj 1 (#801209, BioLegend, 1:1000) diluted in blocking solution overnight at 4 ºC. Cells were then washed twice with 0.1% Triton X-100 in PBS and once with PBS 1x.

2.3.2 Detection of protein aggregates

Protein aggregates present in hippocampal neurons and SH-SY5Y cells were detected using Proteostat Aggresome detection kit (#ENZ-51035-K100, Enzo Life Sciences) according to the manufacturer's instructions. To prepare the Dual Detection Reagent, 1 µL (1:2000) of PROTEOSTAT® Aggresome Detection Reagent and 2 µL (1:1000) of Hoechst 33342 Nuclear Stain were diluted in 2 mL of Assay Buffer 1x. Cells were incubated with Dual Detection Reagent for 30 min at RT. Cells were washed twice with PBS, after which, glass slides were mounted using either ProLong Gold Antifade Reagent (#P36935, Life Technologies) or Dako fluorescence mounting media (#S3023, Palex). The preparations were cured at 4°C, protected from light. After cured, coverslips were sealed with nail polish and kept at 4 °C until microscopy analysis.

2.3.2 Image Acquisition

Image acquisition was performed in the LiM facility of iBiMED, a node of PPBI (Portuguese Platform of BioImaging): POCI-01-0145-FEDER-022122.

The preparations were visualized in a Zeiss LSM 880 confocal microscope. PROTEOSTAT® Detection Reagent according to the manufacturer's instructions and DAPI was exited at 405 nm. Images was acquired in z-stack using a Plan-Apochromat 63x/ 1.4NA

oil DIC (WD = 0.19 mm) objective and Zen Black software. Images were analysed using ImageJ 1.52 software.

2.3.3 Image Analysis and Protein Aggregates' Quantification

Quantification of total area, protein aggregates number and area were performed in Image J 1.52 software.

In SH-SY5Y cells the total area of the cell cluster was calculated by manually selecting the region of interest (ROI) using a manual tracing tool in the Proteostat marker channel and measuring the total area. The number of protein aggregates was calculated by adjusting the brightness and contrast and applying the same value to all conditions. After this, the "analyze particles" plug-in of ImageJ was used to retrieve information on area, perimeter and intensity of each particle.

In hippocampal neurons, the ROI was calculated using the "polygon selection" tool in the Proteostat marker channel and measuring the total area. The number of protein aggregates was calculated by applying the ROI previously calculated. Brightness and contrast were adjusted for each condition and the number, area and perimeter of the protein aggregates were determined using "analyze particles" function. Quantifications were performed blind to the identity of the condition being analyzed.

2.4 Cell Viability Assessment

2.4.1 Hoechst Nuclear Staining

Hoechst Nuclear staining was diluted in Assay Buffer 1x according to the instructions of Proteostat® Aggresome Detection kit manufacturer.

2.4.2 Image Acquisition

Imagens were acquired using an AxioImager Z1 (Zeiss, Germany) epifluorescence microscope with an EC Plan-Neofluar 40x/ 1.3NA oil DIC (WD = 0.21 mm) objective and Zen Blue 2.6 software. Fluorescence of Hoechst Nuclear stain was detected using a filter with excitation wavelength of 365 – 377 nm and an emission of 397 nm.

Image acquisition was performed in the LiM facility of iBiMED, a node of PPBI (Portuguese Platform of BioImaging): POCI-01-0145-FEDER-022122.

2.4.3 Cell Death Quantification

In hippocampal neurons and SH-SY5Y cell line, cell viability was evaluated by counting cells from 8 random fields in each condition.

The percentage of dead cells was determined by dividing the number of dead cells by the total number of cells. The percentage of living cells was determined by dividing the number of living cells by the total number of cells, values were normalized to the control condition.

2.5 Statistical Analysis

Plots and statistical analysis were performed in Graph Pad 6 software. Statistical differences were analyzed by one-way ANOVA using the Bonferroni post-test and treated conditions were compared with control. In Proteostat® optimization experiments, statistical analyzes were performed by a student's t test. Results are presented as averaged values ± standard error of the mean (SEM).

3 Results

3.1 PROTEOSTAT® Protocol Optimization in Hippocampal Neurons

3.1.1 Effect of MG-132 Toxicity

PROTEOSTAT® Aggresome detection kit was, initially, tested on HeLa cell line treated with different proteasome inhibitors to determine protein aggregation. Since our main focus in this study relies on the use of primary neuronal cultures, we first had to optimize the PROTEOSTAT® detection kit to fit our cellular model. MG-132 was used as a positive control for protein aggregation. Rat embryonic hippocampal neurons were treated with MG-132 (for 6-18 hours) in order to determine if there was no significant reduction in cell viability, while observing an increase in protein aggregation. Neurons were cultured until DIV-8, and 10 µM MG-132 added to the culture medium. Cells were treated for 6, 14 and 18 hours. Control cells were treated with DMSO, the solvent used to reconstitute MG-132. Cells were fixed and the nuclei were stained with Hoechst 33342 nuclear marker to asses cell viability by analysing the nuclear morphology. In apoptotic cells the morphology of nucleus is characterized by chromatin condensation and formation of apoptotic bodies (**Fig. 5A**). It was observed that 10 µM MG-132 induces a low but significant percentage of cell death at 14h (19.38% ± 0.86) and 18h (27.70% ± 2.70) when compared with the control (8.40% ± 2.40, 13.27% ± 0.95 respectively) (**Fig. 5C**). In contrast, at 6 hours we did not observe any signs of cell toxicity (16.98% ± 0.50). These observations show that 6 hours is an appropriate stimulation time to induce protein aggregation.

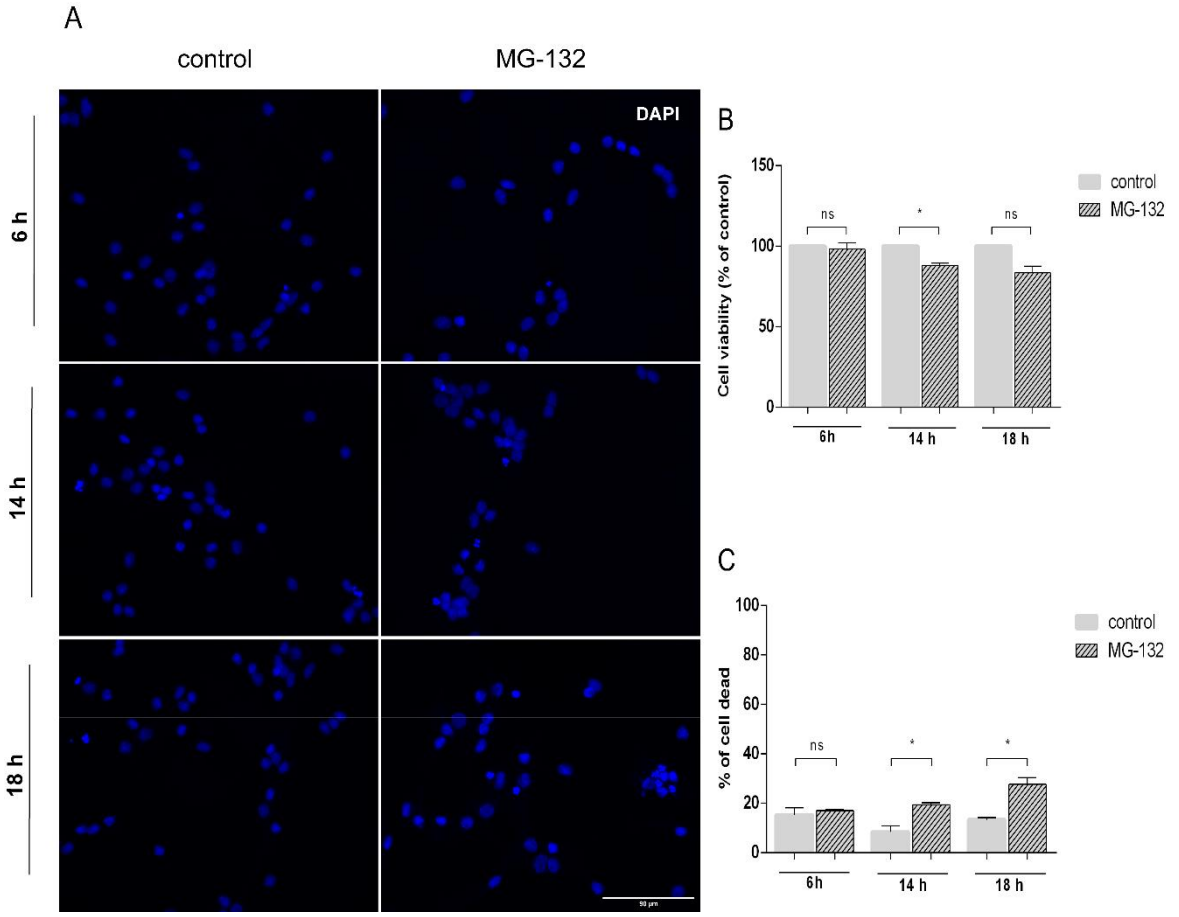

Figure 5. Effect of MG-132 in hippocampal neurons.

(A) Nuclear condensation. At DIV-8, hippocampal neurons were stimulated with 10 µM MG-132 for 6, 14 and 18 hours. DMSO was added to control cells. Nuclei were stained with Hoechst 33342 nuclear dye. The of both live and dying cells, was counted from 4 random fields in experimental replicates, approximately 360 cells, for each experimental condition. Images were acquired using an AxioImager Z1 epifluorescence microscope with a plan-apochromat 40x oil objective. Scale bar: 90 µm.

(B, C) Quantification of cell viability and cell death. Results show that there is a significant percentage decrease in cell viability (B) and a corresponding increase in cell death (C) upon treatment with MG-132 during 14 and 18 hours. Six hours of treatment with MG-132 does not compromise cell viability. Results are presented as the mean ± SEM. Statistical analysis was performed using student's t test. * represents $p < 0.05$ and ns represents non-significant, when compared to control. The different treatment times were compared with respective controls.

3.1.2 Determination of the Optimal Stimulus Time with MG-132

The viability assay shows that 6 hours of treatment with 10 µM MG-132 does not induce toxicity in hippocampal neurons. We next sought to evaluate whether inhibiting the proteasome with MG-132 for 6 hours induces protein aggregation. Hippocampal neurons were stimulated with 10 µM MG-132 at 6, 14 and 18 hours at DIV-8. Protein aggregates were detected using Proteostat® kit **(Fig. 6A)** and quantification of these aggregates was performed in Image J software. Number of aggregates was normalized by cell (**Fig. 6B**) and for the cell area (**Fig. 6C**). MG-132 induces a significant increase in protein aggregation at 6h (309.00% ± 26.00), 14h (202.10% ± 15.44) and 18h (394.90% ± 25.76) (**Fig. 6B**). These results (**Fig. 5** and **Fig. 6**) demonstrate that a 6 hours inhibition with MG-123 does not promote toxicity but it is sufficient to induce protein aggregation, indicating that MG-132-inhibition of the proteasome is an adequate positive control for protein aggregation in hippocampal neurons.

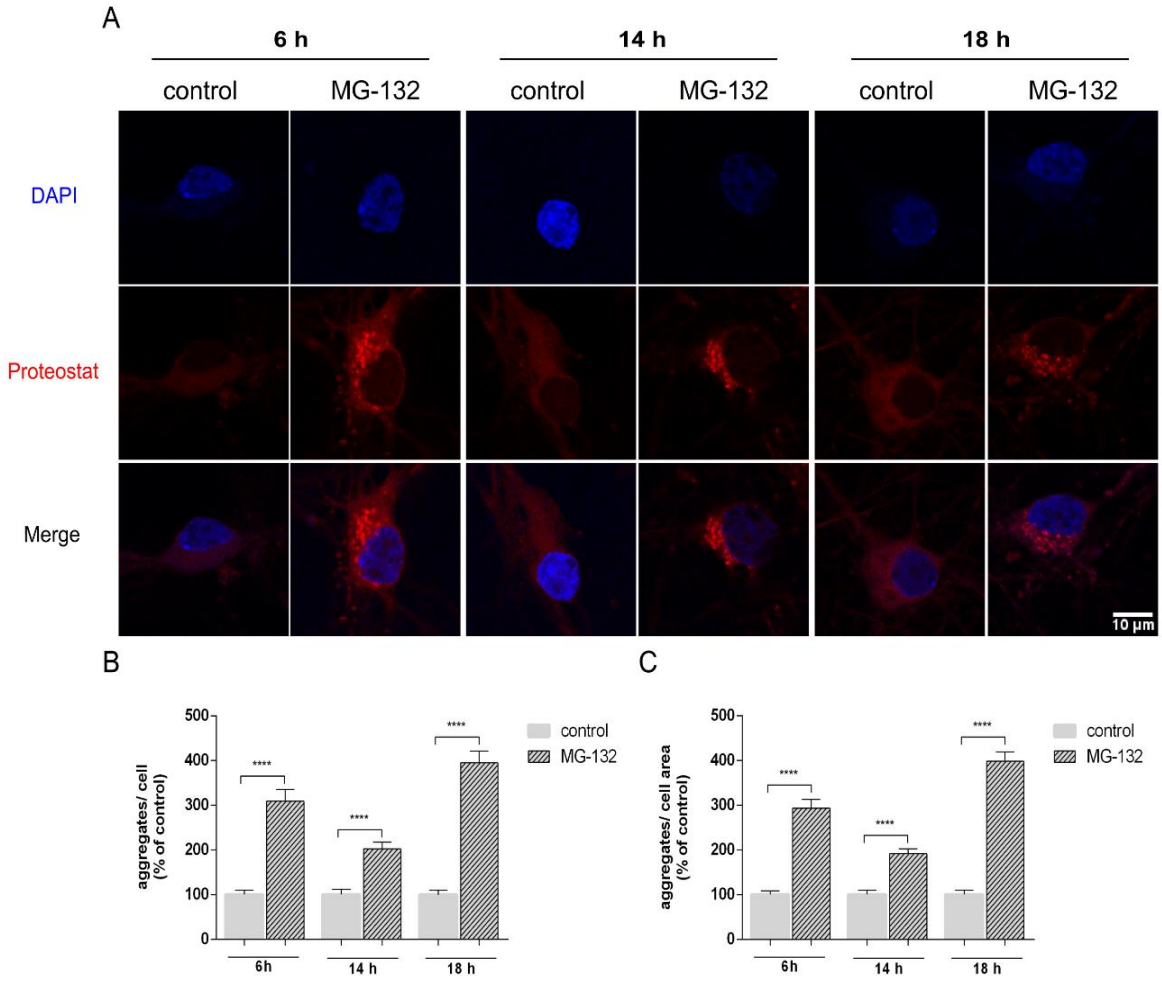

Figure 6. Induction of protein aggregation in hippocampal neurons.
(A) Time-course of proteasome inhibition with MG-132. Hippocampal neurons were incubated with 10 µM MG-132 at 6, 14 and 18 hours, at DIV 8. Control cells were treated with DMSO. Images were acquired from arbitrary neurons using a Zeiss LSM 880 confocal microscope with a Plan-Apochromat 63x oil objective. Scale bar: 10 µM.
(B, C) Quantification of the number of aggregates per cell and per area. Results show that MG-132 induces an increase in protein aggregates/cell (B) and the number of aggregates/area (C) at 6, 14 and 18 hours. Images were analysed in Image J software. For statistical analysis, student's t test was used and **** represents $p < 0.0001$. Results are presented as the mean ± SEM of approximately 40 neurons per condition. The different treatment times were compared with respective controls.

3.2 Effects of Tryptamine in SH-SY5Y Cell Line

3.2.1 Evaluation of Tryptamine Toxicity

It was previously shown that 312 µM (50 µg/ml) tryptamine induces cell death in SH-SY5Y (Paley, Denisova, *et al.*, 2007; Paley, Perry and Sokolova, 2013). Thus, we used SH-SY5Y cell line as a cellular model as a first step to validate the toxic effects of tryptamine. After cell adhesion (approximately 18 hours), different concentrations of tryptamine were applied to the culture for 3 days, 0.27 M HCl was added to the medium as a negative control, as this was the solvent used for tryptamine reconstitution. Nuclei were stained with Hoechst 33342 nuclear marker and alterations in nuclear morphology were analysed by fluorescence microscopy (**Fig. 7A**). We observed that the tryptamine concentrations used do not induce a significant death rate (**Fig. 7B, C**). These observations demonstrate that tryptamine is not toxic in these concentrations. These results are in concordance with previously published results (Paley, Denisova, *et al.*, 2007; Paley, Perry and Sokolova, 2013), since the tryptamine concentrations used are lower than the concentration that induces toxicity.

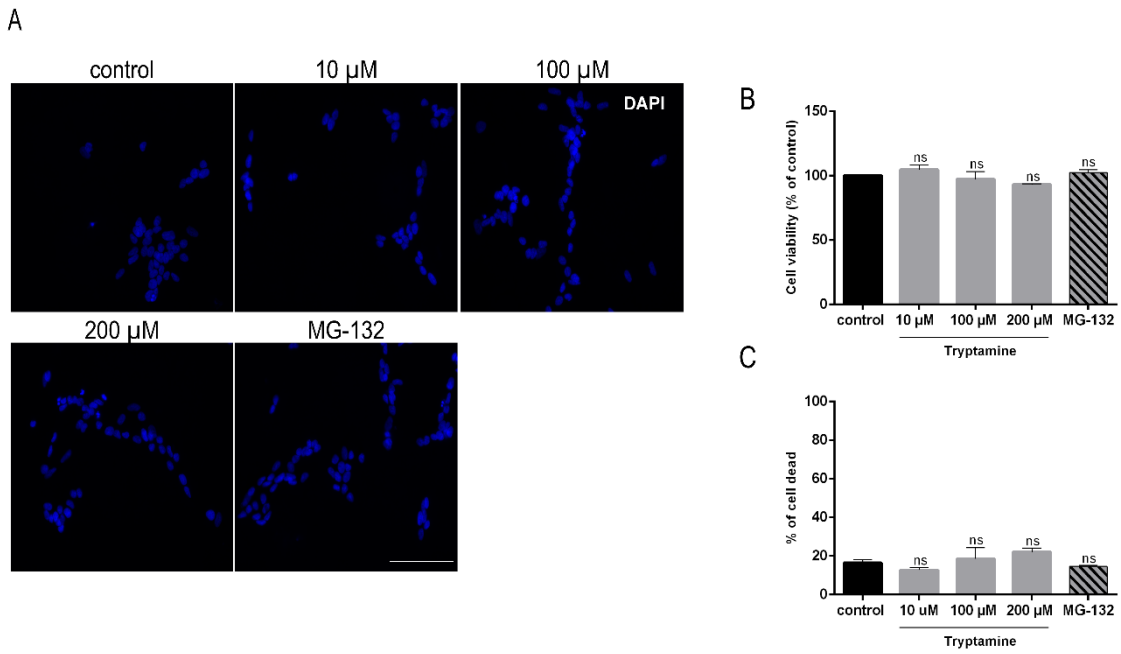

Figure 7. Effect of tryptamine on cell viability in SH-SY5Y cell line.
(A) Analysis of nuclear condensation induced by tryptamine. SH-SY5Y cell line was treated with different tryptamine concentrations for 3 days. 10 µM MG-132 was used for 6 hours as a positive control. Nuclei were stained with Hoechst 33342 nuclear dye. Cell viability was performed by counting the number of living or death cells of 4 random fields per condition from 3 independent experiments. Representative images were acquired used an AxioImager Z1 microscope with Plan-Apochromat 40x oil objective. Scale bar: 90 µM.
(B, C) Percentage of viable and dead cells. Cell viability was calculated by dividing the number of live cells by the total number of cells (B). Cell death was calculated by dividing the number of death cells by the total number of cells (C). Results were normalized to control. Results show that tryptamine does not induce a decrease in cell viability. Graphics are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA analysis of variance using Bonferroni post-test and ns represents non-significant, when compared to control.

3.2.2 Tryptamine Promotes Protein Aggregation

Next, we questioned if these concentrations of tryptamine induce protein aggregation in SH-SY5Y. To test this hypothesis, cells were treated with different concentrations of tryptamine for 3 days. Positive control cells were stimulated with 10 µM MG-132 for 6 hours. Protein aggregates were stained with PROTEOSTAT® kit (**Fig. 8A**). Number of aggregates was normalized by cell (**Fig. 8B**) and by area of cell cluster (**Fig. 8C**).

The increase of aggregates per area and aggregates per cell is proportional to the increase in the concentration of tryptamine. In addition, the number of aggregates is statistically significant for 200 µM tryptamine, 435.59 ± 72.51 (**Fig. 8B**) and 314.85% ± 35.95 (**Fig. 8C**).

In addition, we further characterized the protein aggregates present in SH-SY5Y cells. We evaluated the area (**Fig. 9A**), perimeter (**Fig. 9B**) and intensity (**Fig. 9C**). Area and perimeter of aggregates increases with increasing tryptamine concentration. This increase in area (**Fig. 9A**) and perimeter (**Fig. 9B**) is statistically significant in cells treated with 200 µM tryptamine (234.41 % ± 41.10 and 223.66 ± 35.79, respectively). Although, the aggregates intensity is not statistically significant for 200 µM tryptamine, there is an increase compared to control (**Fig. 9C**). Overall, these results demonstrate that an increase in the concentration of tryptamine leads to an increase in the number of aggregates in SH-SY5Y cells.

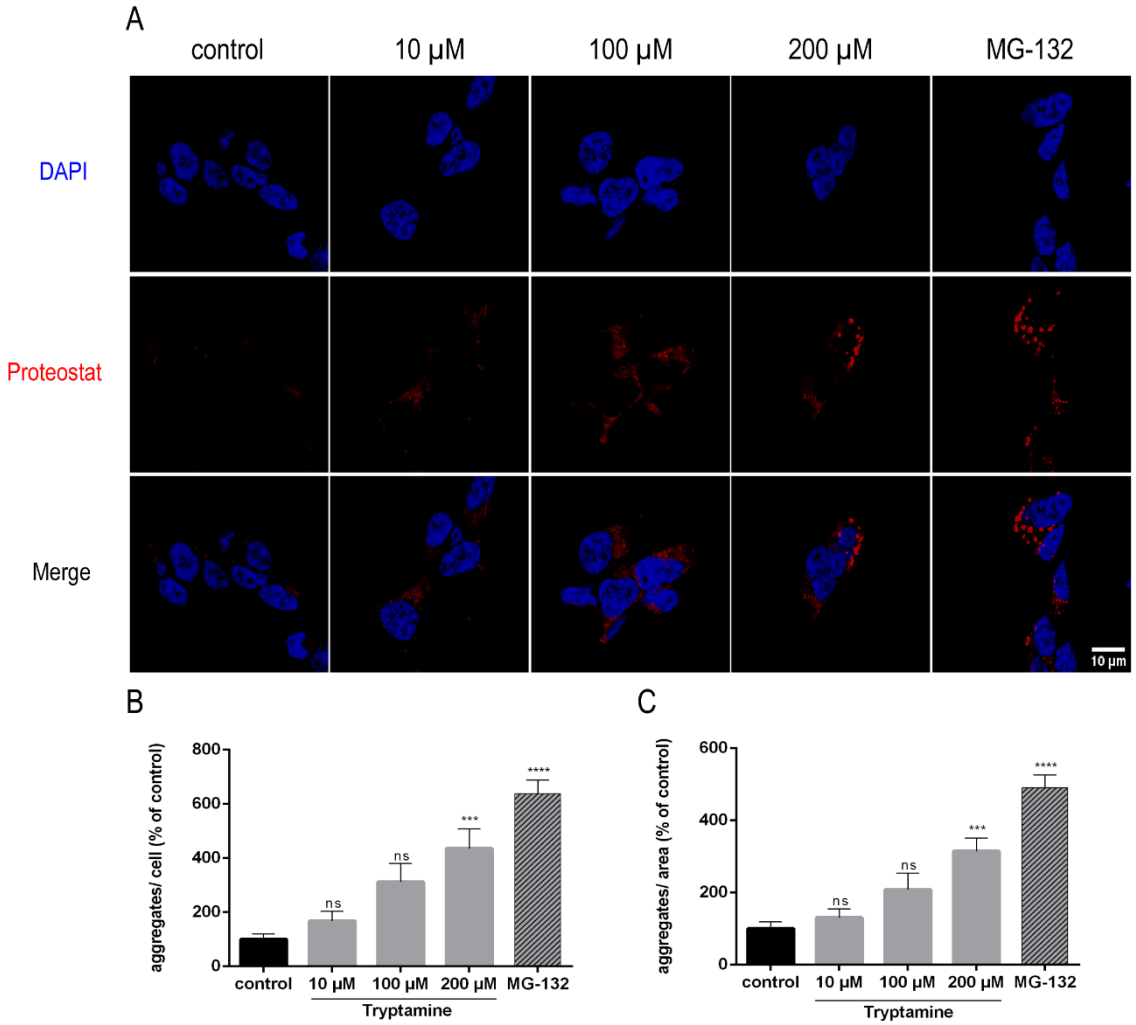

Figure 8. Tryptamine induce protein aggregation in SH-SY5Y.
(A) Analysis of aggregates promoted by tryptamine. SH-SY5Y cells were treated with different concentrations of tryptamine for 3 days. Positive control cells were stimulated with 10 µM MG-132 for 6 hours. Nuclei were stained with Hoechst 33342 nuclear dye and protein aggregates were acquired using the PROTEOSTAT® aggresome detection kit. Imagens were in a Zeiss LSM 880 confocal microscope with a Plan-Apochromat 63x oil objective. Scale bar: 10 µM.
(B, C) Quantification of number of aggregates per cell and area. Results show that tryptamine induces an increase in protein aggregates/cell cluster (B) and in the number of protein aggregates/area of cell cluster (C). Results were normalized for the control condition. Results are presented as mean ± SEM of approximately 10 cell clusters per condition from 3 independent experiments. For statistical analysis was one-way ANOVA analysis by Bonferroni post-test was used. *** represents $p < 0.001$, **** represents $p < 0.0001$ and ns represents non-significant, when compared to control.

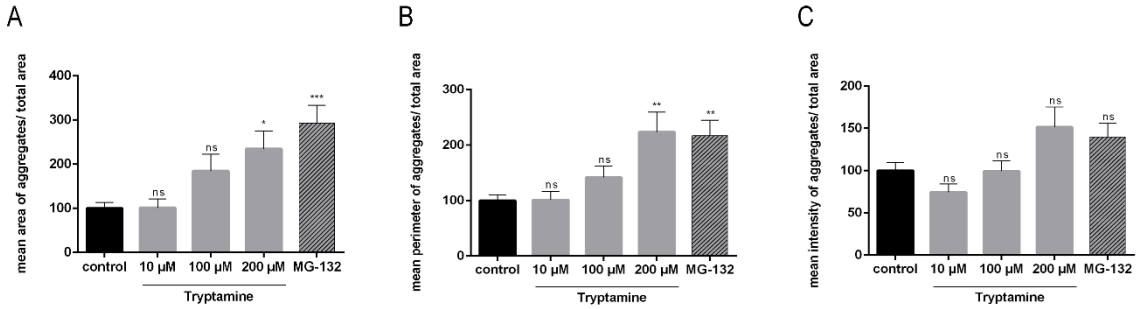

Figure 9. Characterization of proteins aggregates in SH-SY5Y cells.

(A, B, C) Area, perimeter and intensity of protein aggregates were quantified. Mean area of aggregates per total area was calculated by dividing the mean area of aggregates by the total area of cell cluster (A). Mean perimeter of aggregates per total area was determined by dividing the mean perimeter of aggregates by the total area of cell cluster (B). Mean intensity of aggregates per total area was calculated by dividing the mean intensity of aggregates by the total area of cell cluster (C). Results were normalized to control. Results demonstrate that cells treated with 200 µM tryptamine show larger aggregates in area and perimeter compared to control. Bars and plots are presented as mean ± SEM of approximately 10 cells cluster per condition from 3 independent experiments. For statistical analysis one-way ANOVA analyse by Bonferroni post-test was used. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$ and ns represents non-significant, when compared to control.

3.3 Effects of Tryptamine in Hippocampal Neurons

3.3.1 Evaluation of Tryptamine Neurotoxicity

We next evaluated if tryptamine had a similar neurotoxic effect in hippocampal neurons. To evaluate this, rat hippocampal neurons were stimulated at DIV-7/8 with different tryptamine concentrations for 7 days. 0.27 M HCl was added to control cells. Nuclei were stained with Hoechst 33342 nuclear marker (**Fig. 10A**), and nuclear condensation analyzed by fluorescence microscopy. Results demonstrate that, tryptamine in the concentrations tested is not toxic to hippocampal neurons (**Fig. 10B** and **10C**).

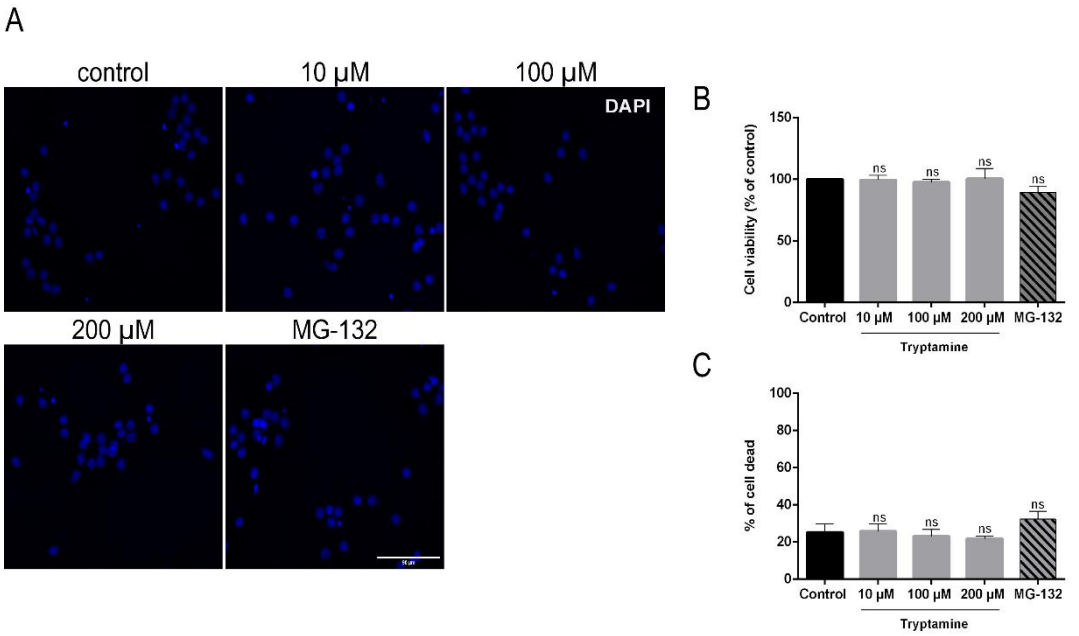

Figure 10. Effect of tryptamine on cell viability of hippocampal neurons.
(A) Nuclear condensation promoted by tryptamine. At DIV-7/8, hippocampal neurons were stimulated with different concentrations of tryptamine. Neurons were treated with tryptamine for 7 days. Positive control was stimulated with 10 µM MG-132 for 6 hours. Nuclei were stained with Hoechst 33342 nuclear dye. Cell viability was evaluated by counting cells from 8 random fields (approximately 300 cells) from 5 independent experiments using an AxioImager Z1 fluorescent microscope with a Plan-Apochromat 40x oil objective. Scale bar: 90 µM.
(B, C) Percentage of viable and dead cells. Percentage of cell viability was calculated by dividing the number of live cells by the total number of cells (B). Percentage of cell death was calculated by dividing the number of live cells by the total number of cells (C). Results were normalized to control. Results demonstrate that tryptamine does not compromise the viability of hippocampal neurons. Bars and plots were represented as mean ± SEM. Statistical analysis by one-way ANOVA analysis of variance using Bonferroni post-test where treated neurons were compared with control and ns represents non-significant.

3.3.2 Tryptamine Induce Protein Aggregates

We next investigated whether tryptamine induced the formation of protein aggregates in hippocampal neurons. Thus, neurons were treated with increasing concentrations of tryptamine for 7 days. As a positive control, neurons were stimulated with 10 µM MG-132 for 6 hours. Protein aggregates were stained with PROTEOSTAT® kit and the nuclei labbled with DAPI (**Fig 11A**), and the number of aggregates per cell (**Fig. 11B**) and per area were analysed (**Fig. 11C**). Number of protein aggregates per cell increases 135.57% ± 9.40 comparatively to control when hippocampal neurons are stimulated with 200 µM tryptamine (**Fig. 11B**). Similarly, at 200 µM tryptamine the number of aggregates per cell area increase 132.24% ± 7.68 comparatively to control (**Fig. 11C**). In hippocampal neurons, there is a significant decrease in the number of protein aggregates per cell and per area when neurons are stimulated with 100 µM tryptamine (63.93% ± 4.26 and 72.25% ± 5.51, respectively).

We further characterized protein aggregates in terms of area (**Fig. 12A**), perimeter (**Fig. 12B**) and intensity (**Fig. 12C**). These parameters do not present significant differences between control and treated neurons (**Fig. 10**). These results demonstrate that tryptamine promotes protein aggregates in neurons without compromising cell viability.

Tryptamine is present at very low concentrations in the CNS (Jones, 1982), but can increase under certain circumstances (Paley, Denisova, *et al.*, 2007; Williams *et al.*, 2014). Our results show that a high tryptamine concentration induces protein aggregation without a visible loss in cell viability. Possibly, in CNS neurons the first consequence of an imbalance in tryptophan metabolism is a formation of protein aggregates.

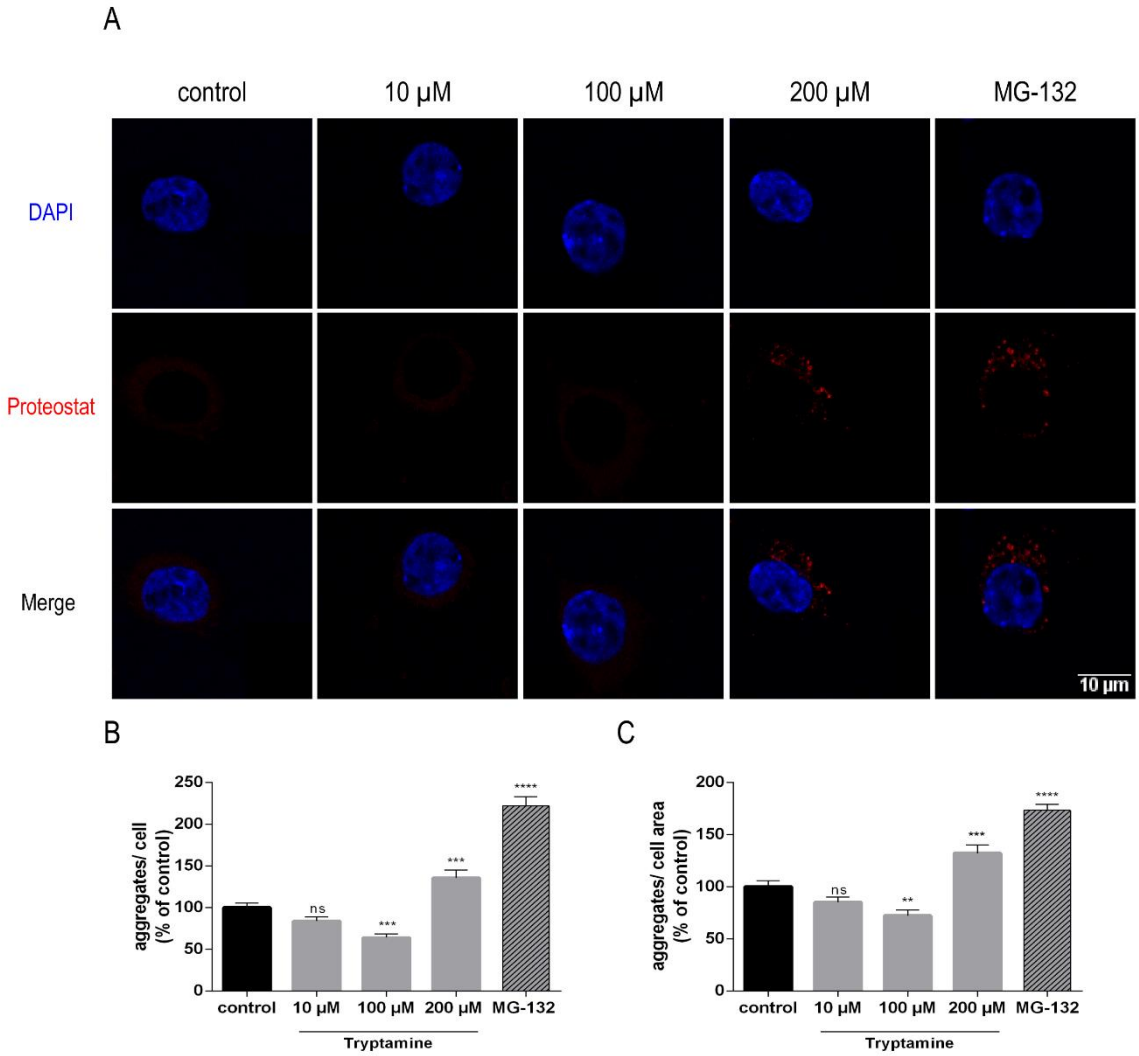

Figure 11. Tryptamine induces protein aggregation in hippocampal neurons.
(A) Analysis of protein aggregation induced by tryptamine stimulation. Hippocampal neurons were treated with different tryptamine concentrations for 7 days. 10 µM MG-132 was used for 6 hours as a positive control. Nuclei were stained with Hoechst 33342 nuclear dye and protein aggregates were stained with PROTEOSTAT® aggresome detection kit. Imagens were acquired in a Zeiss LSM 880 confocal microscope with a PlanAprochromat 63x oil objective. Scale bar: 10 µM.
(B, C) Quantification of number of aggregates per cell and area. Results show that tryptamine induces an increase in protein aggregates/cell cluster (B) and in the number of protein aggregates/area of cell cluster (C). Results show that 200 µM tryptamine induces accumulation of protein aggregates in hippocampal neurons. Protein aggregates were quantified with Image J software. Results are expressed as % of control. Graphics are presented as mean ± SEM of approximately 60 neurons per condition from 3 independent experiments. For statistical analysis, one-way ANOVA analysis by Bonferroni post-test was used. ** represents $p < 0.01$, *** represents $p < 0.001$, **** represents $p < 0.0001$ and ns represents non-significant, when compared with control.

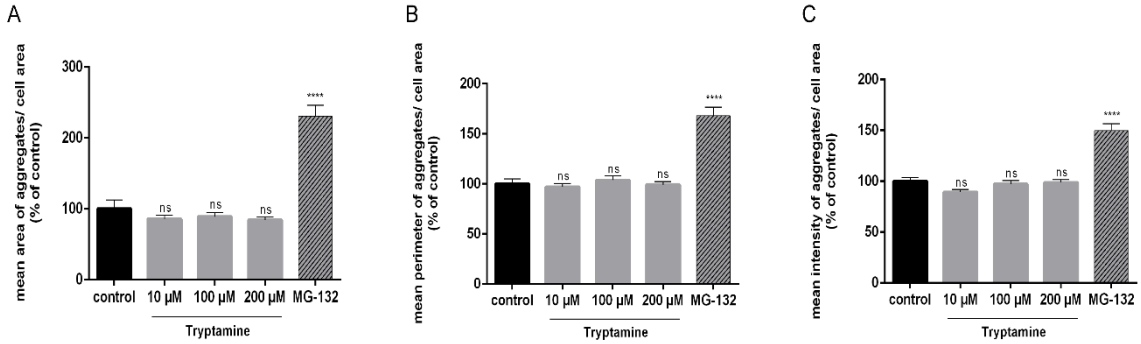

Figure 12. Area, perimeter and mean intensity of protein aggregates in neurons.
(A, B, C) Quantification of area, perimeter and intensity of protein aggregates. Mean area of aggregates per cell area was calculated dividing by the mean area of aggregates by the cell area (A). Mean perimeter per cell area was determined by dividing the mean perimeter by the cell area (B). Mean intensity of protein aggregates per cell area was calculated by dividing the mean intensity by the cell area (C). These parameters were normalized to control. Results show that area, perimeter and intensity of the control neurons is like that of tryptamine-treated neurons. Bars and plots are presented as mean ± SEM of approximately 60 neurons per condition from 3 independent experiments. For statistical analysis was one-way ANOVA analyses by Bonferroni post-test was used. **** represents $p < 0.0001$ and ns represents non-significant, when compared to control.

3.3.3 Tryptamine induce Synaptic Loss

We next investigated if tryptamine influenced synaptic connectivity in hippocampal neurons. Hippocampal neurons were treated with tryptamine for 72 hours. Synaptic clusters were immunostained with antibodies against postsynaptic density protein 95 (PSD95), a postsynaptic marker and vesicular glutamate transporter (VGluT), a presynaptic marker. Dendrites were identified using an antibody against microtubule-associated protein 2 (MAP2) (**Fig. 13A**). The number of PSD95-VGluT clusters per dendritic length decrease approximately 30% comparatively to control, when neurons are stimulated with tryptamine **(Fig. 13B)**. Thus, in hippocampal neurons tryptamine induce a decrease in the number of synapses.

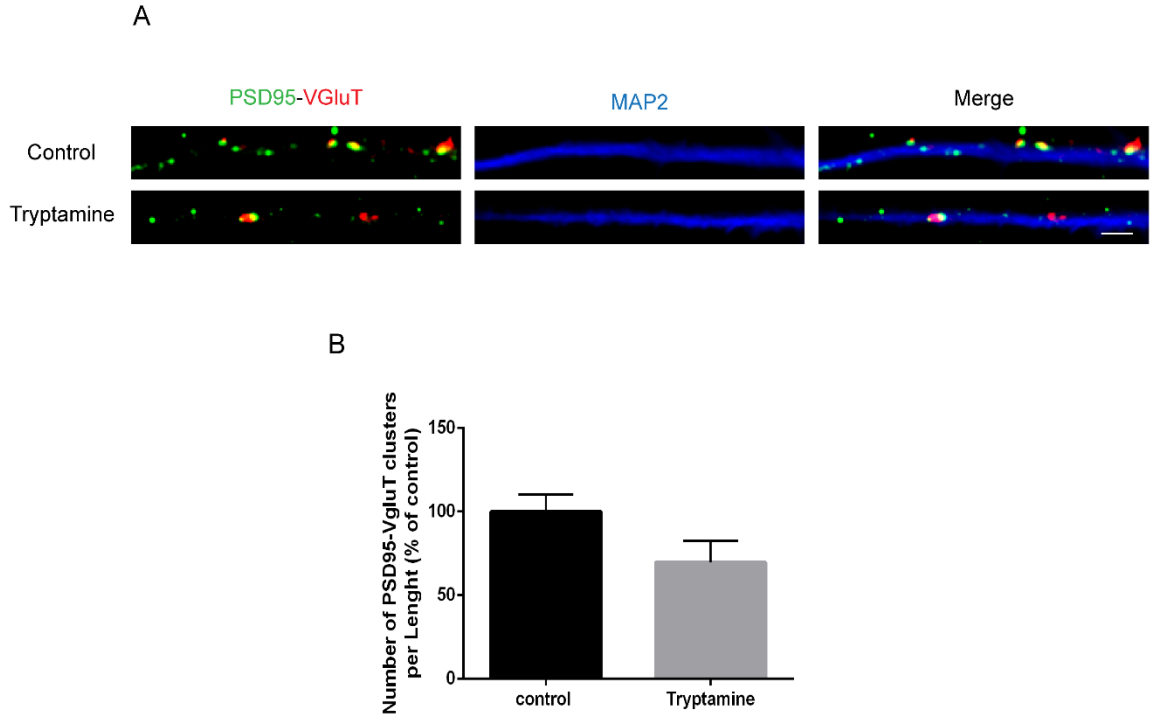

Figure 13. Tryptamine induces synaptic loss in hippocampal neurons.
(A) Effect of tryptamine in synapse formation. At DIV-4, hippocampal neurons were stimulated for 72 hours with tryptamine. The formation of synaptic clusters was assessed by immunocytochemistry using an antibody against PSD95 (green) and VGluT (red), postsynaptic and presynaptic markers, respectively. Dendrites were identified using an antibody against MAP2 (blue). Images were acquired from random dendrites using an AxioObserver Z1 fluorescent microscope with a PlanApochromat 63x oil objective. Scale bar: 2.5 µm.
(B) Quantification of PSD95-VGluT clusters number per dendritic length. Results show that application of tryptamine for 72 hours decreases the number of synaptic clusters, demonstrating that tryptamine stimulation induces synaptic loss in cultured hippocampal neurons. Puncta analysis was performed with Image J software. Results are expressed as % of control. Bars represent the mean ± SEM of 30 randomly selected dendrites of one experiment.

4 Discussion

Tryptamine is one of the catabolites formed after tryptophan metabolism by the gut microbiota. Until recently, the concentration of tryptamine in the brain was considered to depend on the concentration of tryptophan (Warsh *et al.*, 1979). In addition, tryptamine was considered to be obtained only by decarboxylation of tryptophan (Jones, 1982; Facchini, Huber-Allanach and Tari, 2000). Recently, some gut microbiota bacteria have been found that have the ability to synthesize tryptamine by conversion of tryptophan (Williams *et al.*, 2014).

Previous studies demonstrate that tryptamine at physiological concentrations acts as a modulator of brain functions (Berry, 2004). However, tryptamine is a potential competitive inhibitor of tryptophan for tryptophanyl-tRNA synthetase (TrpRS) (Lowe and Tansley, 1984). This enzyme participates in the translational process and is responsible for introducing tryptophan into proteins. Thus, an inhibition of TrpRS leads to an interruption in protein biosynthesis (Paley, Perry and Sokolova, 2013). TrpRS is susceptible to aggregation and this process depends on the tryptamine/tryptophan ratio (Paley, Denisova, *et al.*, 2007; Paley, Smelyanski, *et al.*, 2007).

Aminoacylation reaction by TrpRS is maximal when tryptophan concentration is 10 µM (Paley, Denisova, *et al.*, 2007). However, 500 µM tryptophan induces a total inhibition of TrpRS (Paley, Denisova, *et al.*, 2007). Physiologically, tryptophan to be a precursor, substrate and product must be available in a concentration range of 10 to 50 µM (Paley and Perry, 2018).

Previous studies described tryptamine as being toxic to cells (Herrera *et al.*, 2006; Paley, Denisova, *et al.*, 2007). Since these studies have used immortalized cell lines as a cellular model, we used the SH-SY5Y cell line to investigate if tryptamine induce protein aggregation. We used a tryptamine concentration range of 10 - 200 µM (1.6 - 32 µg/ml).

Based on the viability assays used the results show that in this concentration range tryptamine was not toxic to SH-SY5Y. Other studies reported that 312 µM (50 µg/ml)

tryptamine concentration compromises neuronal viability (Herrera *et al.*, 2006; Paley, Perry and Sokolova, 2013). However, we observed that 200 µM tryptamine (32 µg/ml) induces protein aggregation in SH-SY5Y cells. We might speculate that when present at low and medium concentrations tryptamine induces protein aggregation that does not lead to cell death, while at higher concentrations cell death cascades are activated. It remains to be determined the signaling pathways that are activated under both conditions, and if any overlapping occurs.

Paley and colleagues showed that a tryptamine concentration range of 125-624 µM (20–100 µg/ml), inhibited the enzyme TrpRS activity and, which becomes susceptible to aggregation (Paley, Denisova, *et al.*, 2007). On the other hand, this range of tryptamine concentrations also corresponds to the range in which it is physiologically available through diet (Shalaby, 2000; Swanson *et al.*, 2002; Paley and Perry, 2018). Thus, the results indicate an increase in protein aggregation in cells treated with 200 µM tryptamine are in agreement with previous studies, since this tryptamine concentration is enough to inactivate TrpRS and promote its aggregation.

We next tested the same range of tryptamine concentrations in hippocampal neurons. Cell viability assay show that the tryptamine concentrations used are not toxic effects Tryptamine at a concentration of 200 µM induces protein aggregation.

Interestingly, when hippocampal neurons were treated with 100 µM tryptamine, there was a decrease in protein aggregation that is significantly lower when compared to control. This latter result is in agreement with the literature since 100 µM (16 µg/ml) tryptamine is not a sufficient concentration to inhibit TrpRS activity and aggregation. However, in our studies we cannot distinguish the type of protein aggregates and therefore we do not know if they are mainly TrpRS. Another possible explanation is that neurons treated with 100 µM tryptamine appear to activate a compensatory effect that reduces protein aggregation. This decrease in the number of aggregates with 100 µM of tryptamine was not observed in SH-SY5Y cells. This may be due to the fact that the two cell models used are metabolically different. SH-SY5Y cells are a neuroblastoma cell line and are metabolically more active than hippocampal neurons.

Aggregates resulting from TrpRS inhibition have been described as morphologically similar to the characteristic NFT of AD (Paley, Denisova, *et al.*, 2007). The protein aggregates of TrpRS may be related with the NFT formation in AD. Namely, one of the characteristics of the pathological process in AD is the formation of NFT, helical filaments, constituted by the hyperphosphorylated tau protein. In parallel with this process occurs neuronal loss (Lane, Hardy and Schott, 2018). Thus, decreased cell viability, protein aggregate formation and degeneration are some of the processes that occur in tryptamine-treated cells (Paley and Perry, 2018).

In the CNS, tryptamine in non-physiological concentrations may be neurotoxic, induce protein aggregation and degeneration. In addition, a decrease in tryptophan concentration leads to a decrease in the production of serotonin (Williams *et al.*, 2014). Therefore, in the long-term this dysregulation in tryptamine and tryptophan concentrations may induce degeneration processes that underly behavioral changes.

5 Conclusion

In this thesis project we used two distinct cellular models, SH-SY5Y and hippocampal neurons, that were treated with increasing concentrations of tryptamine in order to understand the main consequences that derive from tryptamine levels dysregulation on neuronal development.

Given the results obtained, we found that the range of tryptamine concentrations used did not compromise cell viability. However, 200 µM tryptamine induces the formation of protein aggregates. Nevertheless, it remains to be determined the composition of these aggregates and if this effect is due to inhibition of TrpRS enzyme. Tryptamine also induces synaptic loss.

We also observed that the effects of tryptamine are dose dependent. It will be interesting to determine if long-term tryptamine may have a neurotoxic effect and induce neuronal degeneration. The imbalance between tryptamine and tryptophan may underlie the neuronal changes that occur with aging.

6 Future Perspectives

Recently, several studies have addressed the mechanisms that influence the gut-brain axis. In this thesis project were studied some of the processes that come from a dysregulation of tryptamine levels in hippocampal neurons. So, as future work it would be important to:

i. Validate our results using an alternative approach, we will use a different method for the detection of protein aggregation. We will clone the heat shock protein 27 fused with green fluorescent protein (HSP27-GFP) and transfect hippocampal neurons with this plasmid. HSP27 has recently been described as a protein with elevated specificity for protein aggregates (Pereira *et al.*, 2018). This plasmid was constructed for detection of protein aggregates in living cells (Pereira *et al.*, 2018).

ii. Study the mechanism of tryptamine transport from the gut to the CNS. For this, it will necessary to generate an *in vitro* circuitry by plating sensory neurons and hippocampal neurons in microfluidic chambers, and then stimulate the axons of sensory neurons with tryptamine.

iii. Determine if tryptamine induces a decrease in mitochondrial density (Paley, Perry and Sokolova, 2013). In aging cells occurs a decrease of mitochondrial function as a consequence of decreased ATP synthesis and oxidative phosphorylation (Bossy-Wetzel *et al.*, 2003). For this, the production of reactive oxygen species (ROS) can be evaluated in tryptamine-treated hippocampal neurons.

iv. Further explore the mechanisms underlying the decrease in the number of synapses after tryptamine treatment.

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