ROMA TRE
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# "THE RELATIONSHIP BETWEEN SEROTONERGIC SYSTEM AND POLYAMINES SYSTEM IN EPILEPSY"


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ROMA TRE
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# “THE RELATIONSHIP BETWEEN SEROTONERGIC SYSTEM AND POLYAMINES SYSTEM IN EPILEPSY”

# “RELAZIONE TRA IL SISTEMA SEROTONINERGICO ED IL SISTEMA DELLE POLIAMMINE NELL’EPILESSIA”.


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# RIASSUNTO

Le poliammine sono piccole molecole ubiquitarie presenti nella maggior parte delle specie viventi. Grazie alla loro carica positiva, sono in grado di interagire con differenti molecole di opposta carica, compresi acidi nucleici, proteine e fosfolipidi. Diversi studi hanno esaminato il coinvolgimento delle poliammine nel cancro e in altre patologie, ciononostante il loro ruolo nel cervello non è ancora chiaro ed è tutt'ora argomento di ricerca. Dati recenti dimostrano che il metabolismo delle poliammine è alterato in diverse malattie neurodegenerative, come Alzheimer, Huntington, Parkinson, sclerosi laterale amiotrofica ed anche a seguito di trauma e ischemia cerebrale. Negli ultimi decenni è emerso anche un coinvolgimento del sistema delle poliammine nella predisposizione alle malattie mentali. Le principali poliammine presenti nei mammiferi sono putrescina, spermidina e spermina. È noto che la spermina, e in alcuni casi la spermidina, possano direttamente modulare l'attività dei recettori ionotropici del glutammato, regolando l'eccitabilità e la plasticità sinaptica. Sono inoltre implicate in diverse patologie, specialmente in condizioni eccitotossiche. L'eccitotossicità è un processo di morte cellulare causato da elevati livelli di amminoacidi eccitatori, che provocano l'apertura dei recettori ionotropici del glutammato, causando una prolungata depolarizzazione dei neuroni, conseguente entrata di calcio e attivazione di meccanismi di morte cellulare. Una eccessiva attivazione dei recettori del glutammato da parte di amminoacidi eccitatori è senza dubbio lo step iniziale di un *pathway* eccitatorio che porta all'epilessia. Dati epidemiologici hanno portato alla luce l'esistenza di una forte comorbidità tra epilessia e malattie mentali. A questo proposito, lo scopo del mio studio è stato valutare se un'alterazione nel livello delle poliammine, specificatamente sovra-esprimendo un enzima catabolico, possa condurre ad una aumentata eccitotossicità ed alla eventuale concomitante predisposizione a malattie mentali. La spermina ossidasi è un enzima, coinvolto nel catabolismo delle poliammine. Catalizza l'ossidazione della spermina in spermidina, 3-amino propanale e perossido di idrogeno. Il gruppo di ricerca, in cui ho svolto il mio lavoro di tesi di dottorato, da anni indaga gli effetti della sovra-espressione della spermina ossidasi, fino ad ora sconosciuti, in un modello murino. Questa linea murina ha mostrato stress eccitotossico dopo iniezione con acido kainico. L'acido kainico mima l'azione del glutammato sul sistema eccitatorio e provoca crisi epilettiche. Al fine di comprendere il ruolo della spermina ossidasi nell'eccitotossicità, ho concentrato la mia attenzione sul sistema inibitorio, il sistema GABAergico. Quest'ultimo esercita un'azione di sedazione sull'attività elettrica neuronale in tutto il sistema nervoso.

Attraverso il GABA, principale neurotrasmettitore inibitorio, assicura il mantenimento di un basso stato di attivazione spontanea nei neuroni, regolando la stabilità di funzionamento dell'intero sistema nervoso. Per i miei studi, ho allevato, genotipizzato e impiegato la linea transgenica murina Dach-SMOX, che sovra-esprime la spermina ossidasi nella neocorteccia. Questa linea murina è stata analizzata sia in condizioni fisiologiche s patologiche. L'induzione del fenotipo epilettico in topi transgenici e di controllo, è stato ottenuto tramite somministrazione del farmaco epilettogeno pentilentetrazolo. La valutazione del comportamento è stato il primo passo di un'analisi preliminare per stabilire l'effetto del farmaco epilettogeno su questa linea murina. I topi transgenici hanno riportato, in seguito ad iniezione, una maggiore sensibilità a crisi epilettiche. Le cortecce cerebrali sono state poi campionate ed analizzate. Per esaminare i possibili cambiamenti nei livelli delle poliammine, ho misurato i livelli di putrescina, spermidina e spermina tramite HPLC, dopo trattamento con soluzione fisiologica o pentilentetrazolo. I risultati hanno mostrato elevati livelli di putrescina e spermidina nei topi transgenici rispetto ai singenici, dopo trattamento con soluzione fisiologica, mentre dopo trattamento con pentilentetrazolo, si è osservato un decremento generale di spermidina e spermina.
Per determinare eventuali alterazioni comportamentali della linea murina, ho effettuato test specifici per evidenziare la presenza di patologie mentali quali depressione ed ansia nonché alterazioni a livello di apprendimento e memoria. Ho effettuato i seguenti test: *light dark box, elevated plus maze, sucrose preference, forced swimming, passive avoidance e novel object recognition*. I risultati delle analisi hanno mostrato che i topi Dach-SMOX hanno un fenotipo ansioso rispetto ai controlli, mentre non hanno presentato alterazioni nei test per la depressione e per la memoria. Una volta appurato che il modello murino Dach-SMOX presentasse un'alterazione dal punto di vista comportamentale, ho effettuato indagini molecolari per caratterizzare ed analizzare il sistema serotoninergico in questa linea murina. Il sistema serotoninergico, infatti, è uno dei sistemi chiave implicato nella fisiopatologia di differenti malattie psichiatriche. Se per anni esso è stato analizzato solo per questo suo ruolo, negli ultimi decenni una sua deregolazione è stata connessa anche con la predisposizione all'epilessia. Ho eseguito l'analisi dei livelli di trascritto tramite *quantitative Real time PCR,* e dei livelli proteici tramite *Western Blot* di differenti recettori della serotonina (5-HT1A, 5-HT1B, 5-HT2A, 5-HT2C) e del trasportatore della serotonina (5-HTT). Le analisi dei risultati hanno evidenziato che i topi Dach-SMOX presentano un'alterazione del sistema serotoninergico. Essi infatti mostrano un significativo decremento, rispetto ai singenici, sia nei livelli di trascritto sia nei livelli

proteici di tutti i recettori analizzati e anche del trasportatore. È noto che il fattore neurotrofico cerebrale, BDNF, promuova lo sviluppo e la funzione dei neuroni serotoninergici e che una sua alterazione possa essere correlata a cambiamenti del sistema serotoninergico, con il quale condivide un meccanismo di mutuale regolazione. La neurotrofina BDNF sembra essere alterata in presenza di stress ossidativo cronico, condizione riscontrabile nel topo Dach-SMOX. Quest'ultimo, a causa della sovra espressione dell'enzima SMOX, ha una sovra produzione di perossido d'idrogeno; ho valutato pertanto se questo stress ossidativo cronico avesse alterato i livelli di BDNF e del suo recettore Trkb. Le analisi non hanno mostrato differenze significative tra i topi transgenici e singenici nei livelli di trascritto di BDNF e Trkb. Al fine di comprendere quali fossero i meccanismi molecolari che nel modello Dach-SMOX mettono in relazione la sovra espressione di SMOX ad un'alterazione del sistema serotoninergico, ho focalizzato le mie indagini sui *pathway* coinvolti nel metabolismo del triptofano.
Il triptofano all'interno della cellula oltre che per la sintesi proteica viene utilizzato come substrato per due differenti *pathway*, quello della serotonina e quello della chinurenina. Lo sbilanciamento a favore del *pathway* della chinurenina determina una deplezione del triptofano disponibile per la sintesi di serotonina con conseguente alterazione del sistema stesso. Recenti dati di letteratura hanno dimostrato come alti livelli di spermidina siano in grado di attivare la Src chinasi, la quale a sua volta attiva l'enzima IDO1 che catalizza il primo step del *pathway* della chinurenina. Ho analizzato tramite *western blot* lo stato di attivazione della Src chinasi riscontrandone uno stato di attivazione maggiore nei topi transgenici. L'analisi dei livelli di trascritto di IDO1 ha altresì evidenziato che esso sia significativamente più alto nei topi Dach-SMOX. Lo sbilanciamento del metabolismo del triptofano a favore del *pathway* della chinurenina potrebbe essere la causa dei cambiamenti osservati nel sistema serotoninergico. In conclusione, tramite questo progetto di ricerca ho investigato come il sistema delle poliammine e quello serotoninergico potessero essere correlati. Le evidenze sperimentali ottenute durante la caratterizzazione del modello murino Dach-SMOX mostrano come questo sistema genetico sia promettente come modello per studiare la comorbidità tra malattie mentali ed epilessia.
Il link tra i *pathway* delle poliammine e della serotonina proposto in questo progetto suggerisce che le poliammine possano essere considerate come un nuovo target di ricerca per il trattamento delle malattie mentali.

# ABSTRACT

Polyamines are small ubiquitous molecules that can be found in most living species. They have central roles in protein synthesis, cell division and cell proliferation. Their positive charge makes them capable of interacting with many different molecules of opposite charge within the cells, including nucleic acids, proteins and phospholipids. Several studies have been conducted to investigate the role of polyamines in cancer and other diseases, but their role in brain physiology is still unclear and is currently under active research. Recent data show that polyamine metabolism is affected in several neurodegenerative disorders, including Alzheimer's disease, Huntington's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis, as well as after cerebral trauma and ischemia. In the last decades, the polyamine system has also been involved in the predisposition to mental illness. The main natural polyamines found in mammal cells are putrescine, spermidine and spermine. It is well known that spermine, and in some cases spermidine, can modulate the activity of ionotropic glutamate receptors, regulating the synaptic excitability and plasticity. They are also implicated in pathological conditions, especially in excitotoxic states. Excitotoxicity refers to a process of neuronal death triggered by elevated levels of excitatory amino acids resulting in the opening of ionotropic glutamate receptors causing prolonged depolarization of neurons, the consequent entry of calcium, and the activation of enzymatic and nuclear mechanisms of cell death. Excessive activation of glutamate receptors by excitatory amino acids is indeed the initial step of the excitotoxic pathway that leads to epilepsy. Over the last few years, several epidemiological studies have shown a strong correlation between epilepsy and different mental illnesses; the comorbidity between epilepsy and psychiatric diseases is increasingly recognized. In this regard, the aim of my project was to understand if an alteration in polyamines levels, by specifically overexpressing a catabolic enzyme, could lead to increased excitotoxicity and to a possible concomitant predisposition to mental illnesses. Spermine oxidase is an enzyme of the polyamine catabolic pathway. It catalyses the oxidation of spermine to produce spermidine, 3-aminopropanal and hydrogen peroxide. The research group I joined to carry out my PhD thesis work, has investigated for years the effects of spermine oxidase overexpression, so far unknown, in a genetic mouse model. This mouse line underwent excitotoxic stress after kainic acid injection. Kainic acid mimics glutamate by acting on the excitatory system and provoking epileptic seizures. In order to understand the role of spermine oxidase in excitotoxicity, I focused my attention on the inhibitory system, the GABAergic system. The latter reduces the neuronal

electrical activity in the whole nervous system. Through GABA, the main inhibitory neurotransmitter, the maintenance of a low state of spontaneous activation in neurons is ensured, regulating the stability of functioning of the entire nervous system. I bred and used the transgenic mouse line Dach-SMOX that overexpresses spermine oxidase specifically in the neocortex. This mouse was analysed both in physiological and in pathological conditions. Induction of the epileptic phenotype on transgenic and control mice was performed by administration of the epileptogenic drug pentylentetrazole, which is strong inhibitor of the GABA(A) receptors. Behavioural evaluation was the first preliminary analysis performed to assess the effect of pentylenetetrazole on this mouse line. Transgenic mice reported a higher sensitivity to seizures, after pentylentetrazole injection. I then harvested and analysed mouse brain cortex to study the alterations of the excitatory pathway. To analyse possible changes in polyamines content following vehicle solution or pentylentetrazole injection, the concentrations of putrescine, spermidine and spermine levels in the neocortex were measured by HPLC. Results showed that putrescine and spermidine levels were higher in transgenic mice compared to syngenic mice in vehicle-injected animals, while there was a general decrease of spermidine and spermine, in transgenic animals, after pentylentetrazole injection. In order to evaluate any behavioural abnormalities in Dach-SMOX mouse model, I carried out a comprehensive battery of behavioural tests. These tests are specific to assess conditions such as anxiety, depression and memory impairment. I performed the following tests: light dark box, elevated plus maze, sucrose preference, forced swimming, passive avoidance and novel object recognition. The results of the behavioural analysis showed that the Tg mice display an anxious phenotype compared to syngenic mice evidenced by the elevated plus maze and light dark box tests, while they have not shown symptoms of depression or memory impairment. Once established that the Dach-SMOX mouse model presented a behavioural and emotional alteration, I carried out molecular investigations to characterise and analyse the serotonergic system in this murine line. The serotonergic system, in fact, is one of the key systems implicated in the pathophysiology of different psychiatric diseases. For years the serotonergic system has been analysed only for this role, while in the last decades its deregulation has been connected also with the predisposition to epilepsy. I performed the analysis of different serotonin receptors (5-HT1A, 5-HT1B, 5-HT2A, 5-HT2C) and of the serotonin transporter (5-HTT). The analysis of transcript levels was performed by quantitative Real time PCR while the analysis of protein levels was performed by Western Blot.

The results showed that Dach-SMOX mice present an alteration of the serotonergic system. In fact, they show a significant decrease, compared to syngenic mice, both in the transcript levels and in the protein levels of all the receptors and of the transporter that were analysed. It is known that the neurotrophic brain factor, BDNF, promotes the development and function of serotonergic neurons and that its alteration may be related to changes in the serotonergic system, with which it shares a mechanism of mutual regulation. BDNF neurotrophin appears to be altered in the presence of chronic oxidative stress, a condition found in Dach-SMOX mice. Transgenic mice, in fact, have an overproduction of hydrogen peroxide due to the over-expression of the SMOX enzyme. I therefore assessed whether this chronic oxidative stress had altered the levels of BDNF and its receptor Trkb. The analyses showed no significant differences between transgenic and syngeneic mice in BDNF and Trkb transcript levels. In order to discover the molecular mechanisms that in the Dach-SMOX model relate the overexpression of SMOX to an alteration of the serotonergic system, I focused my investigations on the pathways involved in tryptophan metabolism. Within the cells two different pathways use tryptophan as a substrate: serotonin and kynurenine pathways. The shift towards the kynurenine pathway leads to a depletion of the tryptophan available for the synthesis of serotonin with consequent alteration of the system itself. Recent literature data have shown how high levels of spermidine are able to activate Src kinase, which in turn activates the IDO1 enzyme. IDO1 catalyses the first step of the kynurenine pathway. I analysed the activation status of Src kinase by western blot, finding a state of greater activation in transgenic mice. The analysis of IDO1 transcript levels also showed that it is significantly higher in Dach-SMOX mice. A higher activation of kynurenine pathway could be the cause of the changes observed in the serotonergic system. In conclusion, with this research project I investigated how the polyamine and serotonergic systems could be correlated. The experimental evidences obtained during the characterization of the Dach-SMOX mouse model show that this genetic system is a promising model to study the comorbidity between mental diseases and epilepsy. The link between the polyamine and serotonin pathways proposed in this project suggests that polyamines can be considered as a new research target for the treatment of mental illnesses.

# INTRODUCTION

## 1. POLYAMINES

Polyamines (PAs) are small endogenous aliphatic polycations, essential for many cellular processes and ubiquitously present in all living cells.

Endogenous PAs play key roles in transcription, proliferation, regeneration differentiation and transformation (Cervelli et al., 2009; Amendola et al., 2009; Cervelli et al., 2014a).

Their structural flexibility and their positive charge let them interact with different negative charged cellular molecules, such as proteins, DNA, RNA and phospholipids.

Total intracellular concentration of PAs is in the millimolar range, but free PAs concentration is much lower because of their binding property to negative charged molecules described before. The most significant source of Pas is the biosynthetic pathway, but a significant contribution is also represented by food uptake (Pegg 2009).

Figure 1 displays the main natural PAs involved in cellular regulation: Putrescine (Put), Spermidine (Spd) and Spermine (Spm) (
Figure ***1***).

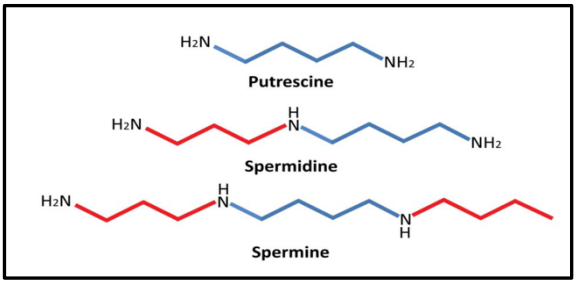


**Figure 1. Structures of the main polyamines found in the organism.** Putrescine (Put), Spermidine (Spd), Spermine (Spm).

Polyamines can be found in all living species, both eukaryotes and prokaryotes, underlining their importance in physiology and cellular viability of all organisms.

## 1.1 Polyamines Metabolism

Since PAs play key roles in several cellular processes, their homeostasis is controlled by a complex regulatory system that includes biosynthesis, degradation and transport. Polyamines concentration can change according to cell status, but it must be maintained within a certain level to preserve normal cell function (Wallace et al., 2003).

Although PAs occur in prokaryotic and eukaryotic cells, from plants and animals (Thomas and Thomas, 2001), the typical PAs synthesized by mammals are Spd and Spm, from the diamine Put (Wallace et al., 2003).

L-Arginine and L-Methionine are the main precursors for Pas synthesis in eukaryotic cells. The enzyme arginase catalyzes the hydrolytic cleavage of Arginine to produce Ornithine. Ornithine is then decarboxylated by ornithine decarboxylase (ODC) to produce Put, which is the precursor of Spd and Spm (Wallace et al., 2003). This represents the first rate-limiting step in PA biosynthesis. Ornithine decarboxylase is tightly regulated at several levels, from transcription to post-translational modifications (Pegg, 2006). The second step-limiting enzyme involved in Pas biosynthesis is S-adenosylmethionine decarboxylase (SAMDC), which provides an aminopropyl group that is then added to Put by Spd synthase (SPDS) to produce Spm. The levels of SAMDC are kept very low in order to control the amount of Spd production. The same mechanism allows the production of Spm, starting from its precursor Spd, by the activity of Spm synthase (Amendola et al., 2009; Polticelli et al., 2012).

Some studies have demonstrated an alternative pathway of PAs synthesis, where agmatine (Agm) can slightly contribute as potential source of Put (Moretti et al., 2014). However, this alternative pathway does not seem to be crucial in the Put synthesis (Coleman et al., 2004). The activity of ODC enzyme is regulated by its binding with a protein inhibitor called antizyme (AZ). The AZ is able to bind ODC in its monomeric form preventing the formation of dimeric active complexes and promoting, at the same time, its degradation through the proteasome 26S machinery. This binding of AZ to ODC can be hampered by the activity of another regulatory protein called antizyme inhibitor (AZIN); this protein can also increase the uptake of PAs from extracellular compartment (Pegg, 2006).

Polyamines anabolism is reversible, the back-conversion pathway of PAs is finely regulated by three enzymes: N1-acetyltransferase (SSAT), polyamines oxidase (PAOX) and spermine oxidase (SMOX). In the first step of the catabolic pathway the SSAT enzyme transfers acetyl groups from acetyl-coenzyme A (acetyl donor) to the N1 position of both Spd and Spm to produce AcSpd and AcSpm, respectively. After this step, these substrates are

oxidized by the flavoprotein N1-acetylpolyamine oxidase (PAOX) to produce Spd and Put, respectively, and 3-aceto-aminopropanal (3-AP) and hydrogen peroxide ($H_2O_2$) (Seiler, 2004; Tavladoraki, 2011).
Another enzyme which is involved in the PAs catabolism is the spermine oxidase (SMOX), discovered at the end of 2002, that specifically oxidases Spm producing Spd, 3-aminopropanal (AP) and $H_2O_2$ (Cervelli et al., 2003, 2012). Spermine oxidase is a member of the FAD-dependent oxidases family and has a high specificity for Spm as substrate (Wang and Casero, 2006). The rate-limiting step for PAS catabolism is catalysed by SSAT, in fact while PAOX is a stable enzyme, SSAT has a rapid turnover that is mediated by ubiquitination and 26S proteasome-dependent degradation (Seiler, 2004).
This complex and redundant system, that controls PA levels, is crucial to maintain PAs concentration stable and effectively control their homeostasis, which can be slightly different according to cell type, age or stress situations. Many enzymes involved in polyamines metabolism have been extensively studied as potential targets in physio-pathological conditions. Figure 2 summarizes the PAs metabolism in mammalian cell.

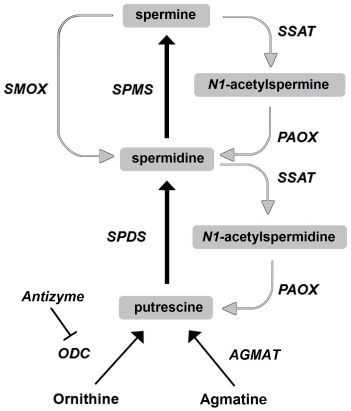


**Figure 2. Mammalian PAs metabolism.** Schematic representation of PAs metabolism showing enzyme network and substrate interconversion pathways. Anabolic and catabolic pathways are indicated by black and grey arrows, respectively.

## 1.2 Polyamines Transport

The main processes that cells use to maintain a suitable level of PAs are their synthesis and degradation. In addition, cells are equipped with an active transport system for the uptake of PAs (Mitchell et al., 2007). As mentioned above, the secondary source of PAs is through the diet; furthermore, the intestinal bacteria produce and excrete considerable amount of PAs. These are absorbed at intestinal level into the blood flow, where they can reach all tissue. The importance of PAs transport should not be underestimated, in fact, an alteration in the biosynthetic pathway or an insufficient production, which could result in a drastic stress and possible cell death, could be overcome by PAs uptake (Kaur et al., 2008).

Antzyme can negatively regulate not only ODC but also PAs uptake (Zhu, 1999); in fact all three different forms of Az (Az1-Az3) have been shown to effectively down-regulate PAs transport. However, the mechanism by which Az affects PAs uptake is still unknown (Mitchell et al., 2007). On the contrary an AZ inhibitor is able to abrogate AZ activity, increasing both ODC production of PAs and PAs uptake (Lòpez-Contreras, 2008).

The identification of the PAs transporter gene is still missing, and little is known about the PAs transporter (PAT)at molecular level. However different studies suggested that proteoglycans, which are required for the binding of positively charged extracellular molecules, play a role in PAs transport (Hougaard, 1992; Welch et al., 2008).

The exact mechanisms and the proteins involved in PAs transport have not yet been fully identified. However, studies in this field suggest that PAs are transported into cells via a specific transporter, the polyamine transport system(s) (PTS). The proteins involved in this system may include membrane transporters of the family of the solute carrier transporters (SLC) (Abdulhussein, 2013). Polyamines, in astrocyte and neurons, are usually stored in vesicles. Recent observation indicated that the SLC18B1 protein functions as a vesicular polyamine transporter (VPAT) responsible for vesicular storage of Spm and Spd (Hiasa et al., 2014).

Polyamines transport is currently studied to better understand its regulation and the precise mechanism involved.

## 1.3 Polyamines and pathologies

Since PAs have a key role in cell progression, differentiation and death, many studies have been carried out to investigate their involvement in a series of different pathologies.

Polyamines concentration increases in proliferating cells (Cervelli et al., 2009), therefore several studies point out their contribution to tumor cells growth (Gerner and Meyskens,2004; Casero and Marton, 2007; Casero et al., 2018). These cells display high activities of ODC and SAMdc decarboxylase (Thomas and Thomas, 2003), calling attention on PAs biosynthesis as a target for antineoplastic therapy (Casero and Marton, 2007). In breast cancer a positive correlation between PAs content and tumor recurrence has been demonstrated (Wallace et al., 2003; Cervelli et al., 2014a). In several cases the use of PAs concentration as a prognostic factor showed a direct correlation between higher PA content and poorer outcome in tumors (Wallace et al., 2003). The interest of scientists, during the last few years, has been to address PAs depletion as a new strategy to inhibit carcinogenesis. The principal irreversible inhibitor of PAs synthesis is the ODC-inhibitor 2-(difluoromethyl) ornithine (DFMO) (Raul, 2007). DFMO is a substrate for ODC and binds permanently within the active site at lys69 and lys360, apart from the ODC cleavage domain (Wallace and Fraser, 2004). In several experiments, the use of DFMO was found to decrease growth rate on both normal and mostly cancer cell lines (Meyskens and Gerner, 1999). However, DFMO and other compounds are poorly efficient as inhibitors of tumor growth in animal models (Gugliucci, 2004), because of the compensatory PA catabolic pathway and cellular PA uptake, which result in a cytostatic rather than cytotoxic effects *in vivo* (Horn et al., 1987). Increased efficiency of DFMO was proved in animal model of cancer when a polyamine-free diet is administrated; this result underlined that to provide an efficient inhibition of cell proliferation both PAs biosynthesis and transport must be targeted (Thomas and Thomas, 2003). Although there is not a full characterization of PAs transport system, a relative number of analogues have been developed to inhibit a PAs uptake (Wang et al., 2003).

Chronic inflammation is often a starting condition of many human cancers (Mueller and Fusenig, 2004). Ulcerative colitis and Crohn's disease are, for example, inflammatory disorders that can lead to colorectal cancer (Seril et al., 2003), and anti-inflammatory treatment is able to reduce cancer incidence (Eaden et al., 2000). Inflammation and degenerative disease have in common the generation of oxidative stress. The balance between reactive oxygen species (ROS) and antioxidant molecules and enzymes is critical to maintain normal cellular viability. High ROS levels, which overcome the detoxifying mechanism, can cause DNA mutations, cell death and apoptosis, and are believed to be chemical effectors in inflammation-driven carcinogenesis (Kundu and Surh., 2008). Oxidative stress and inflammation promote tumor

necrosis factor (TNF-α) production (Beutler, 1999); this cytokine can affect PAs metabolism, enhancing the transcription level of PAOX, SSAT and enhance SMOX activity (Babbar and Casero, 2006). Furthermore, one of the main products of SMOX activity is $H_2O_2$, which can contribute to oxidative stress and DNA damage, and could represent a link between inflammation and cancer.
The ODC inhibitor DFMO has been successfully administrated as an anti-parasitic agent to cure acute infection of *Trypanosoma brucei brucei* (TB) in mammals (Wallace et al., 2003), Because of the slower turnover of the parasite ODC, DFMO was able to effectively inhibit the enzyme without any harm for the host (Heby et al., 2003). DFMO was also able to increase oxidative stress in the parasite preventing the synthesis of trypanothione (the equivalent of glutathione in parasite (Wallace et al., 2003). DFMO is also efficient in the treatment of disease like Chaga's disease, leishmaniosis or malaria (Wallace et al., 2003).
Active research is ongoing to develop new efficient PAs analogues to modulate the enzymes involved in PAs homeostasis and metabolism (Amendola et al., 2005; Casero and Marton, 2007; Cervelli et al., 2014b).

### 1.4 Polyamines and brain

Polyamines and the enzymes involved in their metabolism have been intensively studied both in the biochemical and in the physiological field (Rea et al., 2004; Cervelli et al., 2009; Tavladoraki et al., 2011; Cervelli et al., 2016). In the last years, attention was given to the brain, with experimental designs mainly focused on the response to ischemia, hypoglycemia, epilepsy and trauma (Kauppinen et al., 1995; Casero and Pegg, 2009; Zahedi et al., 2010; Cervelli et al., 2012; Pietropaoli et al., 2018). Even though many results suggested an involvement of PAs metabolism in neurodegeneration, the mechanism by which this takes part in neuronal death still needs to be completely clarified, as well as its role in normal brain function (Capone et al. 2013; Cervelli et al., 2014b; Mastrantonio et al., 2016). It has been proven that alteration of PAs synthesis, for example ODC activity was a response to injuries, such as ischemia, epilepsy or trauma (Paschen, 1992; Henley et al., 1996). Polyamines, especially Spm, interact specifically with several types of ion channels (Williams, 1997; Igarashi et al., 2000; Fleidervish et al., 2008). Intracellular PAs are able to block to block some types of $K^+$ and $Na^+$ channels and the glutamatergic a-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid (AMPA) and kainite (KA) receptors; while extracellular PAs modulate glutamatergic N-methyl-D-aspartate (NMDA) receptors (Williams, 1997; Traynelis et al., 2010). An interesting research

underlined a link between PAs catabolism and calcium influx during brain infarction, resulting in generation of toxic products that could be responsible for brain damage (Takano et al., 2005). Other studies found that PAs catabolic products, specifically acrolein, were involved in brain infarction damage and they were responsible for the increased susceptibility to brain infarction and the increased extent of brain damage in aging (Uemura et al., 2016). In fact, it was found that older mice had an increased concentration of acrolein and a decreased concentration of the major acrolein-detoxifying compound GSH (Uemura et al., 2016). Studies on the role of PAs in brain pathologies involved also neurodegenerative disease. Accumulation of β-amyloid is one of the main characteristics of Alzheimer disease; neuronal damage consequent to Aβ accumulation was found to be directly to induction of PAs synthesis and consequent activation of NMDA receptors and memory impairment (Morrison and Kish, 1995). These effects can be partially prevented inhibiting Spm binding to NMDS receptors and blocking PAs synthesis (Gomes et al., 2014). Through the generation of transgenic mice, it has been shown that ODC overexpression or SSAT overexpression resulted in a certain level of neuroprotection against brain ischemia-reperfusion damage and general neuronal toxicity caused by administration of epileptogenic drugs. Consistent with these data, even ODC overexpression, leading to Put accumulation, is neuroprotective (Jänne et al., 2005).

Moreover, PAs have also been implicated in other important neurodegenerative diseases including Parkinson, Huntington's diseases and amyotrophic lateral sclerosis (Pashen et al., 1992; Morrison and Kish, 1995; Seiler et al., 2000; Rothman et al., 2003; Velloso et al., 2009).
Furthermore, in the last three decades an extensive research has pointed on PAs implication in different psychiatric conditions. In fact, an alteration of PAs content and their metabolic enzymes have been found in different mental illness, such as schizophrenia, mood and anxiety disorders (Andrews, 1985; Fiori and Turecki, 2008; Fiori et al., 2010).

### 1.4.1 Spermine oxidase overexpression in neocortex

Polyamines catabolism, as previously stated, is finely regulated by the combined action of three enzymes: SSAT, PAOX and SMOX (Polticelli et al., 2012; Cervelli et al., 2013a), but over the past years, attention has been focused on SMOX, providing new avenues for cancer research (Goodwin et al., 2008; Cervelli et al., 2010; Chaturvedi et al., 2011; Amendola et al., 2013; Amendola et al., 2014). Spermine oxidase directly oxidases Spm to produce Spd, 3-AP and $H_20_2$ (Cervelli et al., 2012; Cervelli et al., 2013a) (Figure 3).

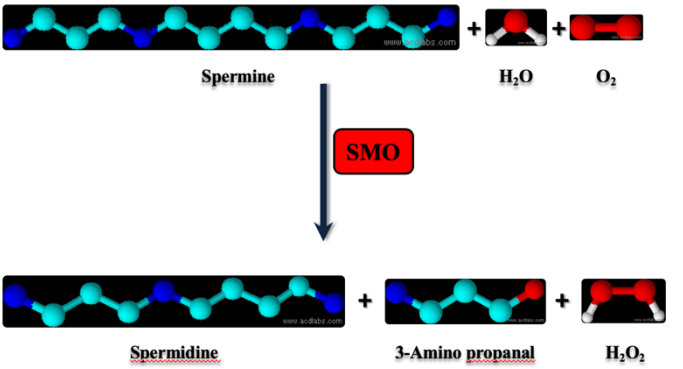


**Figure 3. Spermine oxidase catalytic activity**
Spermine oxidase oxidases spermine to produce spermidine, 3-aminopropanal and hydrogen peroxide.

From a physiological point of view, in the past, SMOX activity has been linked to several pathologies such a chronic inflammation and consequent possible cancer generation (Wang et al., 2005) and to diverse types of cancer generation or progression (Basu et al., 2009; Cervelli et al., 2014a).
Elevated levels of SMOX have been found in the brain (Cervelli et al., 2004), and its activity has important consequences on substrate regulation and products release in this organ. SMOX mRNA increases for 3 to 7 days after traumatic brain injury (TBI) (Zahedi et al., 2010). The late induction correlates with Spd increase, suggesting that SMOX activity might rise at a later stage after injury. Thus, oxidation of essential PAs may also be considered a source of secondary tissue damage, increased inflammation, and apoptotic cell death in the injured brain (Zahedi et al., 2010). Studies on the mechanism related to brain infarction during aging proved that SMOX activity is crucial in determining the extent of the damage (Uemura et al., 2016). This increase in SMOX activity and the simultaneous decrease in reduced glutathione (GSH) levels are one of the main causes for brain stroke in mice (Uemura et al., 2016). Spermine oxidase also plays a significant role in neurotoxicity associated with HIV infection. It was further reported that HIV-Tat elicits SMOX activity upregulation through NMDA receptor stimulation in human SH-SY5Y neuroblastoma cells, thus increasing ROS generation, which in turn leads to GSH depletion, oxidative stress, and

reduced cell viability (Capone et al., 2013; Mastrantonio et al., 2016). Analogously in the recently generated Total-SMOX mice, SMOX overexpression leads to a significant reduction in GSH/GSSG ratio (Ceci et al., 2017). Spermine oxidase has also been studied in mood disorders, founding a correlation between suicide completers and alteration in SMOX gene expression (Klempan et al., 2009).

To investigate the effects of brain SMOX overexpression, so far unknown, in a genetic engineered mouse model, it was generated a mouse model overexpressing SMOX specifically in the brain cortex (named Dach-SMOX), using Cre/loxP-based recombination approach (Cervelli et al., 2013b). conditional activation of SMOX was obtained with a construct (pJoSMOX). It contains a floxed GFP-stop cassette under control of the β-actin/CMV fusion promoter (Niwa et al., 1991), driving ubiquitous expression of the GFP (Green Fluorescent Protein) reporter gene; it also contains an IRES sequence followed by SMOX and lacZ genes. Upon Cre recombination, the GFP-stop cassette, which is surrounded by two loxP sites, was excised, leading to simultaneous expression of SMOX and of the second reporter gene, LacZ (β-galactosidase), via an IRES sequence (Figure 4). The transgenic mouse line generated with this construct was named JoSMOX and the characterization of the mice was carried out to select the founders possessing a single copy inserted transgene by Southern blot analysis and overexpressing GFP in all tissues (Cervelli et al., 2013b). The Dach-SMOX mouse line was obtained crossing JoSMOX with Dachshund-Cre mice, which expresses Cre recombinase and directs the excision in proneural population in the nervous system. The brain of Dach-SMOX mice resulted positive for LacZ expression specifically in the cerebral cortex at E12.5 and E14.5 mouse developmental stages (Cervelli et al., 2013b).

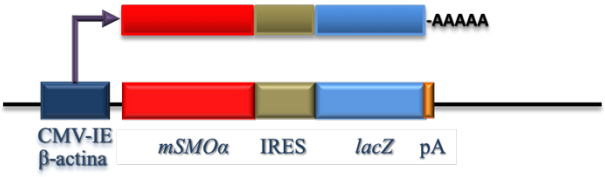


**Figure 4. Dach-SMOX mouse line construct.**
SMOX overexpression (Cervelli et al., 2013b)

## 2. SEROTONIN

Serotonin, also known as 5-hydroxytryptamine (5-HT), was isolated and characterized in 1948 by Maurice Rapport and Irvine Page (Rapport et al., 1948). In 1937, Italian scientist Vittorio Erspamer extracted, from enterochromaffin cells in the gastrointestinal tract, a substance that was responsible for causing smooth muscle contraction which he named enteramine (Erspamer and Asero, 1952). In 1952, was demonstrated that enteramine and serotonin were the same (Reid and Rand, 1952).
Serotonin is present in many mammalian tissues including brain, lung, kidney, platelets, and the gastrointestinal tract (Mohammad-Zahed et al., 2008). Serotonin is a monoamine that acts both in the central and in peripheral nervous systems with different effects since it works like a hormone, a neurotransmitter, and a mitogen.
Its role like neurotransmitter was based on studies that demonstrated the localization of 5-HT receptors to specific areas of the vertebrate brain (Twarog and Page, 1953; Amin et al., 1954). Furthermore, it was elucidated that 5-HT was principally located in the nerve endings of neurons in isolated portions of the mammalian brain (Michaelson and Whittaker, 1963; Zieher and DeRobertis, 1963). These clusters of neurons became known as the serotonergic system (Dahlstrom and Fuxe, 1964). The large number of effects of this neurotransmitter is associated with the extensive projections of 5-HT neurons throughout the brain and the different 5-HT receptor subtypes.

### 2.1 Serotonin metabolism

Serotonin is a biogenic monoamine, similar to epinephrine (EPI), norepinephrine (NET), dopamine (DAT), and histamine (HIT). It is produced in two steps. The essential amino acid tryptophan is hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH). In a second step 5-HTP is decarboxylated to form 5-HT (Clark et al., 1954) (Figure 5). Early pharmacologic studies demonstrated that hydroxylation and decarboxylation occur almost instantaneously in the presence of tryptophan

(Clark et al., 1954). While both enzymes are necessary for the conversion of tryptophan to 5-HT, TPH is considered the rate-limiting enzyme for several reasons. In fact TPH has a relatively high Km ($3 \cdot 10^{-5}$) M; moreover the enzyme has little affinity for other amino acids and its distribution is limited to those tissues containing 5-HT (Noguchi et al., 1973; Tyce,1990; Champier et al., 1997).The specific activity of TPH is in contrast to the nonspecific enzymatic activity of tryptophan decarboxylase (TDC). Tryptophan decarboxylase has affinity for many L-aminoacids, it is present in most tissues, but it is not the limiting factor in 5-HT synthesis; in fact, the inhibition of this enzyme does not result in a reduction in 5-HT levels. Interestingly, the conversion of tryptophan into 5-HT only accounts for 5% of the total metabolism of tryptophan because of the localization of TPH solely to the brain, enterochromaffin cells and, to a much lesser extent, platelets and enzymatic conversion of dietary tryptophan to kynurenine by tryptophan pyrrolase in the liver which accounts for about 95% of tryptophan metabolism (Tyce, 1990).

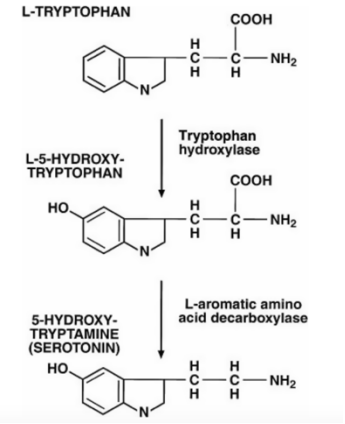


**Figure 5. Conversion of tryptophan to 5-hydroxytryptamine (serotonin).**
(Gwaltney-Brant et al., 2000)

The concentration of 5-HT in tissues is dependent upon the rate of synthesis and the rate of metabolism. The primary metabolic pathway for 5-HT metabolism is by monoamine oxidase (MAO) (McIsaac and Page, 1959). Monoamine oxidase is a ubiquitous enzyme that exists in two major forms, MAO-A and MAO-B. Serotonin is primarily inactivated by MAO-A (Sandler et al., 1981). Metabolism by MAO-B represents a small portion of 5-HT metabolism and is predominant form of MAO in human platelets (Sandler et al., 1981). The major metabolite of MAO metabolism of 5-HT is 5-hydroxyindoleacetic acid (5HIAA) which is excreted primarily in the urine. Major sites of MAO activity include the brain, gastrointestinal tract, lungs, liver, and platelets (Tyce, 1990). Although metabolism occurs very rapidly, storage protects 5-HT against metabolism. In the CNS 5-HT is processed in several ways. Upon neuronal depolarization, 5-HT is released into the synaptic cleft. It can bind to postsynaptic serotonin receptors (5-HTR) or serotonin auto-receptors on the presynaptic membrane (Cerrito and Raiteri, 1979). Binding of 5-HT to the auto-receptor acts as a negative feedback against further release of 5-HT into the synaptic cleft (Cerrito and Raiteri, 1979). The highly selective serotonin transporter (SERT or 5-HTT) located on the presynaptic membrane is responsible for removing 5-HT from the synaptic cleft (Figure 6). Once transported into the presynaptic neuron, 5-HT is recycled back into presynaptic vesicles where it is protected from metabolism. Metabolism by MAO occurs within the cytosol of the neuron. An alternative pathway for 5-HT in the pineal gland is the conversion to melatonin. Serotonin that originates from enterochromaffin cells is released into portal circulation and is quickly eliminated from the plasma via uptake into platelets and metabolism by the liver. Serotonin transporters located on the platelet membrane and enterochromaffin cells are responsible for uptake into those cells. Serotonin that escapes uptake and liver metabolism reaches the lung where is it then metabolized (Tyce, 1990).

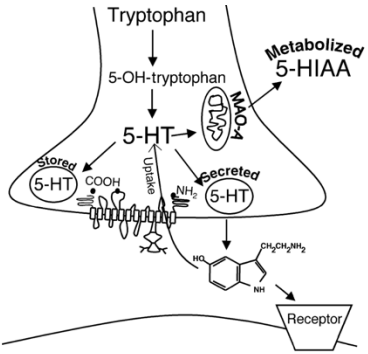


**Figure 6. Serotonergic synapse**
Synthesis, storage, release and uptake of serotonin (Ni and Watts, 2003).

## 2.2 Serotonin receptors and transporter

The different effects of 5-HT are mediated via several 5-HT receptors. The first indication of the existence of more than one type of 5-HT receptor was provided by Gaddum and Picarelli in 1957. Subsequently, pharmacological and neurophysiological studies have shown how 5-HT can act on pre and post synaptic sites and can have both inhibitory and excitatory actions. Today there is molecular and functional evidence for the existence of 16 different subtypes of 5-HT receptors (Naughton et al., 2000). The accepted classification recognizes 7 subtypes of 5-HTreceptors (5-$HT_{1–7}$) (Hoyer et al., 1994). Most subtypes show heterogeneity and are further divided into 5-HT1A, 5-HT1B, etc. Six of these subtypes involve G-protein-coupled receptors. The 5-HT3 receptor is unique in that it involves a ligand-gated $Na^+/K^+$ ion channel similar to gamma-aminobutyric acid (GABA), and N-methyl-D-aspartic acid (NMDA) (Derkach et al., 1989).

5-HT1 and 5-HT5 receptors are negatively coupled with adenylyl cyclase; activation of these receptors downregulates cyclic AMP. 5-HT2 receptor upregulates the inositol triphosphate and diacylglycerol pathways resulting in intracellular $Ca^{2+}$ release. 5-HT4 and 5-HT7 increase cAMP activity. The $Na^+/K^+$ cation channel associated with 5-HT3 results in plasma membrane depolarization (Kandel, 2001).

Moreover, 5-HT receptors are divided into two classes based on their functions: auto-receptors and postsynaptic receptors.

### 2.2.1 Serotonin auto-receptors

5-HT1A and 5-HT1B receptors are two main receptor types that control the firing and release of 5-HT (Hamon et al. 1990). The 5-HT1A receptor is present on serotonergic cell bodies and dendrites (auto-receptor) and on postsynaptic targets of 5-HT release. The stimulation of this auto-receptor inhibits cell firing and 5-HT synthesis (Hamon et al. 1984; Corley et al. 1992). The 5-HT1B receptor is mainly present at sites of 5-HT release and its activation leads to inhibition of 5-HT discharge (Gothert 1990). In addition, the 5-HT1B receptors appears to inhibit other neurotransmitters release such as acetylcholine, glutamate, and GABA (Gothert 1990; Hen 1992). The presynaptic auto- receptors are one of the targets of serotonin reuptake inhibitors. One of the most recognized mechanisms of 5-HT selective reuptake inhibitors (SSRI) function is the down regulation of the $5\text{-HT}_{1A}$ receptor and the subsequent relaxation of negative feedback 5-HT on the serotonergic neurons (Lund et al. 1992; Stahl 1994; Pineyro and Blier 1999).

### 2.2.2 Serotonin postsynaptic receptors

The other 5-HT receptors identified are all postsynaptic (Martin et al. 1998). With the exception of the 5-HT5B receptor, which is expressed in rodents but not in humans, all other 5-HT receptors appear to have similar localization and properties in rodents and primates (human and non-human). The 5-HT3 receptor is an ion channel that belongs to the family of channels gated by acetylcholine, GABA, glycine and glutamate (Saudou and Hen 1994). All other 5-HT receptors are G-protein-coupled receptors. The role of the postsynaptic 5-HT receptors has remained elusive due to their impressive diversity of location, second messenger coupling, and pharmacological specificity. Ultimately, the postsynaptic receptors for 5-HT mediate the variety of actions that 5-HT exerts in the CNS.

### 2.2.3 Serotonin transporter

The strength and duration of signaling on postsynaptic 5-HT receptors are determined by different factors. The major determinant is the abundance of 5-HT in the synaptic cleft; the availability of 5-HT is under the direct control of two mechanisms: the binding of 5-HT to its auto-receptor and the activity of the transporter (5-HTT); both of which are located on the presynaptic membrane (Mohammad-Zahed et al., 2008). The stimulation of the 5-HT auto-receptor leads to negative feedback and to a decrease further release of 5-HT, while 5-HTT actually removes 5-HT from the synaptic cleft.

Serotonin transporter is a member of a general class of monoamine transporters (Torres et al., 2003; Ni and Watts, 2006). It belongs to a family

of proteins with 12 transmembrane domains that includes the dopamine, norepinephrine, GABA and glutamate transporters (Amara and Kuhar 1993). Serotonin transporter uses a sodium gradient of the neurons to drive the active reuptake of released 5-HT, the basic mechanism is the transportation of $Na^+$, $Cl^-$ and the substrate intracellularly in exchange for $K^+$ (Sneddon, 1973; Torres et al., 2003; Ni & Watts, 2006). Serotonin transporter has been identified in the CNS, gastrointestinal tract, pulmonary and peripheral vasculature, and platelets. This transporter is one of the main targets of different drugs including antidepressants and psychostimulants. The mechanism of action of these particular drugs is to slow the transport activity in order to decrease the removal of 5-HT from the synapse.

### 2.3 Serotonin in Central Nervous System

Serotonin is produced by neurons located in the brainstem raphe nuclei and is released from the terminals of serotonergic neurons that project from the raphe nucleus; these projections innervate several cortical brain regions. The serotonergic system, in this way, plays different functions in the CNS. Serotonin regulates a wide repertoire of behaviors, in fact, in the adult, serotonergic neurotransmission modulates many brain functions including emotion, cognition, motor function, and pain sensitivity. Serotonin signaling also influences neuroendocrine functions including food intake, sleep and circadian rhythms, and reproductive activity. In addition to its role as a neurotransmitter, 5-HT plays an important role in brain development via regulation of neurite outgrowth, synaptogenesis, and cell survival (Gaspar et al, 2003). It has been demonstrated that alteration in 5-HT signaling have effects on synaptic plasticity and adult neurogenesis in the hippocampus (Brezun and Daszuta, 1999; Gould, 1999; Santarelli et al, 2003; Djavadian, 2004).Finely tuning levels of 5-HT during early life appears to be essential for normal brain development (Oberlander et al. 2009; Oberlander 2012) through its role in the connective organization of the nervous system, which includes influence on synaptogenesis and dendritic pruning (Whitaker-Azmitia 2001; Gaspar et al. 2003; Daws and Gould 2011; Lesch and Waider 2012)

## 3. MENTAL DISORDERS

According to the World Health Organization (WHO) one in four people will be affected by mental or neurological disorders at some point in their lives; in the U.S.A nearly 50% of the adult population have experienced depression or anxiety disorder, which are the most common and debilitating forms of mental illnesses associated with a substantial decrease of quality life (Elsayed

and Magistretti, 2015). Currently about 450 million people suffer from such conditions, for this reason mental illnesses are one of the worldwide disabilities (World Health Report "Mental Healt: New Understanding New Hope, 2001). In fact, this type of disability is widely recognized as a major responsible of illness indirect costs because of its high economic impact on society (Merikangas et al., 2009). According to the Bureau of Economic Analysis's Health Care Satellite Account, in 2013 in U.S.A around $89 billion have been spent on the treatment of mental illnesses (Kamal et al., 2017).

### 3.1 Serotonin in mental disorders

In the past years, many studies have focused on understanding the mechanisms underlying mental illness; much of the literature has analysed the role of the monoaminergic system, in particular the 5-HT and catecholamine involvement in the aetiology of these pathologies.

Serotonin is one of the most important neurotransmitters influencing mental health (Stanley and Mann, 1983). Most 5-HT is distributed outside of CNS, where it influences a wide range of physiologic processes in several organs (Berger et al., 2009). Serotonin plays a critical role in the etiology of many mental disorders however it is present only for the 2% in the CNS. Both 5-HT receptors and transporter play important roles in synapses. In fact, it has been demonstrated that an alteration in the function of 5-HT receptors and/or transporter be associated with mental disorders.

There is an extensive literature that associates the 5-HT system to pathophysiological conditions and in treatment of mood and anxiety disorders. The different types of diseases that depend on the direct involvement of 5-HT are more than 40. Depression, anxiety, personality disorders, epilepsy, Alzheimer's disease and others are included in the serotonergic diseases (Kudryavtseva et al., 2017).

As demonstrated by several studies, among the different serotonin receptors, described above, some of them appear to be more involved in the etiology of mental disorders (Naughton et al., 2000; Mohammed-Zahed et al., 2008; Albert et al., 2011).

The serotonin receptors 1A is the most abundant among 5-HT receptors in the brain, it is also expressed on 5-HT neurons as an auto-receptors. In these neurons it plays a critical role in the regulation of the activity of the entire 5-HT system (Albert et al., 2011). Serotonin receptor 1A has been implicated in several mental diseases; an overexpression of 5-HT1A receptors has been associated with major depression and suicide and in general, a dysregulation of 5-HT1A receptors levels are frequently observed in depressed patients

(Albert et al., 2011). In post-synaptic cortical regions 5-HT1A receptors are reduced in depression and also in anxiety (Bhagwagar et al., 2004; Sullivan et al., 2005; Shively et al., 2006; Akimova et al., 2009; Stockmeier et al., 2009), while in pre-synaptic regions where they work as auto-receptors an increase of 5-HT1A receptors are closely related with depression (Drevets et al., 2007; Boldrini et al., 2008) In fact, an increase in 5-HT1A auto-receptors leads to a reduction in the activity of 5-HT neurons.

Serotonin' receptor type 2A is predominantly a cortical receptor and it is the most abundant 5-HT receptor in the mammalian cortex (Varnas et al., 2004). Its expression is higher in the cortex than in subcortical structures such as the thalamus, basal ganglia, and hippocampus (Gross-Isseroff et al., 1990; Hall et al., 2000) with minimal expression in the cerebellum and brainstem (Hall et al., 2000). The extensive localization of these receptors in brain areas which are implicated in cognitive functions and social interaction suggests that 5-HT2A receptors could be involved in diseases that affects these functions. Serotonin receptors 2A seem to be involved in several mental illness such as schizophrenia, depression, obsessive compulsive disorder (OCD), and attention deficit–hyperactivity disorder (ADHD) (Raote et al., 2007). Studies have reported a decrease in 5-HT2A receptors levels both in the hippocampus and in the platelets of patients suffering from depression (Mintun et al., 2004). *In vivo* studies, using transgenic mouse knockout for 5-HT2A receptors, show that these receptors are required for modulation of anxiety behavior (Weisstaub et al., 2006).

Serotonin' receptor type 1B is involved in many different physiological effects like locomotor activity, satiety, sleep, sexual behavior and modulation of memory and learning (Buhot et al. 2000; Voigt and Fink 2015).

In the cells, 5-HT1B receptors are principally localized in the presynaptic regions in particular to axon terminals (Varnas et al., 2005). Based on their localization, 5-HT1B receptors may work as auto-receptors by inhibit the 5-HT release (De Groote et al. 2003) or as heteroreceptors, regulating the release of other neurotransmitters (Ruf and Bhagwagar 2009). Evidence suggests that auto-receptorial form of 5-HT1B is the major regulator of 5-HT transmission and emotionality (Donaldson et al., 2014). Animal models, knock out for 5-HT1B receptor gene, show distinct behavioral phenotype, aggressive and less cautious (Saudou et al. 1994; Zhuang et al. 1999). Also, it has been reported that 5-HT1B receptor knockout mice display low anxiety in the elevated plus maze and more activity in the open field tests (Nautiyal et al. 2016). The absence of all 5-HT1B receptors in mice results also in decreased depressive behaviors (Jones and Lucki, 2005; Bechtholt et al, 2008).

Accordingly, in other animal models the activation of 5-HT1B receptors decreases 5-HT levels acting on its release, synthesis and reuptake. These studies, however, were not able to distinguish the contribution of auto-receptors and the other 5-HT1B receptors; these distinct populations can act in different and often opposing roles to modulate behavior (Sari, 2004).
Serotonin' receptor type 2C is extensively expressed within the central and the peripheral nervous system, these receptors seem to play a prominent role in a multitude of behaviors, in fact experimental and clinical researches have shown that 5-HT2C receptors can be a valid pharmacological target in the treatment of many mental illnesses including anxiety, depression and schizophrenia (Chagraoui et al., 2016). Animal models deficient for 5-HT2C receptors showed abnormal feeding behavior, furthermore they were prone to spontaneous death by epileptic seizures (Tecott et al., 1995). In addition, in these mice have been reported behavioral alterations, such as enhanced exploration of a novel environment, dysregulation of sleep patterns and dysregulation of anxiety related behavior (Frank et al., 2002; Heisler et al., 2007). These results obtained *in vivo*, support the involvement of these receptors in the pathophysiology of mental disorders.

### 3.2 Polyamines in mental disorders

The impairment of the monoaminergic system alone cannot explain all the aspects related to mental disorders, since over the years it has become increasingly clear the contribution of other players such as PAs (Fiori et al., 2010). One of the early hints on the PAs neurobiological role was the serendipity discovery that antimalarial drugs, with psychosis side effects, were containing Spd moiety in their structure (Andrews, 1985). As described above, PAs can affect neuronal excitability since they interact with different transmembrane channels (Williams, 1997), in the light of this important role in Central Nervous System (CNS), over the last three decades an extensive research has pointed on their implication in different psychiatric conditions. In fact, an alteration of PA content and their metabolic enzymes have been found in different mental illness, such as schizophrenia, mood and anxiety disorders (Fiori and Turecki, 2008). Schizophrenia animal models and human patients have shown a dysregulation of the PAs metabolism (Das et al., 1989). In patients, the blood' PA content alteration seems to be due to the pharmacological treatment response to neuroleptics drugs since these changes have not been observed in no treated patients and in neuroleptic-resistant schizophrenia patients (Fiori and Turecki, 2008). Early studies have pointed out the involvement of polyamine oxidases in schizophrenia where an increase of these enzymes has been found in schizophrenia patient serums.

The contribution of ODC is still poorly understood, since no differences about its levels were found in patients while an increased ODC activity was observed in cortical neurons from rat model of schizophrenia (Bernstein et al., 1999). Anyway, several studies confirm the importance of ornithine metabolism in the genesis of schizophrenic disease because ornithine aminotransferase (OAT), AZIN1 and ornithine cyclodeaminase (OCD) were found to be decreased in the prefrontal cortex of patients (Middleton et al., 2002). One of the hypotheses proposed to validate the role of brain PAs in the etiology of schizophrenia was that they can modulate dopamine pathway; the latter share with higher PAs (Spd and Spm) the S-adenosyl-methioninamine common precursor in the biosynthetic pathway. Furthermore, NMDAR modulation by Spd and Spm, as described above, could explain the hypofunctional NMDAR signalling in schizophrenia. Alteration in PAs system has also been found in animal models of depression. In rats affected by depression, it has been observed a hippocampal decrease in Put, Spd and Spm while only a decrease in Put levels was observed in the *nucleus accumbens* (Genedani et al., 2001). Putrescine showed to possess antidepressant properties, since its administration by injection is able to reduce immobility time in forced swimming and tail suspension tests. Analysis in plasma of humans suffering from depression, showed high level of Agm that is restored to a normal content after antidepressant treatments, highlighting the critical role of this molecule in depression (Fiori and Turecki, 2008). Recently, it has been proposed for Agm a role of neurotransmitter in the CNS, confirmed by its accumulation in synaptic vesicles and by the ability to be secreted following depolarization (Reis and Regunathan, 2000). Moreover, it has been proved that Agm is a selective antagonist of the NMDA polyamine-binding site (Askalany et al., 2005). All these data are confirming the involvement of the PA system in depression. In a similar way to what was observed in schizophrenic patients, high levels of PAs were also found in the plasma of patients suffering from depression (Dahel et al., 2001). Evidence showed that the transcript and protein levels of different elements of the PAs system are dysregulated in several brain regions of suicide completers; in particular *post-mortem* studies have highlighted changes in the SSAT enzyme which shows a lower level of expression compare to healthy people (Gross and Turecki, 2013). It has been proposed that in the brains of depressed people, the lowering of the expression of SSAT could be a compensatory mechanism to cope with an excessive presence of PAs (Gross and Turecki, 2013). In the last decade, great attention has been focused on the role of PAs in the context of stress response and particularly on its causal relationship with the morbidity of anxiety and psychiatric disorders. This pathological

condition is called PA stress response (PSR), it can be triggered by different types of stressor and this response can be modulated in accordance with the stressors' intensity (Turecki, 2013). In the CNS acute stressors do not increase the concentrations of all PA but rather they lead to an accumulation of Put and Agm as well as an increase of ODC activity. Contrary to the events that have been observed in the CNS, in which the PSR can be activated independently, in the peripheral nervous system PSR trigger occurs only after the activation of the hypothalamic-pituitary axis. Following acute stress, changes are only appreciated in Put and ODC, while Spd and Spm remain unchanged, bringing to an apparently incomplete PSR. When stressors become chronic, they lead to complete PSR and changes are also observed in Spd and Spm levels. However, persistent chronic stressors can activate a maladaptive PSR since cause PAs accumulation. Repeated stress events predispose to an increased risk of developing mental pathologies including depression, anxiety and in suicide, which often has comorbidity with mood and personality disorders (Gross and Turecki, 2013).

## 4. EPILEPSY

Epilepsy is a chronic neurological disorder characterized by the manifestation of spontaneous and repeated seizures (SRS), which are caused by disproportionate and simultaneous electrical activity of neuronal networks (Hirtz et al., 2007). This disorder is one of the most common worldwide neurological diseases; its incidence in the population is estimated to be 1-2% (Naseer et al., 2013). Epilepsy was first described over two thousand five hundred years ago, yet there is still relatively little known about the underlying cause and currently no disease-modifying therapies exist.

In most cases, the disease is idiopathic, since the factors triggering epilepsy are unknown; however, in some cases can be related to hereditary factors or to a brain injury. Epilepsy is classified into different categories: childhood absence epilepsy, benign focal epilepsy, juvenile myoclonic epilepsy and temporal lobe epilepsy (TLE). The latter is the most common epilepsy occurring in adults (Genton and Bureau, 2006; Téllez-Zenteno and Hernández-Ronquillo, 2012). Current treatment options include antiepileptic drugs (AEDs), ketogenic diet, neurosurgical resection, and electrical stimulation of the CNS, which work for some but not all afflicted individuals (Laxer et al., 2014). Thus, there is a clinical need to discover treatments for the entire epileptic population. Most currently available AEDs fail to prevent or control SRS for a sizable percentage of epileptic patients (~30%) (Gu and Dalton, 2017). Therefore, studying epilepsy using laboratory animals exhibiting SRS will provide a valuable tool to explore the underlying mechanism of epilepsy and develop novel therapeutic approaches.

### 4.1 Serotonin and epilepsy

In the last few years, there has been growing evidence that serotonergic neurotransmission modulates a wide variety of experimentally induced seizures (Bagdy et al., 2007). In particular, agents that elevate extracellular 5-HT levels, such as 5-hydroxytryptophan and 5-HT reuptake blockers, leads to inhibition of both focal and generalized seizures (Löscher 1994; Yan et al., 1994). On the contrary, depletion of brain 5-HT lowers the threshold to audiogenically, chemically and electrically evoked convulsions (Statnick et al., 1996). In addition, it has been shown that different anti-epileptic drugs increase endogenous extracellular 5-HT concentration. Also, in epilepsy the different subtypes of 5-HT receptors seem to play an important role, they are expresses in all networks involved in epilepsies. Several knockout mouse models suggest a relation between 5-HT, hippocampal dysfunction, and epilepsy.

Serotonin receptor 1A is the most studied subtype of 5-HT receptors in epileptic seizures, as said before this receptor is linked to a $K^+$ channel and for this reason it is able to hyperpolarize neurons without the involvement of the decrease of cAMP (Andrade et al., 1986).
Generally, stimulation of 5-HT1A receptors decrease the seizures threshold in animal models of absence epilepsy while increase the threshold in other types of epilepsy (Gharedaghi et al., 2013). Other studies conducted in knockout mice support an anti-epileptic role for this receptor types (Tokarski et al., 2002; Clinckers et al., 2004). A study conducted by Sarnyai and colleagues showed that genetic deletion of 5-HT1A receptor resulted in lower seizure threshold and higher mortality rates in kainic acid model of epilepsy (Sarnyai et al., 2000).
The type 2 serotonin receptors are G-Coupled membrane proteins, which increase intracellular levels of $Ca^{2+}$ and lead to an activation of protein kinase C (Goodman et al., 2011). Several studies suggest and inhibitory role for 5-HT2 receptors in seizures. Evidences showed that stimulation of 5-HT2A and 5-HT2C receptors protects against convulsive seizures. Studies conducted in mice knockout for 5-HT2C receptors showed that this animal model has a lower threshold for focal and generalized seizures, in addition these mice undergo sporadic and spontaneous audiogenic seizures which are occasionally lethal (Tecott et al., 1995; Applegate and Tecott 1998)
In general, hyperpolarization of glutamatergic neurons by 5-HT1A receptors and depolarization of GABAergic neurons by 5-HT2C receptors decrease the excitability in most, but not all, networks involved in epilepsies.

### 4.2 Polyamines and epilepsy

Over the years, different research groups have focused their studies on the role that PAs could have in the molecular mechanisms underlying epilepsy. Since the knowledge, derived from clinical human studies, was not sufficient to better understand the epileptic pathways, it was necessary the use of proper animal models. Genetic models helped to define the physiological importance of PAs by offering tools to develop treatment therapies to be applied in epilepsy (Cervelli et al., 2014). The epilepsy animal models utilized provide seizures induction by chemoconvulsants, traumatic brain injury, and electrical or sound stimuli. Among the most used chemoconvulsants are included Pentylenetetrazole (PTZ) and Kainic Acid (KA). Epileptic seizures induced by PTZ and KA have also been used in transgenic mouse model characterized by a deregulation of the PAs system caused by the overexpression of PAs metabolic enzymes. The transgenic mouse line K2 (Halmekytö et al., 1991) overexpressing ODC in the brain, consequently

having a high Put cellular content, showed to be neuro-protected from physically (electroshock) and chemically (PTZ) induced seizure activity (Halonen et al., 1993), but displayed impaired spatial learning, vision, swimming ability and lack of motivation (Halonen et al., 1993). The deficit in spatial learning was demonstrated to be associated with constitutively high Put level since its antagonistic effect on NMDA receptor. Additionally, in transgenic rats overexpressing ODC (Lukkarinen et al., 1998) subjected to transient focal cerebral ischaemia, significantly smaller stroke lesions were observed in comparison with control rats, confirming that induction of ODC and the subsequent accumulation of Put are neuro-protective responses in the transient cerebral ischaemia (Alhonen et al., 2009). The transgenic mouse line ubiquitously overexpressing SSAT and generated by Pietila et al. (Pietila et al., 1997), likewise the ODC over-expressing line mentioned above, displayed high level of Put in the brain as a result of SSAT overexpression. These transgenic mice showed neuroprotection from KA-induced neuronal toxicity (Kaasinen et al., 2000) and an elevated threshold to PTZ-induced convulsions in comparison with wild-type animals (Kaasinen et al., 2003). Neurobehavioral profiling of SSAT overexpressing mice showed impaired spatial learning and revealed to be hypomotoric and less aggressive than wild-type animals (Kaasinen et al., 2004). Albeit Put is a weak antagonist of the NMDA receptor (Williams et al., 1990), its elevated content in the brain could cause a partial blockade of this receptor, thus giving a protection to the transgenic animals from seizure activity, ischaemia reperfusion damage while producing impaired spatial learning (Kaasinen et al., 2003; 2004). A mouse Cre/loxP-based genetic model overexpressing SMOX specifically in neocortex neurons (Dach-SMOX) has been engineered by Cervelli et al. (2013) to investigate the role of this enzyme and its substrate Spm, which is the strongest PA modulator of glutamatergic receptors (GluRs), some types of K+ channels and Na+ channels (Williams 1997; Fleidervish et al., 2008; Traynelis et al., 2010). Interestingly, Dach-SMOX mice showed a phenotype with significant astroglial and microglial activation in the neocortex of old animals, showing a more pronounced brain damage during ageing. Furthermore, in excitotoxic condition induced by KA injection, Dach-SMOX mice resulted to be more sensitive than control animals (Cervetto et al., 2016; Pietropaoli et al., 2018). Compared to transgenic ODC and SSAT overexpressing mice, which displayed a neuroprotective response to different insults, Dach-SMOX animals showed an opposite phenotype, since a higher neurodegeneration was observed during ageing and subsequent to KA injection (Alhonen et al., 2009; Cervelli et al., 2013). The production of $H_2O_2$ and 3-AP, derived from Spm oxidation, together with direct effects of Spm

on AMPA and KA receptors, are synergistically involved in ROS increase and in the end to neuronal degeneration and death. All these engineered transgenic rodent lines may represent useful *in vivo* genetic models for studying PAs metabolism dysregulation in brain pathological conditions due to various physically and chemically induced excitotoxic insults that induce epilepsy (Jänne et al., 2004; Cervelli et al., 2013).

### 4.2.1 Pentylentetrazole, model for epilepsy

The use of animal seizures models is essential in the discovery and development of new drugs for the treatment of epileptic seizures. In the late 1940s and early 1950s, several papers described the properties of the chemoconvulsant pentylentetrazole (PTZ). Since then, the PTZ threshold model, in combination with other models, has been used to identify the majority of agents presently used for the treatment of epileptic seizures (Hansen et al., 2004). Although animal models based on PTZ have been and still are widely used for drug screening, the mechanism by which PTZ elicits its action is not very well understood. One generally accepted mechanism by which PTZ is believed to exert its action is by acting as an antagonist at the picrotoxinin-sensitive site at the g-aminobutyric acid (GABA)A receptor complex (Hansen et al., 2004). Injection of PTZ induces primary generalized seizures, since GABA mediates disruption of inhibition resulting in neuronal excitation (Doi et al., 2009).

While the administration of KA, being a Glu analogue, determines a rise of this excitatory neurotransmitter in the synaptic cleft causing excitotoxicity, PTZ treatment does not involve an administration of further external Glu, but it causes seizures by blocking GABAergic inhibitory system (Naaser et al., 2013). To evaluate seizures intensity behavioural scoring is commonly used in different seizures models. Racine's scale (RS) (Racine, 1972) is frequently chosen as an intensity measurement in experimental seizures or epilepsy models. In the Dach-SMOX model it was used to assess the symptoms of mice treated with KA (Cervelli et al., 2013b). For PTZ-induced seizures, on the contrary, RS is not suitable intensity valuation, given the occurrence of behavioural expressions, not mentioned in RS while it lacks other behavioural stages mentioned by it. As a result, the use of a six-point intensity scale defined by Lüttjohann (Lüttjohann's scale, LS), is more suitable for PTZ-induced seizures (Lüttjohann et al., 2009). This scale has six seizure intensity stages (Figure 7).

| | Lüttjohann behavioral scale |
|---|---|
| 1 | Whisker trembling<br>Sudden behavioral arrest<br>Motionless staring |
| 2 | Facial jerking with muzzle or muzzle and eye |
| 3 | Neck jerks |
| 4 | Clonic seizure in a sitting position |
| 5 | Convulsions including clonic and/or tonic–clonic seizures while lying on the belly and/or pure tonic seizures |
| 6 | Convulsions including clonic and/or tonic–clonic seizures while lying on the side and/or wild jumping |

**Figure 7. Lüttjohann behavioural Scale.**
Seizures scoring according to Lüttjohann behavioural Scale (Lüttjohann et al., 2009)

# MATHERIALS AND METHODS

## 5. ANIMALS

Forty-six syngenic (S)g mice and forty-two Dach-SMOX transgenic (Tg) mice, with a mean body weight of 37 g and at three months old, were analysed. They were initially housed at "Stazione per la Tecnologia Animale" at the University of Tor Vergata (Rome) and then transferred to the animal house of the University of Roma Tre to perform the experiments.
Animals were housed under constant temperature (22 ± 1 °C), humidity (55 ± 10 %) and under a regular light-dark cycle (light 7 a.m.-7 p.m.). These conditions were in accordance with standard laboratory settings that including cage enrichment and *ad libitum* access to food and water. Transgenic animals were obtained crossing JoSMOX (BALB/cx DBA/2) and Dachshund-Cre (DBA/2) mice. Dachshund-Cre mouse line expresses Cre specifically in proneural population of the nervous system and leads to the recombination in the crossed progeny to produce Dach-SMOX mice (Cervelli et al., 2013b). In order to genetically stabilise the Dach-SMOX transgenic line, mice were further genetically stabilised backcrossing ten times with CD1 mice. All the experiments were approved by Italian Ministry of Health (protocol N° 964/2015-PR) and performed in agreement with the guidelines released by the Italian Ministry of Health (D.L 26/14) and the European Community Directive 2010/63/EU.

### 5.1 Pentylentetrazole administration

Pentylenetetrazole (PTZ) (P6500-25G, Sigma, USA) was dissolved in a vehicle solution (50 mM NaPi pH 7.2, 100 mM NaCl) just before the experiment, to avoid the effect of temperature and light, and administered subcutaneously at a dose of 40 mg/kg. As a control, we injected Tg and Sg mice with only the vehicle solution.

### 5.2 Behavioural evaluation of epileptic seizures

Following PTZ administration, animals were singularly placed in an observation box and monitored continuously for 90 minutes for the onset and extent of seizure activity. Seizures were rated accordingly to Lüttjohann's scale (Lüttjohann et al., 2009). This scale has six seizure intensity stages: stage 1, sudden behavioural arrest and/or motionless staring; stage 2, facial jerking with muzzle or muzzle and eye; stage 3, neck jerks; stage 4, clonic seizure in a sitting position; stage 5, convulsions including clonic and/or tonic–clonic seizures while lying on the belly and/or pure tonic seizures; stage 6, convulsions including clonic and/or tonic–clonic seizures while lying on

the side and/or wild jumping. The behavioural assessments described above were performed in a blind manner and the observers had to reach a unanimous agreement regarding the scoring of the behaviour.

**5.3 Genotyping**

Transgenic and Sg mice were restrained and a piece of tissue of 2 mm diameter was punched out of the earlap using an ear-punch and placed with a pair of forceps into a 1.5 mL tube. The ear puncher and the forceps were thoroughly rinsed with distilled water after every biopsy. Mice ear tissues were analyzed with a light fluorescence microscope Axioplan 2 ZEISS, to assess the expression of the green fluorescence protein (GFP). Subsequently all the samples were digested in 450 µl of lysis solution composed of 445.5 µl of lysis buffer (Tris-HCl 1 M pH 8; EDTA 0.5 M pH 8; NaCl 5M; SDS 10%) and 4.5 µl proteinase K (10 mg/ml), respectively. The tissues were incubated overnight at 56°C. The next day the samples were centrifuged for 10 minutes at 16 100 g and NaCl 5M was added to super. After 10 minutes on a rocking platform, the samples were centrifuged again for 10 minutes at 16 100 g. Cold isopropanol was added to the super and centrifuged for 10 minutes at 16 100 g at 4°C. The pellet, containing DNA, was washed with ethanol 80% and suspended in 50 µl of water. The DNA of each mouse was used to perform PCR analysis to identify the genotype, utilizing oligonucleotides of the transgenic sequences of our interest: SMOX-2 5'-AAATATCTCGAGGGAACACATTTGGCAGTGAGG and SMOX-5 5'-TCATCCCCTCGGGCTTCATG, that amplify SMOX fragment of 996 bp; Cre-R2 5'-CTAATGGCCATCTTCCAGCAG and Cre-F1 5'-ATGTCCAATTTACTGACCGTA, that amplify Cre fragment of 1032 bp.

**5.4 Polyamines content determination**

Polyamines concentration was determined as described in Ceci et al. (2017) with minor modifications. Perchloric acid suspension (5%), supplemented with 1.7-diaminoeptane 100 µM as an internal standard, was added to the cortex samples collected from Sg and Tg mice. Samples were then sonicated in ice with Sonics Vibra-Cells to disintegrate the tissue and centrifuged at 16 100 g for 10 minutes. The supernatant was mixed with saturated $Na_2CO_3$ and then with acetone Dansyl chloride solution (7.5 mg/ml). The mixture was incubated overnight, protected from the light at room temperature. The next day, the samples were centrifuged at 16 100 g for 15 minutes at 4°C; proline solution (5%) was added to the supernatant to remove the unreacted Dansyl chloride. After 30 minutes, PAs were extracted with toluene (100%) with vigorous vortexing and then rested for 5 minutes at room temperature in the

dark. The organic phase was dried in a 3 Speedvac Concentrator (Savant Instrument, Inc., New York, USA). The dried Dansyl derivatives were stored at -20°C or dissolved in methanol and immediately assayed. High-performance liquid chromatography technique using the Agilent 1050 system (Agilent Technologies, Germany), was used to detect the PAs content, with an Agilent 1050 photodiode type detector. Continuous on-line quantification of chromatographic peaks was carried out by a fluorimeter Agilent 1200 Spectra-Physics Model SP 4290 and a computing program software "Agilent ChemStation". The separation of Dansyl derivatives was performed on C18 Hypersil BDS 250 X 4.6 mm at constant room temperature 22°C± 1. Two mobile phases were used: (A) water: acetonitrile: methanol (50% :30%: 20%) and (B) acetonitrile: methanol (60% :40%) with the following elution program: 0-5 min: 72% A - 28% B; 5-47 min: 72% A - 28% B; 47-50 min: 36%A - 64% B; 50-55 min: 20% A - 80% B; 55-56 min: 15% A - 85% B; 56-75 min: 72% A - 28% B at flow rate of 1 ml/min.

### 5.5 Behavioural tests

#### 5.5.1 Elevated plus maze test

Apparatus used for the elevated plus maze test consisted of two open arms (50 x 10 x 40 cm, Length x Width x Height) and two closed arms (50 x 10 x 40 cm, L x W x H) connected with a central platform (10 × 10 cm). The maze was placed 50 cm above the ground. Mice were individually placed on the central platform for 5 min and allowed to explore the apparatus. The following parameters were scored using the Observer 3.0 software (Noldus):

- % time spent in the open arms (% TO): (seconds spent on the open arms of the maze/seconds spent on the open + closed arms) × 100
- % open arm entries (% OE): (number of entries into the open arms of the maze/number of entries into open + closed arms) × 100
- Frequency of head dips (frequency with which the animal lowered its head over the sides of the open arms towards the floor).

After testing each mouse, the apparatus was cleaned with 25% ethanol.

#### 5.5.2 Novel object recognition

The object recognition test (ORT) consists in two different phases: training phase and testing phase.

On the training trial, each mouse was individually placed into an open-field arena containing two identical objects (A1 and A2), equidistant from each other, and allowed to explore the objects for 10 min. One hour later, one copy of the familiar object (A3) and a new object (B) were placed in the same

location as during the training trial. Each mouse was placed in the apparatus for 10 min, and the time spent exploring each object was recorded. Each trial was recorded with a camera positioned above the apparatus for subsequent behavioural analysis performed using the Observer 3.0 software (Noldus). The following parameters were scored:

- Time spent sniffing the new object
- Time spent sniffing the old object

The discrimination index was calculated as the difference in time exploring the novel and the familiar objects, expressed as the percentage ratio of the total time spent exploring both objects.

**5.5.3 Forced swimming test**

Apparatus used for the forced swimming test consisted of a Plexiglas cylinder (25 x 13 cm, H x Diameter) filled with water (22°C) up to a height of 10 cm. Mice were placed into the apparatus for 6 min. The percentage of immobility, swimming and climbing time was recorded using the Observer 3.0 software (Noldus).

**5.5.4 Sucrose preference test**

In the sucrose preference test, mice were given a bottle containing water and a second with 2% sucrose solution for successive 3 days with the left-right positions of the bottles alternated daily. Bottles were weighed prior to testing and then every day for the next 3 days. Sucrose preference was expressed as 100 × [(sucrose intake)/ (sucrose intake + water intake)].

**5.5.5 Passive avoidance test**

The passive avoidance test was performed using a 3-day paradigm with two chamber apparatus (King et al, 2003). During the day 1 mice were left free to explore the apparatus for 5 min. In the day 2 mice were placed in the light chamber and received a mild inescapable footshock (0.5 mA, 2 s) after dark chamber entry. During the day 3: mice were placed in the light chamber and time to cross into the dark chamber was measured. Latency to cross was measured on days 2 (train) and 3 (test), with a 5 min maximum.

**5.5.6 Light dark box test**

The apparatus consisted of an arena (45 x 21 x 21 cm, L x W x H), divided into two distinct compartments: one third dark compartment (15 x 21 x 21 cm, L x W x H, under 15 lux illumination) painted black and with a black lid

on top, the remaining two thirds light compartment (30 x 21 x 21 cm, L x W x H, under 85 lux illumination) painted white and uncovered. A small entry within the compartment partition (5×7 cm) allowed each mouse to move between chambers freely. Animals were placed in the dark compartment and their movement tracked for 5 minutes using tracking software (Observer 3.0 software Noldus.) connected to a video camera positioned overhead. The time spent in the dark compartment, the time spent in the light compartment and the number of light/dark transition was recorded. To remove any olfactory cues, the apparatus was cleaned with 70% ethanol after the completion of the trial.

**5.6 RNA isolation, reverse transcription and Real Time PCR**

Total RNA was extracted using using TRIzol® Reagent (Invitrogen, 15596-018) and retro-transcripted into cDNA in two steps by SuperScript™ III First-Strand Synthesis System (Invitrogen, 18080-051) according to the manufacturer's instructions.

cDNA was amplified for the following genes: 5-HT1AR, 5-HT1BR, 5-HT2AR, 5-HT2CR, 5-HTT, BDNF, TrkB, IDO1 and β-actin. The mRNA for the constitutive β-actin was examined as the reference transcript The sequences of primers were reported in Table 1. PCR product quantification was calculated by applying the SYBR-Green method. Reactions were performed in a Rotor gene 6000 machine (Corbett research) using the following program: 40 cycles of 95°C for 2 min, 95°C for 5 sec, 60°C for 30 sec, 72°C for 20 sec. Beta actin mRNA amplification products were present at equivalent levels in tissue samples. The data are calculated relative to the internal housekeeping gene according to the second derivative test (delta–delta Ct (2-ΔΔCT) method.

| Gene | Primers |
|---|---|
| 5-HT1AR | Fwd 5'-CTGTTTATCGCCCTGGATGTG-3'<br>Rev 5'-GGTCCTTGCTGATGGTGCAC-3' |
| 5-HT1BR | Fwd 5'-GATCCACATCCTCGGTCAC-3'<br>Rev 5'-CCAACACACAATAAATGCTCC-3' |
| 5-HT2AR | Fwd 5'-AGAACCCCATTCACCATAGC-3'<br>Rev 5'-TCCTGTAGCCCGAAGACTG-3' |
| 5-HT2CR | Fwd 5'-GCAATAATGGTGAACCTGGG-3'<br>Rev 5'-CGACTATTGAAAGTGCTGGC-3' |
| 5-HTT | Fwd 5'-CAACTCCGGCTTTTCCAATAC-3'<br>Rev 5'-GATGTTCCTATGCAGTAGCC-3' |
| BDNF | Fwd 5'-CCATAAGGACGCGGACTTG-3'<br>Rev 5'-GGCGCCGAACCCTCATAG-3' |
| Trkb | Fwd 5'-GATCTTCACCTACGGCAAGC-3'<br>Rev 5'-TGGCCAAGTTCTGAAGGAGG-3' |
| IDO1 | Fwd 5'-CTCCTGCAATCAAAGCAATCC-3'<br>Rev 5'-CTGCATTTCCAGCCAGACAG-3' |
| β-actin | Fwd 5'-GTGGGAATGGGTCAGAAGG-3'<br>Rev 5'-CTGGGTCATCTTTTCACGG-3' |

**5.7 Tissue Homogenates and Analysis of proteins by Western blotting**

Cortex samples from each Sg and Dach-SMOX mice were mechanically homogenized in 5 vol (w/v) of ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 10% Glycerol, 320 mM Sucrose, 1% Triton, 1 mM PMSF, 1X Complete protease inhibitor cocktail) using Potter homogenizer for 45 seconds. Samples were kept in ice for 30 minutes and then centrifuged at 16 100 g for 15 minutes at 4°C. Supernatants were collected and protein determination was carried out according to Bradford method using bovine serum albumin as standard (Bradford, 1976).

Equal amounts (20 µg proteins/sample) of samples were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) in a 10% polyacrylamide gel and transferred to Nitrocellulose blotting membranes (AmershamTM ProtranTM 0.2 µm NC). Membranes were blocked with 5% non-fat milk or with bovine serum albumin (BSA) 3% and 0.1% Tween 20 in Tris buffer (TBST) and probed with the following antibodies: rabbit polyclonal anti-5-HTT (1:500) (sc-13997, Santa Cruz Biotechnology), mouse monoclonal anti 5-HT2AR (1:1000) (sc-166775, Santa Cruz Biotechnology), rabbit polyclonal anti 5-

HT2CR (1:500) (STJ95764, St John's Laboratory), rabbit polyclonal anti 5-HT1AR (1:1000) (GTX104703 GeneTex), mouse monoclonal anti c-Src (1:1000) (sc-1301124, Santa Cruz Biotechnology), mouse monoclonal anti p-c-Src (h-3) (1:1000) (sc-166860, Santa Cruz Biotechnology); mouse monoclonal anti-vinculin (1:40000) (V9131, SIGMA) or rabbit polyclonal anti-actin (1:2000) (A5060, SIGMA). Membranes were then washed in TBST three times for 10 minutes at room temperature followed by the addition of secondary horseradish peroxidase-conjugated goat polyclonal anti-rabbit IgG antibody (1:5000) (SA00001-2, Proteintech) or goat polyclonal anti-mouse IgG antibody (1:2000) (SA00001-1, Proteintech) diluted in 1% milk TBST or 1% BSA TBST. Membranes were washed in TBST three times for 10 minutes and one time in TBS for 10 minutes. The chemiluminescent signals were detected using BioRad Clairity™ Western ECL Substrate Detection Reagent. Immunoblots were imaged using a ChemiDoc (BioRad) and quantified using Image Lab software 5.2.1 (BioRad). β-actin or vinculin was taken as the reference protein amounts for extracts.

# AIM OF THE PROJECT

Several studies have demonstrated that altered PAs levels are involved in different diseases. In recent years great attention has been given to the involvement of PAs in epilepsy and mental illnesses that are two of the most common neurological diseases. These pathologies affect people of all ages and constitute a huge cost for the health system. Often epilepsy and mental illness are present in the same person; the coexistence of two or more pathologies is defined as comorbidity.

Based on the new evidences of the role of spermine oxidase (SMOX) in neurodegeneration, this research project analysed a novel involvement of this enzyme in epilepsy and mood disorders. The transgenic mouse line Dach-SMOX, with CD1 background, specifically overexpressing SMOX in brain cortex, has been proved to be highly susceptible to epileptic seizures induced by kainic acid. In this project it was investigated the susceptibility of the transgenic mouse model Dach-SMOX, after treatment with the epileptogenic drug pentylentetrazole (PTZ). Furthermore, a large part of this project focused on the characterization of the serotonergic system in Dach-SMOX mice. The central aim of my thesis work was to understand the relationship between the serotonergic system and the PA system in our mouse model and how this relationship could be linked to the comorbidity between epilepsy and mental illness.

The main goals of my project are:

- To investigate the increased susceptibility of Dach-SMOX mice to excitotoxicity;
- To analyse behaviourally the mental emotional state of Dach-SMOX mice;
- To characterize the serotonergic system in an animal model with altered polyamine levels;
- To investigate the molecular pathway and proteins involved in the comorbidity between epilepsy and mental illness.

# RESULTS

## 6. PENTYLENTETRAZOLE TREATMENT

### 6.1 Behavioural evaluation of mice treated with pentylentetrazole

Since a deregulation of polyamines levels lead to an increased susceptibility to excitotoxic injury, as demonstrated with kainic acid treatment of Dach-SMOX mice, I decided to test another epileptogenic drug (PTZ) in order to further characterised this mouse model. A preliminary dose-response study was performed to decide the appropriate dosage of PTZ to administrate. The dosage of 40 mg/kg revealed to be optimum amount of PTZ, since the 60 and 85 mg/kg doses were causing a high mortality. In fact, utilizing the higher doses of 60 and 85 mg/kg, the percentage of deaths was higher than the percentage of survived mice. Based on these results, I have analysed twenty-five Sg and twenty-five Tg mice injected with a single convulsive dose of 40 mg/kg, registering the scores that each mouse showed, according to LS. The behavioural evaluation analysis, for the sake of simplicity, was done by grouping the symptoms into mild (from stages 1 to 3) and severe (from stages 4 to 6).

Figure 8 indicates that 76% of Sg mice showed mild symptoms (stages 1 to 3 of Lüttjohann scale) compared to 44% of Tg mice; on the contrary severe symptoms (stages 4 to 6 of Lüttjohann scale) have been observed in a higher percentage of Tg mice (56%) than Sg mice (24%).

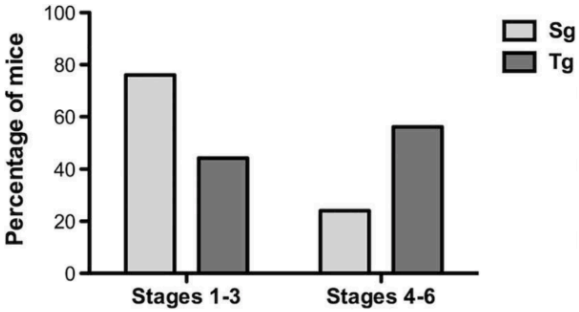


**Figure 8. Behavioral evaluation of Tg and Sg mice treated with pentylentetrazole or vehicle solution.**

The histogram represents the symptoms showed by the 25 Tg and 25 Sg mice after PTZ treatment according to Luttjohann's Scale (LS) (Lüttjohann et al. 2009). The LS has been grouped stages 1–3 (mild symptoms) and stages 4–6 (severe symptoms). The p value was measured with the Chi square test ($p < 0025$). Sg, syngenic mice; Tg, transgenic mice.

### 6.2 Polyamines content in brain cortex of mice treated with pentylentetrazole or vehicle solution

To verify the imbalance of PAs levels - due to the over-expression of SMOX in Dach-SMOX mice - and to examine possible changes in PAs content following vehicle solution or PTZ treatment, I have measured Put, Spd and Spm concentrations from neocortex samples by HPLC (Figure 9). Put and Spd levels were found to be higher in Tg mice compared to Sg mice in vehicle- injected animals, while a general decrease of Spd and Spm was found after PTZ treatment in Tg animals.

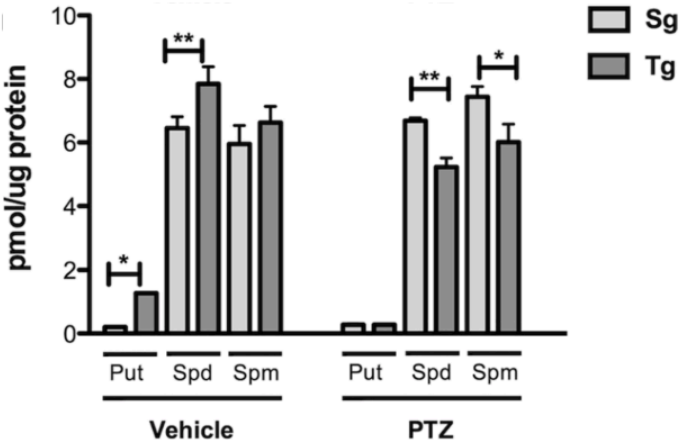


**Figure 9. Polyamines content of Tg and Sg mice treated with pentylentetrazole or vehicle solution.**
PA content from neocortex of Tg and Sg mice were analyzed after 3 days form PTZ treatment. The p values were measured with the two-way ANOVA test and post-hoc test Bonferroni (*, $p<0.05$; **, $p<0,01$). Sg, Syngenic mice; Tg, transgenic mice.

## 7. BEHAVIOURAL CARACHTERIZATION OF DACH-SMOX MICE FOR MENTAL AND MOOD DISORDERS

The SMOX cerebral cortex overexpression in Dach-SMOX mice leads to an imbalance in PAs levels as demonstrated by HPLC. As mentioned above, several studies have correlated a dysregulation of PA content with the onset of mental illness. In order to evaluate any behavioural abnormalities in Dach-SMOX mouse model, I carried out a comprehensive battery of behavioural tests. These tests are specific for assessing conditions such as anxiety, depression and memory impairment.

### 7.1 Sucrose preference test

The sucrose preference test (SPT) is a reward-based assesment, used as an indicator of anhedonia. Anhedonia, or the decreased ability to experience pleasure, represents one of the core symptoms of depression. Rodents are born with an interest in sweet foods or solutions. Reduced preference for sweet solution in SPT represents anhedonia. Sucrose preference is calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake. Nine Sg mice and 10 Tg mice were tested. The figure 10a shows the percentage of sucrose intake by Sg mice in comparison to Tg mice. Figure 10b shows the total intake (g) of sucrose respect to water in Tg and Sg mice. No differences in sucrose intake were observed between Tg and Sg mice, while both genotypes preferred significantly the intake of sucrose.

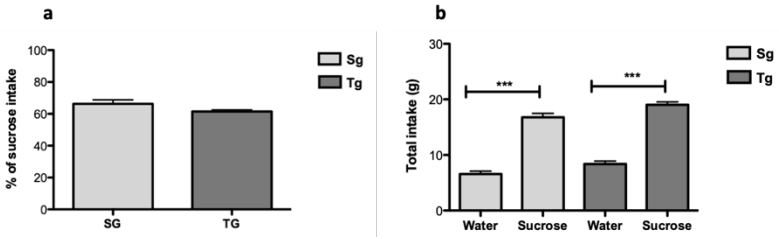


**Figure 10.Sucrose preference test: test for depressive like behaviours.**
(a) Percentage of sucrose intake. The histogram represents th percentage of sucrose intake of Sg and Tg mice. (b) Total intake. The histogram represents the total intake of water and sucrose (expressed in g) of Sg and Tg mice. Data are expressed as mean ± SEM. Differences among experimental group were determined by Student's t-test (***$p<0.001$). Sg, syngenic mice; Tg, transgenic mice.

### 7.2 Forced swim test

The (Porsolt) forced swim test, also known as the behavioural despair test is one of the most commonly used assays for the study of depressive-like behaviour in rodents. The FST is based on the assumption that when placing a mouse in a container filled with water, it will first make efforts to escape but eventually will exhibit immobility that may be considered to reflect a measure of behavioural despair. The experimental session lasts 6 minutes. During the last 4 minutes the following parameters are analysed: floating, swimming, and climbling. Floating in rodents is considered to be a typical depression behaviour. Fourteen Sg and 10 Tg mice were tested. The histogram in Figure 11 show the percentage of time spent floating, swimming and climbing. No significant changes were apparent in floating, swimming and climbing times between Sg and Tg mice; this indicates that there are not significant behavioural despair in both genotypes.

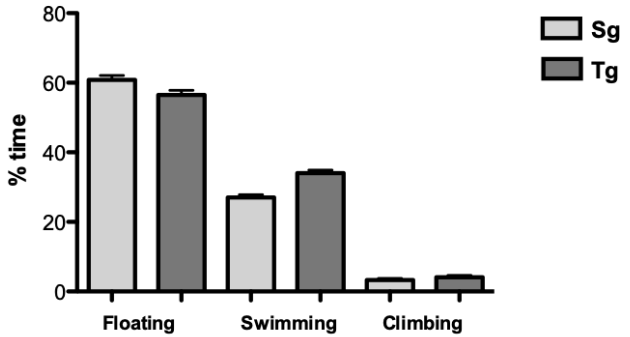


**Figure 11. Forced swim test.**
Time spent floating, swimming and climbing. Data are expressed as mean ± SEM. Differences among experimental group were determined by Student's t-test. Sg, syngenic mice; Tg, transgenic mice.

### 7.3 Elevated plus maze test

The elevated plus maze test is a simple method to assess anxiety-like behaviours in rodents. Mice are placed at the junction of the four arms of the maze, facing an open arm, and entries/duration in each arm are recorded and

observer simultaneously for 5 min. Other ethological parameters (i.e., rears, head dips and stretched-attend postures) can also be observed.
The task is based on an approach-avoidance conflict, meaning that the animal is faced with a struggle between a propensity to explore a novel environment and an unconditioned fear of high and open spaces. Consequently, an anxiety-like state is characterized by increased open arm avoidance compared to a non-anxious animal. Twenty Sg and 11 Tg mice were tested. The figure 12a shows the frequency of head dips of both Sg and Tg mice, the Tg mice display a significant decrease in the frequency of head dips compared to Sg ones (P= 0.0066; T=2.925; Df= 29). The histogram in figure 12b shows the percentage of entries in the open arms, also in this case the Tg mice display a decrease in number of entries in the open arms expressed as percentage (P= 0.0475; T= 2.070; Df= 29). In the figure 12c is shown the percentage of time spent in open arms by both Sg and Tg mice, no significant differences were found. Data obtained through elevated plus maze test indicate that Tg mice have an anxious phenotype compared to Sg mice

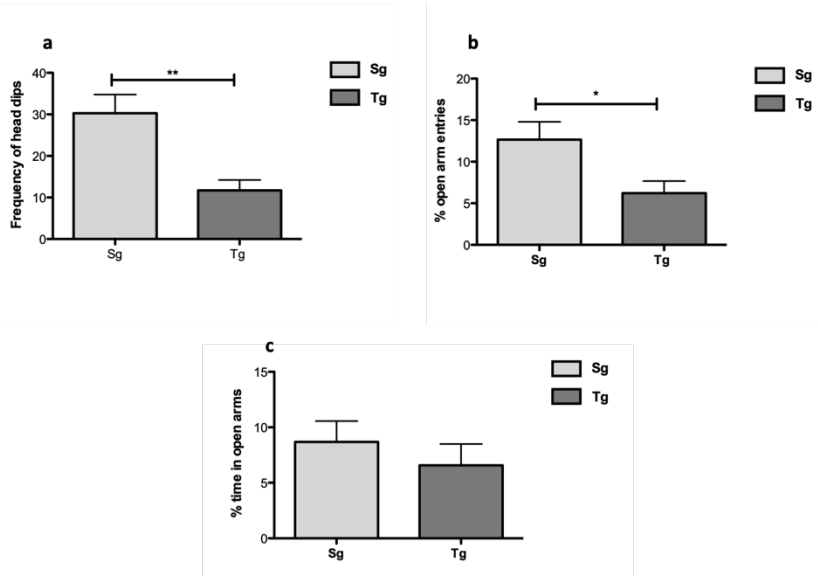


**Figure 12. Elevated plus maze test.**
(a) Frequency of head dips. The histogram represents the frequency of head dips from the open arms of Sg and Tg mice. (b) Percentage of entries in the open arms. The histogram represents the percentage of entries in the open arms of Sg and Tg mice. (c) Percentage time in the open arms. The histogram rapresent the percentage of time spent in open arms by both Sg and Tg mice. Data are expressed as mean ± SEM. Differences among experimental group were determined by Student's t-test (*p<0.05; **p<0.01). Sg, syngenic mice; Tg, transgenic mice.

### 7.4 Light dark box test

The light/dark transition test (LDT) is one of the most widely used tests to measure anxiety-like behaviour in mice. The test is based on the natural aversion of mice to brightly illuminated areas and on their spontaneous exploratory behaviour in response to mild stressors, such as novel environment and light. The test apparatus consists of a box divided into a small (one third) dark chamber (15 lux) and a large (two thirds) brightly illuminated chamber (85 lux). Mice are placed into the lit compartment and allowed to move freely between the two chambers. The first latency to enter

the dark compartment and the total time spent in lit compartment are indices for bright-space anxiety in mice. Transitions (crossing frequency between the two chambers) are index of activity-exploration, because of habituation over time. Fifteen Sg and 11 Tg mice were tested. The Figure 13a shows the time (s) spent by Sg and Tg mice in the light box, Tg mice spent significantly less time in the light box compared to controls (P= 0.0252; T= 2.384; Df=23). The histogram in figure 13b illustrates the time (s) spent by Sg and Tg mice in the dark box, Tg mice spent significantly more time in the dark box respect to Sg mice (P= 0.0258; T=2.384; Df= 23). The histogram in the figure 13c represents the number of crossing (transition) between the two boxes. No differences were found in the number of crossing between Sg and Tg mice. The results obtained by LDT confirm the results obtained by elevated plus

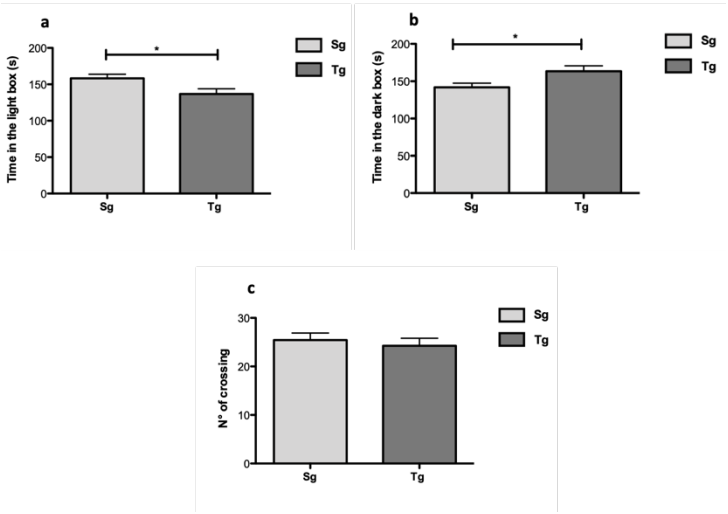


**Figure 13. Dark/light box test.**
(a) Time in the light box. The histogram represents the time spent in the light box by Sg and Tg mice. (b) Time in the dark box. The histogram represents the time spent in the dark box by Sg and Tg mice. (c) Number of crossing. The histogram rapresent the number of crossing between the light and dark boxes. Data are expressed as mean ± SEM. Differences among experimental group were determined by Student's t-test (*p<0.05). Sg, syngenic mice; Tg, transgenic mice.

maze test; the Tg mice are more anxious compared to Sg mice.

### 7.5 Novel object recognition

The object recognition test (ORT) is a commonly used behavioural assay for the investigation of various aspects of learning and memory in mice. The ORT is fairly simple and consists in two different phases: training and testing. On the training trial, each mouse was individually placed into an open-field arena containing two identical objects (A1 and A2), equidistant from each other, and allowed to explore the objects for 10 min. One hour later, one copy of the familiar object (A3) and a new object (B) were placed in the same location as during the training trial. Each mouse was placed in the apparatus for 10 min, and the time spent exploring each object was recorded. The discrimination index was calculated as the difference in time exploring the novel and the familiar objects, expressed as the percentage ratio of the total time spent exploring both objects. Fourteen Sg and 11 Tg mice were tested. The figure 14 shows the time sniffing the old object (a), the time sniffing the new object (b) and the discrimination index (c). Transgenic mice showed a stereotyped behavior with an increase in the time spent to sniff the new object (P=0.043; T= 1.732; Df =22). No memory impairment was found in either of two genotypes.

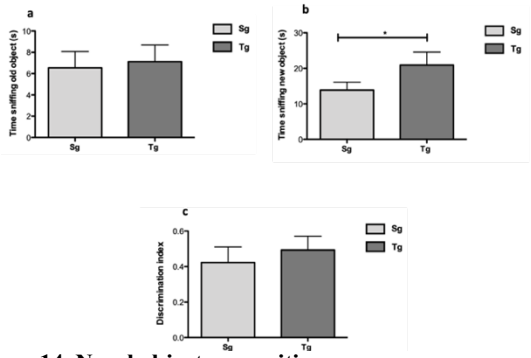


**Figure 14. Novel object recognition.**

(a) Time sniffing old object. The histogram represents the time spent sniffing new object by Sg and Tg mice. (b) Time sniffing old object. The histogram represents the time spent sniffing new object by Sg and Tg mice. (c) Discrimination index. The histogram rapresent the recognition memory evaluated using discrimination index (DI). Data are expressed as mean ± SEM. Differences among experimental group were determined by Student's t-test (*p<0.05). Sg, syngenic mice; Tg, transgenic mice

### 7.6 Passive avoidance test

Passive avoidance is fear-motivated test classically used to assess short-term or long-term memory on small laboratory animals (rat, mice). Passive avoidance paradigm requires the subjects to behave contrary to their innate tendencies for preference of dark areas and avoidance of bright ones. The apparatus chamber used in this test is composed by a black poorly illuminated compartment and a white illuminated compartment.

In the training phase the animal is placed in the white compartment. When the animal innately crosses to the black compartment it receives a mild foot shock. Thus, during the initial phase the animal learns that the moving to the dark compartment has negative consequences. During the test phase the animal is again placed in the white compartment and the passive avoidance response is evaluated. As opposed to an avoidance that entails active movement to avoid an aversive stimulus, the avoidance of the dark compartment requires the animal to remain in the white compartment and, therefore, the absence of movement; namely passive avoidance response.

Memory performance is positively correlated with the latency to escape from the white compartment; the better the recollection, the greater the latency. Fourtee Sg and 11 Tg mice were tested. The figure 15 shows the latency of crossing from the light to the dark compartment during the training day (a) and during the test day (b) by the Sg and Tg mice. No differences were observed.

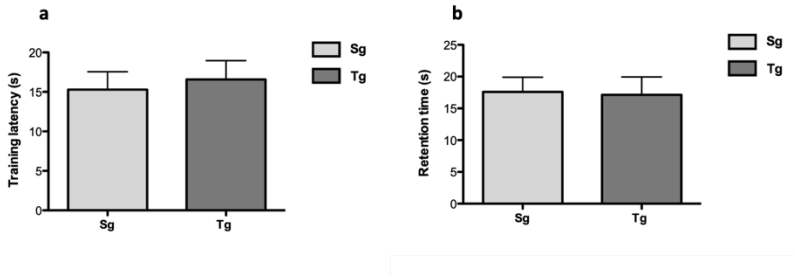


**Figure 15. Passive avoidance test.**

(a) Training latency. The latency of crossing from the light to the dark box prior to a foot shock. (b) Retention time. The latency of crossing from the light to the dark compartment post foot shock by Sg and Tg mice. Data are expressed as mean ± SEM. Differences among experimental group were determined by T-Test. Sg, syngenic mice; Tg, transgenic mice

## 8. MOLECULAR CHARACTERIZATION OF SEROTONIN SYSTEM IN DACH-SMOX MICE

To investigate a possible alteration in serotonin system, a molecular analysis of serotonin receptors and serotonin transporter transcript levels was performed. In particular molecular analysis has focused on those receptors involved both in anxiety and epilepsy (5-HT1AR, 5-HT1BR, 5-HT2AR, 5-HT2CR) and serotonin transporter (5-HTT).

### 8.1 Analysis of serotonin receptors and serotonin transporter transcript levels by quantitative Real Time-PCR (qRT-PCR)

Brain cortex from Sg and Tg mice were harvested and processed to perform qRT-PCR analysis. As shown in figure 16 in Tg mice a significant decrease in both 5-HT1AR and 5-HT1BR auto-receptors was observed compared to controls.

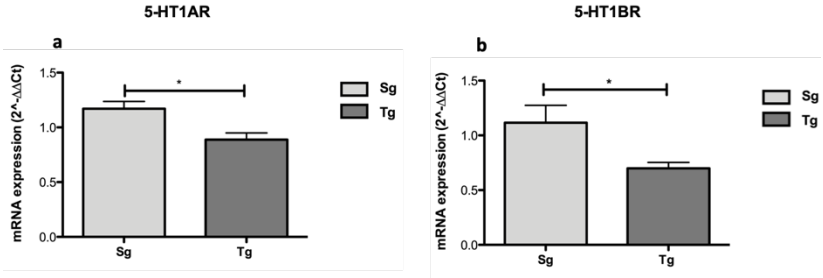


**Figure 16**. **qRT-PCR analysis of 5-HT1AR and 5-HT1BR auto-receptors of Sg and Tg mice**.
Transcript levels of 5-HT1AR (a) and 5-HT1BR (b) receptors from neocortex of Sg and Tg mice were analysed by qRT-PCR. Data are calculated relative to the internal housekeeping gene (actin) and are the means ± SEM from three separate experiments. Student's t-test was used to determine significant differences. *$p<0.05$. Sg, syngenic mice; Tg, transgenic mice.

The Figure 17 shows transcript levels of 5-HT2AR and 5-HT2CR post synaptic receptors, and of 5-HTT serotonin transporter, respectively. The Tg mice display a significant decrease in both receptors and in transporter transcripts levels and in general they seem to be characterized by a general alteration of serotonergic system compared to Sg mice.

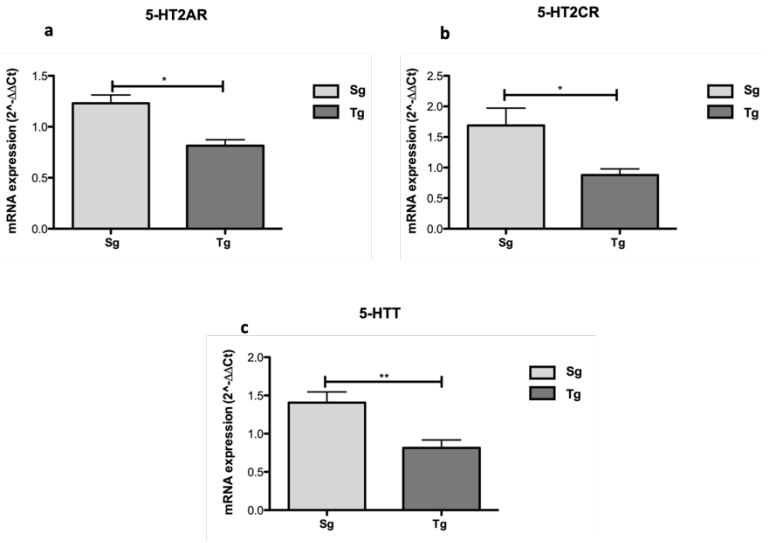


**Figure 17. qRT-PCR analysis of 5-HT2AR, 5-HT2BR receptors and 5-HTT transporter of Sg and Tg mice**.
Transcript levels of 5-HT2AR (a), 5-HT1BR (b) and 5-HTT from neocortex of Sg and Tg mice were analyzed by qRT-PCR. Data are calculated relative to the internal housekeeping gene (actin) and are the means ± SEM from three separate experiments. Student's t-test was used to determine significant differences. *p<0.05; **p<0.01. Sg, syngenic mice; Tg, transgenic mice.

Different studies have been demonstrated that brain derived neurotrophic factor (BDNF) promotes the development and function of serotonergic neurons. This neurotrophin is one of the most abundant and widely distributed in CNS and it exerts its action through Tropomyosin receptor kinase B (TrkB) receptor. Several studies have shown a role of BDNF in the etiogenesis of anxiety and depression, furthermore it has been demonstrated the existence of a mechanism of mutual regulation between the serotoninergic system and BDNF. In order to verify if there could be an alteration of BDNF related to the changes observed in the serotonergic system, I carried out an evaluation of this neurotrophin and TrkB transcript levels. As shown in Figure 18, no significant differences where observed of BDNF and TrkB mRNA levels in both Sg and Tg mice.

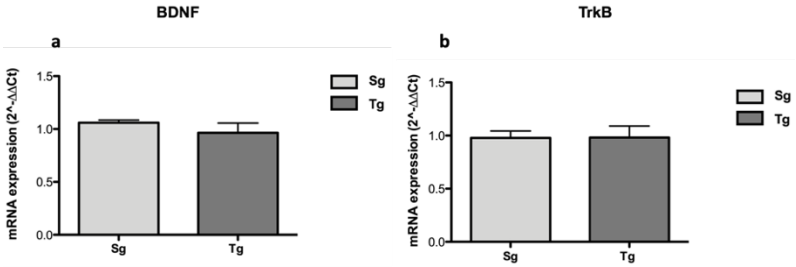


**Figure 18. qRT-PCR analysis of BDNF and TrkB of Sg and Tg mice.**
Transcript levels of BDNF (a) and TrkB (b) from neocortex of Sg and Tg mice were analysed by qRT-PCR. Data are calculated relative to the internal housekeeping gene (actin) and are the means ± SEM from three separate experiments. Student's t-test was used to determine significant differences. Sg, syngenic mice; Tg, transgenic

## 8.2 Analysis of serotonin receptors and serotonin transporter protein levels by Western Blot

Molecular analyses of protein levels of serotonin receptors and serotonin transporter were performed in order to confirm the data obtained by qRT-PCR, and to analyse the protein expression levels of serotonin systems key modulators. Brain cortex from Sg and Tg mice were harvested and processed to perform Western Blot analysis. As shown in Figure 19 the protein expression of serotonin receptor 1A and serotonin transporter are significant lower in Tg mice compared to Sg mice.

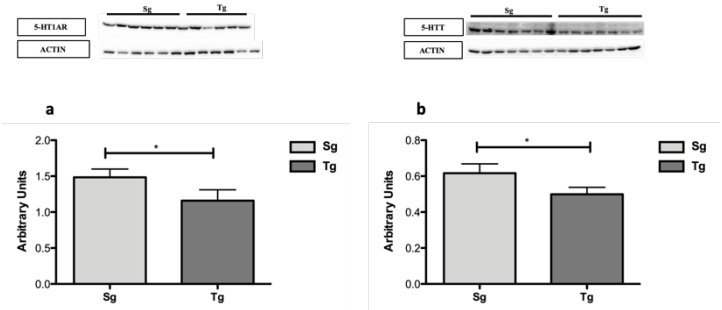


**Figure 19. Western Blot analysis of 5-HT1AR and 5-HTT of Sg and Tg mice.**
Protein levels from neocortex of Sg and Tg mice were analyzed. Actin was used as loading control. The quantification of 5-HT1AR (a) and 5-HTT (b) was determined by densitometry. Arbitrary units represent the normalization ratio between antibodies signal and actin signal. The p value is presented as mean ± SEM from three independent experiments. The p value was measured with Student's t-test (*$p<0.05$). Sg, syngenic mice; Tg, transgenic mice.

Western blot analysis was also performed to evaluate protein expression of 5-HT2AR and 5-HT2CR receptors. The histogram in figure 20 (a) shows a significant reduction in 5-HT2AR protein levels in Tg mice, these mice also display a significant reduction in 5-HT2CR protein content respect to control. Collectively these findings indicate that the overexpression of SMOX in the cerebral cortex of Tg mice leads to an impairment of serotonergic system with a significant reduction in transcript and protein levels of serotonin receptors (1A, 1B, 2A and 2C) and of serotonin receptor.

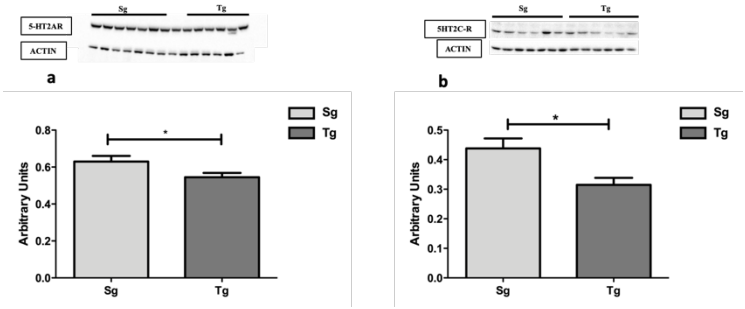


**Figure 20. Western Blot analysis of 5-HT2AR and 5-HT2CR of Sg and Tg mice.** Protein levels from neocortex of Sg and Tg mice were analyzed. Actin was used as loading control. The quantification of 5-HT2AR (a) and 5-HT2CR (b) was determined by densitometry. Arbitrary units represent the normalization ratio between antibodies signal and actin signal. The p value is presented as mean ± SEM from three independent experiments. The p value was measured with Student's t-test (*$p<0.05$). Sg, syngenic mice; Tg, transgenic mice.

## 9. ANALYSIS OF THE BALANCE BETWEEN SEROTONIN PATHWAY AND KYNURENINE PATHWAY IN DACH-SMOX MICE

The results obtained in the behavioural and molecular characterization of Dach-SMOX mice show that they are more susceptible to induced seizures and that they also display an anxious phenotype. Dach-SMOX mice were also found to be characterized by an altered serotonergic system with a significant reduction in serotonergic receptors and in the serotonergic transporter. In order to understand how the overexpression of SMOX could affect the serotoninergic system and determine a predisposition to seizures and mood disorders, the pathway of kynurenine (KYN) was analysed. Kynurenine pathway metabolized the 99% of ingested tryptophan (TRP) not used for protein synthesis and generates a number of neuroactive metabolites

collectively called the kynurenines. In particular, neurokynurenines are involved in anxiety, depression, epilepsy and in other neurologic diseases. The TRP metabolism consists of two pathways: kynurenine and serotonin pathways. When the KYN pathway is activated, it competes with the 5-HT pathway, in fact KYN pathway depletes TRP and debases the 5-HT pathway. Many studies proposed that serotonin deficiency in mood disorders was caused by the TRP shunt towards formation of KYN (kynurenine shunt). Indoleamine 2,3-dioxygenase 1 (IDO1) catalysed the first and rate-limiting step of the kynurenine pathway. Indoleamine 2,3-dioxygenase 1 is activated by a phosphorylation operated by Src kinase, which appears to be activated by high levels of Spd. Spermidine promotes the activation of Src kinase by the dephosphorylation of Tyrosine 530.

**9.1 Analysis of Src kinase protein levels by Western Blot**

Molecular analyses of protein levels of Src kinase were performed by western blot. Figure 21 shows the protein levels of cellular c-Src kinase (a) in brain cortex of Sg and Tg mice. No differences were observed in the amount of total c-Src kinase between Sg and Tg mice. The histogram (b) shows the phosphorylated form in tyrosine 530 of the Src kinase, which indicates the inactive portion of the protein. The levels of p-Src kinase are lower in Tg mice respect to control. Transgenic mice have a significant reduction of inactive Src kinase protein level as shows by the ratio between inactive form of Src kinase and c-Src kinase (c).

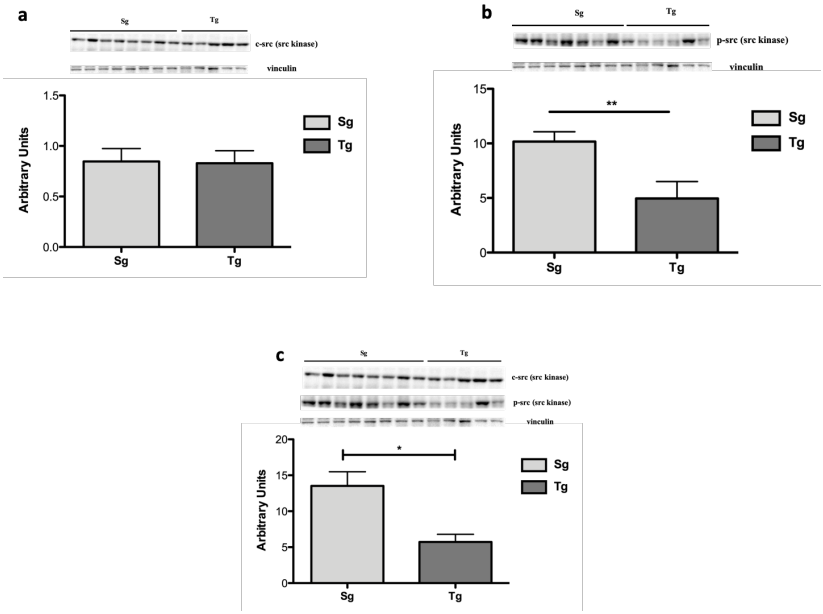


**Figure 21. Western Blot analysis of Src kinases of Sg and Tg mice.**
Protein levels from neocortex of Sg and Tg mice were analysed. Vinculin was used as loading control. The quantification of c-Src kinase (a) phopho (Tyr530) Src kinase, p-Src (b) and the ratio between pSrc/Src kinase (c) was determined by densitometry. Arbitrary units represent the normalization ratio between antibodies signal and vinculin signal. The p value is presented as mean ± SEM from three independent experiments. The p value was measured with Student's t-test (*$p<0.05$; **$p<0.01$). Sg, syngenic mice; Tg, transgenic mice

## 9.2 Analysis of indoleamine 2,3-dioxygenase 1 (IDO1) transcript levels by qRT-PCR

In order to further analyze the KYN pathway in Dach-SMOX mice, IDO1 transcript levels were measured by qRT- PCR. Brain cortex from Sg and Tg mice were harvested and processed to perform the analysis. The Figure 22 shows that Tg mice have higher levels of IDO1 mRNA compared to Sg ones.

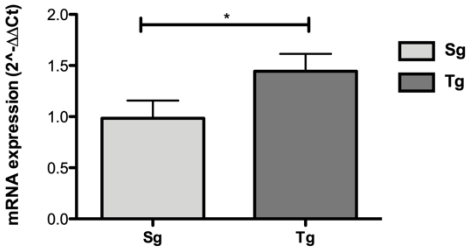


**Figure 22. qRT-PCR analysis of IDO1 of Sg and Tg mice.**
Transcript levels of IDO1 from neocortex of Sg and Tg mice were analyzed by qRT-PCR. Data are calculated relative to the internal housekeeping gene (actin) and are the means ± SEM from three separate experiments. Student's t-test was used to determine significant differences. Sg, syngenic mice; Tg, transgenic mice.

# DISCUSSION AND CONCLUSION

Polyamines have central roles in cellular processes, for example in the regulation of cellular progression, differentiation and death (Casero et al., 2018). Several studies demonstrated that an altered PA metabolism is observed in many different pathologies, including cancer and brain neurodegenerative disorders (Casero et al., 2018). While the role of PA in cancer is well established, the involvement of PAs in brain diseases is still unclear and there are numerous studies aimed at investigating PAs in this field. In order to investigate the role of SMOX enzyme in normal brain functions and during neurodegenerative processes, previous studies were carried out utilizing Dach-SMOX mice, a genetically engineered mouse model. Dach-SMOX mice showed increased vulnerability to excitotoxic injury obtained by KA administration. Kainic acid is an agonist of some GLURs and it is often used as a neurotoxin that mimics the Glu and provokes epileptic seizures. During the analysis of GLURs editing in Dach-SMOX mice it was observed that the transcript levels of serotonin receptors 2C were lower compared to controls.

There is a growing evidence that serotoninergic neurotransmission modulates a wide variety of experimentally induced seizures and is involved in the enhanced seizures susceptibility. In general, it seems that serotonergic neurotransmission, by activating different 5-HTRs, suppresses neuronal network hyperexcitability and seizures activity (Bagdy et al., 2007). The serotonergic system has been studied for decades for its key contribution to the etiology of mental disorders. Over the last few years, several epidemiological studies have shown a strong correlation between epilepsy and different mental illnesses; the comorbidity between epilepsy and psychiatric diseases is increasingly recognized (Harden, 2002; Kanner, 2003; Hamid et al., 2011). The high comorbidity between these two neurological illnesses is strongly suggestive that they share a common background in the central nervous system. In this perspective, this work focused on the role played by SMOX in the brain in physiological and excitotoxic conditions, with the aim to understand its contribution in the comorbidity of epilepsy and mental illnesses. Previous studies showed an increased susceptibility of Dach-SMOX mice after KA treatment (Cervelli et al., 2013b; Pietropaoli et al., 2018). While the administration of glutamate-analogue KA determines an activation of excitatory system (Cervelli et al., 2013b; Cervetto et al., 2016; Pietropaoli et al., 2018), PTZ treatment yields instead epileptic seizures by blocking the GABAergic inhibitory system (Psarropoulourt al., 1994). To evaluate seizure intensity response, behavioral scoring is commonly used according to accepted reference scales. Thus, the use of the six-stage intensity

scale, defined by Lüttjohann LS, results suitable for PTZ induced seizures (Lüttjohann et al., 2009). Based on this scale, Tg mice exhibited higher sensitivity to PTZ as demonstrated by the severity of the symptoms observed. In fact, severe symptoms (stages 4 to 6 of Lüttjohann scale) have been observed in a higher percentage of Tg mice than Sg ones. KA-induced seizures activity in mice brain was demonstrated to clearly activate the PAs interconversion pathway (Cervelli et al., 2013b; Pietropaoli et al., 2018), suggesting a possible contribution of PAs metabolism to neuronal damage, likely due to the production of $H_2O_2$. The results of this investigation indicate that PTZ also stimulates the PAs interconversion pathway in mouse brain. When comparing PAs content between Tg and Sg mice treated with the vehicle solution, it can be noticed that Put and Spd levels where higher in Tg mice if compared to Sg mice, while Spm levels are equivalent. The observed increase of Spd can be explained with Spm oxidation by SMOX, and in turn the increase of Put can be due to an increase of Spd catabolism. No differences can be observed in Spm levels, according to previous work on SMOX overexpression (Cervelli et al., 2013b), presumably because among all PAs, Spm is well buffered in its cellular content, confirming that its homeostasis is crucial for physiological cell life. After PTZ treatment of Dach-SMOX mice, PAs content is further altered because of the whole PAs catabolism activation. Although Dach-SMOX mice are more susceptible to PTZ-induced seizures, when not treated, they display a comparable life expectancy as Sg mice.

Emerging studies show a high frequency of comorbidity in patients between epilepsy and mental illness. Furthermore, it has been demonstrated that epileptic patients with psychiatric comorbidity show a reduced response to pharmacological treatments (Hitiris et al; 2007). Since the Dach-SMOX mice model displays a higher susceptibility to epileptic stimuli demonstrated by treatment with different epileptogenic drugs, I used this model to perform a behavioural characterization. In this regard I conducted a comprehensive battery of behavioural tests specific for assessing conditions such as anxiety, depression and emotional abnormalities. The behavioural analyses showed that Tg mice display an anxious phenotype evidenced by the elevated plus maze and light dark box tests, while they have not shown symptoms of depression or memory impairment. The presence of an anxious phenotype and the absence of other psychiatric pathologies in Tg mice confirms literature data which show that among the many mental illnesses, those prevalent in epileptic patients are mood and anxiety disorders (Chapouthier et al., 2001; Jackson and Turkington, 2005). Since 5-HT is one of the most important neurotransmitters that influences mental health and because 5-HT

neurotransmission has been seen to be involved in the increased susceptibility to epileptic seizures, I analysed the serotoninergic system in Dach-SMOX mice. I performed quantitative real time PCR and Western blot analyses to evaluate possible changes occurred at transcript and protein levels of 5-HTRs and 5-HTT. The results show that Tg mice have an imbalance of the 5-HT system compared to controls. In particular, a reduction in both transcripts and protein levels of serotonin receptors 1A, 1B, 2A, 2C has been found as well as a reduction in 5-HTT. In literature it is known that receptors 1A, 2A and 2C are involved in epilepsy, and that their partial or total reduction (in knock-out genetic systems) can lead to the development of spontaneous epileptic seizures (Goodman et al., 2011; Gharedaghi et al., 2013). Furthermore, a dysregulation of 5-HTT, 5-HT1A, 5-HT1B is common in mouse model of anxiety (Raote et al., 2007; Nautiyal et al. 2016). This alteration could explain the anxious phenotype and the increased susceptibility to excitotoxic stimuli shown in Dach-SMOX mice.

With the aim to understand how an over-expression of SMOX could lead to changes in serotonergic system I analysed the transcript levels of BDNF and of its receptor Trkb. The neurotrophin BDNF promotes the development and function of 5-HT neurons, it could be affected during chronic oxidative stress and its alteration can cause changes in 5-HT system. I analysed the transcript levels of BDNF and Trkb in Tg mice, which are characterized by an overproduction of hydrogen peroxide caused by the overexpression of SMOX. No differences were found in transcript levels of BDNF and Trkb in Tg mice compared to Sg mice.

I therefore focused my investigations on the pathways involved in tryptophan metabolism. Tryptophan, in fact, besides being a substrate for the synthesis of 5-HT, is the substrate for the synthesis of KYN. When the KYN pathway is activated, it competes with the 5-HT pathway, in fact KYN pathway depletes TRP and debases the 5-HT pathway. Many studies proposed that 5-HT deficiency in mood disorders was caused by the TRP shunt towards formation of KYN (kynurenine shunt). I analysed the enzyme that catalysed the first limiting step of KYN pathway, IDO1, and I found that Tg mice display a significant increase of its mRNA.

Recent publication (Mondanelli et al., 2017) has shown that high levels of Spd promote dephosphorylation of Src kinase in tyrosine 530. This dephosphorylation activates the Src kinase. Once activated, Src kinase is able to phosphorylate IDO1 that start and catalyses the first step of the KIN pathway. I decided to analyse the phosphorylation status of tyrosine 530 of Src kinase in Dach-SMOX mice that have, as previously demonstrated, high levels of Spd. The western blot analysis shows that Tg mice have a decreased

in phosphorylated form of Src kinase and therefore a higher activation of this kinase.
Taking together all the results described so far, I can hypothesise the pathway depicted in Figure 23 and described below.

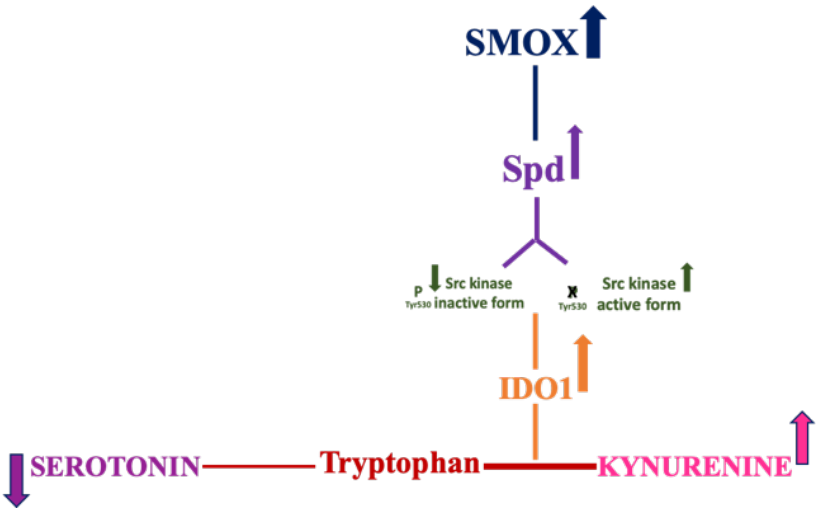


**Figure 23. Scheme of the molecular pathway describing the role of SMOX in the comorbidity between epilepsy and mental illness.**

The overexpression of SMOX in cerebral cortex of Tg mice leads to the production of high levels of Spd. The imbalance in Spd content promotes the dephosphorylation of Src kinase in tyrosine 530 and its consequent activation. This causes the activation of IDO1 which can catalyse the conversion of tryptophan to KYN. The shift of tryptophan metabolism to the KYN pathway may be the cause of the changes observed in the 5-HT system. The imbalance of 5-HT system could be due by the tryptophan depletion available for 5-HT synthesis.

In conclusion, this project has investigated how the PAs and 5-HT systems are related to each other. Experimental evidences obtained during the characterization of Dach-SMOX mice can demonstrate that this genetic system is a promising model to study the comorbidity between epilepsy and mental illnesses. For a long time, the link between epilepsy and mental disorders has been attributed exclusively to the 5-HT metabolism. The monoaminergic system fails to explain all the events or all the linked pathways that lead to the development of mental illnesses, on the other hand it is not possible to state epilepsy is caused only by changes in monoamines. The linking between PAs and 5-HT pathways proposed in this research suggests that PAs may be considered as a new research target for the treatment of these neurological disorders.

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