“STUDY OF THE ROLE OF SPERMINE OXIDASE IN EPILEPSY”

“STUDIO DEL RUOLO DELLA SPERMINA OSSIDASI 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 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 risulta alterato in diverse malattie neurodegenerative, comprese Alzheimer, Huntington, Parkinson, sclerosi laterale amiotrofica ed anche a seguito di trauma ed ischemia cerebrale. Le principali poliammine presenti nei mammiferi sono putrescina, spermidina e spermina. È noto che la spermina, e in alcuni casi la spermidina, possono 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, risultando nell'apertura dei recettori ionotropici del glutammato, causando una prolungata depolarizzazione dei neuroni, la conseguente entrata di calcio e l'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. 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 modificare la regolazione sinaptica. 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, piuttosto che su quello eccitatorio. 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 che patologiche. L'induzione del fenotipo epilettico in topi transgenici e di controllo, è

stata ottenuta tramite somministrazione del farmaco epilettico pentilentetrazolo. La valutazione del comportamento è stato il primo passo di una analisi preliminare per stabilire l'effetto del farmaco epilettico 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 studiare alterazioni della via eccitotossica. 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 modificazioni morfologiche della corteccia cerebrale della linea murina, ho effettuato analisi di immunoistochimica utilizzando anticorpi contro il marcatore neuronale NeuN, il marcatore astrogliale GFAP e il marcatore microgliale Iba-1, in condizioni sia fisiologiche che dopo iniezione con l'epilettico. Usando NeuN, non sono state trovate differenze significative per quanto riguarda il numero dei neuroni tra topi transgenici e singenici, trattati con soluzione fisiologica o pentilentetrazolo. Tuttavia, dopo trattamento con pentilentetrazolo, i neuroni sono apparsi di dimensioni ridotte rispetto ai neuroni trattati con la soluzione fisiologica. Riguardo l'analisi con GFAP, è stato osservato uno scenario simile, dato che non si è avuto un aumento di astrogliosi dopo trattamento con pentilentetrazolo. Al contrario, Iba-1, è stato trovato altamente espresso nella corteccia dei topi transgenici rispetto ai singenici, trattati con soluzione fisiologica, mentre dopo pentilentetrazolo non si sono osservate differenze. Alti livelli di stress ossidativo possono portare a danni critici per la cellula. Per verificare se il topo Dach-SMOX presentasse un incremento del danno ossidativo, ho eseguito un'analisi immunoistochimica, in condizioni sia fisiologiche che dopo danno eccitotossico, misurando i livelli di 8-Oxo-2'-deossiguanosina, un marker biologico di stress ossidativo. Quest'analisi ha confermato che i topi transgenici presentano un aumento dello stress ossidativo in condizioni fisiologiche, e un aumento ancora più evidente dopo trattamento con il farmaco epilettico, rispetto ai topi di controllo. Alcuni studi hanno dimostrato che il sistema xc^- è un trasportatore coinvolto nella risposta antiossidante astrocitaria. In situazioni patologiche, come il glioma, questo recettore è sovra-espresso e in grado di causare eccitotossicità. L'espressione del sistema xc^- è direttamente regolata dal fattore di

trascrizione Nrf2, dimostrato essere più attivo nella linea transgenica. Al fine di verificare se XCT, la subunità catalitica del trasportatore, fosse più espressa nei topi transgenici, ho eseguito un saggio di immunoistochimica. L'espressione di XCT è stata riscontrata aumentata nei topi transgenici rispetto ad i topi singenici, ed ulteriormente incrementata in condizioni eccitotossiche. Dato che l'efflusso di glutammato è normalmente compensato dall'attività dei trasportatori del glutammato astrocitari EAAT-1 e EAAT-2, ho eseguito una analisi dei loro livelli proteici tramite saggio western blot su cortecce cerebrali. I risultati non hanno mostrato differenze significative dei livelli di proteina dei trasportatori, sia in condizioni fisiologiche sia dopo trattamento con 40 mg/kg di pentilentetrazolo. Il persistente stress ossidativo nello spazio sinaptico è in grado di attivare diverse vie cellulari coinvolte nella risposta antiossidante, e per questo, ho analizzato tramite western blot, la protein chinasi c alpha, che può modulare i meccanismi di difesa antiossidanti, funzionando in modi diversi a seconda se opera a livello della trasmissione fisiologica o patologica. La chinasi è risultata aumentata nei topi transgenici trattati con soluzione fisiologica, mentre dopo trattamento con pentiletetrazolo, è stata osservata una riduzione generale del livello della proteina. Infine, per valutare se l'inibizione del sistema xc^- potesse rappresentare una strategia efficace contro l'aumentata sensibilità allo stress eccitotossico dei topi transgenici, ho eseguito una analisi comportamentale su topi trattati con pentilentetrazolo valutando l'attività antiepilettica della sulfasalazina. Il pre-trattamento con sulfasalazina, è stato efficacie nel revertire completamente la aumentata sensibilità all'eccitotossicità del modello Dach-SMOX. Sorprendentemente, nei topi singenici, il pre-trattamento ne ha peggiorato la sensibilità. Ho poi effettuato analisi sulle cortecce, per valutare cambiamenti dei livelli proteici degli EAAT dopo un aumento della concentrazione di pentilentetrazolo (60 mg/kg) e dopo trattamento con sulfasalazina, EAAT-2 è stato trovato significativamente aumentato nei topi transgenici, rispetto ai singenici, trattati con 60 mg/kg di pentilentetrazolo, mentre nei topi singenici e transgenici pre-trattati, non si sono osservate differenze. Una riduzione significativa di EAAT-2 si è vista nei topi transgenici pre-trattati, rispetto ai transgenici trattati con il solo pentilentetrazolo. Analizzare diversi passaggi nel pathway delle poliammine, tra cui biosintesi, degradazione e trasporto, può rappresentare una strategia innovativa per un intervento terapeutico su patologie con trattamenti difficili o con gravi effetti collaterali. In parallelo, quindi, ho espresso la spermina ossidasi in *E.coli*, ho purificato la proteina e iniziato una

caratterizzazione biochimica dell'enzima, con lo scopo di identificare inibitori della spermina ossidasi, da testare sul modello Dach-SMOX, che potrebbero essere usati come farmaci antiepilettici per prevenire o curare l'epilessia.

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 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, and even after cerebral trauma and ischemia. 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. In this regard, my aim was to understand if an alteration in polyamines levels, by specifically overexpressing a catabolic enzyme, could alter synaptic regulation. Spermine oxidase is an enzyme of the polyamine catabolic pathway. It catalyzes the oxidation of spermine to produce spermidine, 3-aminopropanal and hydrogen peroxide. The research group where I carried out my work for the PhD thesis, for years investigated 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. To understand the role of spermine oxidase in excitotoxicity, I focused my attention on the inhibitory system, instead of the excitatory one. I bred and used the transgenic mouse line Dach-SMOX that overexpresses spermine oxidase specifically in the neocortex. This mouse was analyzed both in physiological and in pathological conditions. Induction of the epileptic phenotype on transgenic and control mice was performed by administration of the epileptic drug pentylentetrazole.

Behavioral 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 analyzed mouse brain cortex to study the alterations of the excitatory pathway. To analyze possible changes in polyamines content following vehicle solution or pentylentetrazole injection, 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. To investigate if Dach-SMOX mouse line presented, at a morphological level, changes due to the higher excitability, I performed immunohistochemical analyses using antibodies against the nuclear marker NeuN, the astrogliosis marker GFAP and the microglial marker Iba1 on frozen sections of the neocortex from transgenic and syngenic mice treated with vehicle solution or pentylentetrazole. Using NeuN antibody, no significant differences were found in the number of neurons between transgenic and syngenic mice treated with either vehicle solution or pentylentetrazole. However, after pentylentetrazole treatment, the neurons appeared smaller if compared to the neurons of the neocortex of animals treated with vehicle solution. Regarding the GFAP analysis, a similar scenario was observed since there was no significant increase of astrogliosis after the pentylentetrazole treatment in all samples analyzed. On the contrary, Iba1 was highly expressed in the neocortex of vehicle-injected transgenic mice compared to syngenic animals, while after pentylentetrazole treatment, no differences were observed. To investigate if Dach-SMOX mouse line presented an increased oxidative damage, I performed an immunohistochemical analysis, both in physiological conditions and after excitotoxic injury, evaluating the amount of 8-Oxo-2'-deoxyguanosine, a biomarker for oxidative stress. A significant increase of labelled cells was visible in transgenic mice treated with the vehicle solution compared to syngenic mice and an even greater increase was evident after the treatment. Several studies showed that system xc^- transporter is involved in oxidative stress response in astrocytes. In pathological conditions, such as glioma, this receptor is overexpressed and can cause excitotoxicity. System xc^- transporter expression is regulated by the transcription factor Nrf2, which was proved to be more active in transgenic mice. Therefore, to verify if XCT, the catalytic subunit of the transporter, was more expressed in Dach-SMOX mouse

line, I performed an immunohistochemical analysis. XCT expression was significantly increased in transgenic mice treated with the vehicle solution compared to syngenic mice and a greater increase after pentylentetrazole treatment was evident. Since glutamate efflux is normally compensated by the activity of the astrocytic glutamate transporters EAAT-1 and EAAT-2, to understand if the expression of these transporters was altered in transgenic mice, I performed a western blot analysis on the extracts of brain cortex. Results showed no significant differences in the EAAT-1 and EAAT-2 protein expression levels between Sg and Tg mice, treated with 40 mg/kg of pentylentetrazole or with the vehicle solution. Since persistent oxidative stress in the synaptic cleft can activate several cellular pathways involved in the oxidative stress response, I analyzed the protein kinase c alpha, that might modulate the antioxidant defense mechanism, functioning in a different way according to whether it operates in physiological transmission or in pathology. Protein kinase c was found increased in transgenic mice treated with the vehicle solution, while after pentylentetrazole injection, there was a general reduction of the protein level. To understand if the inhibition of system xc^- activity could represent an efficient strategy to overcome the increased susceptibility to excitotoxicity of the transgenic mouse line, I performed a behavioral analysis on mice treated with pentylentetrazole evaluating the possible anti-epileptic activity of sulfasalazine. Pre-treatment with sulfasalazine completely reverted the increased sensitivity to excitotoxicity of Dach-SMOX mouse line. Surprisingly, pre-treatment of singenic mice, worsened their sensitivity. Analyses were then performed on the harvested brain cortex to evaluate changes in EAAT protein levels, after using a higher concentration of pentylentetrazole and after treatment with sulfasalazine. EAAT-2 was significantly increased in transgenic mice treated with 60 mg/kg of pentylentetrazole in respect to singenic mice, but unchanged between singenic and transgenic mice pre-treated. A significant reduction was observed in pre-treated transgenic mice in respect to the ones treated with pentylentetrazole only. Targeting different steps in polyamine pathway, including biosynthesis, degradation and transport, can represent an innovative strategy for therapeutic intervention in pathologies with difficult or severe side effect treatments. So in parallel, I expressed spermine oxidase in *E.coli*, purified the protein and started the biochemical characterization of the enzyme with the purpose of identifying spermine oxidase inhibitors, to test on the Dach-SMOX mouse model, that could be used as antiepileptic drugs to prevent or cure epilepsy.

INTRODUCTION

1. POLYAMINES

Polyamines (PAs) are low molecular weight aliphatic polycations, positively charged and ubiquitously present in all living cells. Endogenous PAs are essential growth factors for cells and play key roles in transcription, proliferation, regeneration and differentiation (Rea et al., 2004; Cervelli et al., 2009; Amendola et al., 2009; Cervelli et al., 2014a). Figure 1 shows the main natural PAs involved in cellular regulation: Putrescine (Put), Spermidine (Spd) and Spermine (Spm) (Figure 1).

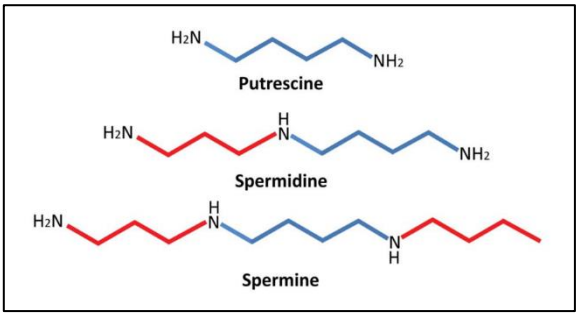

Figure 1. Structures of the main polyamines found in the organism. Putrescine (Put), Spermidine (Spd), Spermine (Spm).

Total intracellular concentration of PAs is in the millimolar range, while free PAs concentration is much lower due to their binding to DNA, RNA, ribosomes, proteins and phospholipids. The most significant source of PAs is the biosynthetic pathway, but a significant contribution is also represented by food uptake (Pegg 2009).
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

Polyamines play key roles in several cellular processes; therefore, their homeostasis is controlled by a complex regulatory system that includes

biosynthesis, degradation and transport. Polyamines concentration can change according to the cell status, but it must be preserved within a certain level to maintain normal cell function (Wallace et al., 2003).

Polyamines naturally synthesized in mammalian cells are Put, Spd and Spm (Fig. 2). 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 PAs biosynthesis. Ornithine decarboxylase expression is tightly regulated at several levels, from transcription to post-translational modifications (Pegg, 2006). The synthesis of Spd requires two different enzymes: S-adenosylmethionine (SAM) decarboxylase (SAMDC), which provides an aminopropyl group that is then transferred to Put by Spd synthase to produce Spm. This represents the second rate-limiting step for PAs synthesis; SAMDC levels are kept very low to control the amount of Spd production. The same mechanism is responsible for the production of Spm, starting from its precursor Spd, by the activity of Spm synthase (Wallace et al., 2003). An alternative pathway for PAs biosynthesis is through agmantine, which is processed by agmantinase to produce urea and Put. However, this alternative pathway does not seem to be crucial in Put synthesis (Coleman et al., 2004). The activity of the 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 resolved by the activity of another regulatory protein called antizyme inhibitor (AZIN); this protein can also increase the uptake of polyamines from extracellular space (Pegg, 2006).
Polyamines anabolism is reversible. There are three classes of important enzymes that are responsible for the back-conversion pathway of PAs. The first step of the catabolic pathway is the acetylation of Spm (or Spd) at N1 position, due to the activity of the Spm/Spd N1-acetyltransferase (SSAT) and its acetyl donor acetylCoA. After this step, acetylspd and acetylspm are oxidized by a FAD-dependent perixosomal N1-acetylpolyamine oxidase (PAOX) producing Spd/Put, 3-acetamido propanal and hydrogen peroxide (Seiler, 2004). Another important enzyme which is involved specifically in Spm catabolism is Spermine

Oxidase (SMOX), discovered at the end of 2002 and it is responsible for the direct oxidation of Spm to produce Spd, 3-amino propanal and hydrogen peroxide. SMOX 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 catalyzed by SSAT. While PAOX is a stable enzyme, SSAT has a rapid turnover that is mediated by ubiquitination and 26S proteasome-dependent degradation (Seiler, 2004).

This complicated and redundant system, that controls polyamines levels, is crucial to maintain their levels 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.

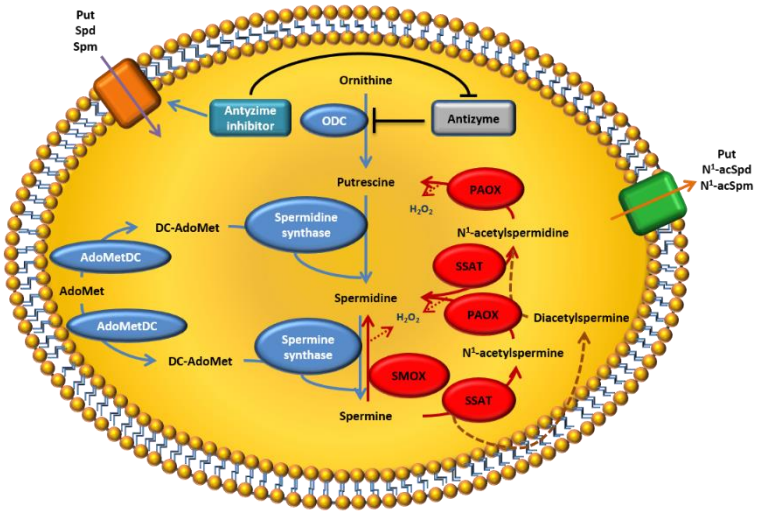

Figure 2. Polyamine metabolism.
This scheme shows the main enzymes involved in PA anabolism (blue) and catabolism (red) in mammalian cells. Modified from Cervelli et al., 2014a.

1.2 Polyamines Transport

Polyamines synthesis and degradation are the main processes that cells use to maintain a suitable level of PAs. However, cells also have an active transport system that is responsible for PAs uptake (Mitchell et al., 2007). The secondary source of PAs, as mentioned above, is through the diet. Furthermore, intestinal bacteria produce and release a significant amount of PAs. These are absorbed at the 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). Antizyme 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 a molecular level. However, several 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). PAs, in astrocyte and neurons, are usually stored in vesicles. Recent observations indicated that the SLC18B1 protein functions as a vesicular polyamine transporter (VPAT) responsible for vesicular storage of Spm and Spd (Hiasa et al., 2014).

1.3 Polyamines and pathologies

The critical role of PAs in cell progression, differentiation and death, brought researcher to focus and investigate their involvement in a series of different pathologies.

Polyamines concentration increases in proliferating cells (Cervelli et al., 2009), and several studies point out their contribution to tumor cell

growth (Casero and Marton, 2007; Gerner and Meyskens, 2004). 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). Using PAs concentration as a prognostic factor, showed a direct correlation between higher PAs content and poorer outcome in tumors (Wallace et al., 2003). The interest of scientists, in 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 cancerous cells but 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), due to the compensatory PAs catabolic pathway and cellular PAs 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 was administrated, underlining 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 polyamines 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 an 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. Overwhelming 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 hydrogen peroxide, which can contribute to oxidative stress and DNA damage, and could represent a link between inflammation and cancer. The biochemical marker of oxidative DNA damage, 8-Oxo-7,8-dihydro-2-deoxyguanosine (8-oxo-dG) has been found augmented in *Helicobacter pylori*-induced gastric tumor (Xu et al., 2004).

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 glutathion in parasite) (Wallace et al., 2003). DFMO is also efficient in the treatment of disease like Chaga's disease, leishmaniasis 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 (Cervelli et al., 2009; Cervelli et al., 2016; Rea et al., 2004; Tavladoraki et al., 2011). In the last decade, 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 et al., 2009; Zahedi et al., 2010; Cervelli et al., 2012). Even though many results suggested an involvement of PAs metabolism in neurodegeneration, the mechanisms by which this takes part in neuronal death still needs to be completely clarified, as well as its role in normal brain function (Cervelli et al., 2014b; Capone et al., 2013; 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, 1991; 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 can block some types of K+ and Na+ channels and the glutamatergic AMPA (a-amino-3-hydroxy-5-

methyl-4-isoxazolepropionicacid) and kainate (KA) receptors; while extracellular PAs modulate glutamatergic NMDA (N-methyl-D-aspartate) receptors (Williams, 1997; Igarashi et al., 2000; 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). 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 linked 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 NMDA 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 epileptic 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 (Paschen et al., 1991; Morrison and Kish, 1995; Seiler et al., 2000; Rothman et al., 2003; Velloso et al., 2009).

2. SPERMINE OXIDASE

Polyamines catabolism, as previously stated, is finely regulated by the combined action of the enzymes, spermidine/spermine-N1-acetyltransferase (SSAT), acetylpolyamine oxidase (PAOX) and the flavoprotein spermine oxidase (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 oxidizes Spm to produce spermidine (Spd), 3-aminopropanal (3-AP) and hydrogenperoxide (H_2O_2) (Cervelli et al., 2012; Cervelli et al., 2013a) (Fig. 3).

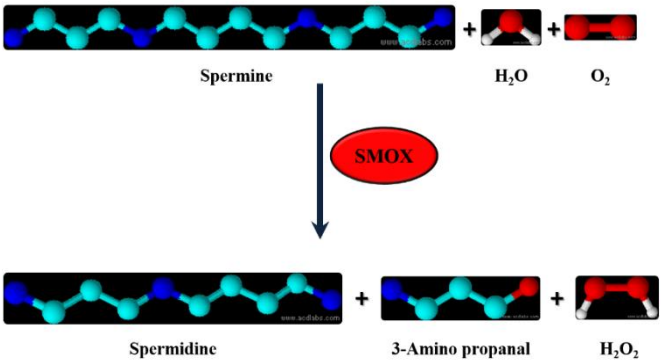

Figure 3. Spermine oxidase catalytic activity.
Spermine oxidase oxidases spermine to produce spermidine, 3-aminopropanal and hydrogen peroxide.

From a biochemical point of view, no crystal structure is available for any mammalian SMOX, and the structural bases for different substrate specificities of the polyamine-oxidizing flavoproteins are not completely understood (Cervelli et al. 2014c). Tertiary structures of the maize polyamine oxidase (PAO) (Binda et al. 1999) and of Fms1 (Huang et al. 2005) are available, while no structure is available for SMOX. The sequences of the mammalian SMOXs and acetylspermine oxidase

(PAOX) display about 20% sequence identity to those of maize PAO and yeast Fms1; despite this the available crystal structures (Binda et al. 1999; Huang et al. 2005) and structural models (Tavladoraki et al. 2011) suggest that they share the same fold of monoamine oxidases (Binda et al. 2002). Fms1 represents the best model to understand the structural basis for the catalytic mechanism and specificity of SMOX (Tavladoraki et al. 2011; Cervelli et al. 2014c). Fms1 is a dimer in the native form and in substrate-enzyme complex crystals, as well as in solution (Huang et al. 2005). The relative position of the two subunits in the dimeric form is the same observed in the native and substrate soaked crystals (Huang et al. 2005). Noteworthy, maize PAO is a monomer in solution, while it is a trimer in the crystal (Binda et al. 1999). Recently, a novel PAOX crystal structure, was obtained by Sjögren et al. (2017), providing a basis for further studies. Therefore, an intriguing open question for experimental investigation is whether SMOX could also have a multimeric nature, and if this is the case, what would be its structure.

From a physiological point of view, in the past, SMOX activity has been linked to several pathologies such as 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 protein 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). SMOX 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 model 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 (Kplempam et al., 2009).

2.1 Spermine oxidase overexpression in neocortex

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 a 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 b-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 (Fig. 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).

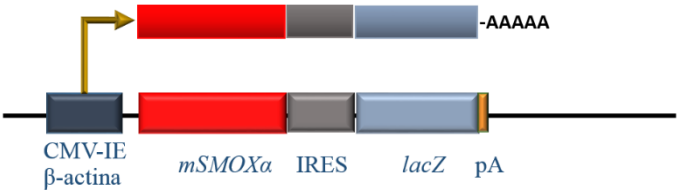

Figure 4. Dach-SMOX mouse line construct.
SMOX overexpression (Cervelli et al., 2013b)

3. EPILEPSY

Epilepsy, a chronic neurological disorder characterized by spontaneous recurrent seizures (SRS), is the fourth most common neurological disorder (Hirtz et al., 2007). 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. Current treatment options include antiepileptic drugs (AEDs), ketogenic diet, neurosurgical resection, and electrical stimulation of the central nervous system (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. SMOX AND EPILEPSY: What is known to date

4.1 Kainic Acid, model for epilepsy

The molecule (2S,3S,4S)-Carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid, also known as kainic acid (KA), is a compound isolated from a red alga named *Digenea*, which is found in tropical and subtropical areas (Coyle, 1987) (Fig. 5).

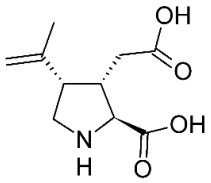

Figure 5. Kainic Acid structure.

Kainic Acid is an analogue of glutamate (Glu) but is far more powerful and able to trigger strong excitotoxic insults (Wang et al., 2005). Its neuroexcitatory activity is related to its capability to mimic Glu action in the synaptic cleft, binding to the major ionotropic Glu receptors (Coyle,

1987). The massive activation of AMPA and KA receptors triggers several pathways that can lead to neurodegeneration. This mechanism is called excitotoxicity and it implies that an excess of stimuli can take to neurotoxicity and apoptosis. An overstimulation of Glu receptors and the change of membrane potential will result in massive depolarization and the entry of a large amount of calcium into the cell. This is the first step of a cascade that affects the whole cell; the cellular stress caused by increasing of ROS and other cellular events can lead to neuronal cell death (Wang et al., 2005). Kainic Acid has been widely used to study the mechanism of excitotoxicity and to study the pathways related to neuronal degeneration in epileptic seizure condition. Kainic acid triggers seizures by its agonist activity to AMPA and KA receptors, inducing the entry of Na^+ and Ca^{2+}. Specifically, the depolarization caused by influx of Na^+ causes the opening of NMDA channels and consequent influx of calcium ions. The Ca^{2+} overload in the cell can activate several calcium-dependent enzymes such as proteases, phospholipases and endonucleases. The increased concentration of Ca^{2+} compromises also the energy metabolism in the mitochondria with consequent ROS formation and loss of electrochemical gradient across the membrane (Sperk et al., 1983). To assess the intensity of seizures, consequent to epileptic drugs administration, the behavioral scale created by Racine (1972) was always used (Racine, 1972). This scale measures the extent of the seizures dividing them in 6 stages, which go from immobility to severe tonic-clonic seizures (Fig. 6).

	Revised Racine behavioral scale
1	Immobility
2	Forelimb and/or tail extension, rigid posture
3	Repetitive movements, head bobbing
4	Rearing and falling
5	Continous rearing and falling
6	Severe tonic-clonic seizures

Figure 6. Racine behavioral Scale.
Seizures scoring according to the Racine behavioral Scale (Racine, 1972).

Previous experimental results from the laboratory of Molecular Biology, where I carried out my thesis work, (Cervelli et al., 2013b; Cervetto et al., 2016; Pietropaoli et al., *in peer review*) pointed out that SMOX overexpressing mice show a slight activation of astrocyte and microglia of the cerebral cortex (Fig. 7).

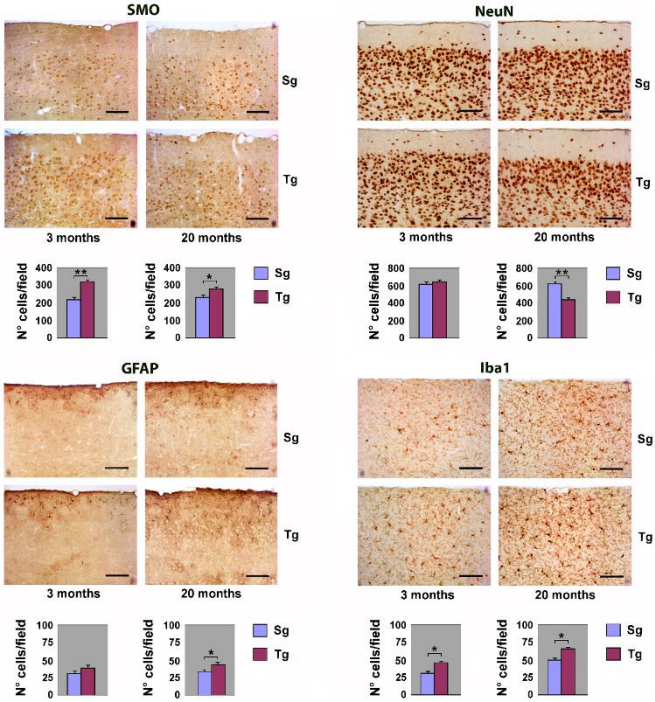

Figure 7. Immunohystochemical analysis of neocortex from Tg and Sg mice. Sagittal brain slices from JoSMOrec mice were stained with antibodies directed against SMO, NeuN, GFAP and Iba1. (*, p,0.05; **, p,0.01). Sg, syngenic mice; Tg, transgenic mice (Cervelli et al., 2013b).

After KA administration, Dach-SMOX mice presented a marked increased vulnerability to epileptic seizures (Fig. 8), compared to the syngenic (Sg) control mice (Cervelli et al., 2013b).

Mouse line	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
Tg %	5.38	3.85	23.08	15.38	7.69	34.62
Sg %	33.33	7.41	22.22	14.81	3.70	18.52

Figure 8. Behavioral evaluation of transgenic and syngenic mice treated with KA. Scored mice are expressed in percentag. Tg, transgenic; Sg, syngenic. (Cervelli et al., 2013b).

Moreover, they displayed a marked neuronal reduction and a reactive astrogliosis with upregulated microglia. (Fig. 9).

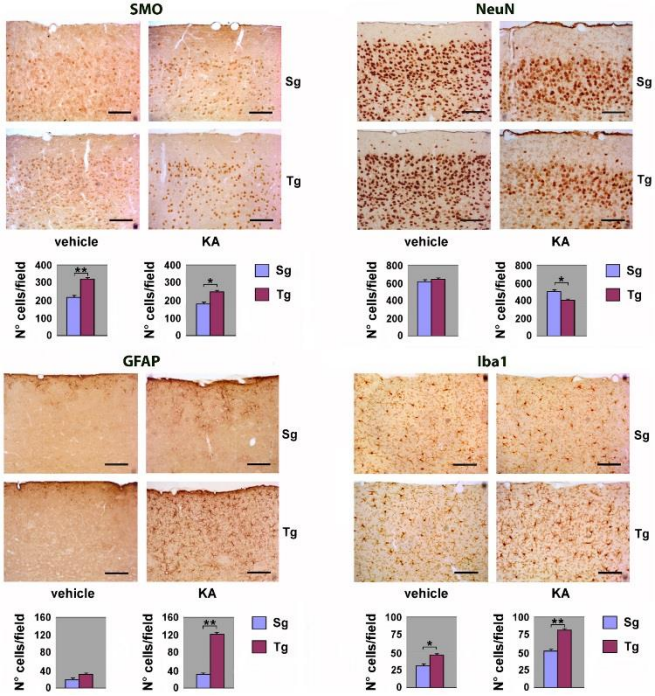

Figure 9. Immunohystochemical analysis of neocortex from Tg and Sg mice stained with antibodies directed against SMO, NeuN, GFAP and Iba1. Slices of neocortex from KA treated 3 months old mice were analyzed. Cell counting is expressed as number of positive cells per 0.24 mm2 area. (*, p,0.05; **, p,0.01). Sg, syngenic mice; Tg, transgenic mice (Cervelli et al., 2013b).

Spermine oxidation products, H_2O_2 and 3-AP, synergistically increase ROS leading to neuronal degeneration (Cervelli et al., 2013b). The latest study on Dach-SMOX mice evidenced an increased efflux of Glu from astrocytes, and increased activity of the oxidative defense enzymes, like superoxide dismutase and catalase (Cervetto et al., 2016) (Fig. 10).

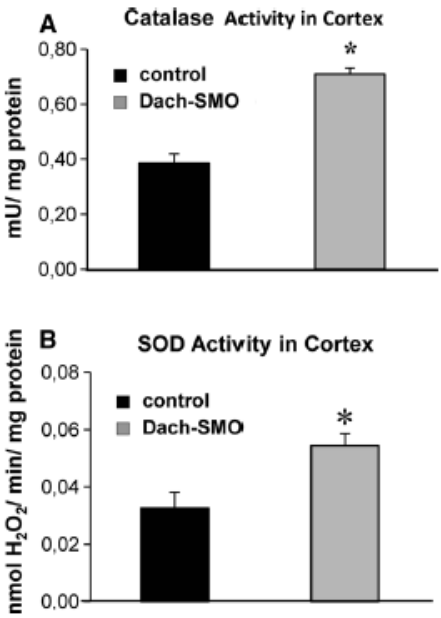

Figure 10. Antioxidant enzyme activities in Dach-SMOX and control mice. Specific activities of catalase (a) (mmoles of decomposed H2O2 per min per mg protein) and SOD (b) (mU of enzyme per mg protein) were quantified by spectrophotometric assays in cortex lysates from both control and Dach-SMO mice. (*, p, 0.05) (Cervetto et al., 2016).

5. THE GLUTAMATERGIC SYNAPSE

Glutamate is the major excitatory neurotransmitter in the nervous central system and Glu receptors are localized throughout the brain and spinal cord in both neurons and glia.

Glutamate concentrations in the extracellular space are kept low and are tightly controlled by many mechanisms at the synaptic level (Okubo, 2010). Alterations to this regulatory system can have harmful effects, such as excess of Glu in the synaptic cleft, which can induce hyperexcitability in post-synaptic neurons and, if persistent, it could lead to excitotoxicity and cell death (Choi, 1994; Doble, 1999). Glutamate is released by presynaptic glutamatergic neurons, as consequence of neuron terminal depolarization, through a vesicular mechanism (Okubo, 2010). Once in the synaptic cleft, Glu can bind to several receptors on the post-synaptic neuron membrane (Siegel et al., 1994).

5.1 Glutamate receptors

Glutamate receptors are various and highly complex; more than 20 Glu receptors have been identified in the mammalian central nervous system. They can be divided in two classes: ionotropic and metabotropic. Ionotropic receptors are fast acting and, once opened, they can produce substantial changes in ion current even if the voltage difference across the membrane is small (Kew and Kemp, 2005). After Glu binds to an ionotropic receptor, the receptor's channel undergoes a change in his shape that allows an immediate influx of extracellular sodium or calcium and an efflux of potassium ions. This triggers membrane depolarization in the post-synaptic cell that is sufficient to induce signal transmission (Pleasure, 2008).

5.2 Polyamines regulation of glutamate receptors

Polyamines, especially Spm, can act as modulators of several type of ion channels. In some cases, the modulation is achieved by a direct binding to the ion channel, in other cases specific binding sites for PAs can modulate receptor activation in a more complex way. Intracellular PAs can directly modulate some AMPARs, specifically the class GLUR2-lacking and the unedited GLUR2, which comprises all the AMPARs permeable to Ca^{2+}. Polyamines, by directly plugging the channel pore, can prevent ion channel opening, after activation by Glu and membrane

depolarization (Williams, 1997). The main PA responsible for the blocking of AMPA ion channel opening is Spm. Its binding site is inside the pore region and it appears to be voltage dependent. Spermine binds to AMPA receptors when the membrane potential is depolarized, blocking the entrance of more Ca^{2+} ions and therefore a further depolarization. Spermine detaches from the channel pore and returns to the cytoplasm when the cell is hyperpolarized. At extreme depolarized membrane potential (more than + 50 mV) Spm can even permeate the ion channel passing into the extracellular space; this mechanism decreases the positive charges inside the cell (Spm has three positive charges at physiological pH) (Williams, 1997). Like for AMPA receptors, also KA receptors undergo pre-mRNA editing and can be modulated by PAs. Spermine can directly plug the channel pore and block the entrance of calcium into the cell, at depolarized membrane potentials. This modulation of AMPA and KA receptors by Spm allows the control of the excitability threshold of glutamatergic synapses containing Ca^{2+} permeable Glu receptors (Guerra et al., 2016) (Fig. 11).

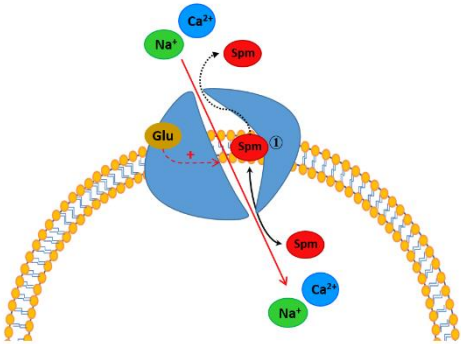

Figure 11. Polyamine regulation of AMPA and KA receptor.
Modified from Williams, 1997

The regulation of the NMDA receptor activity is more complex. They can respond to several modulators at the same time and, according to the circumstances and membrane potential, their activity can be modified. The first effect of extracellular PAs (Spm and Spd) on NMDA receptors

was discovered by Ransom and Stec (1988). They found that this binding caused an increase in channel currents (Ransom and Stec, 1988). Later it was discovered that PAs, specifically Spm modulation of NMDA receptors, could change according to the alternative splicing of the NR1 subunit, the NR2 subunits present in the receptor and the other allosteric modulators that binds the receptor (Guerra et al., 2016). These modulations can be sorted into 4 different macroscopic effects. Spm can potentiate NMDA currents when glycine is present in saturating concentration. This mechanism implies an increase in channel open frequency and a decrease of the receptor desensitization. It is called glycine-indipendent stimulation (Williams, 1997). Another similar effect is called glycine-dependent stimulation, where Spm binding increases the affinity of NMDAR for glycine. The third effect is inhibitory. As for AMPA and KA receptors, Spm can plug the channel pore and block the ion flux. This mechanism is highly voltage dependent: the binding of Spm into the pore is stronger at hyperpolarized membrane potential than at depolarized one (Williams, 1997). The block of NMDA receptor by extracellular Spm is weaker than the block of AMPA receptor by intracellular Spm, but also in this case it can permeate the channel pore. The fourth effect of Spm binding is the decrease in the affinity of the NMDA receptor for the binding of the agonist Glu. This mechanism reduces the time of the Glu stimulation of the receptor, modulating this way the time course of NMDAR response (Guerra et al., 2016) (Fig. 12).

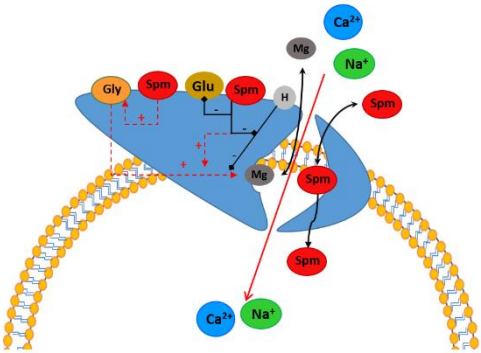

Figure 12. Polyamine regulation of NMDA receptor.
Modified from Williams, 1997

5.3 Glutamate transporters

5.3.1 Excitatory amino acid transporter

After postsynaptic depolarization, it is crucial to restore low levels of Glu in the synaptic cleft. Glutamate transporters are situated on pre-synaptic and post-synaptic neurons, but also on astrocytes, which are the main cells responsible for Glu re-caption. Under physiological conditions, Glu is normally recycled constantly by the interaction between neurons and glia, in the glutamate–glutamine cycle. Glutamate in the synapse is quickly internalized by glial cells via the excitatory aminoacid transporters (EAATs), where it is converted to glutamine by the astrocytic enzyme glutamine synthase. Glutamine is then secreted through specific transporters and then taken up into neurons, where it is hydrolyzed to Glu and ammonia by the enzyme glutaminase (Rothman et al., 2003). Glial cells, under certain pathological conditions, may also release Glu. This kind of reverse transport may be involved in brain damage and stroke (Malarkey and Parpura, 2008). Five different isoforms of EAA transporters have so far been identified: EAAT-1 (GLAST), EAAT-2 (GLT1), EAAT-3 (EAAC1), EAAT4 and EAAT-5. The main role of Glu transporters on glial cells is to keep low concentrations of Glu in the synaptic cleft. However, EAATs are also expressed in other cell types, suggesting an elevated level of cooperation (Shigeri et al., 2004). EAAT-2 is the most abundant Glu transporter: it represents about 1% of the total brain membrane protein, with highest concentrations in the hippocampus and cerebral cortex and it operates the 90% of Glu uptake. Astrocytes express mainly EAAT-1 and EAAT-2 isoforms. Altered expression of EAAT-2 can be found in several pathologies, including amyotrophic lateral sclerosis, which present an excess of Glu levels in the cerebral spinal fluid (Rothman et al., 2003).

Glutamate transport is coupled with the co-transport of 3 Na^+ and 1 H^+ followed by the counter-transport of 1 K^+ (Fig. 13). These proteins also function as Cl^- channels. The Cl^- flux is activated by Glu, but is not thermodynamically coupled to the transport (Balkhi et al., 2014). Increasing evidences prove that astrocytes play a key role in the preservation of the synaptic function and in neuroprotection. Several studies demonstrated that an alteration in glial functionality is responsible for neuron degeneration and *vice versa* in pathological disorders (Balkhi et al., 2014; Shigeri et al., 2004).

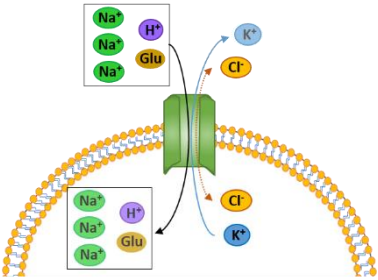

Figure 13. Excitatory aminoacid transporters.
Excitatory aminoacid transporter activity.

5.3.2 System xc⁻ transporter

A significant role of astrocytes is Glu transport, but also, in oxidative stress condition, glutathione production. These two pathways are strictly linked. For glutathione production, a crucial role is played by another important Glu transporter called system xc⁻ transporter. It is involved in the efflux of Glu and influx of cystine, the oxidized dimer form of the amino acid cysteine (Cys-Cys) (Fig. 14). It is an electro-neutral amino acid carrier, so it does not need sodium currents. It is a dimeric protein composed of two different units: a catalytic one (XCT) and a regulator subunit (4F2hc); this second subunit is required for membrane association of the transporter (Balkhi et al., 2014). The expression of this transporter, which in the brain is mostly astroglial, is controlled by the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which is sensitive to oxidative stress. High concentrations of ROS are able to trigger Nrf2 activation and transcription of the Antioxidant Response Element (ARE) genes that are responsible for the antioxidant response. The activity of system xc⁻ transporter is to neutralize ROS. In fact, the internalized cystine is then reduced to cysteine and its concentration is the rate limiting step for the formation of glutathione. In physiological conditions, the efflux of Glu by system xc⁻ transporter is not significant for the total amount of Glu in the synaptic cleft, since the higher affinity for EAAT-2 transporter, which transports it back into the astrocyte. In pathological conditions, such as glioma, deregulation of system xc⁻ transporter activity can result in

excitotoxicity, due to its release of Glu in the synaptic cleft, which contributes to excitotoxic stress.

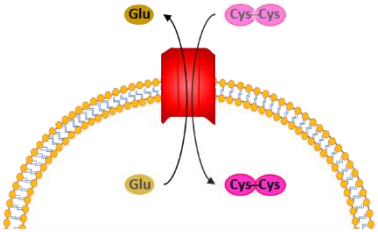

Figure 14. System xc^- transporter.
Activity of system xc^- transporter.

5.4 Glutamate transporters regulation

Excitatory amino acid transporters have several mechanisms of regulation at transcriptional, translational and post translational levels; regulatory molecules can increase or decrease the expression and the activity of the transporters or modify their cellular localization. Different molecules, involved in synaptic signaling and environment, modulate a regulatory mechanism that operates at a translational level of EAAT-2. In particular, both extracellular Glu and H_2O_2 were found to be able to decrease EAAT-2 levels through deregulation of its translation process (Tian et al., 2007). A very important enzyme involved in the regulatory mechanism of EAATs is the Protein Kinase C (PKC). Most of EAATs modification are performed by PKC alpha (PKCα) isoform which is activated by Ca^{2+} and allosteric modulators such as diacylglycerol (DAG) and phorbol esters. Both Ca^{2+} and DAG increase can be the consequence of Phospholipase C (PLC) activity. Several studies proved that PKCα is involved not only in post translational regulations (González-gonzález et al., 2008) but also in transcriptional regulation of some of these transporters. A study proved the involvement of PKCα in the transcription of EAAT-1 demonstrating that this transporter is under Glu-dependent transcriptional control through PKCα (López-Bayghen and Ortega, 2004). Oxidative stress is

often involved in pathological processes of brain disorders and it can trigger several alterations in some protein regulation. In a study by Volterra et al. (1994), the role of H_2O_2 and peroxynitrite in the regulation of Glu transporters activity has been investigated. Results showed that EAAT-1, EAAT-2 and EAAT-3 possess redox-sensitive elements that once oxidized modulate Glu transport in a negative manner (Volterra et al., 1994; Trotti at al., 1998).

The antioxidant defense mechanism mediated by system xc^-, is regulated by PKCα that appears to have a crucial role in this pathway and its deregulation can imbalance the cell redox status (Huang et al., 2000). As mentioned before, the regulation of system xc^- transporter expression is operated by Nrf2. Nrf2 is activated by PKC in response to oxidative stress in the astrocytes and in the surrounding environment. In some pathological conditions, like glioma, deregulation of system xc^- transporter activity can result in excitotoxicity due to Glu release that contributes to excitotoxic stress (de Groot and Sontheimer, 2011). Excessive production of Glu receptors is indeed the initial step of the excitotoxic pathway (Lipton and Rosenberg, 1994) that leads to epilepsy.

5.5 Pentylentetrazole, model for epilepsy

The use of animal seizure 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 pentylenetetrazole (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 analog, 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 the GABAergic

inhibitory system (Naseer et al., 2013). To evaluate seizure intensity behavioral scoring is commonly used in different seizure models. Racine's scale (RS) is frequently chosen as an intensity measurement in experimental seizure 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 a suitable intensity valuation, given the occurrence of behavioral expressions, not mentioned in RS while it lacks other behavioral 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 (Fig. 15):

	Lüttjohann behavioral scale
1	Whisker trembling Sudden behavioral arrest 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 15. Lüttjohann behavioral Scale.
Seizures scoring according to Lüttjohann behavioral Scale (Lüttjohann et al., 2009)

AIM OF THE PROJECT

Several studies have demonstrated altered polyamines levels in many diseases such as cancer, and in brain disorders like epilepsy. Over the past years, many research projects where aimed to understand the role of PAs in normal brain functioning as well as in pathological conditions. Epilepsy is the fourth most common neurological disorder and affects people of all ages. Excitotoxic stress has been associated with epilepsy and it is considered one of the main causes of neuronal degeneration and death. Based on new evidences of the role of spermine oxidase (SMOX) in neurodegeneration, this research analyzes a novel involvement of this enzyme in epilepsy. The transgenic mouse line Dach-SMOX, with CD1 background, specifically overexpressing spermine oxidase 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 epileptic drug pentylentetrazole. There is concern that classical epilepsy animal models may identify only drugs that share characteristics with the existing ones, and are unlikely to influence refractory epilepsies. The aim of this project is therefore to propose the transgenic mouse line Dach-SMOX as a new animal model to study epilepsy and therefore useful to discover new drugs effective on refractory epilepsies.

The main goals of my project are:

- To investigate the cause of the increased sensitivity of Dach-SMOX mice to excitotoxicity
- To understand the involvement of SMOX in excitotoxic stress
- To investigate the molecular mechanism and the proteins involved in epileptic susceptibility
- To evaluate if SMOX enzyme could represent an effective target for prevention of excitotoxicity and neuronal damage, and eventually evaluate if modulation of molecules involved in SMOX downstream cascade can represent an efficient strategy to reduce seizures susceptibility and to prevent or cure epilepsy
- To biochemically characterize SMOX enzyme to better understand its structure to design inhibitors focused on treating epilepsy.

RESULTS

6. PENTYLENTETRAZOLE TREATMENT

6.1 Behavioral evaluation of mice treated with pentylentetrazole

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 the optimum amount of PTZ for injection, 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 this, we analyzed twenty-five Sg mice and sixteen Tg mice injected with a single convulsive dose of 40 mg/Kg, registering the scores that each mouse showed, according to LS. Figure 16a shows the highest symptoms reached: seven Tg mice reached stage 3 as an end point while one displayed stage 4; four Tg mice displayed stage 5 and four Tg mice got to the highest score of 6. No Tg mice showed, as a final stage, the scores 1 and 2 (Fig. 16a). Most of Sg mice reported as a last step, stages 1 and 2 (6 and 10 mice respectively). Three mice exhibited stage 3 and six mice stage 5. The highest score of 6 was not manifested by any of the Sg mice.

The onset latencies for the distinct stages are presented in figure 16b, with on the Y-axis behavioural category and on the X-axis time of onset latency plotted in minutes. For determining whether the observed overall stages can be seen as distinct seizure intensity stages, we examined if the behavioural expressions between Sg and Tg mice, differed regarding their time of first manifestation (onset latency). During the observation period, Tg mice showed the symptoms earlier, in comparison to Sg mice (Fig. 16b). For example, as shown in the graph, Tg mice presented stage 5 after eight minutes from PTZ injection, while one Sg mouse expressed the stage after ten minutes.

A typical PTZ seizure time-course pattern of 90 minutes, of twenty-five Sg mice and sixteen Tg mice, is illustrated in figure 16c, showing the starting points of the different behavioural categories along the time-axis, with time in minutes expressed on the X-axis and clinical symptoms on the Y-axis. It can be appreciated that the diverse behavioural categories present quite dissimilar rate distributions between Tg mice and their controls. Figure 16d illustrates the duration of generalized seizures, considering the same number of Sg and Tg mice, in stages 1 to 3 and in stages 4 to 6 of LS. Among the stages ranging from 1 to 3, Tg mice had

convulsions that lasted 52 seconds while Sg mice had seizures for no more than 3 seconds. The behavioural categories going from 4 to 6, represent the most severe symptoms of PTZ-induced seizures. Transgenic mice, being more sensitive to seizures, undergo convulsions for a longer time (37 seconds) in respect to their controls where seizures last only about 9 seconds (Fig. 16d).

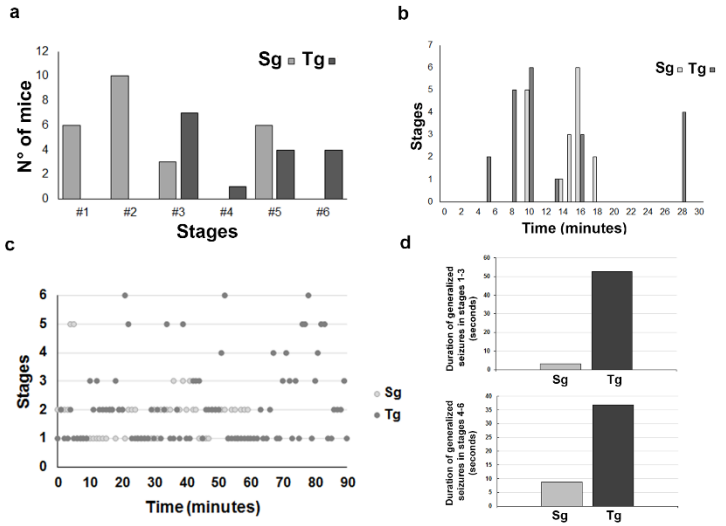

Figure 16. Behavioral evaluation of Tg and Sg mice treated with pentylentetrazole or vehicle solution.
(a) Highest symptoms showed. The histogram represents the highest symptoms reached after PTZ treatment of Tg and Sg mice according to Luttjohann's Scale. The p value was measured with the Chi square test ($p < 0.0005$). (b) Mean first occurrence time. The histogram represents the onset latency of the different behavioral stages displayed by Tg and Sg mice after PTZ treatment, according to Luttjohann's Scale (2009). The p value was measured with the Chi square test ($p < 0.0005$). (c) Seizure time score pattern. Dots in the graph indicate the starting point of all the different behavioral expressions displayed by each Tg and Sg mice after PTZ treatment, according to Luttjohann's Scale (2009). (d) Duration of generalized seizures of stages 1-3 (a) and of stages 4-6 (b) of Sg and Tg mice. The histogram represents Tg and Sg mice expressing different behavioral categories, after PTZ treatment, with different duration of seizures. The p value was measured with the Chi square test (a: $p < 0.03$; b: $p < 0.05$). Sg, syngenic mice; Tg, transgenic mice.

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

To examine possible changes in PAs content following vehicle solution or PTZ treatment, Putrescine (Put), Spd and Spm levels in the neocortex were measured by HPLC (Fig. 17). 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.

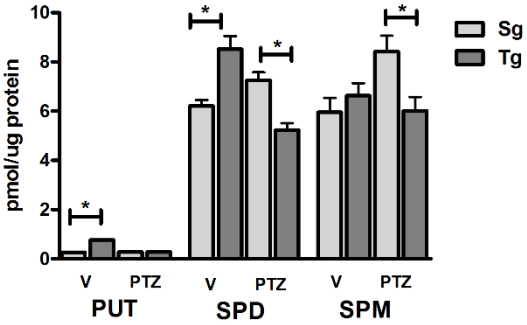

Figure 17. Polyamines content of Tg and Sg mice treated with pentylentetrazole or vehicle solution.
PA content from neocortex of Tg and Sg mice were analyzed after 3 days from PTZ treatment. The p values were measured with the one-way ANOVA test and post-hoc test Bonferroni (*, $p<0.05$). Sg Syngenic mice; Tg, transgenic mice.

6.3 Immunohistochemical analysis of brain cortex

To evaluate, at a morphological level, possible changes due to the higher excitability of Tg mice, immunohistochemical analysis was performed using antibodies against the nuclear marker NeuN, the astrogliosis marker GFAP and the microglial marker Iba1 on serial sagittal frozen sections of the neocortex from Tg and Sg mice treated with a vehicle solution or PTZ and sacrificed 3 days after the treatment. Using NeuN antibody, no differences in the number of neurons between Tg and Sg mice treated with either vehicle solution or PTZ were observed (Fig. 18a). However, after PTZ treatment, the neurons appear smaller if compared to the neurons of

the neocortex of animals treated with vehicle solution. Regarding the GFAP analysis, a similar scenario was observed (Fig. 18b), since there was no significant increase of astrogliosis after the PTZ treatment in all samples analyzed. On the contrary Iba1 is highly expressed in the neocortex of vehicle-injected Tg mice compared to Sg animals, while after PTZ treatment, no differences were observed among Sg and Tg mice (Fig. 18c).

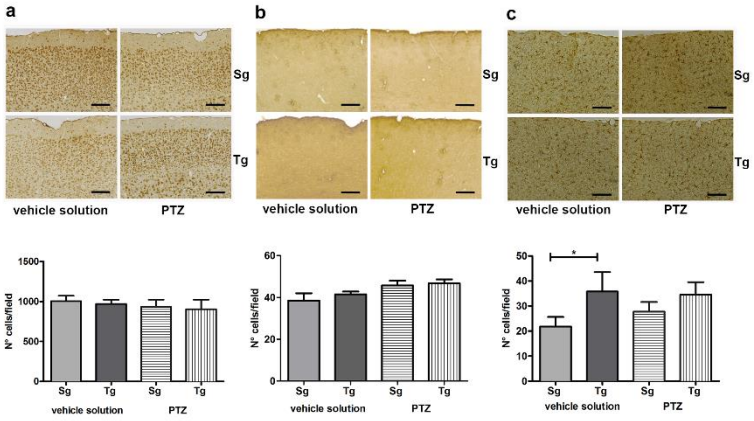

Figure 18. Immunohystochemical analysis of neocortex from vehicle solution and pentylentetrazole treated Tg and Sg mice.
Sagittal brain slices from Dach-SMOX mice were stained with antibodies directed against NeuN (a), GFAP (b) and Iba1 (c). Slices of neocortex from PTZ treated mice were analyzed. Cell counting is expressed as number of positive cells per 0.24 mm2 area. The p values were measured with the one-way ANOVA test and post-hoc test Bonferroni (*, $p<0.05$). Sg, syngenic mice; Tg, transgenic mice.

To measure the oxidative stress in Sg and Tg mice treated with a vehicle solution and PTZ, the immunohistochemical analysis was carried out using antibody against 8-Oxo-dG on animals sacrificed 3 days after the treatment. The 8-oxo-dG is a marker used to determine oxidative stress. A significant increase of labelled cells was visible in Tg mice treated with the vehicle solution compared to Sg mice and an even greater increase was evident after PTZ treatment (Fig. 19a). The catalytic subunit of

system xc^- (XCT), which is a glutamate/cysteine antiporter often found in high concentration in response to oxidative stress for glutathione synthesis (Lewerenz et al. 2013), was also examined by immunohistochemical analysis. A significant increase of XCT was observed in Tg mice treated with the vehicle solution compared to Sg mice and a greater increase after PTZ treatment was evident. This expression pattern matches the 8-oxo-dG response observed (Fig. 19b).

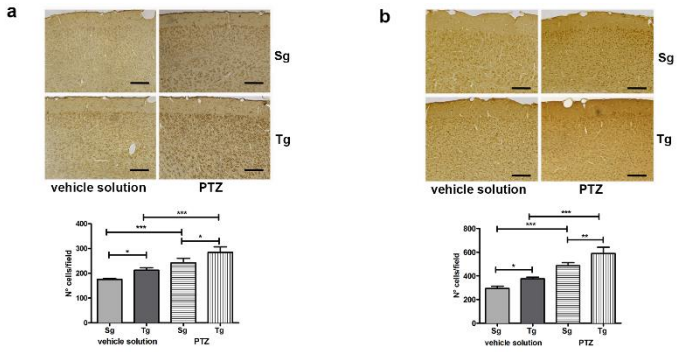

Figure 19. Immunohystochemical analysis of neocortex from vehicle solution and pentylentetrazole treated Tg and Sg mice.
Sagittal brain slices from Dach-SMOX mice were stained with antibodies directed against 8-oxo-dG (a) and XCT (b). Slices of neocortex from PTZ treated mice were analyzed. Cell counting is expressed as number of positive cells per 0.24 mm2 area. The p values were measured with the one-way ANOVA test and post-hoc test Bonferroni (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Sg, syngenic mice; Tg, transgenic mice.

6.4 Western blot analysis after pentylentetrazole administration

To investigate alterations in astrocytic glutamate uptake capability, a molecular analysis of EAAT-1 and EAAT-2 protein expression was performed. Brain cortex from Sg and Tg mice treated with vehicle solution or PTZ were harvested 90 minutes after injection and processed to perform western blot analysis. As shown in figure 20, no significant differences were observed when analyzing EAAT-1 and EAAT-2 protein expression between Sg and Tg mice, with or without treatment.

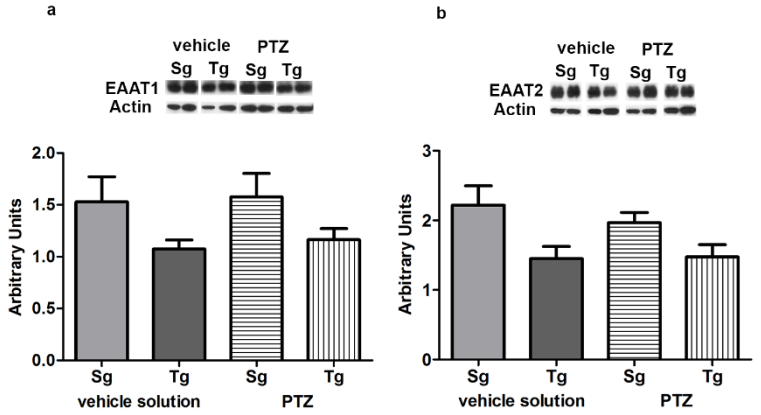

Figure 20. Western blot analysis of EAAT-1 and EAAT-2 of Sg and Tg mice treated with physiological solution or PTZ.
Protein levels from neocortex of Tg and Sg mice were analyzed after 90 minutes of PTZ treatment. Actin was used as loading control. The quantification of EAAT-1 (a) and EAAT-2 (b) was determined by densitometry. Arbitrary units represent the normalization ratio between antibodies signal and actin signal. Sg Syngenic mice; Tg, transgenic mice.

Evaluation of protein kinase C alpha (PKCα) protein expression was performed to correlate the observed decreased levels of EAATs. In Tg mice treated with a physiological solution, there is a significant increase of PKCα in comparison to Sg mice. After PTZ injection, there is a general reduction of the protein level in Sg and Tg mice (Fig. 21).

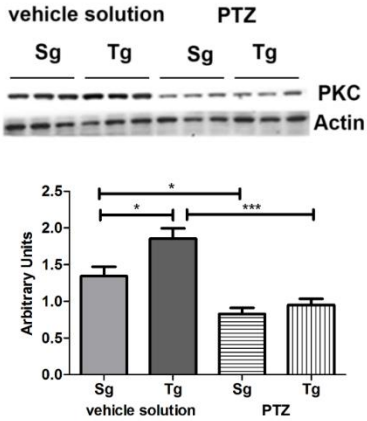

Figure 21. Western blot analysis of PKCα of Sg and Tg mice treated with vehicle solution or pentylentetrazole.
Protein levels from neocortex of Tg and Sg mice were analyzed after 90 minutes of PTZ treatment. Actin was used as loading control. The quantification of PKCα was determined by densitometry. Arbitrary units represent the normalization ratio between antibodies signal and actin signal. The p values are presented as mean ± S.E.M from three independent experiments. The p values were measured with the one-way ANOVA test and post-hoc test Bonferroni (**, $p<0.01$; ***, $p<0.001$). Sg Syngenic mice; Tg, transgenic mice.

7. SULFASALAZINE TREATMENT

7.1 Behavioral evaluation of anti-epileptic activity of sulfasalazine

To evaluate the involvement of system xc^- transporter activity in excitotoxic susceptibility, I performed a behavioral evaluation on Sg and Tg mice injected subcutaneously with PTZ (60 mg/Kg) and some pre-treated with SSA (8 mg) with an intraperitoneal injection 30 minutes before PTZ administration. Transgenic mice confirmed to be more sensitive to PTZ injection (Fig. 22). Pre-treatment with SSA protected Tg mice against seizures. All of Tg mice (100%) showed mild symptoms (stage 1-3), in respect to the group of Tg mice treated only with PTZ (stage 4-6, 100%). Pre-treatment with SSA in Sg mice caused a worsening

of the stages (90% severe symptoms) bringing them to a level similar to the one observed of Tg mice treated with PTZ.

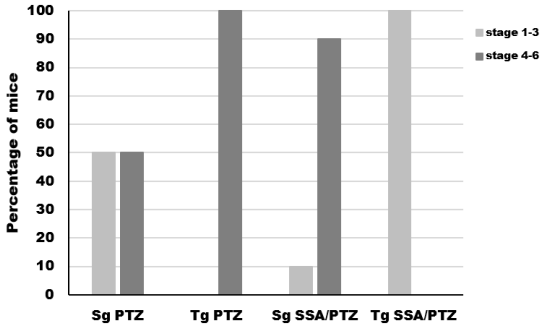

Fig 22. Behavioral evaluation of Sg and Tg mice treated with pentylentetrazole or pentylentetrazole/sulfasalazine.
The histogram represents the highest symptoms reached after PTZ or SSA/PTZ treatment of Tg and Sg mice according to Luttjohann's Scale. The p value was measured with the Chi square test ($p < 0.05$).

7.2 Western blot analysis after sulfasalazine/pentylentetrazole administration

To investigate alterations in astrocytic glutamate uptake capability, after PTZ or SSA treatment, a molecular analysis of EAAT-1 and EAAT-2 protein expression was performed (Fig. 23). Results showed no significantly differences in EAAT-1 after both treatments. EAAT-2 was significantly increased in Tg mice treated with 60 mg/kg of PTZ in respect to Sg, but unchanged between Sg and Tg treated with SSA/PTZ. If comparing Tg mice treated with only 60 mg/kg of PTZ and the ones with SSA pre-treatment (SSA/PTZ), we see a reduction of EAAT-2 in the latter case.

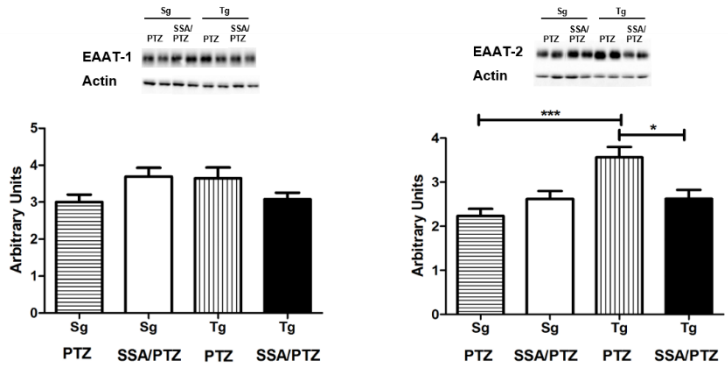

Figure 23. Western blot analysis of EAAT-1 and EAAT-2 of Sg and Tg mice treated with pentylentetrazole or pentylentetrazole/sulfasalazine.
Protein levels from neocortex of Tg and Sg mice were analyzed after 90 minutes of PTZ or SSA/PTZ treatment. Actin was used as loading control. The quantification of EAAT-1 (a) and EAAT-2 (b) was determined by densitometry. Arbitrary units represent the normalization ratio between antibodies signal and actin signal. Sg, syngenic mice; Tg, transgenic mice. The p values are presented as mean ± S.E.M from three independent experiments. The p values were measured with the one-way ANOVA test and post-hoc test Bonferroni (*, $p<0.05$; ***, $p<0.001$). Sg Syngenic mice; Tg, transgenic mice.

8. BIOCHEMICAL CHARACTERIZATION OF SPERMINE OXIDASE

8.1 Gel electrophoresis mobility analyses of spermine oxidase

To investigate the quaternary structure of SMOX, protein mobility and its enzymatic activity were analyzed by native gel electrophoresis. Samples were treated in duplicate with different concentrations of dithiothreitol (DTT) for 15 min (Fig. 24a, b), and with 5 mM DTT for different time (Fig. 24c, d) before loading on two native gels. After electrophoresis, gels were stained for enzymatic activity (Fig. 24a, c) and with Comassie brilliant blue dye (Fig. 24b, d). As shown in Fig. 1a, b, in reducing condition (from 5 to 20 mM DTT), two major bands are clearly visible. The intensity of the upper one decreased with increasing DTT concentration in a dose dependent fashion, but still visible at 20 mM DTT. A similar pattern was observed with a mild (5 mM) DTT treatment for 15 min and 1 h (Fig. 24c, d). In the absence of DTT (lanes C of Fig. 24) a more composite band pattern was visible in both gels, indicating the

formation of additional protein complexes, some with higher molecular weights. Identical protein patterns on the native gel were visible after enzymatic activity staining (Fig. 24a, c) and Coomassie brilliant blue dyeing (Fig. 24b, d), indicating that the formation of protein complexes does not affect the enzymatic activity. We considered the lower band as the SMOX monomer (labelled with * in Fig. 24), while among the slower migrating bands, we assumed as a possible SMOX homodimer the most stained one (labelled with ** in Fig. 24), whose association was disrupted by increasing reducing condition. All the high molecular weight bands present in the control lanes (lanes C), as well as a minor and barely visible band whose mobility is similar to the SMOX homodimer, disappeared in all the DTT treated samples, even at low DTT concentration or short incubation periods, suggesting that they are more labile than the putative dimer. Further control experiments demonstrated the covalent nature of the SMOX homodimer. In particular, the dimer band did not disappear in denaturing conditions (6 M urea), both at room temperature and after heating at 95 °C for 15 min. Interestingly, the amount of dimer did not increase after aerobic incubation of the SMOX samples for 3 h under vigorous stirring (not shown). Since the reduction with mercaptans, as well as dithiothreitol, in dissociating solvents is the choice procedure for attaining disulfide cleavage, in this study we used this methodology, together with heat treatment.

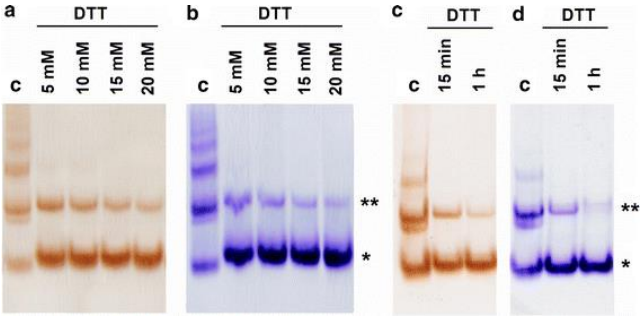

Figure 24. Purified recombinant spermine oxidase (SMOX) protein analyses. Nondenaturing PAGE analysis of the recombinant SMOX (0.05 units) treated with different (5–20 mM) DTT concentrations for 15 min (a, b). Stained with the enzymatic activity buffer (a) and with brilliant blue Coomassie (b). Nondenaturing PAGE analysis of the recombinant SMOX (0.05 units) treated with 5 mM DTT for 15 min and 1 h (c, d). Stained with the enzymatic activity buffer (c) and with brilliant blue Coomassie (d); ** homodimer, *monomer. (Cervelli et al., 2016).

8.2 Expression of the mutant protein $SMOX_{Cys263\ Cys429}$ in *E. coli*

To increase the knowledge on the SMOX structure and stability, a SMOX recombinant mutant was prepared. The three-dimensional model of SMOX (obtained both by homology modelling and threading techniques; Tavladoraki et al. 2011) suggests that 12 out of its 14 cysteine residues are exposed on the protein surface. We reasoned that eliminating the surface-exposed Cys residues could shed light on the structural bases of SMOX quaternary structure, in particular with respect to the possibility of generating a dimeric structure. We then produced a SMOX recombinant mutant, substituting the external Cys residues with Ser and maintaining only the two internal Cys263 and Cys429 residues, named $SMOX_{Cys263\ Cys429}$ (Fig. 25).

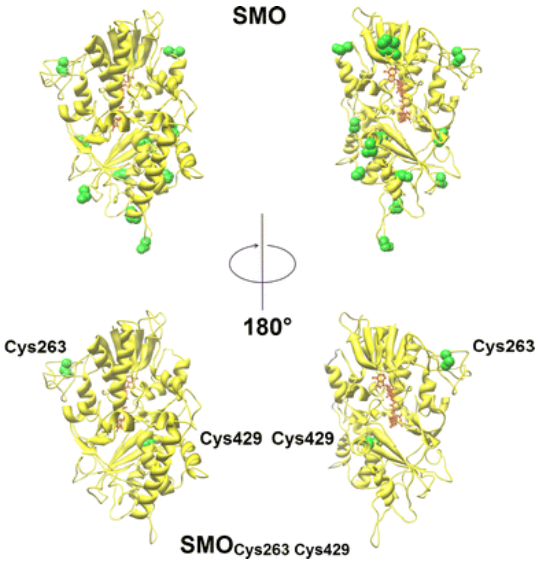

Figure 25. Schematic representation of the structural models of wild type. SMOX (top panels) and $SMOX_{Cys263\ Cys429}$ (bottom panels). FAD moiety is shown in red and Cys residues in green. Left and right panels are related by a 180° rotation around the axis indicated in the figure. (Cervelli et al., 2016).

The recombinant $SMOX_{Cys263\ Cys429}$ was expressed in the E. coli heterologous system, purified and biochemically characterized. Although the protein resulted to be less stable than the wild type (its activity decreased by about 90 % after 1 day from the preparation, and storage at 6 or –20 °C), the freshly prepared SMOX mutant was subjected to biochemical characterization, whose results are reported in Table 1.

Enzyme	K_M (μM)	k_{cat} (s^{-1})	k_{cat} (s^{-1})/K_M (μM)
SMOX	90 ± 18	4.50 ± 0.02	0.050
$SMOX_{Cys263\ Cys429}$	20 ± 10	0.32 ± 0.08	0.016

Table 1. SMOX and $SMOX_{Cys263\ Cys429}$ catalytic properties.
(Cervelli et al., 2016).

In particular K_M and k_{cat} resulted to be, respectively, 20 μM and 0.32 s−1. The mutant $SMOX_{Cys263\ Cys429}$ was also analyzed by gel electrophoresis. A single band was observed after staining for enzymatic activity and with Comassie brilliant blue dye, slightly slower migrating compared to the SMOX monomer band (Fig. 26). This strongly supports the predicted structural model and the hypothesis that the exposed Cys residues in SMOX protein complexes are responsible for the formation of the SMOX covalent homodimer observed in the wild type protein.

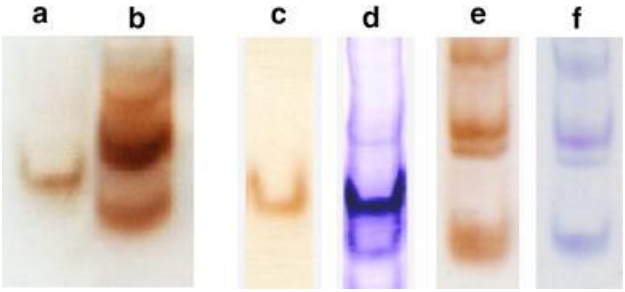

Fig 26. Purified recombinant mutant $SMOX_{Cys263\ Cys429}$ and SMOX protein analysis.
Nondenaturing PAGE analysis of the recombinant $SMOX_{Cys263\ Cys429}$ (0.05 units) (a, c, d) and wild type SMO (0.05 units) (b, e, f). Stained for enzymatic activity (a–c, e) and with brilliant blue Coomassie (d, f).

8.3 Differential scanning calorimetry on spermine oxidase

To get additional structural information, a study was also carried out on SMOX enzyme by differential scanning calorimetry (DSC). The thermal and chemical stability of both enzymes was studied using a VP-DSC MicroCal instrument. DSC allows detection of overall changes in protein structure correlated with modifications in thermal stability of one or more calorimetric domains. This is particularly important in the case of SMOX, a protein for which only scanty structural information are available. Our studies demonstrated that the thermal denaturation profile of the SMOX molecule displays one single, endothermic peak (T_m 62.8 °C) (Fig. 27a).

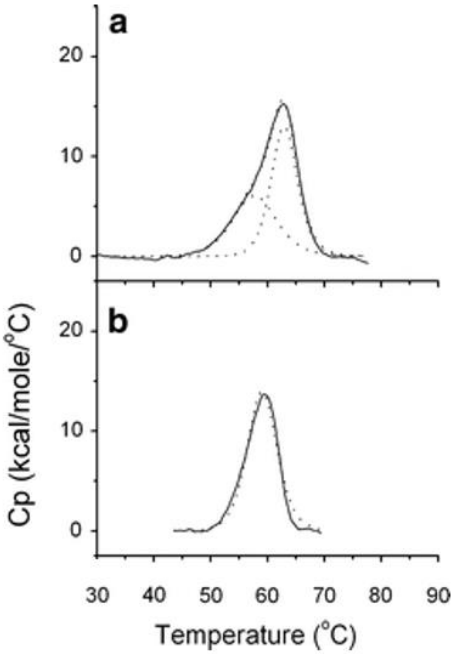

Figure 27. Thermal denaturation profiles of SMOX

Thermal denaturation profiles of SMOX (a), SMOX treated with β-mercaptoethanol (b). The dotted lines show the best fit obtained by deconvolution of each thermogram in non-two state transitions. (Cervelli et al., 2016).

The peak may be deconvoluted into two transitions and reveals the presence of two non-similar size calorimetric domains: the lowest temperature domain is described by a two-state transition while the highest temperature domain is described by a non two-state transition. The data show that in this case the ratio of ΔHvH to ΔHc, which should approximate unity for a two-state transition (Privalov 1986), is 1.3 for peak I and 1.8 for peak II. This suggests that peak I, within the limits of experimental uncertainty, represent two-state transitions of domains. Peak II, instead, represents a more complex process, presumably the denaturation of more than one folding domain in a single cooperative unit. The thermodynamic values derived for the transition components are given in Table 2.

SMOX	T_m °C	ΔH_c kcal/mol	ΔH_{vH} kcal/mol
Native			
Overall	62.8	136.9	
I	57.7	62.4	84.5
II	62.9	80.1	145.8
+βME			
Overall	59.6	94.7	
I	59.0	96.8	126.0

Table 2. Thermodynamic parameters and best fit for SMOX.
T_m temperature of maximum excess heat capacity for each simulated transition, ΔH_c calorimetric enthalpy change, ΔH_{vH} van't Hoff enthalpy change. (Cervelli et al., 2016).

Spermine oxidase enzyme treated with 0.1 mM β-mercaptoethanol displays one narrow transition, centered at a temperature of 59.6 °C at pH 8.3. The deconvolution of the thermogram showed non two-state transitions, as reported in Table 2 and represented in figure 27b. The thermal denaturation is irreversible after heating to 100 °C, as indicated by the flat thermogram obtained in a subsequent heating cycle (data not shown).

8.4 Enzymatic activity as a function of temperature

A further study on the stability of both enzymes was carried out spectrophotometrically at several temperatures. The SMOX enzyme in Tris–HCl 0.1 M pH 8.3 buffer was heated at a rate of 60 °C/h. The experiment revealed that 50 % of the enzymatic activity was lost at about 59.0 °C, in agreement with the *T*m value of 57.7 °C, obtained by DSC. The enzymatic activity of SMOX was plotted as a function of the temperature (Fig. 28).

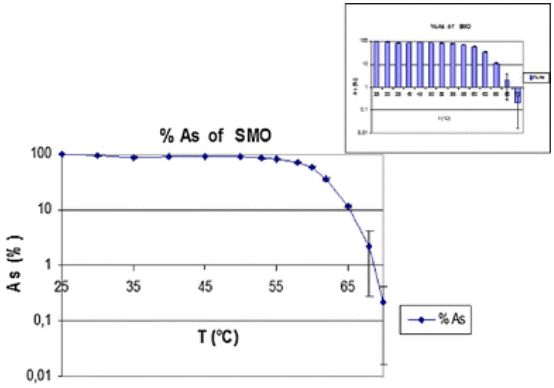

Figure 28. Heat inactivation of SMOX.
The SMOX enzyme in Tris–HCl 0.1 M pH 8.3 buffer was heated at a rate of 60 °C/h. The residual activity was measured in the standard assay (for further details see "Materials and methods"). Means and SDs are shown from three experiments. When not shown, SDs lie within symbols. (Cervelli et al., 2016).

8.5 FAD fluorescence analysis

Additional experiments were carried out to monitor the variation of FAD fluorescence with temperature. FAD can be excited by 450 nm light, and its emission spectrum is centered around 530 nm. It is known that FAD quantum yield increases when FAD is released from its binding pocket, i.e., following the denaturation of the FAD-binding domain. This phenomenon is due to dequenching events (Munro and Noble, 1999; Ahou et al. 2014) and can be easily followed fluorimetrically. Figure 29 illustrates the fluorescence versus temperature profile of a SMOX sample.

FAD fluorescence increased of about 70 %, following a sigmoidal transition, with *T*m = 57.6 ± 0.3 °C, in good agreement with the activity assay (59 °C) as well as with the transitions recorded by DSC measurements (Table 1).

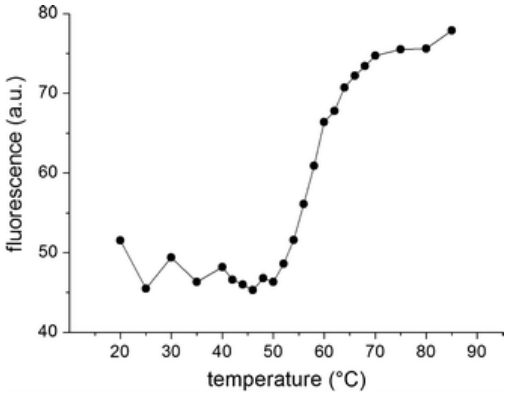

Figure 29. Variation of FAD fluorescence with temperature of SMOX.
FAD emission spectra were recorded after excitation at 450 nm; the fluorescence intensity at 530 nm is plotted against temperature. (Cervelli et al., 2016).

DISCUSSION AND CONCLUSION

Polyamines have central roles in cellular processes, for example in the regulation of cellular progression, differentiation and death (Cervetto et al., 2016). Several studies demonstrated that an altered PA metabolism is observed in many different pathologies, including cancer and brain neurodegenerative disorders (Cervetto et al., 2016). While the role of PA in cancer is well established, the role of PA in brain diseases is still unclear and the attempts to study PA in this field are numerous. In this perspective, this work focuses on the role played by SMOX enzyme in the brain and its contribution to physiological and excitotoxic conditions, by exploring the PTZ-induced seizure threshold and severity in Dach-SMOX mice analyzing which molecular pathways are involved in this response. A previous study showed an increased susceptibility of Dach-SMOX mice after KA treatment (Cervelli et al., 2013b). While the administration of glutamate-analogue KA determines an activation of the excitatory system (Cervelli et al., 2013b; Cervetto et al., 2016), PTZ treatment yields instead epileptic seizures by blocking the GABAergic inhibitory system (Psarropoulou et 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, by the onset latency of each stage and by the duration of generalized seizures in 1-3 and 4-6 stages. KA-induced seizure activity in mice brain was demonstrated to clearly activate the PAs interconversion pathway (Cervelli et al., 2013b), 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 are higher in Tg mice if compared to Sg mice, while Spm levels are comparable. The observed increase of Spd can be explained with Spm oxidation by SMOX, and 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. In fact, the reduction of Spm and Spd levels in Tg mice can cause rectification of AMPA and KA receptors acting as internal cell blockers of these channel receptors and reducing their activity while a reduction of Spd and Spm could increase the receptors response (Williams, 1997). This could explain the higher sensitivity of Tg mice to the PTZ treatment. Although Dach-SMOX mice are more susceptible to PTZ-induced seizures, when not treated, they display a normal behavior and a comparable life expectancy as syngenic mice. Morphological changes were instead found in Tg mice, when analyzing the cortex immunohistochemically, observing an increased number of positive cells for the microglial marker Iba1, which is usually upregulated during activation of microglia. Glia has a key role in brain functioning, representing an important antioxidant defense in oxidative stress conditions (de Groot and Sontheimer, 2011). Oxidative stress has long been recognized as a key mechanism in several neurological disorders (Patel, 2004). The oxidized derivative of deoxyguanosine, 8-oxo-dG, is often used as a marker for oxidative stress. Spermine oxidase overexpression can affect Tg phenotype leading to a pronounced brain damage by H_2O_2 overproduction, as previously demonstrated (Cervelli et al., 2013b). In fact, Tg mice displayed a higher amount of 8-oxo-dG, both in the vehicle solution and after PTZ-treatment, evidence of a chronic oxidative stress.

Emerging studies confirm that oxidative stress can increase in response to a seizure insult, and it can become, in turn, the cause of epileptogenesis (Patel, 2004; Puttachary et al., 2015). Since Dach-SMOX mice display a higher ROS production, this could explain its increased susceptibility to epileptic stimuli. In this regard, we analyzed whether system xc^- is involved as a defensive antioxidant mechanism (Bridges et al., 2012). The expression of the catalytic subunit of system xc^- (XCT) was found enhanced in Dach-SMOX mice where it probably tries to compensate the high oxidative stress of the surrounding environment, since the oxidized cysteine imported into the cell is required for glutathione synthesis (Bannai, 1986; Sato et al., 1999). On the other hand, Glu exported by the system xc^- contributes to the extracellular Glu concentration in the brain. The increase of the level of the system xc^- in turn rises the extracellular Glu, responsible for hyper excitability of neurons in Tg mice after either KA or PTZ treatment. On the contrary, no differences were found in the Glu uptake contribution of EAATs levels. Therefore, system xc^- acts like a bridge that links the antioxidant defense with neuronal excitability. The concomitant increase in Glu efflux and the production of H_2O_2, enhances

oxidative stress in the glutamatergic synapse of Tg mice. Reactive oxygen species formation is a key event in the development of neuronal injury in several acute and long-term neurodegenerative diseases (Volterra et al. 1994). Recent studies reported that glioma cells highly express the system xc^- through which they release cytotoxic levels of Glu into the extracellular space (de Groot and Sontheimer, 2011; Buckingham et al., 2011; Buckingham and Robel, 2013).

One of the regulatory mechanisms of system xc^- is operated by Nrf2. Nrf2 is activated by the PKCα pathway in response to oxidative stress in the astrocytes and in the surrounding environment (Huang HC et al., 2000). As reported by Mastrantonio et al. (2016) in a HIV cellular model, SMOX can trigger an antioxidant cell response through Nrf-2 induction.

Western blot analysis showed an increased level of PKCα in Dach-SMOX mice that is thought to be linked to the increased oxidative stress that activates directly Nrf2 and consequently the defense mechanism (Qaisiya et al., 2014). The rapid loss of PKCα levels after PTZ treatment is a common consequence of brain damage, as also observed after cerebral ischemia induced by various global and focal stroke models (Tremblay et al., 1999). Thus, all these events yield to oxidative stress and cell death termed as “oxidative glutamate toxicity”, increasing neuronal excitability and reducing seizure threshold (Lewerenz et al., 2013; Puttachary et al., 2015). For a schematic overview, see Figure 30.

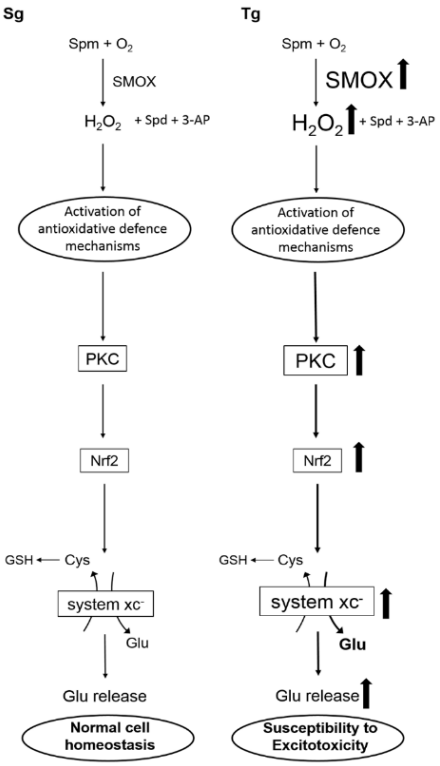

Fig 30. Proposed model of the role of SMOX in excitotoxicity comparing Dach-SMOX (Tg) and syngenic (Sg) mice.
SMOX produces H_2O_2 and its overexpression leads to oxidative stress. Oxidative stress activates PKC that is able to trigger an antioxidant response through the transcriptional induction of Nrf2-dependent ARE genes, like system xc-. System xc^- uptakes cystine and releases Glu. In Sg mice, there is a normal cell homeostasis, while in Tg mice, the unbalanced system xc- creates an accumulation of Glu that creates excitotoxicity. For more details see text.
Abbreviations: ARE, antioxidant-response element; Glu, glutamate; H_2O_2, hydrogen peroxide; Nrf2, nuclear factor erythroid 2-related factor 2; PKC, protein kinase c; Sg, syngenic; SMOX, spermine oxidase; Tg, transgenic.

To counteract this increased "oxidative glutamate toxicity", I focused my attention on reducing Glu concentration in the synaptic cleft.

Sulfasalazine is a Food and Drug Administration (FDA) approved drug, normally prescribed for the treatment of rheumatoid arthritis, ulcerative colitis and Crohn's disease (Lichtenstein et al., 2009). To understand if SSA-mediated block of system xc^- transporter could have an effect *in vivo* on Dach-SMOX mice in excitotoxic conditions, I performed a behavioral evaluation of Tg and Sg mice injected with PTZ (60 mg/Kg); a subgroup of both animals was pre-treated with SSA (8mg). Dach-SMOX mice confirmed to be more sensitive to PTZ injection, as previously reported. Pre-treatment with SSA protected Tg mice against seizures. All mice (100%) showed only mild symptoms (stage 1-3), in respect to the group of Tg mice treated only with PTZ (100%). This means that inhibition of system xc^- transporter with SSA is effective in preventing the excitotoxic effect of PTZ administration *in vivo* in Tg mice. Data show that SSA completely reverts the increased susceptibility of Dach-SMOX mice, lowering the sensitivity to PTZ induced seizures. Surprisingly, pre-treatment with SSA in Sg mice caused a worsening of the stages (90% severe symptoms) bringing them to a level similar to the one observed of Tg mice treated with PTZ. This unexpected scenario still needs further investigation.
To evaluate if a change occurred at a protein level of EAAT-1 and EAAT-2 after a higher dose of PTZ (60mg/kg) and after pre-treatment with SSA, I performed a western blot analysis. This revealed that EAAT-2 was significantly increased in Tg mice treated with 60 mg/kg of PTZ in respect to Sg, but unchanged between Sg and Tg treated with SSA/PTZ. This higher concentration of PTZ could represent a threshold effect. Beneath the concentration of 60 mg/kg, the effect could not create a strong oxidative stress likely to modify the protein. From 60 mg/kg and higher, induced oxidative stress has a greater effect on SMOX and an increase of EAAT-2 can be observed. This increase is counterbalanced by pre-treatment with SSA. If comparing Tg mice treated with only 60 mg/kg of PTZ and the ones with SSA pre-treatment (SSA/PTZ), we see a reduction of EAAT-2 in the latter case.

At present, some of the patients with epilepsy have seizures although treated with antiepileptic drugs, which produce physical harm, psychosocial dysfunction and physiological damage (Carmona-Aparicio et al., 2015). Moreover, the existing animal models may identify only drugs that share characteristics with existing drugs, and are unlikely to have an effect on refractory epilepsies. Therefore, it is of foremost importance to continue exploring the mechanisms underlying epilepsy to

identify new targets for therapies that are more effective in the refractory population and that may finally lead to an effective cure in susceptible individuals. In this regard, focusing on SMOX gene as a potential target by using specific inhibitors could be encouraging to discover new antiepileptic drugs. Collectively, the results obtained with PTZ presented in this work and previous works utilizing KA (Cervelli et al., 2013b; Cervetto et al., 2016), can demonstrate that Dach-SMOX mouse is a promising new genetic model to investigate epilepsy and therefore useful to discover new drugs effective on refractory epilepsies.

To further comprehend the biochemical characteristics of the SMOX enzyme, and find inhibitors that could interact with SMOX to prevent epilepsy, in this work I characterized in detail some of the physicochemical properties of the SMOX enzyme. Dimerization using disulfide bonds is a common evolutionary strategy for protein stabilization often observed in nature (Vinther et al., 2011). The quaternary structure of the SMOX protein was firstly analyzed by native gel electrophoresis, which revealed the formation of protein complexes in solution. These multimeric complexes all showed enzymatic activity, indicating that the SMOX protein present in quaternary structures maintains its ability of catalyzing the SPM oxidation reaction. In these protein complexes, there are two classes of disulfide bonds, those that can be reduced under mild reducing conditions to give SMOX monomer (*) and those that require stronger reducing conditions (**). In fact, after mild treatment with DTT, two prominent bands remain visible and were inferred as monomer (*) and homodimer (**), the latter disappearing with increasing concentration of DTT and prolonged incubation time. The SMOX homodimer (**) is a disulfide bond-based covalent assembly, as demonstrated by its persistence in non-reducing denaturizing conditions. These observations support the prediction that some of the twelve externally positioned Cys residues, as hypothesized by molecular modeling, play a central role in the generation of SMOX complexes. Albeit we cannot exclude that intramolecular disulfide bonds can occur, affecting the monomeric structure and influencing the dimer/complex formation. The recombinant $SMOX_{Cys263\ Cys429}$ mutant was engineered, substituting all Cys with Ser residues, but conserving the two internal Cys263 and Cys429 according to the SMOX structural model. This recombinant $SMOX_{Cys263\ Cys429}$ resulted to be reasonably stable, though

less stable than wild type SMOX, conserving its tertiary structure, as confirmed by CD analyses (data not shown), and displaying catalytic activity. While *K*M values of the mutant and wild type SMOX were of the same order of magnitude, a 14-fold reduction of *k*cat is observed for the mutant enzyme. The $SMOX_{Cys263\ Cys429}$ was analyzed by nondenaturing gel electrophoresis, which revealed no protein complexes formation, confirming that external Cys residues present in the SMOX protein are involved in protein complexes formation. Concerning the thermal denaturation of SMOX, this enzyme is irreversible after heating to 100 °C, as shown by the flat thermogram obtained in a subsequent heating cycle (data not shown). However, it can be observed that the first peak area decreased only by 36 % when scanning was performed after previous heating to 59 °C and cooling, but showed no reversibility after heating to 69 °C and rescanning. The second peak area decreased by 67.5 % when scanning was performed after previous heating to 69 °C and cooling. The protein unfolding was observed at 59 °C with 50 % decrease of the activity. A further decrease was observed at 63 °C, while the enzyme was inactivated at 68 °C. The thermal denaturation pattern of native SMOX shows a single broad transition. Its deconvolution in two bands, is represented in Fig. 4a. Both transitions display ΔHc lower than ΔHvH. The first transition at 57.7 °C is a two-state one and is reversible (62 %), in agreement with the transition from folded to unfolded state. The second one is non two-state and it is irreversible, as it is observed in presence of protein complexes, or when the denaturation occurs in several steps. In the absence of reducing agents, activity-based as well as spectrofluorometric assays coherently show that the transition at about 57.7 °C is clearly associated with the denaturation of the FAD-binding domain and the following FAD release. This transition is also related with the loss of 50 % of the enzymatic activity and associated with the beginning of the denaturation process. Instead, the transition at 63 °C relates to the denaturation of the domain with higher stability and corresponds to the loss of the residual enzymatic activity (about 30 %). A final consideration should be added about the physiological significance of these novel observations. Comparing different preparations of SMOX all gave approximately the same SMOX homodimer-to-monomer molar ratio. Trying to artificially increase this ratio by keeping the samples in presence of oxygen, did not give any variations in the homodimer-to-

monomer ratio. It remains an open question whether the SMOX homodimer is formed during the purification steps or whether its formation occurs biologically in the expression host (*E. coli*). In the latter case, it would be interesting to verify whether the SMOX homodimer is functionally relevant in animal cells and if it could be a turning point to further correlate SMOX and the pathways that take place during excitotoxicity.

REFERENCES

Abdulhussein AA & Wallace HM (2014). Polyamines and membrane transporters. *Amino Acids*. **46**, 655-660.

Ahou A, Martignago D, Alabdallah O, Tavazza R, Stano P, Macone A, Pivato M, Masi A, Rambla JL, Vera-Sirera F, Angelini R, Federico R, Tavladoraki P (2014). A plant spermine oxidase/dehydrogenase regulated by the proteasome and polyamines. *J Exp Bot*. **65**, 1585–1603

Amendola R, Bellini A, Cervelli M, Degan P, Marcocci L, Martini F, Mariottini P (2005). Direct oxidative DNA damage, apoptosis and radio sensitivity by spermine oxidase activities in mouse neuroblastoma cells. *Biochim. Biophys. Acta Rev. Cancer*. **1775**, 15-24.

Amendola R, Cervelli M, Fratini E, Polticelli F, Sallustio DE, Mariottini P (2009). Spermine metabolism and anticancer therapy. *Current Cancer Drug Targets*. **9**, 118–130.

Amendola R, Cervelli M, Fratini E, Sallustio DE, Tempera G, Ueshima T, Mariottini P, Agostinelli E (2013). Reactive oxygen species spermine metabolites generated from amine oxidases and radiation represent a therapeutic gain in cancer treatments. *Int J Oncol*. **43**, 813-820.

Amendola R, Cervelli M, Tempera G, Fratini E, Varesio L, Mariottini P, Agostinelli E (2014). Spermine metabolism and radiation-derived reactive oxygen species for future therapeutic implications in cancer: an additive or adaptive response. *Amino Acids*. **46**, 487-498.

Babbar N, Casero RA. Jr (2006). Tumor Necrosis Factor-A Increases Reactive Oxygen Species by Inducing Spermine Oxidase in Human Lung Epithelial Cells: A Potential Mechanism for Inflammation-Induced Carcinogenesis. *Cancer Res*. **66**, 11125-11130.

Balkhi HM, Gul T, Banday MZ, & Haq E (2014). Glutamate Excitotoxicity: An Insight into the Mechanism. *International Journal of Advanced Research*. **2**, 361-373.

Bannai S (1986). Exchange of cystine and glutamate across plasma membrane of human fibroblasts. J. *Biol. Chem*. **26**, 2256–2263.

Basu HS, Thompson TA, Church DR, Clower CC, Mehraein-Ghomi F, Amlong CA, Martin CT, Woster PM, Lindstrom MJ & Wilding G (2009). A small molecule polyamine oxidase inhibitor blocks androgen-induced oxidative stress and delays prostate cancer progression in the transgenic adenocarcinoma of the mouse prostate model. *Cancer research*. **69**, 7689-7695.

Beutler BA (1999). The role of tumor necrosis factor in health and disease. *J. Rheumatol. Suppl*. **57**, 16-21.

Binda C, Coda A, Angelini R, Federico R, Ascenzi P & Mattevi A (1999). A 30 Å long U-shaped catalytic tunnel in the crystal structure of polyamine oxidase. *Structure*. ***7***, 265-276.

Binda C, Mattevi A, Edmondson DE (2002). Structure-function relationships in flavoenzyme dependent amine oxidations. A comparison of polyamine oxidase and monoamine oxidase. *J Biol Chem*. **277**, 23973–23976.

Bridges RJ, Natale NR, Patel SA (2012). System xc^- cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS. *Br J Pharmacol*. **165**, 20-34.

Buckingham SC, Campbell SL, Haas BR, Montana V, Robel S, Ogunrinu T and Sontheimer H (2011). Glutamate Release by Primary Brain Tumors Induces Epileptic Activity. *Nature Medicine*. **17**, 1269–1274.

Buckingham SC, Robel S (2013). Glutamate and tumor-associated epilepsy: Glial cell dysfunction in the peritumoral environment. *Neurochemistry International*. **63**, 696–701.

Capone C, Cervelli M, Angelucci E, Colasanti M, Macone A, Mariottini P, Persichini T (2013). A role for spermine oxidase as a mediator of reactive oxygen species production in HIV-Tat-induced neuronal toxicity. *Free Radic Biol Med*. **63**, 99-107.

Carmona-Aparicio L, Pérez-Cruz C, Zavala-Tecuapetla C, Granados-Rojas L, Rivera-Espinosa L, Montesinos-Correa H, Hernández-Damián J, Pedraza-Chaverri J, Aristides III Sampieri, Coballase-Urrutia E, Cárdenas-Rodríguez N (2015). Overview of Nrf2 as Therapeutic Target in Epilepsy. *Int. J. Mol*. **16**, 18348-18367.

Casero RA Jr, Marton LJ (2007). Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. *Nature Rev. Drug Discov*. **6**, 373-390.

Casero RA, Pegg AE (2009). Polyamine catabolism and disease. *Biochem J*. **421**, 323–38.

Ceci R, Duranti G, Leonetti A, Pietropaoli S, Spinozzi F, Marcocci L, Amendola R, Cecconi F, Sabatini S, Mariottini P, Cervelli M (2017). Adaptive responses of heart and skeletal muscle to spermine oxidase overexpression: Evaluation of a new transgenic mouse model. *Free Radic Biol Med*. **103**, 216-225.

Cervelli M, Bellini A, Bianchi M, Marcocci L, Nocera S, Polticelli F, Federico R, Amendola R, Mariottini P (2004). Mouse spermine oxidase gene splice variants. Nuclear subcellular localization of a novel active isoform. *Eur J Biochem*. **271**, 760-770.

Cervelli M, Fratini E, Amendola R, Bianchi M, Signori E, Ferraro E, Lisi A, Federico R, Marcocci L, Mariottini P (2009). Increased spermine oxidase (SMO) activity as a novel differentiation marker of myogenic C2C12 cells. *Int J Biochem Cell Biol*. **41**, 934–944.

Cervelli M, Bellavia G, Fratini E, Amendola R, Polticelli F, Barba M, Federico R, Signore F, Gucciardo G, Grillo R, Woster PM, Casero RA Jr, Mariottini P (2010). Spermine oxidase (SMO) activity in breast tumor tissues and biochemical analysis of the anticancer spermine analogues BENSpm and CPENSpm. *BMC Cancer*. **10**, 555.

Cervelli M, Amendola R, Polticelli F, Mariottini P (2012). Spermine oxidase: ten years after. *Amino Acids*. **42**, 441–450.

Cervelli M, Salvi D, Polticelli F, Amendola R, Mariottini P (2013a). Structure–Function Relationships in the Evolutionary Framework of Spermine Oxidase. *J Mol Evol*. **76**, 365-370.

Cervelli M, Bellavia G, D'Amelio M, Cavallucci V, Moreno S, Berger J, Nardacci R, Marcoli M, Maura G, Piacentini M, Amendola R, Cecconi F, Mariottini P (2013b). A New Transgenic Mouse Model for Studying the Neurotoxicity of Spermine Oxidase Dosage in the Response to Excitotoxic Injury. *PLoS ONE*. **8**, e64810.

Cervelli M, Pietropaoli S, Signore F, Amendola R, Mariottini P (2014a). Polyamines metabolism and breast cancer: state of the art and perspectives. *Breast Cancer Res Treat*. **148**, 233-248.

Cervelli M, Angelucci E, Germani F, Amendola R, Mariottini P (2014b). Inflammation, carcinogenesis and neurodegeneration studies in transgenic animal models for polyamine research. *Amino Acids*. **46**, 521-530.

Cervelli M, Angelucci E, Stano P, Leboffe L, Federico R, Antonini G, Mariottini P and Polticelli F (2014c). The Glu(216)/Ser(218) pocket is a major determinant of spermine oxidase substrate specificity. *Biochemical Journal*. **461**, 453–459.

Cervelli M, Leonetti A, Cervoni L, Ohkubo S, Xhani M, Stano P, Federico R, Polticelli F, Mariottini P, Agostinelli E (2016). Stability of spermine oxidase to thermal and chemical denaturation: comparison with bovine serum amine oxidase. *Amino Acids*. **48**, 2283-2291.

Cervetto C, Vergani L, Passalacqua M, Ragazzoni M, Venturini A, Cecconi F, Berretta N, Mercuri N, D'Amelio M, Maura G, Mariottini P, Voci A, Marcoli M, Cervelli M (2016). Astrocyte-dependent vulnerability to excitotoxicity in spermine oxidase-overexpressing mouse. *Neuromol. Med*. **18**, 50–68.

Chaturvedi R, Asim M, Romero-Gallo J, Barry DP, Hoge S, de Sablet T, Delgado AG, Wroblewski LE, Piazuelo MB, Yan F, Israel DA, Casero RA Jr, Correa P, Gobert AP, Polk DB, Peek RM Jr, Wilson KT (2011). Spermine oxidase mediates the gastric cancer risk associated with Helicobacter pylori CagA. *Gastroenterology*. **141**, 1696-1708.

Choi DW (1994). Calcium and excitotoxic neuronal injury. *Annals of the New York Academy of Sciences*. **747**, 162-171.

Coleman CS, Hu G & Pegg AE (2004). Putrescine biosynthesis in mammalian tissues. *Biochem. J*. **379**, 849-855.

Coyle JT (1987). Kainic acid: insights into excitatory mechanisms causing selective neuronal degeneration. *In Selective Neuronal Death*. Wiley New York. pp, 186-203

De Groot J & Sontheimer H (2011). Glutamate and the biology of gliomas. *Glia*. **59**, 1181-1189.

Doble A (1999). The role of excitotoxicity in neurodegenerative disease: implications for therapy. *Pharmacology & therapeutics*. **81**, 163-221.

Doi T, Ueda Y, Nagatomo K, Willmore LJ (2009). Role of glutamate and GABA transporters in development of pentylenetetrazol-kindling. *Neurochem Res.* **34**, 1324-1331.

Eaden J, Abrams K, Ekbom A, Jackson E & Mayberry J (2000). Colorectal cancer prevention in ulcerative colitis: a case–control study. *Aliment Pharmacol. Ther*. **14**, 145-153.

Fleidervish IA, Libman L, Katz E, Gutnick MJ (2008). Endogenous polyamines regulate cortical neuronal excitability by blocking voltage-gated Na+ channels. *Proc Natl Acad Sci USA*. **105**, 18994–18999.

Gerner EW, Meyskens FL Jr (2004). Polyamines and cancer: old molecules, new understanding. *Nat. Rev. Cancer*. **4**, 781-792.

Gomes GM, Dalmolin GD, Bär J, KARpova A, Mello CF, Kreutz MR & Rubin MA (2014). Inhibition of the polyamine system counteracts β-amyloid peptide-induced memory impairment in mice: involvement of extrasynaptic NMDA receptors. *PloS one*. **9**, e99184.

González-gonzález IM, García-tardón N, Giménez C & Zafra F (2008). PKC-dependent endocytosis of the GLT1 glutamate transporter depends

on ubiquitylation of lysines located in a C-terminal cluster. *Glia*. **56**, 963-974.

Goodwin AC, Jadallah S, Toubaji A, Lecksell K, Hicks JL, Kowalski J, Bova GS, De Marzo AM, Netto GJ, Casero RA Jr (2008). Increased spermine oxidase expression in human prostate cancer and prostatic intraepithelial neoplasia tissues. *Prostate*. **68**, 766-772.

Gu B & Daltone KA (2017). Models and detection of spontaneous recurrent seizures in laboratory rodents. *Zoological Research*. **38**, 171.

Guerra GP, Rubin MA & Mello CF (2016). Modulation of learning and memory by natural polyamines. *Pharmacological research*. **112**, 99-118.

Gugliucci A (2004). Polyamines as clinical laboratory tools. *Clin Chim Acta*. **344**, 23-35.

Hansen SL, Sperling BB & Sánchez C (2004). Anticonvulsant and antiepileptogenic effects of GABA A receptor ligands in pentylenetetrazole-kindled mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. ***28***, 105-113.

Heby O, Roberts SC & Ullman B (2003). Polyamine biosynthetic enzymes as drug targets in parasitic protozoa. *Biochem. Soc. Trans*. **31**, 415-419.

Henley CM, Muszynski C, Cherian L & Robertson CS (1996). Activation of ornithine decarboxylase and accumulation of putrescine after traumatic brain injury. *Journal of neurotrauma*. **13**, 487-496.

Hiasa M, Miyaji, T, Haruna Y, Takeuchi T, Harada Y, Moriyama S, Yamamoto A, Omote H & Moriyama Y (2014). Identification of a mammalian vesicular polyamine transporter. *Scientific reports*. **4**, 6836.

Hirtz D, Thurman DJ, Gwinn-Hardy K, Mohamed M, Chaudhuri AR, Zalutsky R (2007). How common are the "common" neurologic disorders? *Neurology*. **68,** 326-337.

Horn Y, Schechter PJ & Marton LJ (1987). Phase I–II clinical trial with difluoromethylornithine an inhibitor of polyamine biosynthesis. *Eur. J. Clin. Oncol*. **23**, 1103-1107.

Hougaard DM (1992). Polyamine cytochemistry: localization and possible functions of polyamines. *International review of cytology*. **138**, 51-88.

Huang HC, Nguyen T, and Pickett CB (2000). Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *PNAS*. **97**, 12475–12480.

Huang Q, Liu Q, Hao Q (2005). Crystal structures of Fms1 and its complex with spermine reveal substrate specificity. *J Mol Biol.* **348**, 951–959.

Igarashi K, Kashiwagi K (2000). Polyamines: mysterious modulators of cellular functions. *Biochem Biophys Res Commun*. **271**, 559–564.

Jänne J, Alhonen L, Keinänen TA & Pietilä M (2005). Animal disease models generated by genetic engineering of polyamine metabolism. *Journal of cellular and molecular medicine*. **9**, 865.

Kauppinen RA, Alhonen LI (1995). Transgenic animals as models in the study of the neurobiological role of polyamines. *Progr Neurobiol*. **47**, 545–563.

Kaur N, Delcros JG, Archer J, Weagraff NZ, Martin B & Phanstiel IV O (2008). Designing the polyamine pharmacophore: influence of N-substituents on the transport behavior of polyamine conjugates. *J. Med. Chem*. **51**, 2551-2560.

Kew JN & Kemp JA (2005). Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology*. **179**, 4-29.

Klempan TA, Sequeira A, Canetti L, Lalovic A, Ernst C, ffrench-Mullen J & Turecki G (2009). Altered expression of genes involved in ATP biosynthesis and GABAergic neurotransmission in the ventral prefrontal cortex of suicides with and without major depression. *Mol. Psychiatry*. **14**, 175-189.

Kundu JK, Surh YJ (2008). Inflammation: Gearing the journey to cancer. *Mutat. Res*. **659**, 15-30.

Kupferberg H (2001). Animal models used in the screening of antiepileptic drugs. *Epilepsia*. ***42***, 7-12.

Laxer KD, Trinka E, Hirsch LJ, Cendes F, Langfitt J, Delanty N, Resnick T, Benbadis SR (2014). The consequences of refractory epilepsy and its treatment. *Epilepsy & Behavior*, **37**, 59-70.

Lewerenz J, Hewett SJ, Huang Y, Lambros M, Gout PW, Massie KA, Smolders I, Methner A, Pergande M, Smith SB, Ganapathy V, and Maher P (2013). The Cystine/Glutamate Antiporter System xc- in Health and Disease: From Molecular Mechanisms to Novel Therapeutic Opportunities. *ANTIOXIDANTS & REDOX SIGNALING*. **18**, 522-555.

Lichtenstein GR, Hanauer SB, Sandborn WJ (2009). Management of Crohn's disease in adults. *The American journal of gastroenterology*. **104**, 465-483.

Lipton SA, Rosenberg PA (1994). Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med*. **330**, 613-622.

López-Bayghen E & Ortega A (2004). Glutamate-dependent transcriptional regulation of GLAST: role of PKC. *Journal of neurochemistry*. **91**, 200-209.

López-Contreras AJ, Ramos-Molina B, Cremades A & Peñafiel R (2008). Antizyme inhibitor 2 (AZIN2/ODCp) stimulates polyamine uptake in mammalian cells. *Journal of Biological Chemistry*. **283**, 20761-20769.

Lüttjohann A, Fabene PF, van Luijtelaar G (2009) A revised Racine's scale for PTZ-induced seizures in rats. *Physiology & Behavior*. **98**, 579–586.

Malarkey EB & Parpura V (2008). Mechanisms of glutamate release from astrocytes. *Neurochem. Int*. **52**, 142-154.

Mastrantonio R, Cervelli M, Pietropaoli S, Mariottini P, Colasanti M, Persichini T (2016). HIV-Tat Induces the Nrf2/ARE Pathway through NMDA Receptor-Elicited Spermine Oxidase Activation in Human Neuroblastoma Cells. *PLoS One*. **11**, e0149802.

Meyskens FL Jr & Gerner EW (1999). Development of 2-difluoromethylornithine (DFMO) as a chemoprevention agent. *Clin. Cancer Res*. **5**, 945-951.

Mitchell JLA, Thane TK, Sequeira JM, Marton LJ & Thokala R (2007). Antizyme and antizyme inhibitor activities influence cellular responses to polyamine analogs. *Amino acids*. **33**, 291-297.

Morrison LD & Kish SJ (1995). Brain polyamine levels are altered in Alzheimer's disease. *Neuroscience letters*. **197**, 5-8.

Mueller MM & Fusenig NE (2004). Friends or foes—bipolar effects of the tumour stroma in cancer. *Nature Reviews Cancer*. **4**, 839-849.

Munro AW, Noble MA (1999). Fluorescence analysis of flavoproteins. In: Chapman SK, Reid GA (eds) Methods in molecular biology, vol. 131 (Flavoprotein Protocols). *Humana Press Inc. Totowa*. pp 25–48.

Naseer MI, Ullah I, Al-Qahtani MH, Karim S, Ullah N, Ansari SA, Kim MO, Bibi F (2013). Decreased GABABR expression and increased neuronal cell death in developing rat brain after PTZ-induced seizure. *Neurol Sci*. **34**, 497–503.

Niwa H, Yamamura K & Miyazaki J (1991). Efficient selection for high-expression transfectants with a novel euKARyotic vector. *Gene*. **108**, 193-199.

Okubo Y (2010). Imaging extrasynaptic glutamate dynamics in the brain. *Proc. Natl. Acad. Sci*. **10**7, 6526-6531.

Paschen W (1991). Polyamine metabolism in reversible cerebral ischemia. *Cerebrovascular and brain metabolism reviews*. **4**, 59-88.

Patel M (2004). Mitochondrial dysfunction and oxidative stress: cause and consequence of epileptic seizures. *Free Radical Biology and Medicine*. **37**, 1951–1962.

Pegg AE (2006). Regulation of ornithine decarboxylase. *J. Biol. Chem*. **281**, 14529–14532.

Pegg, AE (2009). Mammalian polyamine metabolism and function. *IUBMB life*. **61**, 880-894.

Pleasure D (2008). Diagnostic and pathogenic significance of glutamate receptor autoantibodies. *Arch Neurol*. **65**, 589–592.

Polticelli F, Salvi D, Mariottini P, Amendola R and Cervelli M (2012). Molecular evolution of the polyamine oxidase gene family in Metazoa. *BMC Evolutionary Biology*. **12**, 90.

Privalov PL and Potekhin SA (1986). Scanning microcalorimetry in studying temperature-induced changes in proteins. *Meth. Enzymol*. **131**, 4–51.

Psarropoulou C, Matsokis N, Angelatou F, Kostopoulos G (1994). Pentylenetetrazol-induced seizures decrease gamma-aminobutyric acid-mediated recurrent inhibition and enhance adenosine-mediated depression. *Epilepsia*. **35**, 12–19.

Puttachary S, Sharma S, Stark S, and Thippeswamy T (2015). Seizure-Induced Oxidative Stress in Temporal Lobe Epilepsy. *BioMed Research International*. 2015: 745613.

Qaisiya M, Zabetta CDC, Bellarosa C & Tiribelli C (2014). Bilirubin mediated oxidative stress involves antioxidant response activation via Nrf2 pathway. *Cellular Signalling*. **26**, 512-520.

Racine RJ (1972). Modification Of Seizure Activity By Electrical Stimulation: II. Motor Seizure. *Electroencephalography and Clinical Neurophysiology*. **281.**

Ransom RW & Stec NL (1988). Cooperative modulation of [3H]MK-801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. *J. Neurochem*. **51**, 830-836.

Raul F (2007). Revival of 2-(difluoromethyl)ornithine (DFMO), an inhibitor of polyamine biosynthesis, as a cancer chemopreventive agent. *Biochem Soc Trans*. **35**, 353-5.

Rea G, Bocedi A, Cervelli M (2004). Question: What is the biological function of the polyamines? *IUBMB Life*. **56**, 167–169.

Rothman DL, Behar KL, Hyder F & Shulman RG. (2003). In vivo NMR studies of the glutamate neurotransmitter flux and neuroenergetics: Implications for brain function. *Annu Rev Physiol*. **65**, 401-427.

Sato H, Tamba M, Ishii T, Bannai S (1999). Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J. Biol. Chem*. **274**, 11455–11458.

Seiler N (2004). How important is the oxidative degradation of spermine? Minireview article. *Amino Acids*. **26**, 317-319.

Seril DN, Liao J, Yang GY & Yang CS (2003). Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. *Carcinogenesis*. **24**, 353-362.

Shigeri Y, Seal RP & Shimamoto K (2004). Molecular pharmacology of glutamate transporters, EAATs and VGLUTs. *Brain research reviews*. **45**, 250-265.

Siegel GJ, Agranoff BW, Albers RW & Molinoff PB (1994). Basic Neurochemistry: Molecular, Cellular, and Medical Aspects. *AMERICAN JOURNAL OF NEURORADIOLOGY*. **27**, 465.

Sjögren T, Wassvik CM, Snijder A, Aagaard A, Kumanomidou T, Barlind L & Fjellström O (2017). The Structure of Murine N 1-Acetylspermine Oxidase Reveals Molecular Details of Vertebrate Polyamine Catabolism. *Biochemistry*. **56**, 458-467.

Sperk G, Lassmann H, Baran HK, Kish SJ, Seitelberger F & Hornykiewicz O (1983). Kainic acid induced seizures: neurochemical and histopathological changes. *Neuroscience*. **10**, 1301-1315.

Takano K, Ogura M, Nakamura Y & Yoneda Y (2005). Neuronal and glial responses to polyamines in the ischemic brain. *Current neurovascular research*. **2**, 213-223.

Tavladoraki P, Cervelli M, Antonangeli F, Minervini G, Stano P, Federico R, Mariottini P, Polticelli F (2011). Probing mammalian spermine oxidase enzyme-substrate complex through molecular modeling, site-directed mutagenesis and biochemical characterization. *Amino Acids*. **40**, 1115-1126.

Thomas T & Thomas TJ (2003). Polyamine metabolism and cancer. *J. Cell. Mol. Med*. **7**, 113-126.

Tian G, Lai L, Guo H, Lin Y, Butchbach M, Chang Y & Lin CLG (2007). Translational control of glial glutamate transporter EAAT2 expression. *Journal of biological chemistry*. **282**, 1727-1737.

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R (2010). Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev* **62**, 405–496.

Trotti D, Danbolt NC & Volterra A (1998). Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? *Trends in pharmacological sciences*. **19**, 328-333.

Uemura T, Watanabe K., Ishibashi M, Saiki R, Kuni K, Nishimura K, Toida T, Kashiwagi K, Igarashi K (2016). Aggravation of brain infarction through an increase in acrolein production and a decrease in glutathione with aging. *Biochem Biophys Res Commun*. **473**, 630-635.

Velloso NA, Dalmolin GD, Gomes GM, Rubin MA, Canas PM, Cunha RA & Mello CF (2009). Spermine improves recognition memory deficit in a rodent model of Huntington's disease. *Neurobiol Learn Mem*. **92**, 574-80.

Vinther TN, Norrman M, Strauss HM, Huus K, Schlein M, Pedersen TA, Kjeldsen T, Jensen KJ, Hubálek F (2011). Novel covalently linked insulin dimer engineered to investigate the function of insulin dimerization. *PLoS One*. **7**: e30882.

Volterra A, Trotti D, Tromba C, Floridi S & Racagni G (1994). Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. *The journal of neuroscience*. **14**, 2924-2932.

Wallace HM, Fraser AV & Hughes A (2003). A perspective of polyamine metabolism. *Biochem. J*. **376**, 1-14.

Wallace HM & Fraser AV (2004). Inhibitors of polyamine metabolism: review article. *Amino Acids*. **26**, 353-365.

Wang C, Delcros JG, Biggerstaff J & Phanstiel IV O (2003). Molecular requirements for targeting the polyamine transport system. Synthesis and biological evaluation of polyamine-anthracene conjugates. *Journal of medicinal chemistry*. **46**, 2672-2682.

Wang Q, Yu S, Simonyi A, Sun GY & Sun AY (2005). Kainic acid-mediated excitotoxicity as a model for neurodegeneration. *Molecular neurobiology*. **31**, 3-16.

Wang Y & Casero RA Jr (2006). Mammalian polyamine catabolism: a therapeutic target, a pathological problem, or both? *J. Biochem*. **139**, 17-25.

Welch JE, Bengtson P, Svensson K, Wittrup A, Jenniskens GJ, Ten Dam GB, Van Kuppevelt TH & Belting M (2008). Single chain fragment anti-heparan sulfate antibody targets the polyamine transport system and attenuates polyamine-dependent cell proliferation. *International journal of oncology*. **32**, 749-756.

Williams K (1997). Interactions of polyamines with ion channels. *Biochem J*. **325**, 289–297.

Xu H, Chaturvedi R, Cheng Y, Bussiere FI, Asim M, Yao MD, Potosky D, Meltzer SJ, Rhee JG, Kim SS, Moss SF, Hacker A, Wang Y, Casero

RA Jr, Wilson KT (2004). Spermine oxidation induced by Helicobacter pylori results in apoptosis and DNA damage: implications for gastric carcinogenesis. *Cancer Res.* **64**, 8521-8525.

Zahedi K, Huttinger F, Morrison R, Murray-Stewart T, Casero RA, Strauss KI (2010). Polyamine catabolism is enhanced after traumatic brain injury. *J Neurotrauma*. **27**, 515-525.

Zhu C, Lang DW & Coffino P (1999). Antizyme2 is a negative regulator of ornithine decarboxylase and polyamine transport. *Journal of Biological Chemistry*. **274**, 26425-26430.