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Characterization of transgenic murine model overexpressing spermine oxidase (SMO)

Caratterizzazione del modello murino transgenico sovraesprimente la spermina ossidasi (SMO)

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ABSTRACT

Natural polyamine (PAs), putrescine (Put), spermidine (Spd) and spermine (Spm) are positively charged aliphatic amines present in all tissues of almost all living organisms.
The notion that PAs are absolute required for several cell functions has led to the study of their metabolism as a strategy for therapeutic interventions. Recently, efforts are addressed to understand the link between PAs and brain physiology. This interest originates from growing data indicating that PA metabolism is affected in several neurodegenerative disorders (Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrofic lateral sclerosis) or after neurotrama and cerebral ischemia.
Spermine oxidase (SMO) is an enzyme involved in PA catabolic pathway; it oxidises Spm to produce Spd and two cytotoxic compounds: 3-aminopropanal and hydrogen peroxide. The *SMO* gene has been found to be highly expressed in the brain organ in physiological conditions, on the other hand its altered expression in some human organs is often associated to pathological disorders. Therefore, in this study I investigated whether SMO enzyme could play a role in the pathological *status* such as excitotoxic condition, found to be a common mechanism in neurodegenerative disorders.
To this end, I exploited Dach::SMO mouse line which overexpresses SMO specifically in the brain cortex. Trangenic mice treated with kainic acid (KA) were more vulnerable to this neurotoxin and showed a higher neuronal cell death and gliosis and alteration of PA metabolism compared to syngenic controls.
Moreover, *in vitro* study performed on corticostriatal slice cultures has suggested that SMO inhibition in excitotoxic condition could be partially neuroprotective.
These data point out that SMO enzyme can be considered as a potential and additional therapeutic target against neurodegeneration induced by excitotoxicity.

1 Introduction

This section describes polyamine metabolism focusing especially on the brain. It is here reviewed the link between this metabolism and neurodegenerative diseases. In particular, the mechanism of KA excitotocity is discussed.
Moreover, organotypic brain slice cultures were described as a useful tool in order to study the mechanisms undergoing in pathological conditions.

1.1 POLYAMINES

1.1.1. ROLE OF POLYAMINES IN MAMMALS

Natural polyamines (PAs), putrescine (Put), spermidine (Spd) and spermine (Spm) are positively charged aliphatic amines present in all tissues of almost all species (Fig. 1.1) (Wallace, 2000; Wallace et al., 2003).
The total intracellular concentration of the PAs is in the milllimolar range; however, free PA concentrations are considerably lower as they are mostly ionically bound to various anions in the cell including DNA, RNA, proteins and phopholipids (Casero & Marton, 2007). The major source of PAs in the majority of mammalian cells is via *de novo* biosynthesis, with the uptake by diet playing a significant, but lesser, role. The smallest contribution to intracellular PA pools is made by the gut microflora (Wallace, 2009).
The notion that PAs are absolute required for mammalian cellular functions has been already largely demonstrated. Infact, it is well-known that they play important roles in cell growth and proliferation, signal transduction, modulation of transcription and translation, as well as in the regulation of neurotransmitter receptors (Fig. 1.2) (Tabor & Tabor, 1984; Cohen, 1998; Thomas & Thomas, 2003; Williams, 1997; Wallace, 2003; Oredsson, 2003).

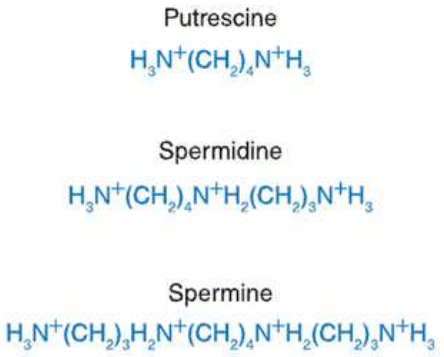


FIG. 1.1 STRUCTURE OF THE ENDOGENOUS PAS.

(from Wallace, 2009)

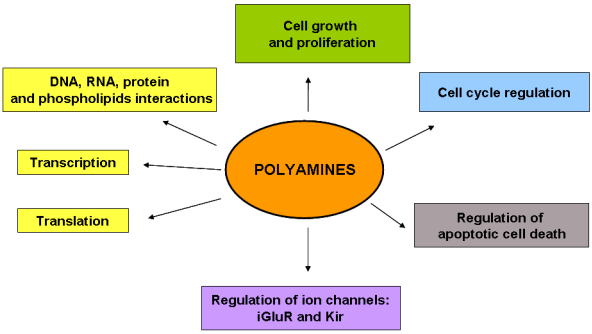


FIG. 1.2 FUNCTIONS OF PAS.

Due to their positive charge distributed along whole structure, PAs can interact with several molecules with opposite charge in the cell (nucleic acids and phospholipids). They are also involved in important processes which are required for cell functions.

1.1.2. PA METABOLISM IN MAMMALS

PA homeostasis in mammalian cells is achieved by a complex network of regulatory mechanisms concerning synthesis and degradation, as well as by membrane transport of PAs (Persson, 2009). It is clear that the cellular PAs have to be kept within certain levels for normal cell function. Infact, if the induction of cell growth is usually associated with an increase of cellular synthesis and PAs levels, on the other hand, too high concentrations of these molecules may be toxic to the cells, inducing cell death or apoptosis. Thus, cellular PA concentrations are usually maintained within rather narrow limits. PA metabolism is outlined in figure 1.3 (Wallace et al., 2003).

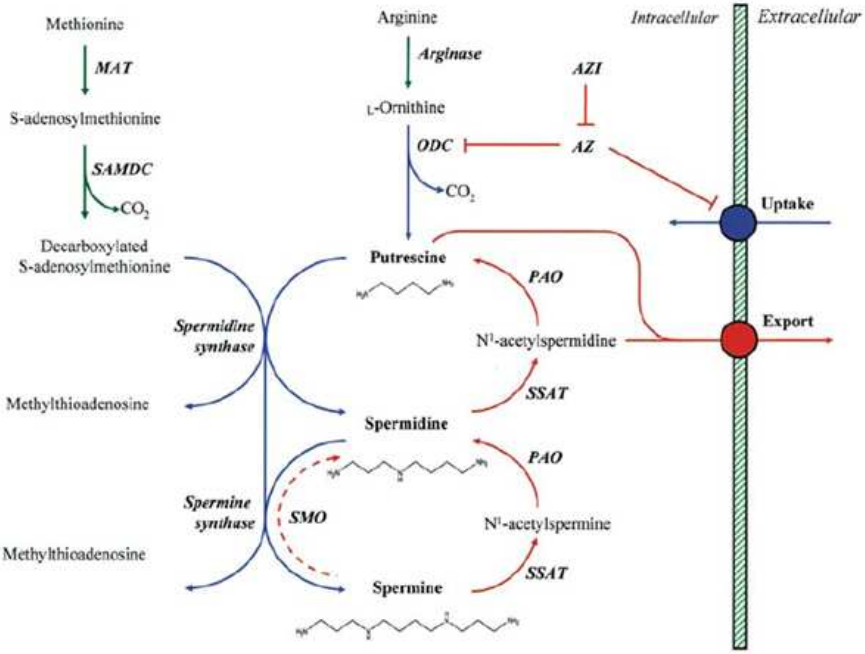


FIG. 1.3 PATHWAY OF PA METABOLISM.

Biosynthetic and catabolic pathways of PAs are represented with blu and red arrows respectively. Acetylated PAs as well as Put can be exported from the cell. Together these mechanisms cooperate to PAs homeostasis (from Wallace, 2003).

PA synthesis

The PA biosynthetic pathway includes four different enzymes, ODC (ornithine decarboxylase), SAMDC (S-adenosylmethionine decarboxylase; also referred as AdoMetDC), spermidine synthase and spermine synthase.
ODC catalyzes the production of Put by decarboxylation of ornithine. ODC expression is tightly regulated at several levels from transcription to post-translational modification (Pegg, 2006).
Two aminopropyl groups are then added consecutively to Put to form Spd, and to Spd to produce Spm. The aminopropyl groups are provided by decarboxylated S-adenosylmethionine (dcSAM), which is itself produced from the activity of SAMDC. Spermidine synthase and spermine synthase catalyze the aminopropyltransferase reactions. These synthases are stable enzymes that

are expressed constitutively with little inducibility (Ikeguchi et al., 2006). This is in contrast with the expression of both ODC and SAMDC enzymes, which have extremely high turnover rates in the cell (Hayashi et al., 1996; Coffino, 2001; Yerlikaya & Stanley, 2004), and that are readily induced by a range of agents. The rate-limiting step in biosynthesis of PAs appears to be either the decarboxylation of ornithine or the decarboxylation of S-adenosylmethionine (SAM).
Owing to the fast turnover of ODC and SAMDC, the cellular enzyme levels and thus the corresponding activities are rapidly altered when the synthesis and/or degradation of the enzymes are changed. Both enzymes are subject to a strong feedback control by PAs. ODC and SAMDC are rapidly up-regulated when cells become depleted of their PA content. On the other hand, both enzymes are down-regulated when cells are exposed to an excess of PAs.

PA degradation

Spd and Spm may be degraded to Put and Spd respectively, in a two-step process usually referred to as 'the PA interconversion pathway' (Seiler, 2004). The first step in the interconversion process is the acetylation of the N1-nitrogen of Spd and Spm producing N1-acetylspermidine or N1-acetylspermine (N1-acetylSpd, N1-acetylSpm). This reaction is catalysed by the enzyme spermidine/spermine N1-acetyltransferase (SSAT).
The second step is the oxidation of acetylated PAs producing Put and Spd respectively. It is catalyzed by a FAD-dependent peroxisomal PA oxidase that is nowadays referred to as the N1-acetylpolyamine oxidase (APAO), to distinguish it from the lastly discovered spermine oxidase (SMO). Infact, towards the end of 2002, an oxidase was cloned that converts directly Spm back into Spd, without the need for an acetylation step and producing 3-aminopropanal (3AP) and hydrogen peroxide (Wang et al., 2001; Vujcic et al., 2002). This enzyme has been termed SMO.
Like APAO, SMO is a FAD-dependent oxidase but, unlike the former enzyme, SMO has a high specificity for Spm as a substrate. In great contrast with APAO, SMO is highly induced by several PA analogues, indicating an important role in PA homoeostasis (Wang et al., 2001). However, the regulation of SMO appears to be mainly at the level of mRNA transcription-stability, rather than translation/post-translation (Wang et al., 2005).
The products of APAO activity are Put and Spd respectively, as well as 3-acetamidopropanal and hydrogen peroxide. Put may be further oxidized by diamine oxidase, whereas Spd may undergo another round in the interconversion pathway. The acetylated derivatives of Spd and Spm may also be excreted from the cells. The exact mechanisms by which the acetylated PAs are excreted are still not clear.

The rate-limiting step in the PA interconversion pathway is catalyzed by SSAT. Like ODC and SAMDC, SSAT has a very fast turnover with a half-life as short as 15 min, whereas APAO is a stable enzyme (Casero & Pegg, 1993). The rapid turnover of SSAT is mediated by the 26S proteasome and is dependent on ubiquitination of the protein (Coleman & Pegg, 2001). SSAT is strongly induced by a variety of stimuli, including various toxins and hormones.
Thus, similar to ODC and SAMDC, SSAT plays an important role in PAs homoeostasis.

PA uptake

In addition to regulating PA levels by synthesis, degradation and efflux, cells are equipped with an active transport system for the uptake of PAs (Mitchell et al., 2007). Large amounts of PAs are found in the food. Bacteria in the intestinal system produce and excrete considerable quantities of PAs. It is conceivable that a large fraction of these exogenous PAs are absorbed from the intestines and later taken up and used by cells in the body. However, to what extent cells rely upon endogenous compared with exogenous PAs is not yet clear. Nevertheless, in situations when the PA biosynthetic machinery is insufficient, cells would certainly be more dependent on extracellularly derived PAs. The exact mechanisms and the proteins involved in PA transport are still not identified. Whether there are individual transport systems for the various PAs or only a single transporter, capable of transporting all of the PAs, is not clear. Results obtained indicate that uptake of PAs by mammalian cells, at least partly, occurs by a mechanism involving cell-surface heparin sulfate proteoglycans and endocytosis (Welch et al., 2008). The polysaccharides of the proteoglycans are negatively charged and may interact with the positively charged PAs with affinities even stronger than the interaction between DNA and PAs. Moreover, it was recently demonstrated that PA uptake in human colon cancer cells follows a dynamin-dependent and clathrin-independent endocytic route, which is negatively regulated by caveolin-1 (Roy et al., 2008). A cell surface protein, capable of transporting both Put and Spd, has been cloned from the protozoan pathogen *Leishmania major* (Hasne & Ullman, 2005).
Mitchell et al., (2007) have shown that the activity of the PA transporter is partly regulated by cellular PA levels. Cellular depletion of PAs results in a marked increase in the cellular uptake of exogenous PAs. On the other hand, the PA transporter is down-regulated when cells are exposed to an excess of PAs. This feedback regulation is dependent on protein synthesis and involves a protein with a very fast turnover. Interestingly, antizyme (Az), which is an inhibitor of ODC enzyme, induced by an excess of PAs, also appears to negatively regulate the cellular PA transporter (Mitchell et al., 2007). Cells in

which Az is expressed to high levels exhibit a marked reduction in PA uptake. All three different forms of Az (Az1–Az3) have been shown to effectively down-regulate PA transport. However, the mechanism by which Az affects PA uptake is so far unknown.

1.2. PAs AND BRAIN: PHYSIOLOGICAL CONDITIONS

The functional role of the natural PAs in the normal and pathological states of the brain is under active research.
Several studies have led research to suppose a particular role of PAs in mammalian brain. It is intriguing to imagine PAs could play a peculiar function in the central nervous system (CNS) in addition to an universal role in the regulation of cell proliferation and growth, as seen in other organs.
Infact, endogenous PAs exert a number of key regulatory functions in the CNS ranging from cell proliferation to interactions with ion channels (Paschen, 1992; Scott et al., 1993; Kauppinen & Alhonen, 1995; Seiler et al., 1996; Williams, 1997). While the higher PAs Spd and Spm, are present at submillimolar total concentrations (300-500 µmol/kg), Put is expressed in the brain only following stimulatory factors as a consequence of ODC activation (Lukkarinen et al., 1998).

1.2.1. EFFECT OF PAs ON ION CHANNELS

In the last decade it has been demonstrated that PAs play important roles in the regulation of ion channels. Infact specific interactions of PAs with a number of distinct types of cation channels has been described. PAs have multiple modulatory effects on ionotropic glutamate receptors (iGluRs), they also interact with inwardly rectifying potassium channels (Kir) as well as some other channels that affect intracellular Ca^{++} signaling or Na^{+} transport (Williams, 1997; Fleidervish et al., 2008).

PA interaction with ionotropic Glutamate Receptors (iGluR)

iGluRs are major mediators of rapid excitatory neurotransmission in the mammalian CNS.
These receptors comprise a family of ligand-gated ion channels that include the amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate (KA), and N-methyl-D-aspartate (NMDA) receptors (Fig 1.4).
All iGluRs are thought to share a similar overall structure, with four homologous subunits arranged around a central cation selective pore. The

subunits themselves share a common membrane topology, with three transmembrane domains (termed M1, M3, and M4) and a reentrant loop similar to the P loop found in potassium channels (termed M2).
PAs influence GluRs mediating both fast responses at excitatory synapses, such as AMPA receptors and KA receptors and slow voltage-dependent responses such as NMDA receptors (Dingledine et al., 1999).

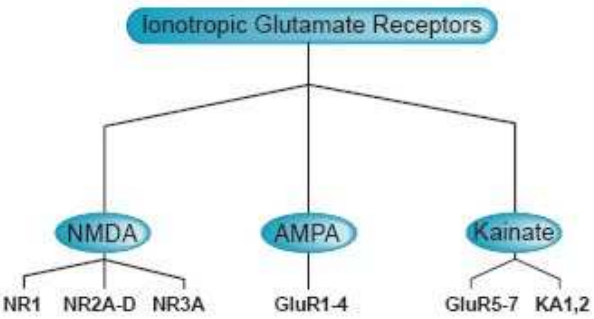


FIG. 1.4 CLASSIFICATION OF IONOTROPIC GLUTAMATE RECEPTORS (IGLURS).

Ionotropic Glutamate Receptors (iGluR) are divided in three main subfamilies: NMDA, AMPA and Kainate receptors. Each group is characterized by different glutamate receptor subunits (from: http://www.bris.ac.uk/synaptic/receptors).

AMPA and Kainate Receptors

Recent studies have shown that *intracellular* PAs have a profound effect on some subtypes of AMPA and KA receptors, where PAs control rectification of these receptor channels.
Native AMPA and KA receptors are heterooligomers composed of combinations of GluR and KA subunits.
AMPA receptors are ligand-gated channels composed of four possible subunits (GluR1–4) and a large number of them have been cloned (Hollmann & Heinemann, 1994). They are responsible for fast excitatory neurotransmission in the CNS.
KA receptors are formed of the GluR5-7 and KA1-2 subunits (Hollmann & Heinemann, 1994). GluR5-7 form the channel where KA1-2 are peptides that alter the biophysical characteristic of the KA channel complex. They are similar to AMPA receptors but have unique roles in synaptic function in

sensing pain, neuronal development and synaptogenesis and in supporting plasticity processes that are the cellular bases of learning and memory.
Most native AMPA and KA receptors gate Na+, but are relatively impermeable to Ca2+ and have I-V relationships that are close to linear. However, a subset of receptors show a high Ca2+ permeability and pronounced inward rectification (Ilno et al., 1990; Burnashev et al., 1992).
The *intracellular* PAs are able to interact with the subset of AMPA and KA receptors that gate Ca2+. They act by blocking the pore of the these receptors to prevent the flux and Ca2+ as the membrane potential is depolarized (Fig.1.5) (Bowie & Mayer, 1995).
The sensitivity of AMPA and KA receptors to PAs depends on their subunit composition and on RNA editing of the GluR subunits. Infact, the diversity of GluR subunits is extended by alternative splicing of their mRNAs and by editing of the pre-mRNA (Hollmann & Heinemann, 1994).
The RNA editing occurs at a so-called 'glutamine/arginine (Q/R) site' of the GluR2 (AMPA subunit) and GluR6 (KA subunit) mRNAs (Kohler et al., 1993; Seeburg, 1996). It is a crucial event because it controls the ionic property of these channels. The presence of an arginine residue (R) in the pore channel results in receptors that have low permeability to Ca2+, whereas the presence of a glutamine (Q) residue leads to receptors with higher Ca2+ permeability.
Therefore, AMPA receptors, those lacking the GluR2 subunit or with GluR2 (Q) unedited form, are permeable to Ca2+ ions and are subject to a block by endogenous intracellular PAs (predominantly Spm). Binding of Spm occurs within the pore region of the channel and is voltage dependent. Infact, at depolarized potential Spm blocks the channel, and as the cells is hyperpolarized, it unbinds and returns to the cytoplasm. At extreme depolarized potentials (more positive than + 50 mV), Spm can permeate the ion channel and can pass through to the extracellular side. This interaction confers inward rectification at these channels.
Likewise, the GluR6 subunit of KA receptors can also be edited and this determines the extent of KA receptors that allow the permeation of Ca2+ ions and that are sensible to the blockage by intracellular PAs at positive potential. The degree of RNA editing for GluR6 is developmentally regulated. In the early embryonic rat brain, the GluR6 (Q) form is found exclusively, and GluR6 (R) is co-expressed (≅ 50% of GluR6) at birth. In adult rats, the edited GluR6 (R) represents 70-85% of GluR6 in the hippocampus (Bernard & Khrestchatisky, 1994). Thus KA receptors in the foetal and neonatal nervous system may be particularly sensitive to PAs, which could play a role in synapse formation, plasticity, and elimination during development.
Therefore, PAs may regulate the amount of Ca^{2+} flux and the excitability threshold at synapses by the interaction with containing PA-sensitive AMPA/KA receptors.

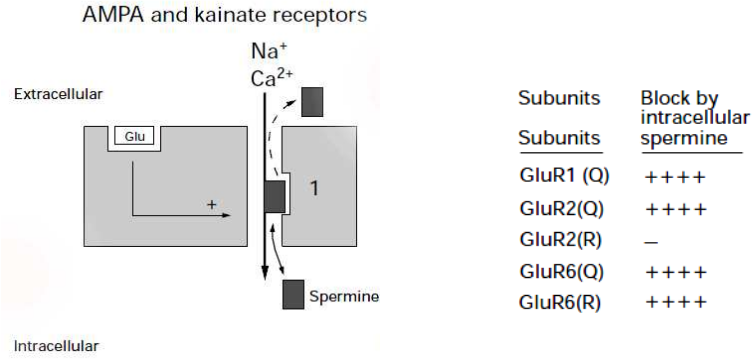


FIG. 1.5 PA INTERACTION WITH AMPA/KA RECEPTORS.

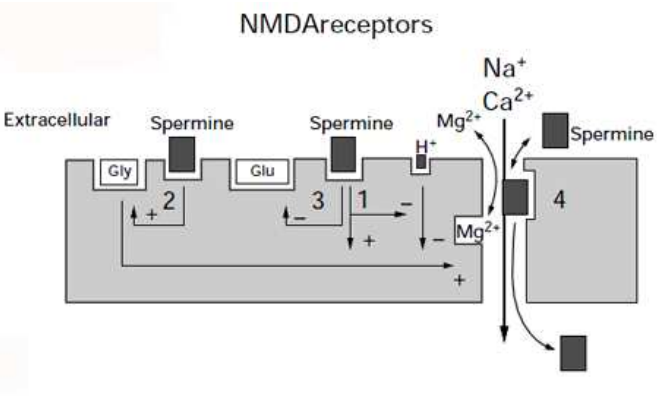
Intracellular Spm (dark rectangles) can block AMPA and KA receptors at depolarized potentials and, as the cell is hyperpolarized, Spm unbinds and returns to the cytoplasm (solid arrow). At extreme depolarized potentials (more positive than +50mV), Spm can permeate the ion channels and pass through to the extracellular side (broken arrow) (from Williams, 1997, modified).

NMDA Receptor

The NMDA receptors are involved in synaptic plasticity and may also play a role in seizure activity (Collingridge & Lester, 1989; McBain & Mayer, 1994).
NMDA receptors have a number of characteristics that distinguish them from other GluRs. Activation of NMDA receptors requires binding not only of the agonist glutamate but also of a co-agonist, glycine, at a separate site on the receptor. NMDA channels are blocked in a voltage-dependent manner by extracellular Mg2+, leading to a decreased conductance at negative potentials, with little or no current flow below about -70 mV.
Two families of NMDA receptor subunits, termed NR1 and NR2, have been cloned. The NR1 subunit is the product of a single gene that is transcribed as eight alternatively spliced mRNAs. The NR2 subunits, NR2A-NR2D, are distinct gene products with different regional and temporal patterns of expression (Hollmann & Heinemann, 1994). Native NMDA receptors are probably hetero-oligomers composed of combinations of NR1 and NR2 subunits.
NMDA receptors may contain two copies of the NR1 subunit in each receptor complex (Behé et al., 1995), and at least some NMDA receptors contain two different types of NR2 subunit (e.g. NR2A and NR2B) within a single hetero-oligomer (Luo et al., 1997; Wafford et al., 1993). Most studies of recombinant

NMDA receptors have focused on 'binary' receptors containing NR1 and one type of NR2 subunit (e.g. NR1/NR2B).
Modulation of NMDA receptors by PAs was first reported in 1988 by Ransom and Stec (Ransom & Stec, 1988) who showed that Spm and Spd increased the binding of [^{3} H] MK801 (an open-channel blocker of NMDA receptors) to the brain membranes. In electrophysiological studies, extracellular Spm was found to enhance NMDA-induced whole-cell currents in cultured neurons (Williams et al., 1990; Benveniste & Mayer, 1993).
Multiple and sometimes opposing effects of *extracellular* Spm on these channels have now been described (Williams, 1997).
Spm has four macroscopic effects that are differentially controlled by alternative splicing of exon 5 in the NR1 subunit and by the various NR2 subunits.
One is termed "glycine independent stimulation" because Spm potentiates NMDA currents in the presence of saturating concentrations of glycine. It involves an increase in the frequency of channel opening and a decrease in the desensitization of NMDA receptors (Benveniste & Mayer, 1993, Lerma, 1992; Rock & Macdonald, 1992).
The second effect is a "glycine-dependent stimulation" which produces an increase in the affinity of the receptor for glycine.
At physiologic pH, both stimulatory effects are seen at NR1/NR2B receptors but not at NR1/NR2A receptors (Han et al, 2008).
The third effect is a voltage-dependent inhibition. Spm inhibition of NMDA receptor is strongly voltage-dependent, being more pronounced at hyperpolarized than at depolarized membrane potentials. It occurs via interactions in the outer vestibule of the channel pore. Mutations affecting this interaction have been mapped (Jin et al., 2008).
It is conceptually similar to the block of AMPA channels and Kir channels (see below) by intracellular PAs, except that, in the case of NMDA receptors, the channels are blocked by *extracellular* Spm, although the block is very weak compared with the block of Kir and AMPA channels by *intracellular* Spm.
The fourth effect is a decrease affinity for agonist glutamate; it has been observed at some recombinant NMDA receptors (Williams, 1994).
The mechanism underlying this effect is not known, but it may reflect an increased rate of dissociation of glutamate from the receptor in the presence of Spm. In this case, Spm could have a marked effect on the time course of NMDA responses at the synapse, where the duration of the response is dependent on the rate of unbinding of glutamate from the receptors (Clements et al.,1992; Lester et al., 1990).
All these effects of PAs on NMDA receptors activity are schematized in figure1.6.
Spm and Spd are present in high concentrations in the CNS, and uptake and depolarization-induced release of PAs from brain slices has been reported

(Harman & Shaw, 1981; Fage et al., 1992). Thus it is possible that PAs are released from neurons or glia in the brain and can reach concentrations in the synaptic cleft that are sufficient to influence the activation of NMDA receptors, although there is as yet no direct evidence that this happens *in vivo*.
Excessive activation of NMDA receptors leads to neurodegeneration, and it is conceivable that excessive release of PAs, for example from injured cells, could exacerbate neuronal injury by potentiating the activity of NMDA receptors.



Effect of spermine	Sununit composition NR1A/ NR2A	NR1A/ NR2B	NR1A/ NR2C	NR1A/ NR2D	NR1A/ NR2B
1. 'Glycine-independent stimulation	–	+	–	–	–
2. 'Glycine-dependent stimulation (increase affinity for agonists)	+	+	–	–	+
3. Decrease affinity for agonists	–	+	–	–	?
4. Voltage-dependaent inhibition	+	+	–	–	+

FIG. 1.6 MODULATIONS OF NMDA RECEPTORS BY EXTRACELLULAR PAs.

Extracellular Spm has multiple effects on NMDA receptors ranging from stimulation to inhibition according to the subunit composition. Spm can block NMDA channels at negative membrane potentials (solid arrow), and at extreme negative potentials Spm can permeate the NMDA receptor to pass into the cell (broken arrow) (from Williams, 1997, modified).

PA interaction with Kir channels

Different forms of K+-selective ion channels have been identified, based on their biophysical, pharmacological and molecular properties. These include inward rectifier potassium channels (Kir) (Doupnik et al., 1995).
The term 'inward rectifier' refers to the ability of the channel to conduct ions in the inward direction at negative membrane potentials, but to show a greatly decreased outward conductance at membrane potentials positive to the potassium equilibrium potential (Ek ;Fig. 1.7). Kir channels, which are present in both excitable and non-excitable cells, are crucial for maintaining the resting membrane potential close to Ek (Doupnik et al., 1995).
The Kir gene family consists of 7 subfamilies (Kir1-7). These channels are tetramers of pore-forming subunits with two transmembrane domains (M1 and M2) separated by a P-region. The P-loop and a segment of the M2 domain form the transmembrane pore. There is also a cytoplasmic pore containing the binding site for the ligands and other regulators and controlling access to the transmembrane pore.
Voltage-dependent block by *intracellular* PAs is the common mechanism underlying the inward rectification in all the Kir channels. All of the natural PAs can bind and have some effects in experimental conditions but the affinity increases from Put to Spd to Spm (Stanfield et al., 2003; Guo et al., 2003). This block has been most intensively studied in some strongly inward rectifying channels such as Kir2.1 and Kir6.2 (Yan et al., 2005; Kurata et al., 2008). Evidence for different states in these channels with low and high affinity blocks has been published (Ishihara et al., 2007). Two different regions with negatively charged micro-environments are critical for binding PAs and determining inward rectification characteristics; one is in the cytoplasmic pore and the other in the transmembrane pore (Guo et al., 2003; Kurata et al., 2007). Models in which pre-positioning of PAs at the cytoplasmic pore then facilitates entry of PAs into a deeper binding site located within the membrane pore, are consistent with the experimental data using PAs and experimental analogs (Kurata et al., 2007, 2008).
Mice with a deletion in the *spermine synthase* gene (also referred as Gyro mice), have no Spm, show elevated Spd levels, and are also totally deaf. These mice have an almost complete loss of endocochlear potential. This phenotype may be explained by effects of the PAs imbalance on the cochlear lateral wall-specific Kir4.1 channel, which is known to play a critical role in the maintenance of this potential (Becerra-Solano et al., 2009).

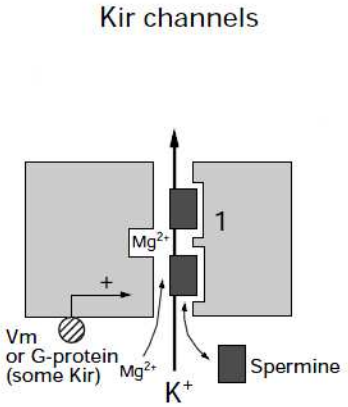


FIG. 1.7 BLOCKAGE OF KIR CHANNELS BY PAs.

Two molecules of intracellular Spm can enter deep within the ion-channel pore and may block the channel simultaneously (from Williams, 1997, modified).

PA interaction with voltage-gated Na+ channels

Voltage-gated Na+ channels are critical elements of action potential initiation and propagation in excitable cells because they are responsible for the initial depolarization of the membrane.
They are formed by a single long polypeptide that have four domains (I-IV). The four domains join together to form an acqueous pore of the channel.
The membrane excitability and input/output properties of neurons are largely determined by the spatial distribution and availability of their voltage-gated Na+ channels. In neocortical pyramidal cells, Na+ channels are present not only in the axon, where voltage threshold for action potential (AP) generation is lowest, but also in soma and dendrites.
Recently, it has also been reported a novel neuromodulatory mechanism that links the availability of Na+ channels to metabolism of PAs in the cerebral cortex. Fleidervish and collegues (2008) have demonstrated that PAs, which are normally present in the intracellular and extracellular compartments, are endogenous blockers of Na+ channels in layer 5 pyramidal cells. Because the blockade is activity-dependent, it is particularly effective against Na+ channels which fail to inactivate rapidly and thus underlie the persistent Na+ current. These data suggest that changes in PA levels, whether associated with normal brain states or pathological conditions, profoundly modify the Na+ availability

and thereby shape the integrative behaviour of single neurons and neocortical circuits (Fleidervish et al., 2008).

1.2.2. PA transport in the brain

Since PAs have the ability to modulate the activity of iGluRs and thus synaptic transmission *in vivo*, it is reasonable suppose that they can be released from neurons or glia and rapidly reincorporate into those cells in the brain. However, little is known about mechanisms underlying transport, uptake and release of PAs in neurons and glia.

It has been demonstrated PAs are taken up in cerebral cortex slices (Harman and Shaw, 1981), cultured cerebellar astrocytes (Dot et al., 2000) and synaptosomes (Gilad and Gilad, 1991). Masuko et al., (2003) characterized PA transport systems in synaptic vesicles, synaptosomes and glial cells, as well as the release of spermine from hippocampal slices. They have suggested that PAs transporters have broad spectra of substrate specificity and recognize agmatine, histidine and histamine as well as PAs (Spm, Spd and Put) in synaptosomes and glial cells. By contrast, PA transport system is rather selective for histamine only, beside PAs, in synaptic vesicles (Masuko et al., 2003). Moreover, another report has shown that agmatine can be transported by a PA transporter in NIH3T3 cells (Satriano et al., 2001).

A speculation regarding the possible traffic and action of PAs at synapsis was suggested by Takano and collegues (2005). They have hypothisized PAs could be released into synaptic clefts from neurons to facilitate the opening of NMDA receptor channels permeable to Ca2+ ions in response to particular pathological stimuli. On the other hand, intracellular PAs may inhibit the influx of cations across AMPA and KA receptor channels through plugging of ion channel pores at the intracellular domains. Then, extracellular PAs would be incorporated into intracellular spaces through particular transporters expressed by neurons, astrocytes and/or microglia. (Fig. 1.8) (Takano et al., 2005).

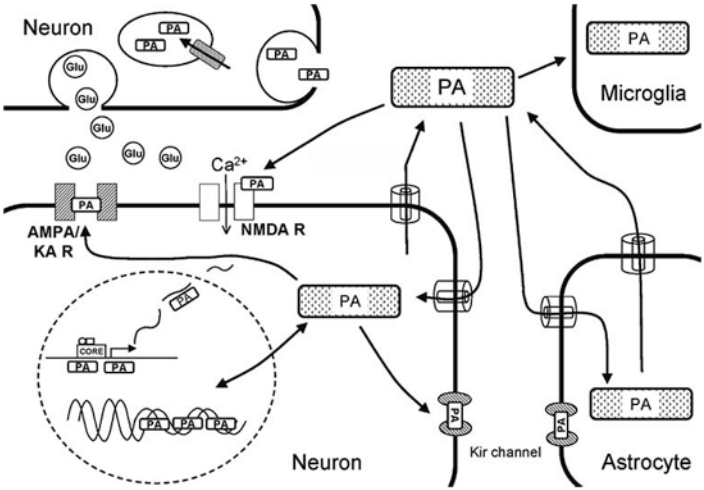


FIG. 1.8 MODEL OF PA ACTION AT SYNAPTIC CLEFT.

PAs could be release into synaptic clefts from neurons to facilitate the opening of NMDA receptor channel permeable to Ca+2 ions in response to particular pathological stimuli. Intracellular PAs may inhibit the influx of cations across AMPA and KA receptor channels through plugging of ion channel pores at the intracellular domains. Extracellular PAs woul be incorporated into intracellular spaces through particular transporters expressed by neurons, astrocytes and/or microglia (from Takano et al., 2005).

1.3. PAs AND BRAIN: PATHOLOGICAL CONDITIONS

In different neurodegenerative disease it has been found a change in PA metabolism. It often consists in an alteration of the delicate equilibrium among the three PAs. The mechanism of homeostasis is affected, and maybe it cannot work to restore the proper PA content in the cell.
Considering the several and additional roles that PAs play in neurons, a misregulation of this pathway can affect dramatically neuron functioning.
PAs concentrations increase in the brain of neurodegenerative disorders, such as Alzheimer's disease (AD) and ischemia (Morrison and Kish, 1995; Paschen et al., 1987). Elevated levels of immunoreactive ODC protein are demonstrated in neocortex of AD patients (Bernstein and Muller, 1995). The β-amyloid induces ODC activity and also stimulates PA uptake (Yatin et al., 1999). Recently, it has been reported the accumulation of AZ inhibitor 2 (AZIN2) and consequently ODC activation in neurons of hippocampus of AD patients (Makitie et al., 2010). Morrison et al. (2003), have shown an increased activity

of SAMDC, the key rate-limiting enzyme of Spd and Spm byosynthesis, in autopsied brains of patients with AD.

One of the most widely used animal models of Huntington's disease (HD) is based on the intrastriatal administration of quinolinic acid (QA). Spm treatment protects against the impairment of recognition memory and prevents the astrogliosis but not neuronal damage in the striatum of rat model of HD (Velloso et al., 2009).

In addition PAs are also implicated in the pathogenesis of ischemic brain damage (Zhang et al., 1994; Harman and Shaw, 1981; Bergeron et al., 1996; Glantz et al., 1996; Anderson et al., 1994).

PA biosynthesis is increased after the onset of cerebral ischemia, for example due to an ischemia-mediated induction of ODC (Kindy et al., 1994; Marton and Pegg, 1995; Lovkvist-Wallstrom et al., 1995, Pegg et al., 1994; Paschen, 1992). The administration of inhibitors of ODC prevents the development of ischemic brain damage, suggesting that the accumulation of PAs plays an important role in the pathogenesis of stroke in the ischemic brain (Kindy et al., 1994).

Several data have also contributed to point the attention on PA catabolism as potential target of a neuroprotective strategy.

It has been shown in the normal brain that approximately 70% of Put is derived from PA interconversion pathway, whereas only approximately 30% is formed by *de novo* synthesis from ornithine (Seiler, 1995).

Treatment of rats with inhibitors of PA oxidases prevents the production of 3-aminopropanal (3 AP), and significantly protects against the development of ischemic brain damage *in vivo*. 3 AP, as toxic product of APAO activity, has been proposed as a mediator of the brain damaging sequelae of cerebral ischemia, which can be therapeutically modulated (Ivanova et al., 1998). It is worthy to underline that now it is known that another enzyme, SMO, contributes to the production of 3 AP as well as hydrogen peroxide (H_2O_2). It has also found a relationship between PA catabolism and traumatic brain injury (TBI). Inhibition of PA oxidase with MDL 72,527 is neuroprotective against edema formation and necrotic cavitation after TBI (Dogan et al., 1999). PA back-conversion is enhanced after TBI; SSAT and SMO expression increased in brain damaged as well as Spd, Put and acetylated Spd content. In particular they have observed a late induction of SMO (from 3 to 7 days post-injury) which correlates very well with Spd increases, suggesting that SMO activity may be elevated at later times post-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.

A possible mechanism through which enhanced PA back-conversion may contribute to cerebral injury is through production of toxic metabolites. Increases of aminoaldehyde and pro-oxidant metabolites, such as H_2O_2 and 3-acetyl-aminopropanal, are damaging to vulnerable brain tissue (Li et al., 2003; Mello et al., 2007; Seiler, 2000; Takano et al., 2005; Wood et al., 2006a, 2006b,

2007). Therefore, the onset of PA back-conversion and PA synthesis after brain injury, together with concomitant elevations of oxidative metabolites and acetylated PAs, likely instigate secondary tissue injury. Thus, treatments that retard PA catabolism and reduce these cytotoxic oxidative byproducts may be expected to reduce secondary tissue damage in brain regions that demonstrate enhanced PA catabolism after TBI (Zahedi et al., 2010).

1.4. KA-MEDIATED EXCITOTOXICITY

1.4.1. Definition of excitotoxicity

In 1957, Lucas and Newhouse first described the neurotoxic effect of monosodium glutamate on retina of the mouse. Twenty years later, the explanation for this effect was formalised in the concept of excitotoxicity by John Olney (1978). Excitotoxicity refers to a process of neuronal death caused by excessive or prolonged activation of excitatory amino acid receptors. Infact in normal synaptic functioning, activation of these receptors is a transitory event. However, if, for any reason, receptor activation becomes excessive or prolonged, the target neurons become damaged and eventually die.

1.4.2. The excitatory amino acid: kainate

Kainic acid (KA) (2-carboxy-4-isopropenylpyrrolidin-3-ylacetic acid), also known as alga *kaininso*, is isolated from *Digenea*, a red alga found in tropical and subtropical waters (Coley et al., 1987). KA has been used for centuries as an anthelminthic compound for removal of worms in the gut. Subsequent studies indicated KA as a nondegradable analog of glutamate and it is 30-fold more potent in neurotoxicity than glutamate (Bleakman & Lodge, 1998). This neuroexcitant can bind to the AMPA/KA receptors in the brain (Bleakman & Lodge, 1998). Activation of KA receptor has been shown to elicit a number of cellular events, including the increase in intracellular Ca2+, production of ROS, and other biochemical events leading to neuronal cell death (Sun et al., 1992; Candelario-Jalil et al., 2001). In recent years, neurodegeneration caused by systemic injection of KA has been widely used in studies to investigate mechanisms of excitotoxicity (Wang et al., 2005).

1.4.3. Molecular mechanisms in excitotoxicity

Excessive stimulation by excitatory amino acid (EAAs) can cause death of the postsynaptic neuron. Addition of an EAAs, such as KA, stimulates both

AMPARs and KARs, which mediates fast synaptic potentials, and results in opening of cation channels permeable to Na^+, K^+ and sometimes Ca^{2+} depending on the subunit composition (Arundine & Tymianski, 2003; Wang et al., 2005).

Thus, the initial response is a large influx of Na^+ escorted by water and Cl^- which makes neurons appear swelled (Choi, 1992).

Continuous stimulation leads to an increase of the intracellular Ca2+ concentration. Active AMPA and KA receptors can enable Ca2+ to flow into the cell, however, the Na+ influx is substantial to generate depolarization of the cell, which is a prerequisite for activation of the slow potential NMDA receptors (Standaert et al., 1999). In this conditions, NMDA receptors release their magnesium block and allowing Ca2+ to flow through the channel. NMDA activation represents the major source of calcium entry into the cell.

Ca2+ overload can activate a series of enzymes including proteases, endonucleases and phospholipases that are Ca2+-dependent and when triggered contribute to protein breakdown and DNA fragmentation (Wang *et al.*, 2005; Sanchez et *al.*, 2008). Additionally, energy metabolism in the mitochondria is compromised, and the resulting lack of ATP hamperes ATP-dependent ion pumps in the plasma membrane, which causes destruction of the electrochemical gradient across the cell membrane. Formation of reactive oxygen species (ROS) increases and furthermore, nitric oxide synthase (NOS) is activated. In collaboration with ROS, NOS generate reactive nitrogen oxide species (RNOS) that cause destruction of cell membrane integrity and attack membrane lipids, proteins and DNA (Rego & Oliveira, 2003; Wang et al., 2005a; Estrada Sanchez et al., 2008).

These conditions might lead to neuronal death by apoptosis or necrosis, with further release of glutamate as a consequence. Then glutamate may act on neighboring cells by same mechanism. Contrary to KA, glutamate induces all types of iGluRs and mGluRs (metabotropic glutamate receptors) in the postsynaptic neuron, which additionally contribute to an increase in intracellular Ca2+ concentration (Choi, 1992). This self-propagating pattern can thus cause extensive neuronal death.

However, an increase in intracellular Ca2+ concentration does not always denote neuronal death. It appears that a combination of the severity of an insult and the increase in Ca2+ concentration determines the fate of the neuron. Thus, a thin line separates the outcome for the neuron and might also decide if death is caused by apoptosis or necrosis (Choi, 1995). Therefore, changes in glutamate concentration, shifting receptor-density in the cells, variations in subunit composition of GluRs and signaling properties, may all be cellular events that alter the severity of the insults and affect the outcome of excessive EAA stimulation.

1.4.4. Oxidative Stress Mediated by KA

Several studies linking oxidative stress to KA-mediated neurotoxicity.
Studies have demonstrated the production of free radicals after KA administration *in vivo* (Sun et al, 1992) and in neuron cells *in vitro* (Chen & Sun, 1994). KA also stimulated the release of lactate dehydrogenase (LDH), an indication of loss of cell membrane integrity, and a decrease in 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), suggesting a decrease in mitochondrial function.
Exposure of rat brain homogenates to KA can significantly increase the production of malondialdehyde and 4-hydroxy-alkenals, suggesting an increase in lipid peroxidation (Candelario-Jalil & Sonia Leon 2003). In addition to the increase in lipid peroxidation, systemic administration of KA also caused a decrease in glutathione (GSH) levels in the hippocampus, cerebellum, and amygdala/piriform cortex (Wang et al, 2004). The increase in superoxide production and oxidative DNA damage following KA administration are indications of KA induced mitochondrial and oxidative damage (Patel & Li, 2003). Injection of KA, also, causes the increase in nitric oxide synthase (NOS) in neurons (Yasuda et al, 2001).

1.4.5. KA Induces Apoptotic Neuronal Cell Death

The opening of Ca+2-AMPA/KA receptor channels in the postsynaptic terminal permits rapid Ca2+ influx and stimulates oxidative pathways resulting in the generation of ROS. There is sufficient evidence that ROS generation could lead to mitochondrial dysfunction and subsequent apoptotic or necrotic cell death pathways. Apoptotic pathways can be triggered as a result of the collapse of the mitochondrial membrane potential ($m\Delta\psi$) and the opening of mitochondrial permeability transition pores (MPT) that allow the release of cytochrome-*c* into the cytoplasm. In turn, cytochrome-*c* in cytoplasm is coupled with the apoptotic-inducing factor (AIF), which subsequently leads to activation of the caspase cascade (Weiss & Sensi, 2000). Rats treated with KA showed degenerating neurons in the hippocampus. Neurons were intensely stained with *in situ* nick end labelling (ISNEL) and displayed pathological features suggesting both necrosis and apoptosis (Nishiyama et al., 1996). Intraventricular infusion of KA into adult mouse brain also caused neuronal morphological changes, with condensed nuclei reflecting of apoptosis in the pyramidal layer of the hippocampal formation (Osaka et al., 1999). In organotypic hippocampal slice cultures, KA-mediated neuronal damage was associated with complete reduction of rhodamine 123 fluorescence, an indication of mitochondrial membrane potential dissipation, and increased

levels of cytochrome-*c* and caspase-3 in the cytosol. Cyclosporin A, an inhibitor of MPT opening, partially prevented cytochrome-*c* release, caspase activation, and neuronal death. Inhibition of caspase-3 activity by an inhibitor, z-VAD, also partially protected neurons from KA-induced cytotoxicity (Liu et al., 2001).

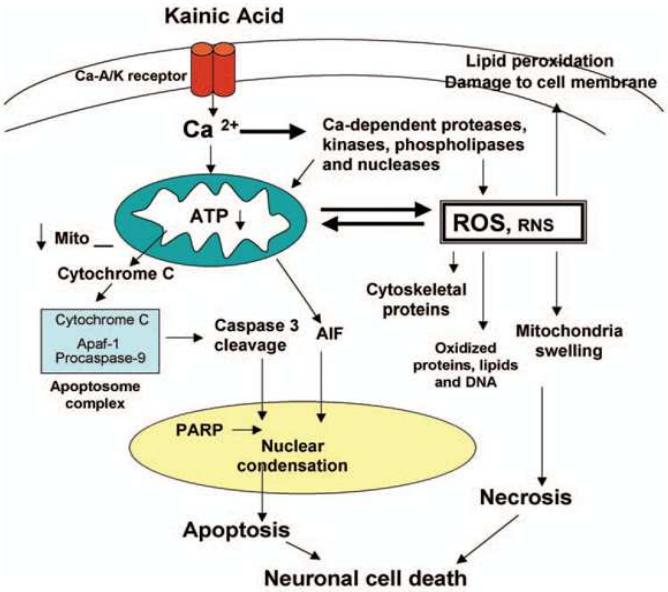


FIG. 1.9 NEURONAL CELL DEATH PATHWAY INDUCED BY KA.

KA stimulates the Ca2+-AMPA/KA receptors, leading to rapid Ca2+ entry; (2) activation of Ca2+-dependent enzymes and generation of ROS; (3) excessive Ca2+ and ROS lead to collapse of mitochondrial membrane potential (mΔΨ) and opening of mitochondrial permeability transition pores (MPT); (4) release of mitochondrial factors (e.g., cytochrome-c and apoptotic-inducing factor (AIF); (5) cytochrome-c binding to Apaf-1 and caspase-9 to form apoptosome complex and activation of caspase-3 pathway; (6) nuclear condensation and DNA fragmentation. Alternatively, intense Ca2+ overload could directly cause mitochondrial swelling and damage, decrease in ATP, and increase in ROS, which oxidize protein, lipid, and DNA, causing acute neuronal necrosis (from Wang et al., 2005).

1.4.6. Glial Cell Activation After KA-Induced Injury

Activation or recruitment of glial cells (astrocytes and microglial cells) is a common event associated with neuronal injury. Astrocytes comprise the major cell type in the brain and they are known to play multiple functional roles in support of neurons (Aschner, 1998; Zhang et al., 2000). Both astrocytes and microglial cells are immune active and become activated under pathological conditions. Reactive astrogliosis and microgliosis are intimately associated with many neurodegeneration processes and contribute to the increase in pro-inflammatory factors and ROS. Astrocytes are able to respond to pro-inflammatory cytokines, which stimulate transcription factors and cause induction of a number of genes, including inducible nitric oxide synthase (iNOS) (Li et al., 1999; Akama & Van Eldik, 2000), cyclooxygenase-2 (COX-2) (Kyrkanides et al., 2002), and secretory phospholipase A2 (sPLA2) (Li et al., 1999; Dorandeu et al., 1998; Wang & Sun 2000). Increase in PLA2 has been regarded as an important factor underlying a number of neurodegenerative diseases, and PLA2 inhibitors have been shown to protect against neurotoxicity induced by oxidative stressors (Xu et al., 2002; Farooqui et al., 1997). Systemic injection of KA to rats was shown to enhance cytosolic PLA2 immunoreactivity in the hippocampal area (Farooqui et al., 2004). Systemic injection of KA to rats resulted not only in neuronal cell death in the hippocampal area but also a large increase in reactive astrocytes and microglial cells (Wang et al, 2004).
These results further demonstrate the intimate relation between glial cell activation and neuron cell death in excitotoxic injury in the brain.

1.4.7. PA metabolism and excitotoxicity

PA property to interact with iGluR lead to hypothesize a link between these molecules and excitotoxicity phenomenon. However if it is known the cascade of events occurring during excitotoxicity it has not been understood the mechanism that may correlate excitotoxicity and PAs.
PA interconversion pathway is rapidly activated in limbic area following kainate-induced seizures activity. This was demonstrated both by an increase in SSAT activity, as well as in actetylated PA levels following the inhibition of PA oxidase. The results suggested that the increase in Put levels reported in several types of insults are due not only to the increased expression of ODC and the biosynthetic pathway but also to the stimulation of the interconversion via. As the catabolic pathway generates H_2O_2 it is conceivable that activation of this pathway contributes to the pathological manifestations of seizure activity,

such as neuronal damage and glial proliferation, as oxygen radicals have been suggested to be involved in the excitotoxicity (Baudry & Najm, 1994).
An overshoot of Put after different kind of injuries has been documentated. For instance, a concurrence between increases in Put concentrations and hippocampal or frontal cortex damage was observed in rats treated with GluR agonists in order to induce seizures. This relationship supports the proposed role of Put as a biochemical marker of acute brain damage (Camon et al., 2001; de Vera et al., 2002). It is interesting to underline that KA increases brain Put levels whereas picrotoxinin or pentilenetetrazol-induced seizures did not modify PA levels, suggesting that epileptic seizures *per se* do not induce Put increases (Martinez et al., 1990).

1.5. ORGANOTYPIC SLICE CULTURES

1.5.1. General characteristics

The culturing of slices of developing brain tissue dates back to over two decades. In 1981 Gahwiler (Gahwiler, 1981) introduced the roller drum technique to culture a number of different brain areas (Gahwiler et al., 1992). This method required that slice cultures were embedded in either a plasma clot or in a collagen matrix on a glass coverslips, placed inside tubes. The tubes contained a small amount of culture medium and were placed in a slowly rotating drum that periodically immersed the cultures in medium.
Slice performed by the roller drum method flattened to almost a monolayer (Gähwiler et al., 1997; Gähwiler et al., 1999).
Later a relatively simple method of culturing hippocampal slices on semiporous membranes was introducted by Stoppini (Stoppini et al., 1991).
Cultures grow on semi-porous membranes as an intermediate between medium and humidified air (Stoppini *et al.*, 1991; Gähwiler *et al.*, 1999).
In contrary to the roller drum method, the cultures remain multilayered demonstrating a higher degree of 3-dimensional organization (Stoppini et a*l.*, 1991; Gähwiler et al., 1997; Gähwiler et al., 1999). Illustrations of both techniques are given in figure1.10.
The characteristic of preserving the cytoarchitecture as well as the physiological properties of the tissue of origin justifies the term “organotypic”. Thus, organotypic slice cultures, maintaining the network of synaptic connections and the interactions between neurons and glia cells, mimick closely the *in vivo* situations. They represent a good intermediate between *in vitro* cell cultures and *in vivo* models.
The most commonly used donors of brain tissue for organotypic slice culturing have been rats and mice, lately including transgenic mice (Teter et al., 1999; Olsson et al, 2004), but also rabbits (Savas et al., 2001), pigs (Meyer et al., 2000) and human fetal brain tissue (Bauer et al., 2001; Walsh et al., 2005) have been used. Cultures are usually derived from early post-natal (P0-P7) animals, although attempts to culture adolescent or adult rat brain tissue have been recently made (Xiang et al., 2000; Hassen et al., 2004). It has also been reported that neurons and glial cells can survive for weeks in slice cultures prepared from adult human brains with a postmortem delay of maximally 8 hours (Verwer et al., 2002).
Hippocampal slice cultures are well characterized with regard to their neuronal and connective organization and electrophysiological properties (Gahwiler, 1984, 1988; Gahwiler et al., 1997; Zimmer and Gahwiler, 1984, 1987; Frotscher et al., 1990; Finsen et al., 1992; Torp et al., 1992), but also cortex-

striatum slice cultures are well studied (Ostergaard, 1993; Ostergaard et al., 1995; Plenz and Aertsen 1996; Plenz and Kitai, 1996, 1998).

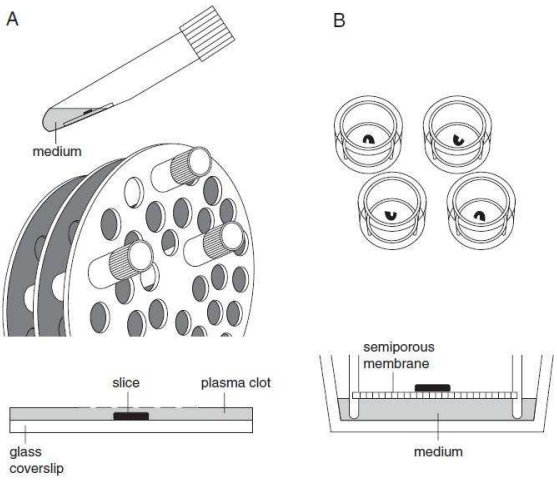


FIG. 1.10 ILLUSTRATION OF THE TWO PRINCIPLE METHODS OF GROWING BRAIN SLICES.

The roller drum method (a) utilizes slices placed in a plasma cloth in glass tubes added medium (slices are drawn side-view as bold back line). The tubes rotate, thus slices are covered half of the time in media and half of the time in air. In the interface method (b) slices are grown on semi-porous membranes inserted in culture trays with permanent access to humidified air on top of the slice and medium to the bottom of the slice (from Gähwiler et al., 1999)

1.5.2. Applications

Organotypic brain slice cultures are increasingly been used as models to investigate mechanisms and treatment strategies for neurodegenerative disorders like stroke (Newell et al., 1995; Strasser and Fischer 1995; Bonde et al., 2002; Bonde et al., 2005; Montero et al., 2007; Cui et al., 2009) or direct exposure to excitotoxins (Vornov et al., 1991; Zimmer et al., 2000; Kristensen et al., 2001; Noraberg, 2004; Aguirre & Baudry 2009), Alzheimer's disease (Bruce et al., 1996; Lambert et al., 1998; Selkoe, 2008; Wei et al., 2010), Parkinson's disease (Madsen et al, 2003; Jakobsen et al., 2005; Larsen et al., 2008), Huntington's disease (Storgaard et al., 2000; Zhang et al., 2005), amyotrophic lateral sclerosis (Rothstein et al., 1993; Elliott, 1999; Birgbauer et al., 2004; Harrer et al., 2009), and epilepsia (Thompson 1993; Poulsen et al.,

2002; Albus et al., 2008) for review see (Noraberg et al., 2005; Sundstrom et al., 2005; Cho et al., 2007; Cimarosti & Henley 2008; Lossi et al., 2009).
The slice cultures are also used in studies of non-excitotoxic neurotoxic compounds (Noraberg et al., 1998; Noraberg & Zimmer 1998; Kristensen et al. 2003; Barron et al., 2008), HIV neurotoxicity (Brana et al., 1999; Prendergast et al., 2002; Self et al., 2004), meningities (Stringaris et al., 2002; Gianinazzi et al., 2005), traumatic brain injury (TBI) (Adamchik et al., 2000; Morrison et al., 2005) and neurogenesis (Raineteau et al., 2004; Poulsen et al., 2005; Lossi et al., 2009).

1.5.3. Model for study of excitotoxic neurodegeneration

Several studies are focusing their attention on discovery of neuroprotective compounds against neuronal cell death occurring in excitotoxic conditions (Choi, 1992). Infact the mechanism of neurodegeneration seems to be common to many neurodegenerative diseases such as Alzheimer's, Huntington's, Parkinson's disease as well as amyotrofic lateral sclerosis, multiple sclerosis and HIV-associated dementia.
Organotypic slice cultures are become a useful model to study excitotoxic neurodegeneration. It has been shown that both hippocampal and corticostriatal cultures constitute a feasible test system for studies of the neurotoxic and neuroprotective effects of glutamate receptor agonists (AMPA, KA and NMDA) and antagonists. Futhermore, the slice cultures have responded with more *in vivo*-like patterns of excitotoxicity than primary neuronal cultures (Kristensen et al., 1999; 2001).
This method has been proposed as a valuable alternative model for the screening of neuroprotectans, which would provide to significantly limit the use of *in vivo* tests in animal (Ring et al., 2010). For instance, FK506, an inhibitor of calcineurin, has been found to protect against KA-excitotoxicity in organotypic hippocampal cultures (Lee et al., 2010). The AMPA antagonist PNQX (9-methyl-amino-6-nitro-hexahydro-benzo(F)quinoxalinedione) has been demonstrated to have a neuroprotective effect in mouse hippocampal slice cultures subjected to oxygen and glucose deprivation (OGD) (Montero et al., 2007).

1.5.4. Advantages and critical aspects of the method

Organotypic slice cultures satisfy several needs in the study of neurodegenerative disease.
This method represents a useful tool to grow brain slices for several weeks. It enables to study the effects of compounds on CNS lesions in a complex *in vitro*

system over a period of days to weeks in a context that reflects organ characteristics. It means, also for the first time, the possibility to monitor neuronal circuits with “long-term” live-imaging studies (Gogolla et al., 2006).
The unique property to preserve synaptic interactions as they are *in vivo*, makes possible to use electrophysiological approaches to study the neuronal netwok activity in these brain slices to gain valuable information on the structure-activity relationship. Organotypic systems have been often used with multi-electrode arrays, where the culture are simply placed on top of a grid of electrodes (De Bouard et al., 2002).
Another interesting aspect is that the explantation of the tissue of interest allows addition to the culture medium of drugs that would not cross the blood brain barrier. It means the possibility to study the effects of such compounds on signalling pathways.
Several groups have taken advantage of the potential for growing organotypic cultures from transgenic mice, effectively producing a model knockout system for functional genomic studies (Duff et al., 2002) or using transgenic mice expressing fluorescent proteins in subpopulation in neurons or glial cells as donors (Noraberg et al., 2007). Recent studies have also shown that brain slice cultures can be relatively easily transfected using either biolistic (Wirth & Wahle, 2003) or viral vectors (Glover et al, 2002).
There is growing demands for models which can replace or reduce animal experiments (Prieto et al. 2006). This is particularly important in the screening of neurotoxic and gliotoxic compounds. Infact a very significant sparing of animals is achieved as ~ 30 slices that can be produced from a single donor animal, allowing the generation of multiple data points from cultures derived from a single animal.
However, organotypic brain slice cultures are not without limitations. Brain slice cultures, for instance, can currently be produced only from juvenile donor animals (typically up to 12 days postnatal), and it is known that juvenile animals are more resistant to ischaemic damage than adults (Towfighi et al., 1997).
Another disadvantage is that not all areas of the brain are amenable to culture. The organotypic method is ideal for brain regions with a lamellar structure that can be aligned parallel to the plane of slicing, such as the rat striatum, cerebellum, hippocampus and cortex as well as various brain nuclei. The production of slice cultures from other regions with significantly out-of-plane projections, such as the nigro-striatal pathway, remains challenging. Lastly, organotypic brain slice cultures do not have a functional vascular compartment. Therefore, the effects of drugs that act on vascular or systemic components may not be accurately modelled in these systems, although this can be used as an advantage when trying to dissociate direct actions on neuronal tissue from indirect actions on the cardiovascular system (Sundstrom et al., 2005).

1.5.5. Corticostriatal slice cultures

Corticostriatal brain slices are well studied (Ostergaard, 1993; Ostergaard et al., 1995; Plenz and Aertsen 1996; Plenz and Kitai, 1996, 1998) for characterization of their basic cellular, connective and functional organization, or for experimental manipulation, including application of neurotrophic factors or potentially toxic compounds (Zimmer et al., 2000).
Cultured slices of neocortex seem to preserve a laminar organization both cultured together with striatum in co-culture and when cultured alone (Vogt Weisenhorn et al., 1996; Plenz & Aertsen, 1996; Petersen, 1997). The corticostriatal border is easy to see and the cortical tissue usually flattens much less than the striatal tissue. The corticostriatal projection which develop in the coculture system are thought to share common features with the projection system *in vivo* (Plenz & Aertsen, 1996).
In addition, a sustained function of corticostriatal pathway in such co-culture of cortex and striatum have been demonstrated by patch-clamp technique (Thomas *et al.*, 1998). However, for cholinergic neurons (ChAT-ir) and parvalbumin-ir neurons there were slight differences in electric conduction (Plenz & Aertsen, 1996b, a). Still these experiments suggest that there exists a strong resemblance between cortico-striatal brain slice cultures and the corresponding tissues *in vivo*.
Because slice cultures are derived from neonatal brain, the tissue has not achieved yet its final degree of maturation and development at the time it is explantated. In neonatal brain, neurons are still elaborating their dendritic trees, axons are growing and synaptogenesis is under way, cells may be migrating, and (in some brain regions) cells may still be undergoing in mitosis. The degree to which the culture retains its “organotypic” organization is therefore determined primarily by the age and maturity of the tissue at the time of the explantation: older slices generally attain a more organotypic state (Pitkanen et al., 2006). Corticostriatal cultures achieve their maturity after 3 weeks in culture.
Furthermore, expression of KA and AMPA receptors subunits (GluR1-7 and KA1-2) begins at an embryonic stage and continues through development and into adulthood at varying levels (Lilliu et al., 2002). By RT-PCR analysis all KA receptor and AMPA receptor subunits were found to be transcribed in medium-high and high levels (medium-high: 57%-75%, high: 76%-100% of the maximal level) in rat striatum at P0, with the exception of GluR6 that was expressed at medium-low levels (26-50% of maximal level) (Lilliu et al., 2002).

2 State of art

This section gives more information about spermine oxidase (SMO), protein of interest of this project.

2.1. SPERMINE OXIDASE (SMO)

SMO enzyme is involved in the recycling pathway of PAs. Infact, it mediates the back-conversion of the longest PA Spm to Spd, and producing 3-aminopropanal (3AP) and hydrogen peroxide at the same time (Fig.2.1).

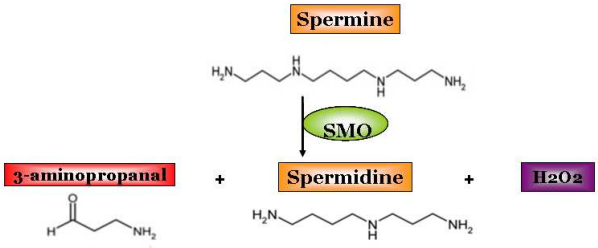


FIG. 2.1 SMO CATALITIC REACTION.

SMO enzyme oxidises spermine (Spm) to produce spermidine (Spd), 3-aminopropanal (3AP) and hydrogen peroxide.

2.2. SMO discovery

SMO discovery as an additional member of PA catabolism was made fortuitously during an attempt to clone APAO, in fact Wang and colleagues found a PA oxidise, initially called as PAOh1, able to oxidise preferentially Spm instead of the corresponding acetylated form. Subsequently, Vujcic et al. (2002) confirmed the preference of PAOh1 for Spm and renamed it as spermine oxidase utilizing the acronym SMO (Wang et al., 2001; Vujcic et al., 2002).

2.3. General characteristics

The single copy mammalian *SMO* gene encodes for many splice variants, both in human and mouse (Murray-Stewart et al., 2002; Cervelli et al., 2004). In particular, in mouse, among nine isoforms isolated, the SMO isoforms, *alfa* (SMOα) and *mu* (SMOμ), are the only splice variants with catalytic activity. Interestingly, these two proteins have different subcellular localization, SMOα localizes in the cytoplasm, SMOμ proved to be also nuclear localized (Cervelli et al., 2004).

However, the structural requirements for nuclear translocation is controversial (Bianchi et al., 2005; Murray-Stewart et al., 2008).
The predominant human splice variant, SMO1 (SMO/PAOh1), codes for a 61 kD protein containing 555 amino acids. The purified, recombinant protein shows a *K*m for Spm of 8 µM and a *K*cat of 7.2 s−1 (Wang et al., 2003). It has also been characterized the murine SMO with a calculated Mr of 61,8523 (Cervelli et al., 2003). Amino acid sequence alignment between mSMO and SMO/PAOh1 has revealed that they share a 95.1% sequence identity (Wang et al., 2001). SMO expression appears to be regulated predominantly at the level of transcription and somewhat by transcript stabilization (Wang et al., 2005).

During the last 25 years, much efforts has been invested in synthesizing PA analogues and derivatives in order to modulate PA metabolism as a possible therapeutic target. The availability of selective inhibitors for SMO would allow investigation of the role of this enzyme in PA metabolism.

2.4. MDL 72,527: a well characterized SMO inhibitor

MDL 72,527 (N_1,N_4-bis(2,3-butadienyl)-1,4-butanediamine) is a Spm analogue lacking terminal amino groups (Fig. 2.2).
It represents the most studied mammalian SMO and APAO inhibitor. In fact, it inhibits both SMO and APAO activities, showing a comparable K_i value, of 6.3 x 10^{-5} M and 2.1 x 10^{-5} M for SMO and APAO, respectively (Bianchi et al., 2006).
Some studies have shown that MDL 72,527 could have a lysosomotropic effect in some cell lines, as shown in baby hamster kidney (BHK) and CaCo-2 cells (Brunton et al., 1991; Seiler et al., 2000). In the course of these investigations became clear that the cytotoxic effect of this inhibitor was independent of its ability to inactivate APAO and SMO (Seiler et al., 2005).
Dai et al. (1999) demonstrated also an high sensitivity of transformed haematopoietic cells to MDL 72,527. Vacuole formation and an induction of apoptosis was also shown for colon carcinoma-derived SW 480 and SW 620 cell lines (Seiler et al., 2005; Duranton et al., 2002) and MDR LoVo human colon adenocarcinoma (Agostinelli et al., 2006) and M14 melanoma cells, when they are pretreated with MDL 72,527.
However, in contrast with the latter observation on LoVo and M14 cells, vacuole formation in leukemia cells was not reversible in the presence of MDL 72,527 (Dai et al., 1999).
Moreover, in neuroblastoma cell line the dosage requested to inhibit APAO and SMO activities did not influence cell survival (Amendola et al., 2005).

In contrast with these data, it has been demonstrated a beneficial effect and an effectiveness of MDL 72,527 treatment in human prostate cancer cells.
In fact, MDL 72,527 blocks androgen-induced ROS production in human prostate cancer (CaP cells) and delays prostate cancer progression in the transgenic adenocarcinoma of mouse prostate model (Basu et al., 2009).
Taken together, these data suggest that MDL 72,527 still represents a suitable SMO and APAO inhibitor. Since its action results in a decrease of the major pathway for ROS production, MDL 72,527 can play an important role in cancer etiology, occurrence and progression. But, in order to exploit this compound as antitumour molecule it needs to consider also its potential lysosomotropic effect as observed in some tumour cell lines.

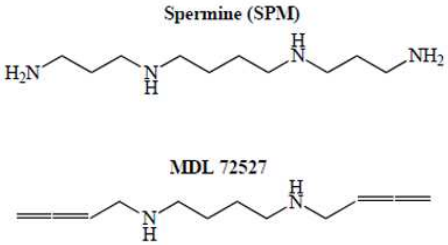


FIG . 2.2 CHEMICAL STRUCTURES OF SMO SUBSTRATE AND INHIBITOR.

Chemical structure of Spm, the specific substrate of SMO enzyme (above). Structure of the SMO inhibitor: MDL 72,527 (below).

2.5. SMO activity and cellular response to PA analogues

Unlike APAO, SMO is highly inducible by a variety of stimuli including some antitumor PA analogues (Devereux et al., 2003; Hacker et al., 2008).
As the ROS H_2O_2 is a product of SMO activity, the rapid increase in SMO expression subsequent to analogue exposure has the potential to play a significant role in the tumor cell response to analogue treatment.
The role of H_2O_2 in the selective cytotoxic response of human non-small cell lung cancers to the bis(ethyl)polyamine analogues was first proposed by Woster and collegues (Woster et al., 1997).
Before the awareness of the existence of SMO it was hypothesized that the increase of SSAT in response to analogue produced sufficient substrate for APAO which would have been responsible of H_2O_2 production within the tumor cells.
This interpretation was re-evaluated after SMO discovery. Although it was clear that induction of PA catabolism by PA analogues resulted in both H_2O_2 production and oxidative DNA damage, it was not clear as to the origin of the H_2O_2.
Using a stable, short hairpin RNA (shRNA) knockdown strategy it was demonstrated in a human breast cancer model that SMO was the primary source of the cytotoxic H_2O_2 produced in response to bis(ethyl)norspermine (BENSpm) treatment (Pledgie et al., 2005). This data suggested that SMO activity is the major mediator of the cellular response of breast cancer cells to BENSpm and that APAO plays little or no role in this response.
Recently, it has been shown by RT-PCR and enzyme activity analyses that SMO expression is significantly lower in breast cancer tissue than in nonneoplastic tissues, (Cervelli et al., 2010). Thus, it is tempting to speculate that the significant decreases in SMO activity observed in breast cancer tissue may contribute to tumor growth through a decreased rate of endogenous apoptosis resulting from decreases in the local concentrations of H_2O_2.
In this context the development of analogues able to induce SMO activity and to target selectively to cell death in tumor cells may be a therapeutic strategy.

2.6. Induction of SMO by various stimuli associated with human pathologies

Recently, an increasing interest has been posed on the SMO enzyme activity, since its ability to produce oxidative stress able to drive cells to death. In fact, as ROS are thought to play a role in the etiology of several pathologies, physiological relevance of SMO activity must be considered. Parchment and Pierce originally suggested that PA oxidation and the subsequent production of H_2O_2 were an important tissue-remodeling events in early embryogenesis (Parchment & Pierce, 1989; Parchment, 1993). They postulated that PA oxidase-produced H_2O_2 leads to apoptosis of cells as a natural process during development. Although this process is obviously necessary for normal development it also has the potential to produce a pathological outcome if not carefully controlled.
It has been found that SMO expression is highly induced in several human pathological conditions, most of them associated to an unbalanced ROS content They range from stroke, infections, inflammations and carcinogenesis.

Ischemia reperfusion injury

Recent studies in several laboratories indicates that the PA catabolic enzymes, SSAT, APAO and SMO, play a significant role in the production of ROS in myocardial infarct, kidney IRI (ischemia reperfusion injury), and in stroke. Zahedi and colleagues have implicated H_2O_2 production through PA catabolism as a source of injurious ROS in kidney IRI (Zahedi et al., 2003).
Although the studies do not provide data to show a mechanistic link between increased PA catabolism and stroke injury, the studies do suggest that the PA oxidases, APAO and SMO, in addition to the metabolite acrolein (produced by conversion of 3AP) provide useful biomarkers for the diagnosis of stroke. It will, however, be necessary to define any existing molecular links between ischemic stroke and PA catabolism to determine if this pathway represents a useful therapeutic target for treatment or prevention of cerebral strokes.

Infection

Several recent lines of evidence suggest that SMO plays a significant role in carcinogenesis and maybe one of the direct molecular links between infection, inflammation, and carcinogenesis. The first evidence of this possibility was the observation that gut macrophages infected with *Helicobacter pylori*, the causative agent in gastric ulcers and stomach cancer, highly induced SMO and

resulted in apoptosis of the affected macrophages (Chaturvedi et al., 2004). These results suggested a mechanism by which *H. pylori* infection could escape the immune system by killing the immune cells responsible for eradicating the infection. The mechanism of apoptotic death induced by *H. pylori* in the macrophages was directly associated with the H_2O_2 produced by SMO and subsequent fatal DNA damage (Chaturvedi et al., 2004).
Specifically, if *H. pylori* infection leads to increased SMO expression in the gastric epithelial cells, the chronic, sub-lethal production of H_2O_2 could result in the necessary mutagenic DNA damage required for neoplastic transformation. Inhibition of SMO by MDL 72,527 or the reduction of its expression through the use of siRNA targeting SMO greatly reduced the DNA damage produced by *H. pylori* exposure.
When taken together, these results point directly to an association of *H. pylori* infection, SMO induction, and DNA damage and the data strongly indicate a molecular mechanism directly linking infection with the production of carcinogenic ROS.

Inflammatory cytokines

Approximately 20-30% of epithelial cancers are thought to have an inflammatory component. Therefore, it has been investigated if SMO enzyme could be induced by inflammatory stimuli likewise to infection agents.
Using TNFα, a pleiotropic inflammatory cytokine, it was possible to demonstrate that this general mediator of inflammation was capable of significant induction of SMO in two non-tumorigenic human lung epithelial cell lines (Babbar & Casero, 2006). The increased SMO resulted in substantial production of H_2O_2 production accompanied by oxidative DNA damage. Both the level of ROS generated and the amount of DNA damage produced could be effectively inhibited by either MDL 72,527 treatment or by specific knockdown of SMO mRNA. Additionally, it is important to note that exposure of the lung epithelial cells to the cytokine IL-6 produced similar results with respect to SMO induction, indicating that the results are not limited to a single inflammatory cytokine.

Prostate cancer

Another human epithelial cancer in which inflammation has been strongly implicated in carcinogenesis is prostate cancer. DeMarzo, Nelson and colleagues have provided strong evidence that inflammation is intimately associated with prostate carcinogenesis (DeMarzo et al., 2003; Palapattu et al., 2005).

PAs, particularly Spm, have long been known to be present in high concentrations in the prostate (Pegg & Ashman, 1968; Pegg et al., 1970).
As stated above, it has been demonstrated that increased SMO expression was coincident with the early lesions thought to be precursors of prostate cancer (Basu et al., 2009). These data suggest that overexpression of SMO is an early event in the development of prostate cancer.
The high expression of SMO in response to various inflammatory stimuli, its ability to produce mutagenic ROS, and its demonstrated ability to damage DNA suggest that SMO may represent a legitimate target for chemoprevention. The demonstration that SMO is one direct molecular link between inflammation and carcinogenesis would provide an excellent starting point for chemopreventive intervention.

3 Aim of research

The aim of the project is reported in this section.

The notion that PAs are absolute required for several cell functions has led to the study of their metabolism as a strategy for therapeutic interventions. In fact, as crucial molecoles within the cells, many processes can be impaired by an alteration of PA homeostasis contributing to a pathological *status.*
Recently, efforts are addressed to understand the link between PAs and brain. Indeed, the neurobiological role of PAs in under active research. This interest originates from growing data indicating that PA metabolism is affected in several neurodegenerative disorders (Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrofic lateral sclerosis) (Bernstein & Muller 1995; Paschen et al., 1991; Yatin et al., 1999; Gomes-Trolin et al., 2002; Virgili et al., 2006; Velloso et al., 2009; Lewandowski et al., 2010;) or after neurotrama and cerebral ischemia (Gilad & Gilad, 1992; Koenig et al., 1989, Seiler et al., 2000).
It has been shown that excitotoxicity is a common pathway of neuronal cell death in neurodegenerative disorders (Doble, 1999).
Stated that, during my PhD I wondered if PAs could have a further role in the brain in addition to the "traditional" one already seen in other non neuronal cells.
Moreover, considering that the substrate of SMO, Spm, is involved in modulation of some ion channels (iGluRs, Kir and Na+ channels) and that SMO expression is high in brain, consequently it is conceivable that SMO could act as an important player in neuron function.

In this context, the general goal of this project was to gain knowledge about this SMO enzyme under pathological conditions and the major aims were:

1) To investigate the effect of SMO overexpression in the brain cortex using Dach::SMO transgenic mouse line after KA treatment.

2) To study the effect of SMO inhibition on organotypic brain slice cultures in KA-induced excitotoxicity.

The *in vivo* study was performed exploiting two transgenic mouse lines.
The first one, GFP-SMO line has been generated in my laboratory in order to investigate the role of SMO enzyme. It constitutively expresses the Green Fluorescent Protein (GFP) but not SMO, and its construct is outlined in figure 3.1 A.
The second one, Dach-CRE line is characterized by the *Dach* promoter which specifically addresses the CRE recombinase expression on the brain cortex. Crossing the GFP-SMO line with the Dach-CRE line, it is possible to obtain in the offspring double transgenic mice overexpressing SMO only in the brain cortex. Indeed, CRE recombinase mediating the GFP excision, allows SMO

gene, as well as LacZ, to be under control of the strong promoter leading to SMO overexpression.

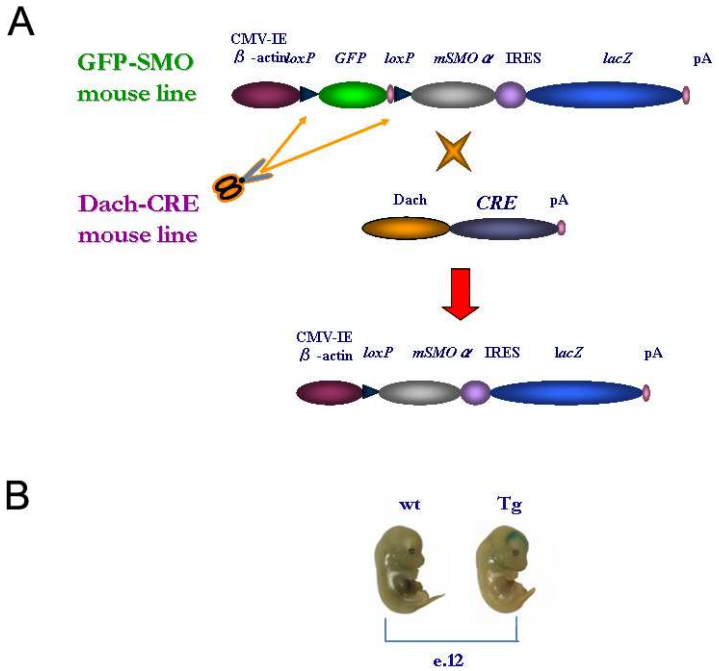


FIG. 3.1 DACH::SMO TRANGENIC MICE OVEREXPRESSING SMO ENZYME IN THE BRAIN CORTEX.

(A) Crossbreed between GFP-SMO line and Dach-CRE line to obtain Tg mice overespressing SMO in the brain cortex. Tissue-specific expression of Tg embryos is shown in picture (B) by the expression of the reporter gene LacZ. e.12: embrionic day.

On the other hand, corticostriatal slice cultures were performed from wild type newborn donor mice to elucidate the mechanism that might link SMO activity and KA excitotoxicity.

4 Results

Results are reported in this section and organized in two parts. The first part presents all data obtained on Dach::SMO transgenic mice after kainic acid (KA) treatment. The second part shows the study of the role of PA catabolism in excitotoxic conditions using organotypic slice cultures from wild type donor mice.

4.1. EFFECTS OF KA TREATMENT ON DACH::SMO TRANSGENIC MOUSE LINE

According to Benkovic et al. (2005), adult C57BL/6 mice were treated systemically with KA at dose of 25 mg/Kg whereas control animals were treated with saline solution (SA).
After a behavioural analysis, treated mice were used for different experimental purposes. Some of them were processed for immunoistochemical analysis and a subset for the analysis of PA metabolism in terms of PA content and of enzymatic activity.

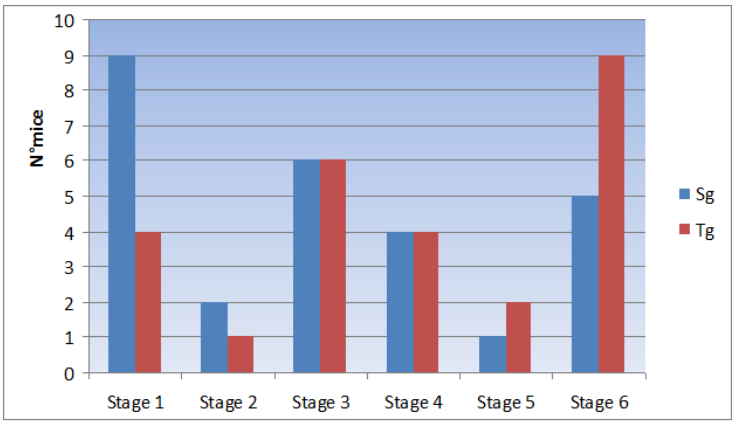
4.1.1. Behavioural evaluation

KA treated animals were monitored continuously for 6 h for the onset and extent of seizures activity. The induced seizures were scored according to a scale previously defined by Racine (Racine, 1972). Figure 4.1 shows the Racine's scale. Behavioural response is represented in figure 4.2.
Most of syngenic (Sg) mice (~ 63 %) displayed a milder phenotype than transgenic mice (Tg) ranging from stage 1 to 3. In fact, they were immobile for all time of monitoring or within 1h, they exhibited repetitive movements and head bobbing. On the contrary, within 30 min of injection 58% of Tg mice showed progressive seizures characterized by rearing and falling (stage 4 and 5), over the next hour some animals displayed also continuous tonic-clonic seizures (stage 6). No seizure activity was observed in sham animals (treated with saline).
Summarizing, most of Sg mice showed a behavioural response that is equivalent to the lowest stages of the scale. On the other hand, Tg mice, were more sensitive and vulnerable to KA treatment than Sg mice. The behavioural seizures mainly ranged from stage 4 to stage 6.
After 6 h of monitoring, all mice showed again a normal behaviour and were kept up to 1 day or 3 days before to be sacrificed according to the following experimental procedures.

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

FIG. 4.1 CLASSIFICATION OF SEIZURE PARAMETERS AFTER SYSTEMIC ADMINISTRATION OF KA.

Seizure scoring according to Racine's scale (Racine, 1972).



	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
Sg %	33,33	7,41	22,22	14,81	3,70	18,52
Tg %	15,38	3,85	23,08	15,38	7,69	34,62

FIG. 4.2 BEHAVIOURAL EVALUATION FOLLOWING KA TREATMENT.

Syngenic mice (blue) showed mainly a mild phenotype ranging from stage 1 to stage 3. On the contrary, transgenic mice (red) displayed severe behavioural seizures, equivalent to stage 3 to 6. No surviving animals were not included in this graph. Sg: syngenic; Tg: transgenic.

4.1.2. Immunoistochemical analysis

Three days after KA injection, some mice were used for immunoistochemical analysis. Sagittal brain slices were stained for spermine oxidase (SMO) as protein of interest, for NeuN (Neuronal Nuclei) as marker of neurons and for GFAP (Glial fibrillary acid protein) and Iba1 (ionized calcium binding adapter molecule) markers of astrocytes and microglia, respectively.

SMO

Figure 4.3 shows slides of cortex of Sg and Tg mice stained for SMO antibody. In detail, the panel shows layers I-IV of cortex. As expected Tg mice presented an higher expression of SMO protein compared to Sg one. After KA treatment the number of SMO immunoreactive cells decreased in both animals, Sg and Tg, compared to the animals injected only with saline. But Tg treated mice displayed a number of positive cells higher than Sg treated animal. Figure 4.4. shows the cell counting expressed as number of positive cells per 0,24 mm^2 area.

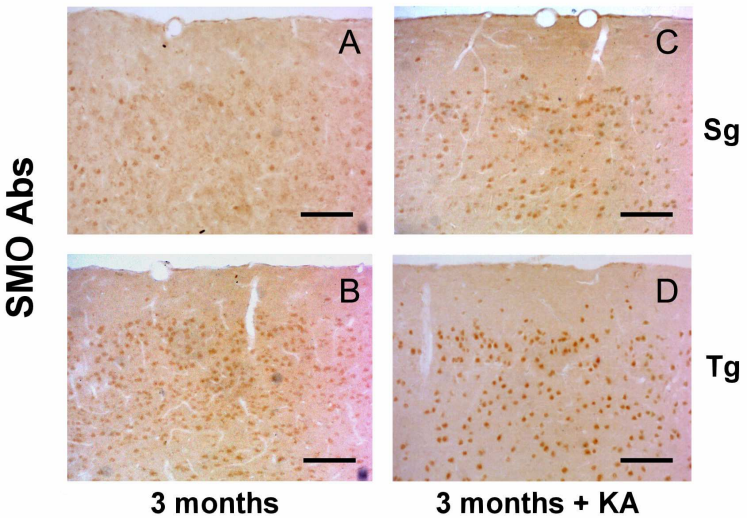


FIG. 4.3 REPRESENTATIVE IMAGES OF SMO-IMMUNOREACTIVE CELLS.

In no treatment conditions Tg mice (B) displayed an higher number of positive cell to SMO compared to Sg one (A). KA treatment induced a decrease of positivity in both groups of animal (C-D). KA: kainic acid; Sg: syngenic; Tg: transgenic. Scale bar: 1mm.

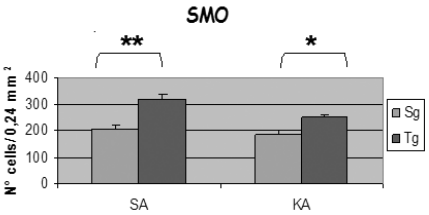


FIG 4.4. DECREASE OF SMO POSITIVE CELLS AFTER KA TREATMENT.

Cell counting was performed on cortex slides by three independent individuals for each group. This analysis confirmed the results observed by IHC: an higher number of SMO positive cells in Tg mice than controls under no treatment conditions. After KA injection a decrease of number of cells was displayed in both groups. To note that even after treatment Tg mice showed a major number of SMO positive cells. SA: saline; KA: kainic acid. Student's *t*-test (∗=P<0.05; ∗∗=P<.0.01).

NeuN (Neuronal Nuclei)

NeuN is an excellent marker for neurons infact, its expression is observed in most neuronal cell types throughout the CNS and PNS (peripheral nervous system) in both embryos and adult mice. Therefore, in the present study NeuN antibody was used to study the extent of neuronal damage in the cortex after KA treatment.

No differences are found between Tg and Sg mice under physiological condition (saline) (Fig. 4.5: A-B). But the KA administration induced a reduction of number of neurons in both groups of animals (Fig. 4.5: C-D), indicating neuronal cell death occurred. In particular, Tg mice showed an higher loss of neurons as shown also by the neuronal counting (Fig. 4.6).

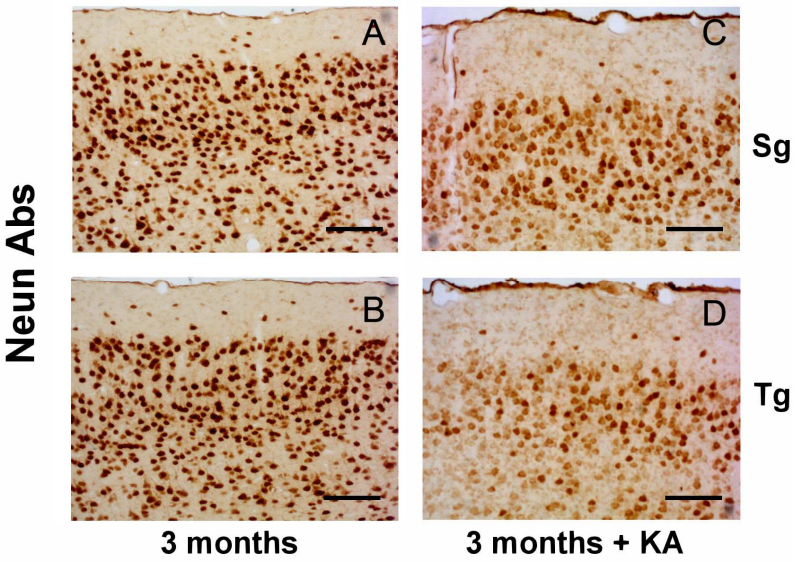


FIG. 4.5 REPRESENTATIVE IMAGES OF NEUN-IMMUNOREACTIVE CELLS.

No differences between Sg (A) and Tg (B) mice were observed under no treatment conditions. KA treatment induced a high neuronal cell loss in both animals; however the degree of neuronal cell death is higher in Tg (D) compared to Sg (C) mice. KA: kainic acid; Sg: syngenic; Tg: transgenic. Scale bar: 1mm.

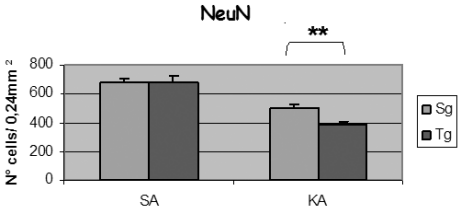


FIG. 4.6 NEURONAL CELL LOSS INDUCED BY KA TREATMENT.

Cell counting was performed on cortex slides by three independent individuals for each group. This analysis. No differences were found in the number of neurons between Sg and Tg mice. But a lower number of NeuN positive cells resulted in Tg than Sg mice after KA treatment. This data indicated a major neuronal cell loss in Tg animals. SA: saline; KA: kainic acid. Student's *t*-test (**=P<.0.01).

GFAP (Glial fibrillary acid protein)

GFAP, a class-III intermediate filament (IF), is a cell-specific marker that, during the development of the CNS, distinguishes astrocytes from other glial cells. In response to injury of the CNS, astrocytes become reactive and express high levels of the intermediate filament (IF) proteins such as GFAP. Moreover, it is also well-known that one of the onset of KA-induced excitotoxicity is astrogliosis. Thus, immunohistochemical analysis with GFAP antibody was performed to observe the effect of the treatment on astroglial population in our transgenic model.

No treated animal (Sg and Tg) showed a little GFAP positivity, especially in the first and in the second layers of the cortex (Fig. 4.7: A-B). Treated Tg mice displayed a strong reactivity to GFAP antibody, higher than Sg mice and it also involved all layers of the cortex whereas in Sg mice only some astrocytes of first layers seemed activated (Fig. 4.7). A considerable astrogliosis occurred in Tg mice after treatment.

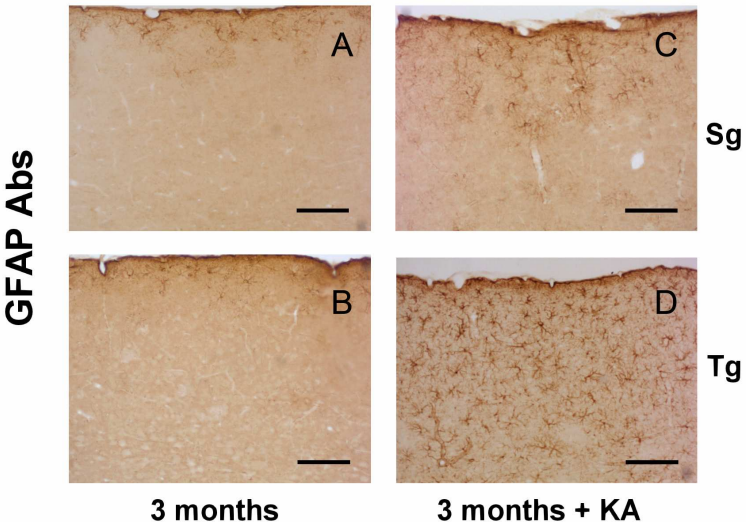


FIG. 4.7 REPRESENTATIVE IMAGES OF GFAP-IMMUNOREACTIVE CELLS.

In all sham animals (A-B) was observed a little positivity to GFAP-Abs in the first two layers of cortex. KA treatment induced astrocyte activation in both groups (C-D), but it resulted in strong gliosis in Tg (D). KA: kainic acid; Sg: syngenic; Tg: transgenic. Scale bar: 1mm.

Iba1(ionized calcium binding adapter molecule)

Iba1 (ionized calcium binding adaptor molecule 1) is a protein specifically expressed in microglia and is upregulated during activation of these cells.
Microglia are a type of glial cells that are the resident macrophages of the brain and spinal cord, and thus act as the first and main form of active immune defence in the CNS. Microglia is exquisitely sensitive to brain injury and disease, altering their morphology and phenotype to adopt a so-called activated state in response to patho-physiological brain insults. Microglial responsiveness to injury suggests that these cells have the potential to act as diagnostic markers of disease onset or progression, and could contribute to the outcome of neurodegenerative diseases.
Iba1 staining indicated that at physiological conditions there was already a little microglia activation in Tg mice compared to Sg mice (Fig.4.8: A-B). This effect was highly enhanced after KA treatment. Tg mice showed a stronger microgliosis compared to that observed in Sg mice (Fig.4.8: C-D).

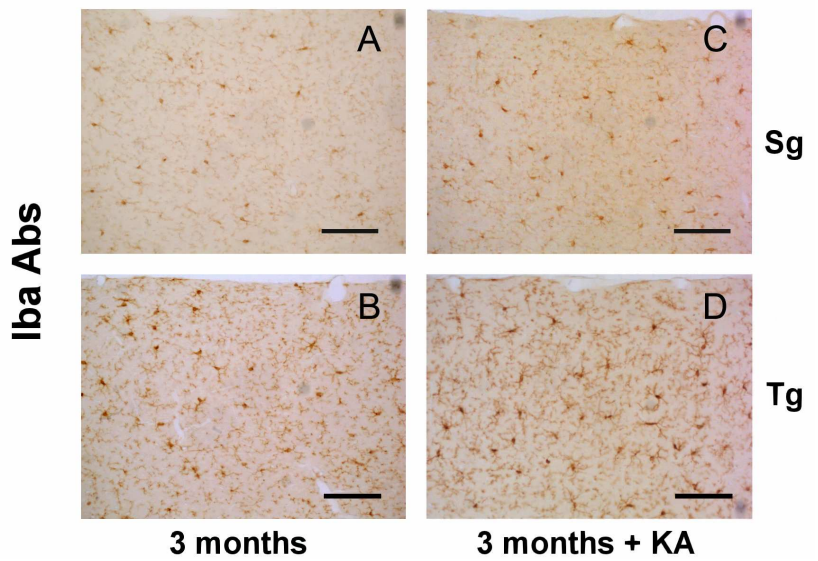


FIG. 4.8 REPRESENTATIVE IMAGES OF IBA1-IMMUNOREACTIVE CELLS.

In no treatment conditions Tg mice (B) displayed an higher number of positive cell to Iba1 compared to Sg one (A). Under KA conditions it was observed still an higher positivity in Tg mice (C) than Sg one (D). This result indicated also a considerable microglia activation in Tg mice. KA: kainic acid; Sg: syngenic; Tg: transgenic. Scale bar: 1mm.

4.1.3. Analysis of enzymatic activity of the key enzymes of PA metabolism

A subset of mice undergoing KA treatment were used for the analysis of the most representative enzymes of PA metabolism.
To this purpose, animals were sacrificed 1 day, and 3 days from the beginning of the experiment, in order to record possible changes following the drug administration. SMO, APAO, SSAT and ODC enzymatic activities were measured in cerebral cortex samples.

SMO

I like to recall that SMO catalytic reaction produces together with Spd and 3AP also H_2O_2.
Consequently, in order to analyse the SMO enzymatic activity the production of H_2O_2 was measured following the addition of the substrate Spm. Under no treatment condition Tg mice displayed an higher level of SMO activity than Sg mice. The KA treatment induced an increase of the enzymatic activity in both groups (Tg and Sg) at 1day after. In particular as shown in figure 4.9 SMO activity was considerable higher in Tg mice compared to control. No differences were found at time of 3 day after.

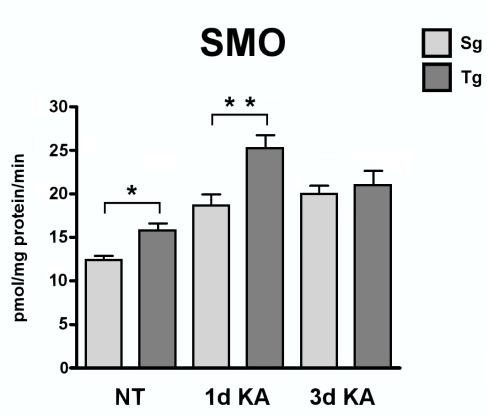


FIG. 4.9 SMO ACTIVITY AFTER KA TREATMENT.

Tg mice showed an higher level of SMO activity than Sg mice under no treatement conditions. The KA administration induced an increase of the enzymatic activity in both groups of animals. But this increase resulted in Tg significant higher compared to controls within 1 day (1d KA). No differences were observed at 3 days from KA (3d KA). NT: no treated; KA: kainic acid; Sg: syngenic; Tg: transgenic. Student's *t*- test (**=P<0.01).

APAO

Likewise SMO, the enzymatic activity of APAO was based on the production of H_2O_2 using acetylSpm as substrate.
KA treatment induced a significant increase of APAO activity in Tg mice within 1 day after, whereas no differences were observed at 3 days from the injection (Fig. 4.10).

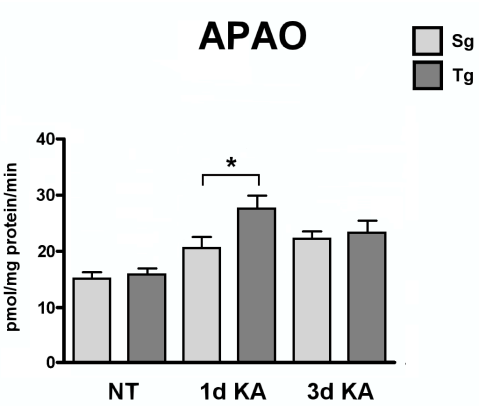


FIG. 4.10 APAO ACTIVITY AFTER KA TREATMENT.

The KA administration induced an increase of APAO enzymatic activity in Tg mice which was higher than Sg one within 1 day (1d KA). No differences were observed in NT animals and at 3 days from KA (3d KA). NT: no treated; KA: kainic acid; Sg: syngenic; Tg: transgenic. Student's *t*-test (**=P<0.01).

SSAT

SSAT acts in the PA interconversion pathway acetylating Spm and Spd to produce the equivalent acetylated PAs.
In sham-animals, in which vehicle alone was injected, the level of SSAT activity by itself was higher in Tg animals than Sg (Fig. 4.11).
KA treatment resulted in a massive increase of the SSAT activity at 1 day and remained significantly high at 3 days in the Tg mice. This increase was more than double compared to Sg mice.

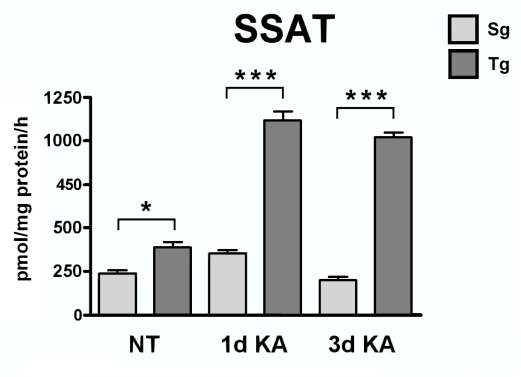


FIG. 4.11 SSAT ACTIVITY AFTER KA TREATMENT.

All Tg mice displayed a higher level of SSAT activity than Sg one in both conditions under no treatment and after KA administration. This increase significantly differed from Sg mice at 1 day and 3 days from injection. NT: no treated; KA: kainic acid; Sg: syngenic; Tg: transgenic. Student's *t*-test (∗=P<.0.05; ∗∗∗=P<.0.001).

ODC

ODC is the first enzyme in the biosynthetic pathway of PA metabolism. It catalyzes the production of the smallest PA: the Put.
ODC activity was always measured at 1 day and 3 days after KA injury.
No differences were found between Tg and Sg mice in physiological conditions and after KA treatment (Fig. 4.12). Therefore at this time points no ODC induction was recorded.

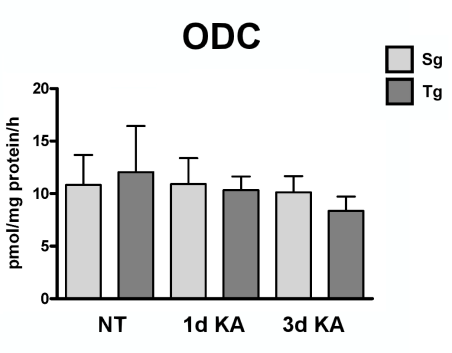


FIG. 4.12 ODC ACTIVITY AFTER KA TREATMENT.

No differences were found among all groups and in both conditions in ODC activity. NT: no treated; KA: kainic acid; Sg: syngenic; Tg: transgenic.

4.1.4. Analysis of PA content

To understand if PA metabolism could be affect by KA treatment, and if any change was occurred the content of three PAs, Put, Spd and Spm was measured by HPLC.
Figure 4.13 shows the concentrations of Put, Spd and Spm in the cortex in no treatment condition and after KA administration.
Put concentration was higher in Tg mice compared to Sg mice in sham animal group. But, KA treatment resulted in a decrease of Put content in Tg mice at 1 and 3 days, whereas in Sg mice no differences were found.
As regards Spd concentration, it was higher in all Tg mice (sham and KA-treated animals) compared to Sg mice. But, a general decrease of Spd content was observed following the treatment in either Sg or Tg mice particularly at time of 3 days.
No modifications in Spm concentration were found between Sg and Tg mice in sham and in treated animals. However, KA administration induced a significant reduction of Spm content in Sg and Tg mice after 3 days of treatment.

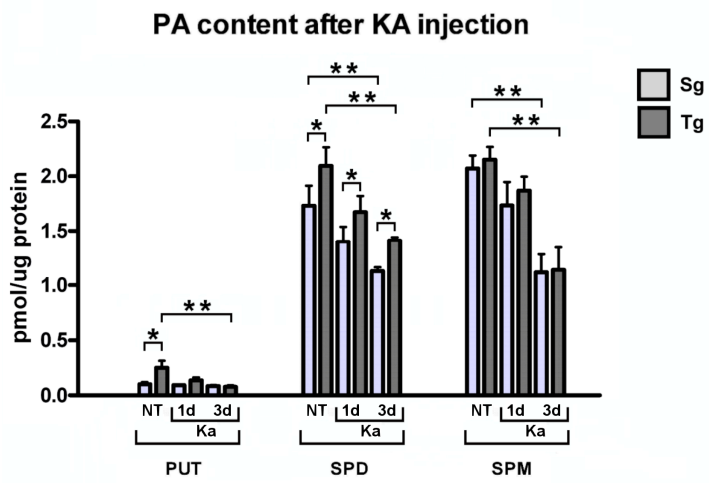


FIG. 4.13 PA CONTENT IN THE CEREBRAL CORTEX AFTER KA INJECTION.

The general effect of KA treatment on PA content was a decrease of all PAs (Put, Spd, Spm) in Tg mice, which was particular significant at 3 day after. It can also be observed an accumulation of Spd content in Tg animals, whereas no differences between Tg and Sg in Put and Spm content after KA injection. Sg: syngenic; Tg: transgenic. Student's *t*-test (∗=P<0.05; ∗∗∗=P<0.001).

4.2. ORGANOTYPIC SLICE CULTURES AS A TOOL FOR STUDYING THE LINK BETWEEN PA CATABOLISM AND KA EXCITOTOXICITY

To better understand the link between PA interconversion pathway and KA excitotoxicity, organotypic brain slice cultures were exploited.
This method allows to study the effects of compounds in an *in vitro* model system which maintains the neuronal network and the cell interactions as they are *in vivo*, thus these cultures were called "organotypic".

4.2.1. Corticostriatal slice cultures

To study the hypothetical role of SMO in KA excitotoxicity, and in particular in the cerebral cortex, corticostriatal slice cultures were performed.
The brain slice preparation is outlined in figure 4.14. Slices were obtained from P0-P1 (postnatal day) donor mice and then cultured for 3 weeks.

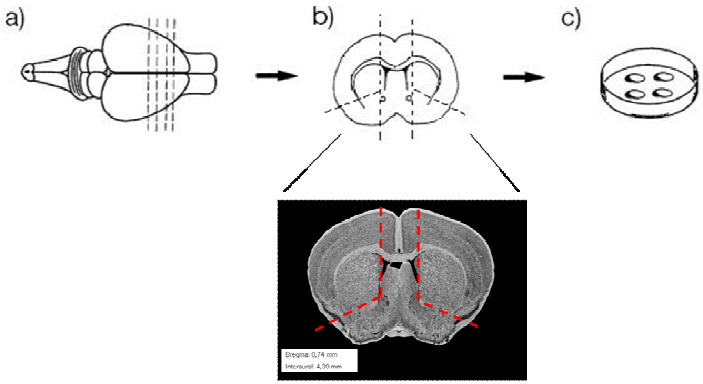


FIG. 4.14 DEMONSTRATION OF BRAIN SLICE PREPARATION.

Four 350 µm thick slices were obtained from the area corresponding to 1,32 mm to 0,40 mm anterior to bregma in the adult mice. The approximate location in the neonatal mouse is sketched in (a). Slices were trimmed and subventricular zone (SVZ) and ventral parts of striatum eliminated, hence two cultures were gained from each brain slice (b). Four randomly selected slices were placed at equal distance on semi-porous membrane and allowed to grow in incubator at 36°C (c). (Modified from Franke et al., 2003 and below from http://www.mbl.org/atlas170/atlas170_frame.html).

It is well known that corticostriatal cultures represent a suitable model to study the effect of KA induced lesions.
Taking in mind that our transgenic mice, overexpressing SMO in the cerebral cortex, are more vulnerable to KA injury than Sg one, it is conceivable that on the contrary the inhibition of SMO could result in a neuroprotective effect in excitotoxic conditions. This hypothesis is supporting by a previous work in which Liu et collegues showed that inhibition of PA oxidation decreased KA-induced cell death in organotypic hippocampal cultures (Liu et al., 2001).
The idea was to perform a similar experiment in our tissue of interest to study the effect of SMO inhibition in KA-induced excitotoxicity in corticostriatal slice cultures.
The inhibitor used was MDL 72,527 (N1, N4-bis (2,3-butadienyl)-1,4 butanediamine) that acts as competitive inhibitor of SMO (Belleli et al., 2004). Unfortunately, MDL 72, 527 is not a specific inhibitor of SMO, indeed, it was first characterized as the most irreversible and selective inhibitor of APAO enzyme, which oxidises acetylated PAs. At present, a selective SMO inhibitor SMO has not been identified yet.

4.2.2. Effect of MDL 72,527 (SMO inhibitor) on KA excitotoxicity evaluated by propidium iodide (PI) uptake and toluidine blue (TB)

PI

In several studies, the cellular uptake of the fluorescent dye propidium iodide (PI) has been used as a marker for dead or dying cells in organotypic slice cultures (Noraberg et al., 1999).
In this study PI uptake was used as marker for quantification of KA induced lesions. The experimental strategy is schematized in figure 4.15.
Corticostriatal cultures were performed and cultured for 3 weeks before starting the experiment. Then a preincubation of 24h with MDL72,527 preceded the time of KA exposure (48h). A set of pictures was taken every 24h to record the PI uptake as marker of cell death in the slices cultures.

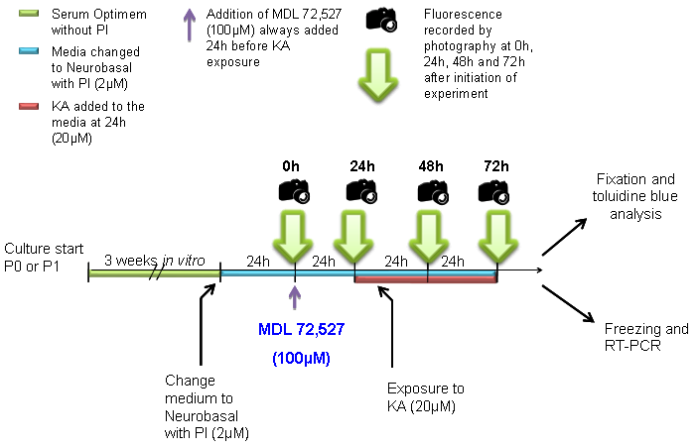


FIG. 4.15 EXPERIMENTAL PROTOCOL TO INVESTIGATE CELL DEATH BY ADDITION OF KA AND MDL72,527.

At equivalent postnatal days (EPD) 21 medium was changed to serum free neurobasal medium with the fluorescent marker PI that enters dead or dying cells. Thus, cell death was recorded by photography at 0h before adding any compounds and 24h, 48h and 72h corresponding to 48h of KA exposure. MDL 72,527 (100 µM) was added 24h before KA exposure as indicated with purple arrows. Experiments were terminated by fixation of tissue for immunohistochemical analysis or by freezing for RT-PCR. KA, kainic acid; MDL 72,527, N,N1-bis(2,3-butadienyl)-1,4-butanediamine; P0-1, Postnatal day 0-1; PI, propidium iodide.

As shown in the panel of pictures (Fig. 4.16: A-D) control slice cultures looked healthy and show a very low PI uptake. In order to study the effect of MDL 72,527 without lesion, a group of slices was treated only with this compound. MDL 72,527 induced by itself a basal PI uptake. But the degree of cell death occurring was almost the same during the 72h (Fig. 4.16: E-H).

KA treated slices displayed an high PI uptake in all cortex, indicating a widespread cell death. This effect was particularly clear at 72 h corresponding to 48 h of exposure to the neurotoxin (Fig. 4.16: I-N). The addition of MDL 72,527, before the KA administration, resulted in high PI uptake in the cortex as seen in slices treated only with KA at 48h and 72h (Fig. 4.16: O-R). On the other hand, a very low and not significant PI uptake was found in the striatum of all groups.

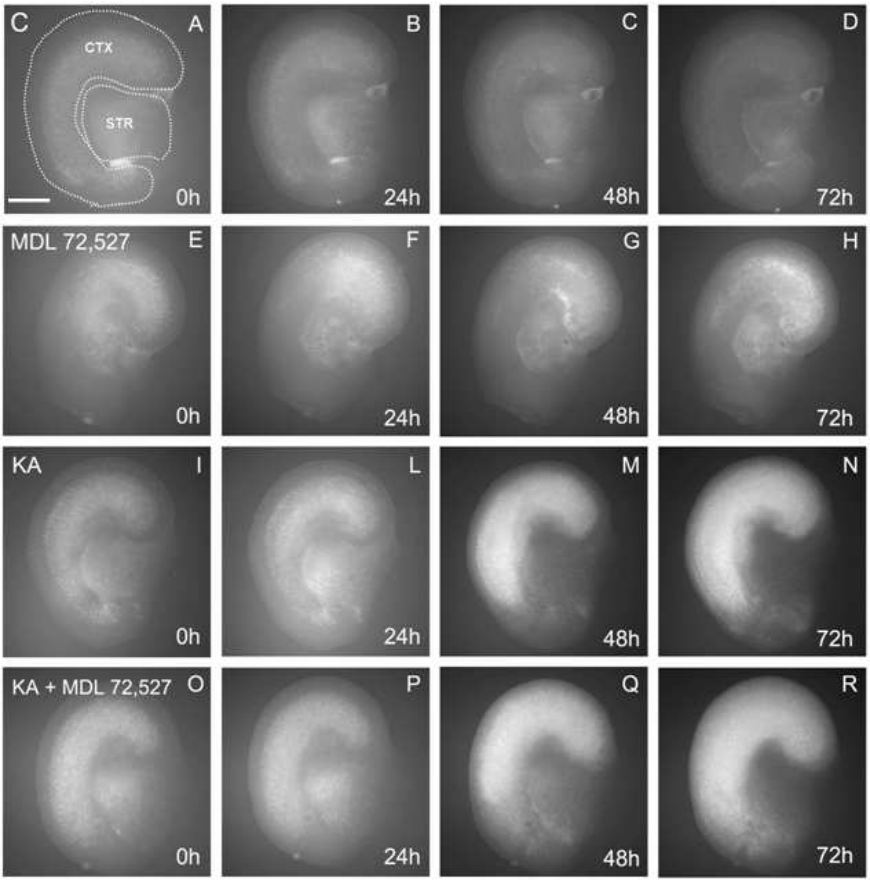
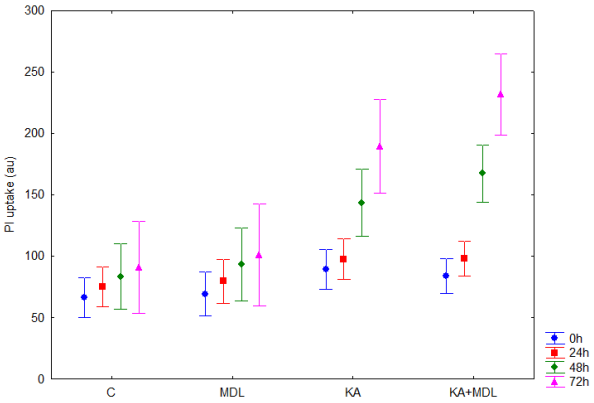


FIG. 4.16 PROPIDIUM IODIDE (PI) UPTAKE IN MOUSE CORTICOSTRIATAL SLICE CULTURES.

Slice cultures were grown for 21 days in vitro (DIV) prior to beginning of experiment, and accumulation of PI was recorded by fluorescent microscopy at different time points: 0h before exposure to any compounds and at 24h, 48h and 76h after. The first row shows control cultures (A-D) with no or very low PI uptake. The second row displays the effect of MDL 72,527 *per se* (E-H). At 24 h (F) a localized PI uptake can be observed in the cortex and it seemed the same during all time of exposure (G-H). The third row displays KA treated cultures (I-N) with high PI uptake at 48h and 72h (M-N) corresponding to the time of exposure. The last row shows the effect of the addition of MDL 72,527 together KA treatment (O-R). High PI uptake is also observed in slice cultures treated with both MDL 72,527 and KA. Example of delineation of striatum and cortex is demonstrated in top left corner. Scale bar= 1 mm applicable to all pictures. CTX, cortex; STR, striatum.

The densitometric analysis of PI uptake allowed to quantify the degree of cell death in all slices. Both tissues, cortex and striatum, were selected to measure the intensity of fluorescence indicating the PI uptake.
In cortex (Fig. 4.17) slices treated with KA showed an higher PI uptake (almost double) compared to control cultures as well as to those treated only with MDL 72,527. Fluorescence surveyed in slice treated with MDL72,527 and KA was higher than all groups at 48 h and 72 h. Statistical analysis indicated differences in all groups considered ($F_{12,272}$=6.7095, P<0.01) (Fig. 4.17: A). The pairwise comparisons performed by the post hoc Tukey test are reported in figure 4.17 B. In particular KA vs KA + MDL 72,527 was never significant at both time 24h and 48h KA exposure.
Similarly to the qualitative observation of pictures, in striatum no significant differences were found comparing all groups considered ($F_{12,251}$=1.6142, ns) (Fig. 4.18).

A



B

0h	MDL	KA	KA-MDL
C	0,995737	0,204072	0,361845
MDL		0,358203	0,565469
KA			0,964483

24h	MDL	KA	KA-MDL
C	0,981675	0,226214	0,157512
MDL		0,473529	0,391244
KA			0,999966

48h	MDL	KA	KA-MDL
C	0,963246	0,013061	0,000186
MDL		0,071382	0,001160
KA			0,558512

72h	MDL	KA	KA-MDL
C	0,983931	0,002220	0,000139
MDL		0,012246	0,000153
KA			0,345494

FIG. 4.17 DENSITOMETRIC MEASUREMENTS OF PI UPTAKE IN THE CORTEX EXPOSED TO KA AND MDL72, 527.

Quantitative analysis of PI uptake did not show significant differences between KA and KA + MDL 72,527 groups. Data are shown as arbitrary unit of PI uptake. Error bars indicate SEM (A). Significant values are indicating in red (B).

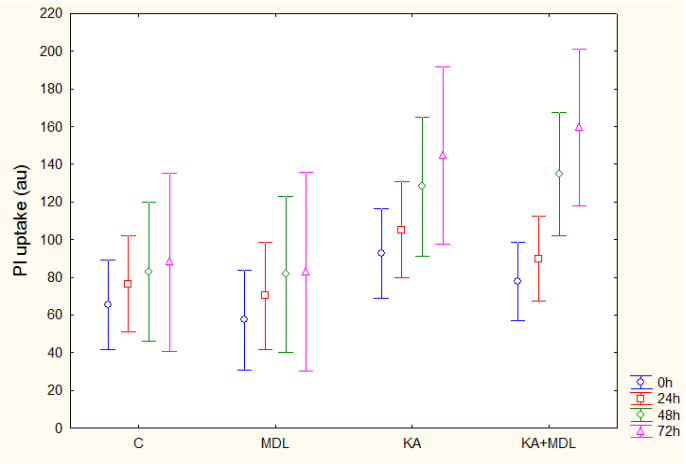


FIG. 4.18 DENSITOMETRIC MEASUREMENTS OF PI UPTAKE IN THE STRIATUM EXPOSED TO KA AND MDL72, 527.

Quantitative analysis of PI uptake did not show significant differences among all groups. Data are shown as arbitrary unit of PI uptake. Error bars indicate SEM.

TOLUIDINE BLUE

Toluidine blue (TB) is a blue nuclear counterstain, frequently employed to demonstrate Nissl substance. Neurons contain Nissl substance, which is primarily composed of rough endoplasmic reticulum, with the amount, form, and distribution varying in different types of neurons. By varying the pH and the degree of differentiation, both Nissl substance and nuclei may be stained. Nissl substance is lost after cell injury and if the axon degenerates.
The extent of damage was examined by applying this dye.
Corticostriatal cultures were cut at cryostat and stained with TB. This dye enables to look at morphology of the slices and at the same time it gives information in regard to cell loss.
The staining was performed on the same groups used for PI analysis. The cultures were compared qualitatively for any differences in staining intensity as well as structural dissimilarities.
There were no differences between cultures treated with MDL 72,527 and control slices (Fig. 4.20: A-D). They maintained the layer organization of cortex and a deep blue staining.
Slices cultures with KA induced lesion, displayed a high degree of cell loss in deeper layers (V-VI) , and pycnotic (necrotic) cells in layer III-IV as it can be observed by a lither staining (Fig. 4.20: E-F).
Opposing to the result obtained with PI measurements, KA + MDL 72,527 treated slice cultures showed a cell loss not comparable to that in KA group. The extent of damage was lower as indicated by the intensity of staining, suggesting a neuroprotection effect (Fig. 4.20: G-H).
Likely to PI analysis, the TB staining of striatum do not show differences between all groups considered.

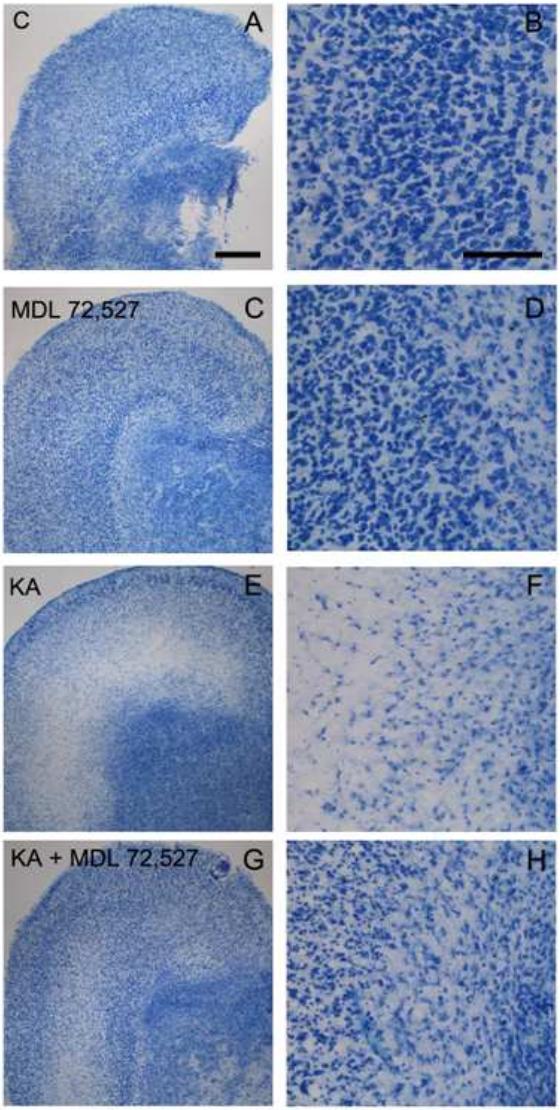


FIG. 4.20 TOLUIDINE BLUE STAINING OF CORTICOSTRIATAL CULTURES TREATED WITH KA.

No differences were found in intensity of staining and structure between MDL 72,527 treated slices (C-D) and control cultures (A-B) in the cortex. Slices with KA lesion displayed a high cell loss especially in the deeper layers of cortex (E-F). KA + MDL 72,527 slices showed a lighter staining than control in some layers (III-IV) of cortex, but the cell death was definitely less compared to KA treated cultures (G-H). Scale bar = 1 mm is applicable to all pictures.

4.2.3. RT-PCR

At the end of the experimental protocol a group of corticostriatal cultures were used for RT-PCR assay in order to study how PA metabolism was affected by KA treatment.
For this analysis only KA treated and no treated (control) slices were considered. Slice cultures were frozen and then cortex tissue was isolated from each slice. RT-PCR was performed for key enzymes of PA metabolism: SMO, APAO, SSAT, ODC. As shown in figure 4.21 A the expression of SMO, APAO and SSAT was induced under KA conditions. The densitometric analysis is reported in figure 4.21 B.

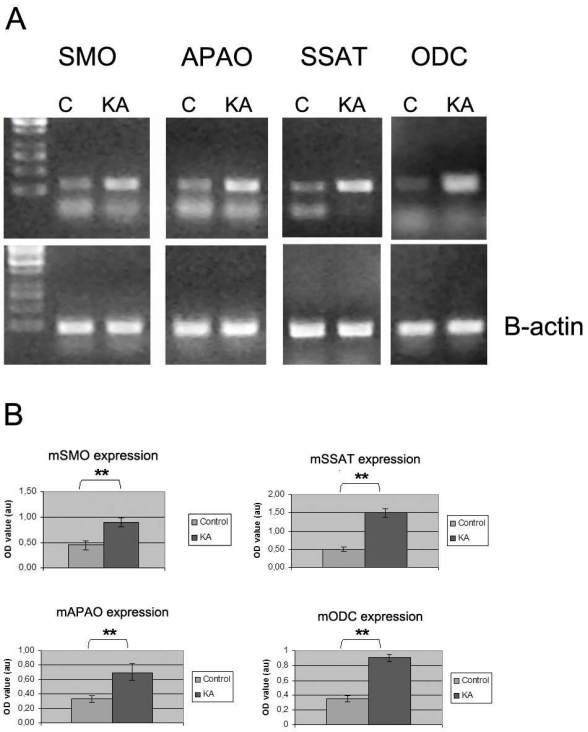


FIG. 4.21 RT.PCRS DETERMINATION OF SELECTED GENES IN CORTEX.

Cortex samples from control and KA treated slice cultures were analyzed for the expression of key enzymes of PA metabolism. (A) Representative RT-PCR experiments are shown. (B) Densitometric analyses of PCR gel bands. Gene of interest/β-actin ratios have been used for normalization. An arbitrary densitometric unit is shown. The P values were measured with the Student's *t*- test (**=P<0.001).

5 Discussion and Further Perspectives

Alteration of PA metabolism has been found in several pathological conditions ranging from neurodegenerative disorder to stroke (Morrison et al., 1993; Bernstein & Muller 1995; Paschen et al., 1991; Yatin et al., 1999; Virgili et al., 2006; Velloso et al., 2009; Gilad & Gilad, 1992; Koenig et al., 1989, Seiler et al., 2000).

These evidences have been addressed research to focus attention on this pathway in order to gain knowledge about the link between PAs and brain.

In this perspective the present work aimed to better understand the contribution of SMO enzyme, highly expressed in the brain, to neurobiological functions of PAs.

To test the hypothesis of key role of SMO under pathological conditions in the brain I have performed *in vivo* study on transgenic mice overexpressing SMO specifically in the cortex (Dach::SMO transgenic line).

Kainic acid (KA) treatment was used as strategy to induce excitotoxic conditions, which occur in several brain disorders.

Behavioural evaluation based on a well-defined scale (Racine et al., 1972) suggested a major vulnerability of Tg mice. Their behavioural response resulted mainly in repetitive falling and severe seizures which correspond to the highest stages of the scale. On the other hand, Sg mice displayed phenotypes such as immobility, rigid posture and head bobbing, typical of the lowest stages.

This differential sensitiveness to KA was reflected also looking at semithin cortical slides performed by IHC analysis.

Infact, Tg mice overexpressing SMO showed major number of SMO-immunoreactive cells if compared with Sg one. KA treatment induced a decrease of SMO positive cells in both groups of animals. The observed reduction of SMO positivity seemed depend on neuronal cell loss as showed by NeuN immunoistochemistry. It is considerable that Tg mice displayed an higher cell loss compared to control animals suggesting KA has a major and harmful effect in neurons overexpressing SMO.

This major extent of damage in Tg animals was also confirmed by glial response. Indeed, after treatment Tg mice were characterized by a strong gliosis observed by GFAP and Iba1 antibodies.

The analysis of PA metabolism after the administration of drug highlighted an induction in Tg mice of the key enzymes of the catabolic pathway (SMO, APAO and SSAT). On the contrary, the ODC activity did not show differences between Tg and Sg mice. This result seems in contradiction with literature which demonstrated ODC induction after brain injury (Agnati et al. 1985; Bernstein & Muller 1995; Pegg et al. 1995; Marton & Pegg 1995; Reed and De Belleroche, 2006).

However, ODC is known to be a high inducible enzyme with a half-life that is among the shortest of any known protein (10-30 minutes in mammalian

system). The range of analysis (1 day and 3 days from KA) could not allow to record a change on the activity of this enzyme.
The decrease of PA content seen in Tg mice after treatment reflected this enhancement of PA catabolism which not results in an accumulation of Spd and Put. Since KA induced a notable increase of SSAT activity in Tg mice it is assumable a massive acetylation process of Spm and Spd. Acetylated PAs (N^1-acetylSpd > N^1-acetylSpm) may be exported from cell as well as Put, may be exported from cell.
This could justifies the reduction of all the three PAs observed after KA injection.
If SMO overexspression resulted in a major susceptibility to KA, the question raised was: could SMO inhibition lead to a neuroprotection in excitotoxic condition?
To answer this query an *in vitro* approach was carried out. Organotypic brain slice cultures were the model system chosen to this purpose.
Use of this kind of cultures allowed to perform this study on a system able to maintain the complex network between neurons, thus mimicking better the *in vivo* environment.
In this project, corticostriatal cultures were exploited to test the potential neuroprotective effects of MDL 72,527, a well-defined SMO inhibitor, against KA neurotoxin.
Concerning the cortex, PI uptake showed (Fig 4.16) a considerable cell death at 48h and 72h in KA treated slices as well as in those pre-treated also with MDL 72,527. The addition of SMO inhibitor seemed not to decrease, unexpectedly, the cell death occurring after KA lesion. Moreover, statistical analysis did not pointed out differences between these two groups, but only with control cultures and MDL 72,527 alone. Interestingly, MDL 72,527 induced by itself a basal PI uptake which seemed to not increase during the experiment.
As striatum concerns, no differences were found in PI uptake among all groups as also confirmed by statistic analysis suggesting no effect on this tissue.
A possible explanation of no reactivity of striatum to KA administration could find in a differential response between these two tissues. Indeed, in spite of corticostriatal cultures were well characterized as feasible method for screening the neurotoxic effects of glutamate receptor agonists, for instance KA, all studies were performed on rats. Therefore it is possible to hypothesize that cortex and striatum display a differential sensitiveness to KA in mouse animal model. This different response might be due to AMPA and KA subunits composition which may differ in the two brain regions. Striatum of mouse corticostriatal cultures could express AMPA and KA receptors not mature after three weeks in culture.
Stated these interpretations, corticostriatal cultures derived from mice might require an higher concentration of KA than that used in the present work

(>20µM). Furthermore, the TB staining (data not shown) did not disclose any cell loss in this area that, on the contrary, could explain no PI uptake.
TB staining on the cortex showed no differences between control and MDL 72,527 slices and it also confirmed the high cell loss in KA treated slices. However, differently from PI pictures, looking at slices exposed to KA and MDL 72,527 cell death was strongly lower than KA treated slice, suggesting a potential neuroprotective effect.
The discrepancy observed in cortex between PI uptake and Nissl staining (TB) requires a comparison of both methods used.
Indeed, PI can be considered a "dynamic" dye which allows us to record dying cell in a certain time (0h, 24 h, 48h, 72 h) and therefore it cannot give informations about all time of experiments. Additionally, PI might enter cells that are transiently undergoing membrane disruption, without dying and make them appear as dying cells. It also must be considered that corticostriatal cultures are slices 350 µm of thickness, therefore the intensity of fluorescence is the result of several layers. On the contrary, TB staining was performed on semithin slices (20 µm) and it might better reflect the final effect of the combination of KA and MDL 72,527.
The hypothesis of the potential neuroprotective effect of MDL 72,527 is also supported by data in literature.
On organotypic hippocampal slice cultures (Liu et al., 2001) has been demonstrated that treatment with MDL 72,527 provides significant, albeit partial, neuronal protection against KA excitotoxicity. The protection was more pronounced in CA1 than CA3 and was associated with decreases in lipid peroxidation and activation of glial cells. *In vivo* treatment with MDL 72,527 has been found to be neuroprotective against edema and delayed cell damage after traumatic brain injury (TBI) (Dogan et al., 1999).
But further analysis are needed to validate the potential neuroprotective role of MDL 72,527 in excitotoxic condition and brain injury as well.
In conclusion, these data stress the idea that PA catabolism in strongly involved in KA-induced neurodegeneration.
In particular, SMO involvement in this pathway might depend on the double implication of its enzymatic activity. Either production of toxic metabolites (H_2O_2, 3-aminopropanal and consequently acrolein) or modulation of Spm content in neurons could exacerbate the KA-induced excitotoxicity. Together these two effects could contribute to speed up the neuronal cell death induced by different stimuli. This speculation might account for the major sensitiveness of Tg mice to KA treatment and for astrogliosis as well.
It would be interesting to perform corticostriatal cultures on Dach::SMO mice in order to study if SMO inhibition could revert the higher sensitiveness observed in Tg animals.
In order to better understand the role of SMO in neuron function, knowledge of subcellular localization of SMO and Spm is crucial in these cells. Since Spm is

stored within synaptic vesicles, it becomes interesting to understand if SMO enzyme localizes also in the synaptic area and moreover, in which conditions SMO could interact with its substrate.
These results much more point out SMO as therapeutic target against neurodegeneration induced by excitotoxicity. Treatments that reduce SMO cytotoxic byproducts may be expected to protect against neurodegeneration.
Hopefully a specific SMO inhibitor may help in this direction, allowing to better define the exact mechanism by which this enzyme participates to excitotoxicity.

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Materials

Standard compounds spermine (spm), N1-acetyl-spermine (N1-AcSpm), 1,6-diaminohexane (1,6-DIA) and kainic acid (KA) were obtained from Sigma-Aldrich, St. Louis, MO. Dansylchloride from Applichem. MDL72,527 was a generous gift from Hoechst Marion Roussel Inc. Antibodies: Anti-SMO was produced in our laboratory, anti-NeuN was from Milllipore, anti-GFAP was from Dako and anti-Iba1 was from BIOCARE-Medical. Reagents for PCR (DreamTaq polymerase, Green Taq Buffer and dNTP) were purchased from Fermentas. Other compounds were from Sigma-Aldrich, Bio-Rad (Bio-Rad Italia, Milano, Italy) and J. T. Baker (Baker Italia, Milano, Italy).

Culture Media

Serum containing media (100ml)

25ml Horse serum	GIBCO
50ml Opti-MEM® 1 reduced serum medium	GIBCO
25ml Hanks balanced salt solution	GIBCO
1ml 50% glucose	

Defined serum free medium (500ml)

490ml NEUROBASAL™ Medium	GIBCO
10ml B-27 supplement	GIBCO
2500µl L-glutamin	GIBCO

NEUROBASAL™ Medium : Contains no L-glutamine, L-glutamic acid, or aspartic acid.

Animals

Male C57BL/6 strain mice were housed four per cage in standard laboratory conditions (food and water ad libitum, 22±2°C and 12:12 h light: dark cycles).

Genotyping of mouse lines

Mouse genotype was determined by PCR technique on total DNA isolated from tail biopsies.

DNA extraction from mouse tail

A fragment of tail was cut from mice and place in 1.5 mcf tube for digestion. Scissors was washed with 75% EtOH between cuts to prevent cross contamination.

Tail samples derived from both GFP-SMO line and Dach::SMO line were first observed at fluorescence microscope in order to verify the presence of the transgene *GFP* and consequently of *SMO*.

Afterwards, samples were digested in 450 µl of lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS) containing proteinase K (1 mg/ml; SIGMA) and incubated o/n at 56°C. Then samples were centrifuged 10 min. at 13200 rpm and supernatants were used for DNA extraction.

PCR analysis

Presence of different transgenes was detected by PCR reactions.

PCR Mix

0,8 µl	DNA sample
2.5 µl	GreenTaq 10X buffer (Fermentas)
0.5 µl	primer forward (100ng/µl)
0.5 µl	primer reverse (100ng/µl)
0.5 µl	dNTPs (10mM; Fermentas)
0.625 U	DreamTaqGreen polymerase (Fermentas)
17.5 µl	water

GENE	Primer name	Primer sequence
SMO	SMO-2	5'-AAATATCTCGAGGGAACACAT TTGGCAGTGAGG-3'
	SMO-5	5'-TCATCCCCTCGGGCTTCATG-3'
Cre	CRE-F1	5'-TGTCCAATTTACTGACCGTA-3'
	CRE-R2	5'-CTAATGGCCATCTTCCAGCAG-3'

Table I: Primer sequences

PCR Profile for SMO-2 and SMO-5 = CRE F1- CRE R2

94ºC	2 min.	
94ºC	30 sec.	
60ºC	30 sec.	35/30 cycles for SMO and CRE respectively
72ºC	90 sec.	
72ºC	10 min.	
4ºC	forever	

PCR products were separated in eletroforetic agarose gel 1%

KA administration and Behavioural analysis

KA was dissolved in 0.9% phosphate-buffered saline (PBS). On the day of the experiment, mice were injected with saline solution (n=26) or with KA (n=31) 25 mg/kg. They were monitored continuosly for 6 h to perform the behavioural evaluation. The precence of *status epilepticus* was evaluated according to the behavioural scale proposed by Racine, 1972.
They were returned to their cages and sacrificed 1 or 3 days later according to the experimental procedures.

Immunoistochemestry staining

Treated mice (n=3) were selected only for histological examination, which was performed following transcardiac perfusion.
Mice were anaesthetized with urethane 25% and then perfused with saline (0,856 % NaCl, 0,025 % gr KCl, 00,2 % NaHCO3), followed by a solution of 4% paraformaldeyde (pH 7.2). The brains were removed and divided in two emipheres by making a sagittal cut; the two half were immersed in cryoprotective solution (0,9 % NaCl, 30% sucrose) at 4°C for three days, with a change of the solution 24 hours later.
The fresh brains were cut in slices (14 µm) by freezing microtome (Microm HM 400). Slices were collected in a multi-well plate (6 wells): 4-6 slices per well, containing phosphate buffer 0,1M. The staining with the different antibodies was performed directly in the wells. After staining the slices were coverslipped with glycerol/phosphate buffer 0,4 M (3:1) mounting solution. Immunohistochemical staining was performed using monoclonal mouse antibody specific for SMO and NeuN, and policlonal mouse antibody for GFAP and Iba1. Biotinylated anti-mouse IgG or anti-goat were used as secondary antibodies, followed by a preformed **A**vidin

and **B**iotinylated horseradish peroxidase macromolecular **C**omplex (Vector Laboratories; ABC Elite). The reaction was developed using diaminobenzidine (DAB; Vector Laboratories), as chromogen substrate and 0.01% H2O2. Endogenous peroxidise activity was blocked by 3% H2O2.

Polyamine determination

Polyamine pools were quantified using dansyl chloride labelling followed by HPLC. Briefly the cortex samples were homogenised by sonication (tissue/volume=1/5) in perchlorid acid PCA 5% containing the internal standard (1,6 diaminohexane, 100 μM) and incubated at 4°C overnight. The samples were centrifuged for 10 min at 13200 rpm. Supernatants were processed for dansyl chloride labelling (Martinez et al., 1991). For HPLC analysis a C18 Hypersil BDS (250 x4,6 mm, particle size 5μm) was used and the fluorescence detection for dansylated derivatives of polyamines was performed at 365 and 510 nm for excitation and emission wavelengths, respectively. The elution was performed with a gradient consisting of a solvent A: MeOH and a solvent B: H2O2. Initial conditions (A: 40%) were maintained for 2 min., and then a 20 min. linear gradient from 60 % to 95 % of solvent A was run. Final conditions were maintained for 6 min. The solvent flow rate was adjusted to 1 ml/min.

Determination of SMO and PAO enzyme activities

In cortex samples SMO and APAO enzymatic activities were determined by measuring the production of H2O2 as pmol produced/mg protein/h, following the oxidation of their specific substrates as reported previously (Wang et al., 2003). Protein concentration was estimated by the method of Bradford (Bradford, 1976). Briefly 250 μg of tissue sample was used for the assay utilizing Spm and N1-acetylated Spm as substrates for SMO and APAO, respectively. In details, enzyme activity was assayed in 83 mM glycine buffer, pH 8.0, 5.0 nmol luminol, 20 μg horseradish peroxidase, 0.2 mM 2-bromoethylamine (copper-containing amine oxidase inhibitor/catalase inhibitor), 15 μM deprenyl (mitochondrial oxidase B inhibitor), 0.15 mM clorgyline (mitochondrial oxidase A inhibitor) and 250 μM Spm or alternatively N1-acetylated Spm as the substrate. All reagents with the exception of substrate were combined in a volume of 250 μl and incubated for 2 minutes at 37°C, transferred to the luminometer where substrate was added, and the resulting chemiluminescence was integrated over 40 seconds.

Spm + SMO --> H_2O_2 + luminol --(POD)--> Light

N1-acetilSpm + APAO --> H_2O_2 + luminol --(POD)--> Light

Determination of SSAT and ODC enzyme activities

Both ODC and SSAT activities were determined as pmol CO2 produced/mg protein/h by using 14C-labeled substrate and scintillation counting of end metabolized products. Cortex samples were sonicated and centrifuged at 22,000 g for 10 min at 4°C. The SSAT enzyme activity was determined as described by Chen et al. (Chen et al., 2003). In particular, 75 µg of homogenate tissue sample in a final volume of 50 µl included 10 µl of 5.5 M Bicine buffer (pH 8.0), 5 µl of 30 mM Spd, 10 µl of doubly distilled water, 5 µl of 0.1 mM [14C]acetyl-CoA (53 mCi/mmol; Sigma) and 20 µl of each sample. The mixture was incubated for 5 min at 37°C. The enzyme reaction was stopped by the addition of 20 µl 0.5 M hydroxylamine hydrochloride, and the mixture was heated in boiling water for 3 min. The resulting samples were centrifuged (22,000 g), and an aliquot of 50 µl was spotted onto Whatman P81 phosphocellulose discs and counted for radioactivity in a liquid-scintillation counter. The ODC enzyme activity was determined as follows: 100 µg of homogenate tissue sample in a final volume of 100 µl including 20 mM Tris Buffer (pH 7.5), 1 mM EDTA, 0.1 mM Piridoxal 5' POH, 5 mM DTT, 0.1 mM [14C] L-Ornithine-carboxy (55 mCi/nmol; Sigma) and 0.4 mM L-Ornithine was put in a screwedcap plastic "bijoux" vial. A disk of filter paper, embedded with 30 µl of 2N NaOH, was screwed with the cap on the top of the vial. The mixture was incubated for 30 min at 37°C. The enzyme reaction was stopped by the addition of 200 µl of 10% TCA, then the mixture was further incubated for 10 min at 37°C to ensure complete CO2 adsorption on the filter paper that was finally counted for radioactivity in a liquid-scintillation counter.

Organotypic slice cultures

Newborn to one day old mice (postnatal day: P0-1), were killed by decapitation at the foramen magnum level. The entire brain was cut in coronal slices (350 µm) by a McIlwain tissue chopper (Mickle laboratory, Cambridge, UK). Only slices comparable to levels between 1,32 mm anterior to 0.40 mm posterior to the bregma in adult mice were used (Paxinos & Watson, 1995). From each mouse, an average of 3 slices was collected (Figure Ia). To obtain the cortico-striatal cultures, the slices were placed in Gey's balanced salt solution with glucose (GBSS, GIBCO) and

dissected by cutting through the dorsal cortex (Figure Ib), eliminating subventricular zone (SVZ) and a second cut angled about 45° from the ventral horizontal plane to avoid inclusion of tissue from medioventral parts of the neostriatum, globus pallidus and ventral striatum. This was done on both sides of the hemisphere to achieve 6-4 cortico-striatal cultures per brain.

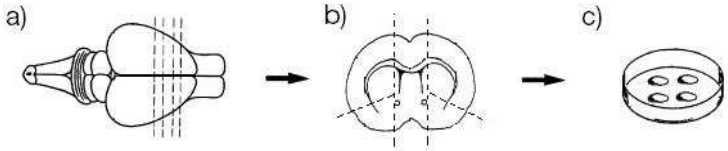

Figure I. Demonstration of brain slice preparation

Three 350 µm thick slices were obtained from the area corresponding to 1.32 mm anterior to 0.40 mm posterior to bregma in the adult mouse. The approximate location in the neonatal mouse is schematize in (a). Slices were trimmed and subventricular zone (SVZ) and ventral parts of striatum eliminated, hence two cultures were gained from each brain slice (b). Four randomly selected slices were placed at equal distance on semi-porous membrane and allowed to grow in incubator at 36°C (c) (Modified from Franke et al., 2003).

Four cortico-striatal slices were randomly placed at equal distance on porous (0.4µm) transparent membrane (Ø: 30mm, Millicell-CM, Milipore Corporation) (Fig. Ic) inserted in culture trays with 6 wells (Corning Costar, Corning, NY, USA) containing 1 ml serum containing media. Finally, the cultures were left grow in an incubator with 5% CO2 and 100% humidity in atmospheric air at 36°C. Serum containing media was changed 2 times a week during the three weeks of culturing and no antimitotic or antibiotic drugs were used at any stage. The age of the culture is denoted as equivalent postnatal days (EPD) corresponding to the age of animal at the time of slice preparation, and the total numbers of days *in vitro*. The whole procedure of preparation of the cultures was done sterile.

Toluidine blue cell staining

For histological evaluation of the corticostriatal slice cultures, TB staining was performed on cryostat sections of cultures previously fixed in 4% PFA and cryoprotected in 20% sucrose. The sections were thawed at RT for 30 min, washed in Tris-buffered saline, pH 7.4 (TBS) for 15 min followed by rinsing in distilled water for 2 min before incubated in theTB solution for 10 min followed by 3×5 min washes indistilled water to get rid of the excess of TB

solution. After dehydration in 99% ethanol 3×5 min, sections were cleared in xylene 3×5 min, and coverslipped with DePex mounting medium.

Image analysis and calculation

The fluorescent compound propidium iodide (PI or 3,8-Diamino-5-[3-(diethylmethyl-ammonio)propyl]-6-phenylphenanthridinium diiodide, Sigma) was used to monitor cell death. PI is a stable fluorescent dye that absorbs blue-green lights at 493 nm and emits red light at 630 nm. Being a polar compound it only enters cells with damaged cell membranes and once inside the cells it binds to nucleic acids. It is basically non-toxic to neurons (Hsu et al., 1994; Pozzo Miller et al., 1994; Rudolph et al., 1997; Sakaguchi et al., 1997; Kristensen et al., 2007) and is therefore thought to accumulate in dead or dying cells thus providing the investigator with a momentary estimation of cell death at the time of recordings. By modifying previously schedules used to examine cell death in cortico-striatal cultures (Kristensen et al., 1999; Noraberg et al., 1999) the protocol demonstrated was used. 24 h prior to experiment initiation, at 21 in vitro days (DIV), the culture medium was changed from serum containing medium to chemically defined serum-free Neurobasal medium supplemented with B27, containing 2µM PI to allow for a sufficient accumulation in the damaged cells. Slice cultures were exposed to the KA (Sigma), in a concentration of 20µM for a total of 48 h. PI accumulation was recorded by inverted fluorescence microscope (Olympus IMT-2, 4x magnifications) with a standard rhodamine filter and a digital camera (Sensys KAF 1400 G2, Photometrics, Tucson, AZ, USA) with at an exposure time of 0.75 sec. The first photograph was taken just before the cultures were exposed to KA (21DIV) to record the initial cell death and then at intervals of 24 h for a total of 72 h. Finally, at each experiment, a picture of the semi-porous membrane itself was taken daily as a measure of background PI staining. After last photography slice cultures were fixated for a total of 30 min for later immunohistochemical analysis. First 4% PFA was added to the bottom of the wells for 5 min and then slices were covered by PFA and allowed to react for additional 25 min.

RT-PCR

Slices were frozen in liquid Nitrogen and then placed in a new sterile multi–well plate. The cortex was separated from striatum by cutting with a blade and transferred in eppendorf tube. Total RNA was extracted using the NucleoSpin RNA II kit (Machery-Nagel). RNA quantification was performed with

NanoDrop ND 1000 Spectrophotometer. cDNAs were obtained by using High Capacity cDNA Reverse Transcription kit (Applied Biosystems).
The cDNA were amplified using the primers reported in table II and as a loading control, β-actin cDNA was also amplified.

GENE	Primer name	Primer sequence
SMO	SMO-F	5'-GTACCTGAAGGTGGAGAGC-3'
	SMO-R	5'-TGCATGGGCGCTGTCTTGG 3'
PAO	PAO-F	5'-AATATCTGGAGAAGGCCAGGTCTCTGGAGG-3'
	PAO-R	5'-AAATATCTCGAGAAGCTACCAGTAAGATCCC-3'
SSAT	SSAT-F	5'-CGTCCAGCCACTGCCTCTG-3'-
	SSAT-R	5'-GCAAGTACTCTTTGTCAATCTTG-3'
ODC	ODC-F	5'-TCCAGGTTCCCTGTAAGCAC-3'
	ODC-R	5'-CCAACTTTGCCTTTGGATGT-3'
ACTIN	ACTIN-F	5'-TGTTACCAACTGGGACGACA-3'
	ACTIN-R	5'-AAGGAAGGCTGGAAAAGAGC-3'

Table II: Primer sequences

PCR Profile for SMO/APAO/SSAT/ODC

94ºC	4 min.	
94ºC	30 sec.	} 35 cycles/
60ºC	30 sec.	
72ºC	60 sec.	
72ºC	10 min.	
4ºC	forever	

Statistical analysis

Densitometric measurements of PI uptake were expressed as mean±SEM, and differences of $p<0.05$ were considered significant. Statistical significance was determined by using ANOVA followed by Bonferroni's post hoc analysis of relevant experimental groups. All other data significance was assessed with Student's t-test.