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# The subthalamic dopamine D5 receptors in Parkinson's disease

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**Abstract.** Parkinson's disease is a neurological disorder characterized by the manifestation of the cardinal motor symptoms, which are attributed to the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc). It has been reported that the burst firing is a pathological signature of STN neurons in Parkinson's disease, however, the origin of bursts remains unknown. Here we tested the hypothesis that dopamine D5 receptors, characterized by a high constitutive activity, may contribute to the emergence of burst firing in the STN and therefore in the manifestation of parkinsonian motor deficits. We tested this hypothesis and have shown that application of an inverse agonist inhibits D5 receptors constitutive activity within the STN and reduced burst firing of STN neurons in brain slices. Moreover, it converted pathological STN burst firing into physiological tonic activity in the 6-OHDA rat model of Parkinson's disease. These results are the first to demonstrate that subthalamic D5 receptors are involved in the pathophysiology of Parkinson's disease and that administering an inverse agonist to these receptors may alleviate motor symptoms.

**Keywords** Parkinson's disease – Subthalamic nucleus – Dopamine D5 receptors

## 1 Introduction

Parkinson's disease is a neurological disorder characterized by the manifestation of the cardinal motor symptoms, akinesia, rigidity and tremor at rest. These motor disorders are attributed to the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc), which results in dopamine depletion in the striatum and also in extrastriatal nuclei such as the subthalamic nucleus (STN) (Benazzouz et al., 2014). The STN is a basal ganglia structure involved in the control of movement and plays a critical role in the pathophysiology of Parkinson's disease. Previous studies have shown that the tonic regular pattern of STN neurons in a normal situation becomes bursty after dopamine neuron degeneration in animal models (Bergman et al.,

1994; Ni et al., 2001) and also in patients with Parkinson's disease (Benazzouz et al., 2002). Lesioning the STN has been shown to reverse the motor deficits induced by MPTP in non-human primates (Bergman et al., 1990). However, the beneficial effect was accompanied by dyskinetic abnormal movements. Nevertheless, to avoid these irreversible side effects, we proposed to replace the lesion by high frequency electrical stimulation of the STN, which dramatically alleviated Parkinsonian-like motor deficits in MPTP-intoxicated monkeys (Benazzouz et al., 1993) and later in patients with advanced stage Parkinson's disease (Limousin et al., 1995). It is now accepted that the burst firing is a pathological signature of STN neurons in Parkinson's disease; however, the origin of bursts and their causal link with motor deficits remain unknown. In our recent study we hypothesized that the constitutive activity of dopamine D5 receptors may be involved in the genesis of burst firing in the STN and consequently in the development of the associated motor deficits.

## 2 Methods

In vitro and in vivo electrophysiological studies as well as behavioral experiments associated with local infusion of dopamine agents in the STN were performed in the rat.

## 3 Findings and argument

The STN dopamine D5 receptors, which display a high agonist-independent constitutive activity are able to potentiate burst firing of STN neurons in in vitro rat brain slices (Baufreton et al., 2003). More recently, we have shown that local microinjection of an inverse agonist of D5 receptors reduced burst activity of STN neurons in vitro and transformed the burst firing in the regular tonic activity in vivo. This normalization of firing pattern was associated with the improvement of motor deficits in the 6-OHDA rat model of Parkinson's disease (Chetrit et al., 2013).

## 4 Conclusion

These results are the first to demonstrate that the constitutive activity of dopamine D5 receptors, located in the STN, are at least in part at the origin of the development of the burst activity and therefore are involved in the pathophysiology of PD. They provide evidence that selective action on this receptor subtype might lead to better-targeted drug therapy for the disease. This opens up new avenues for therapeutic approaches based on specific pharmacological agents.

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## Long Term High Fat Diet Elicits Anxio-Depressive Like Symptoms: Involvement of the Serotonergic system

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**Abstract.** Although epidemiological studies suggest the existence of a bidirectional link between type 2 diabetes (T2D) and major depression, the neurobiological substrates and the mechanisms underlying such comorbidity are still poorly understood. To study the putative relationship between T2D and psychiatric disorders, we validated a metabolic and an emotionality z-score in mice integrating relevant parameters. Interestingly a strong correlation between the intensity of T2D and behavioural anomalies were observed, thereby strengthening the hypothesis that both pathologies are intertwined. The present results also suggest that body weight gain observed in high fat diet (HFD) fed mice is not involved in the anxio-depressive like phenotype. Finally, because the monoaminergic systems plays a pivotal role in major depression, we next asked whether T2D altered the serotonergic neurotransmission in the hippocampus, a brain region involved in emotionality. Remarkably HFD-induced T2D resulted in a significant decrease in hippocampal extracellular 5-HT levels. The latter results are of particular importance because they might predict a resistance of currently available serotonergic antidepressant drugs in animal models of comorbid T2D and depression, but also in patients suffering from both pathologies.

**Keywords** type 2 diabetes – anxiety – depression – serotonin – behaviour – in vivo intracerebral microdialysis

## 1 Introduction

Type 2 diabetes (T2D) and depressive disorders are major health concerns, each affecting 350 million people

worldwide. Remarkably, results of recent epidemiological studies strongly support the existence of a bidirectional relationship between both diseases. Indeed, major depression (MD) is associated with a 60 % increased risk of (T2D) (Mezuk et al., 2008) while 10-30 % of T2D individuals suffer from MD (Ali et al., 2008). Although the mechanisms underlying this association are not clearly understood, a recent study showed that a long-term high fat diet (HFD) abolished serotonin (5-HT)-induced activation of the Akt/GSK3 $\beta$  cascade in the DG of the hippocampus (Papazoglou et al., 2014). These results strongly suggested an involvement of the serotonergic system in T2D and depression and make the hippocampus an important region to study such comorbidity.

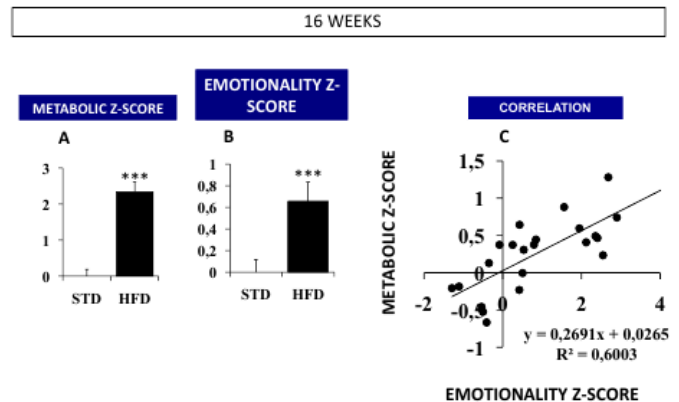
## 2 Methods

To study the relationship between T2D and MD, we used HFD-induced T2D. C57BL6J male mice were fed either a standard (STD) or a HFD (hyper-lipidic: 40 % of fat) for 16 weeks and then subjected to a full comprehensive behavioural analysis including tests recapitulating diverse anomalies related to depressive state. An emotionality z-score (Guilloux et al., 2011) was calculated from the performances of mice in paradigms evaluating anxiety, despair and carelessness in the open field (OF), tail suspension (TST) and splash test (ST); respectively. In the same way a metabolic z-score integrating body weight gain, fasting glycaemia and glucose tolerance was calculated in mice in order to establish putative correlations with behavioural impairments. In a second part of this study, anesthetized mice were implanted with microdialysis probes in the ventral hippocampus and the next day ( $\sim 20$  hours after the surgery), dialysate samples were collected every 15 minutes for 2 hours to compare the basal extracellular levels of 5-HT between freely moving animals fed a STD and HF diet.

## 3 Findings and argument

Our data indicated that mice fed a HFD displayed increased body weight gain relative to control STD animals as shown by their increased body weight gain (11(1) vs 6.0(5) gr;  $p < 0.01$ ), their increased fasting glycaemia (165(17) vs 130(5) ng/dl;  $p < 0.05$ ) and glucose intolerance. Remarkably, this HFD also elicited significant anomalies in mood-related behaviours, such as increased anxiety detected in the open field (time in the center: 109(11) vs 175(20) sec;  $p < 0.05$ ), as well as decreased self-care evaluated in the splash test (time of grooming: 125(15) vs 160(10) sec;  $p < 0.05$ ). In an attempt to correlate the intensity of T2D and anxio-depressive-like symptoms, we therefore established a separate metabolic and emotionality z-score 1. Our analysis showed that the higher the metabolic score, the

higher depression severity with a significant correlation between both scores ( $R^2=0,6$ ). Interestingly, such a correlation persisted when the body weight gain parameter was removed from the metabolic z-score ( $R^2=0,57$ ).



**Figure 1:** T2D induced anxio-depressive like phenotype. Data are means  $\pm$  SEM of the “metabolic z-score” (including, body weight gain, fasting glycaemia and glucose tolerance in the oral glucose tolerance test (OGTT) and of the “emotionality z-score” (including animals performances in the OF, TST and ST); respectively. (C) Correlation between metabolic z-score and emotionality z-score.

With respect to the serotonergic neurotransmission, a lower basal extracellular 5-HT levels was detected in the hippocampus of HFD mice compared to STD controls (8.0(9) vs 5.0(3) fmol/samples;  $p < 0.05$ ).

## 4 Conclusion

These results suggest that metabolic disorders could negatively reverberate on mood and may dampen antidepressant drug response, underlying the necessity to adapt treatment in patients with comorbid diabetes and depression. Further experiments are now required to better understand how T2D alters 5-HT neurotransmission, particularly by evaluating the functional activity of relevant targets such as the 5-HT<sub>1A</sub> or 5-HT<sub>1B</sub> autoreceptors or the 5-HT transporter.

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## Modelling gene-environment interactions in aggression

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**Abstract.** Gene-environment interactions have been shown to play a critical role in the development of aggression and other neuropsychiatric disorders. Several independent studies have highlighted that pathological aggression in males is often linked to the interaction of early-life abuse and/or neglect with allelic variants associated with low activity of monoamine oxidase (MAO) A, the key enzyme for the degradation of brain serotonin and norepinephrine.

To explore the neural underpinnings of the interaction between early stress and low MAO A, we tested the impact of early stress (ES) in MAO A<sup>Neo</sup> mice, a newly generated line of MAO A hypomorphic mutants. While ES did not significantly affect the aggressive behavior in either MAO A knockout (KO) or wild-type (WT) mice, the same manipulation resulted in a robust enhancement of fighting responses in MAO A<sup>Neo</sup> mice, to a level comparable with that of MAO A KO counterparts.

These data parallel epidemiological findings on the interaction of low-MAO A allelic variants and early stress in males with respect to the development of antisocial behavior; furthermore, our findings provide a powerful translational platform to investigate the pathophysiology of aggression on a highly isomorphic murine model.

Further studies in our laboratory are beginning to elucidate the neurodevelopmental mechanisms, supporting the interaction of early stress and MAOA genetic variants in reactive aggression and related emotional disturbances.

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**Keywords** Monoamine oxidase A – aggression – gene x environment interactions

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# Modulation of the Glutamatergic System to Novel Potential Therapeutics

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**Abstract.** Glutamate is the main excitatory neurotransmitter in the CNS, and its actions are mediated by metabotropic and ionotropic receptors (iGluRs, Kainate, AMPA and NMDA receptor subfamilies). The GluK1 subunit of Kainate receptors is predominantly associated in pain pathways. We focused on the development of AMPA/KA selective ligands based on a bicyclic pyrimidinedione scaffold. The selectivity for modulation of the single iGluRs subunits, together with the varying intrinsic activity of the developed ligands, have been extensively examined and correlated to the specific decoration of the core scaffold. The essential contribution given to the design by the recent resolution of the crystal structures of a series of our analogues in complex with the target receptor subunits (GluK1, GluK3 or GluA2), coupled to molecular modeling application, represent a further and determinant support for pursuing our research objective. Preliminary in vivo data (formalin test) have demonstrated an antinociceptive effect for our potent and selective GluK1 ligands.

**Keywords** Glutamate receptors – AMPA – Kainate – Pain – X-ray – Molecular modelling

## 1 Introduction

Glutamate (Glu) plays an important role in neuronal plasticity, neurotoxicity, and in degenerative disorders, its actions are mediated by metabotropic and ionotropic receptors (iGluRs). The excitatory amino acid trans-

porters (EAATs), which are essential for terminating synaptic excitation and for maintaining extracellular L-Glu concentration below toxic levels, represent further key target proteins of the glutamatergic system (Campiani et al., 2003). The iGluRs consist in the Kainate, AMPA and NMDA receptor subfamilies. The GluK1 subunit of the Kainate receptors (KARs) is predominantly associated with pain pathways, as shown in different areas of the CNS, and the use of GluK1 competitive antagonists allowed the demonstration of its involvement in pain signalling (Dolman et al., 2007).

Our research group acquired, over a number of years, strong experience in the selective targeting of either i) transporters (Campiani, De Angelis et al., 2001), and ii) iGluRs (GluA2 or GluK1 selective agonists and antagonists) (Campiani, Morelli et al., 2001; Butini et al., 2008; Venskutonyte et al., 2011).

The development of selective KARs agonist/antagonists may lead to compounds potentially able to treat neuropathic pain. In this frame, we developed AMPA/KA selective ligands based on a bicyclic pyrimidinedione scaffold (Figure 1, (Campiani, Morelli et al., 2001). The selectivity for the modulation of the single iGluRs subunits (Butini et al., 2008; Venskutonyte et al., 2011), together with the varying intrinsic activity of the developed ligands, has been successfully correlated to the particular decoration of the core scaffold. The essential contribution given to the design by the recent resolution of the crystal structures of a series of our analogues in complex with the target receptor subunits (GluK1, GluK3 or GluA2) shed light on the selectivity determinants for our compounds. Molecular modeling studies are in progress to correlate the displacement of key water molecules of the receptor subunit apoforms, for the design of novel, extremely selective ligands. In particular, our focus will be directed on identifying selective GluK3 ligands with respect to the other receptor subtypes in order to promote synaptic plasticity and cognitive processes by fine-tuning KARs.

## 2 Methods

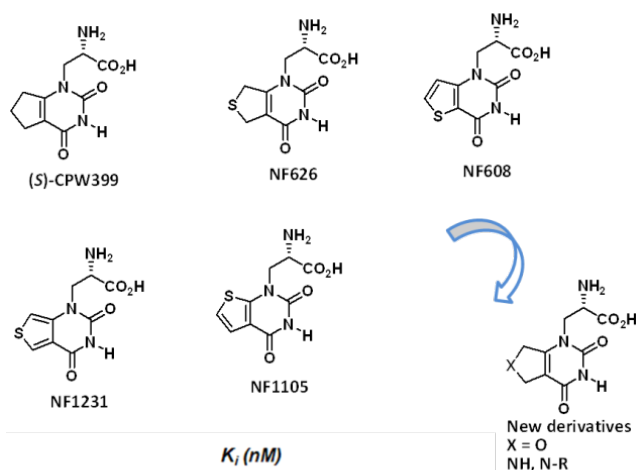
The application of an array of computer-aided drug design (CADD) tools allowed the identification of potent and selective GluK1 ligands. Moreover, through molecular modeling techniques we further explored the features (steric and electronic components that are indispensable for biological activity) and the shape that the known substrates show when bound to the site of interest.

Standard organic chemistry approaches and ad-hoc developed synthetic methodologies have been exploited for the construction of the selected structural motifs. Automated flash chromatography, HPLC separations

have been performed. Spectroscopic characterization of the compounds was performed with NMR (400, 300 and 200 MHz), MS and tandem MS instruments. X-ray diffraction data for GluA2 LBD and GluK3 LBD were collected at the I911-3 beamline (MAX-Lab, Lund, Sweden).

Two data sets were collected to the following resolutions: 1.0 Å resolution and 1.15 Å resolution for the GluA2 LBD in complex with two derivatives. All the X-ray data was processed using XDS118 and the CCP4 suite of programs. The structures were solved by molecular replacement using PHASER implemented in CCP4.120.

The GluA2 LBD structure in complex with (S)-CPW399 was used as search model for the GluA2 LBD in complex with our compounds. Furthermore, the amino acid residues of GluA2 LBD were built using Autobuild in PHENIX. Ligand coordinates were prepared by using the PRODRG server and fitted into the electron density. Topology and parameter files were obtained by using eLBOW after geometry optimization. After PHENIX refinements structures of the GluA2 complexes were validated in PHENIX as well as using the PDB validation server.



Cmpds	$K_i$ (nM)		
	GluA2	GluK1	GluK3
(S)-CPW399	223	44	5,090
NF626	80	5.20	>1,000,000
NF608	112	5.29	>1,000,000
NF1231	465	3.90	1,022
NF1105	503	5.89	4,750

Figure 1: Structure of representative compounds

### 3 Findings and argument

X-ray crystal structure of NF626 (Figure 1) in complex with GluA2 LBD shows similar binding mode as

that of a previously developed ligand (S)-CPW399 (Figure 1). Docking studies in the GluK1 cleft traces out this binding mode. Based on these findings we designed a new series of compounds, bearing different heteroatoms (N or O) as bioisosteric replacement of the sulfur atom (Figure 1). For two of these compounds we were able to get crystal structures at 1.0 Å and 1.15 Å resolution for the GluA2 LBD. The X-ray studies with GluA2 LBD were used for a structure-activity relationship (SAR) analysis. Analysis of superimposed crystal structures of the two compounds and (S)-CPW399 in complexes with GluA2 LBD reveals that the  $\alpha$ -amino acid moiety of all three compounds establishes similar contacts with surrounding residues (Pro478, Thr480, Arg485, Ser654 and Glu705) and two water molecules (W1 and W2). Interactions with water molecule W4 (present in two different positions) was observed for one compound; interestingly, this interaction was not found in (S)-CPW399 crystal structure. But the most crucial difference in the X-ray complexes of the new compounds was the conformation of Glu402 which was different from that in the X-ray structure of our lead (S)-CPW399. This may be the relevant feature responsible for the different affinity of these compounds. In addition, the number of water molecules near the GluA2 LBD was quite similar for the analyzed compounds also in comparison with the X-ray structures resolved in complex with the GluK3 LBD (despite the different aminoacidic composition). Taken together all the observed similarities and differences will drive the computational studies needed for rationalizing the recently determined differences in affinity and selectivity of the new compounds.

### 4 Conclusion

Based on the structure of (S)-CPW399 we rationally designed a series of pyrimidinedione-related analogues which displayed different potencies and selectivity towards AMPA and KA receptor subtypes. These differences were analyzed by means of computational studies and crystallography. Preliminary in vivo data (formalin test) have demonstrated an antinociceptive effect for our potent and selective GluK1 ligands.

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## Synthesis, Pharmacological assessment and Molecular Modeling of Acetylcholinesterase/Butyrylcholinesterase inhibitors: Effect against Amyloid-beta - induced Neurotoxicity

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**Abstract.** The synthesis, molecular modeling, and pharmacological analysis of phenoxyalkylamino-4-phenylnicotinates (**2-7**), phenoxyalkoxybenzylidene-malononitriles (**12-13**), pyridonepezils (14-18), and quinolinodonepezils (**19-21**) are described. Pyridonepezils **14-18** were found to be selective and moderately potent regarding the inhibition of hAChE, whereas quinolinodonepezils **19-21** were found to be poor inhibitors of hAChE. The most potent and selective hAChE inhibitor was ethyl 6-(4-(1-benzylpiperidin-4-yl)butylamino)-5-cyano-2-methyl-4-phenylnicotinate (**18**) [IC<sub>50</sub> (hAChE)=0.25(2) μM]. Pyridonepezils 14-18 and quinolinodonepezils **20-21** are more potent selective inhibitors of EeAChE than hAChE. The most potent and selective EeAChE inhibitor was ethyl 6-(2-(1-benzylpiperidin-4-yl)ethylamino)-



5-cyano-2-methyl-4-phenylnicotinate (20-21) [ $IC_{50}$  (EeAChE)=0.0167(2)  $\mu$ M], which exhibits the same inhibitory potency as donepezil against hAChE. Compounds **2**, **7**, **13**, **17**, **18**, **35** and **36** significantly prevented the decrease in cell viability caused by A $\beta$ 1-42. All compounds were effective in preventing the enhancement of AChE activity induced by A $\beta$ 1-42. Compounds **2-7** caused a significant reduction whereas pyridonepezils **17** and **18**, and compound **16** also showed some activity. The pyrazolo[3,4-b]quinolines **36** and **38** also prevented the upregulation of AChE induced by A $\beta$ 1-42. Compounds **2**, **7**, **12**, **13**, **17**, **18** and **36** may act as antagonists of VSCC since they significantly prevented the  $Ca^{2+}$  influx evoked by KCl depolarization. Docking studies show that compounds **16** and **18** adopted different orientations and conformations inside the active-site gorges of hAChE and hBuChE. The structural and energetic features of the **16**-AChE and **18**-AChE complexes compared to the **16**-BuChE and **18**-BuChE complexes account for a higher affinity of the ligand toward AChE. The present data indicate that compounds **2**, **7**, **17**, **18** and **36** may represent attractive multipotent molecules for the potential treatment of Alzheimer's disease.

**Keywords** Pyridonepezils – Quinolinodonepezils – AChE/BuChE inhibitors – A $\beta$  peptide – Alzheimer's disease.

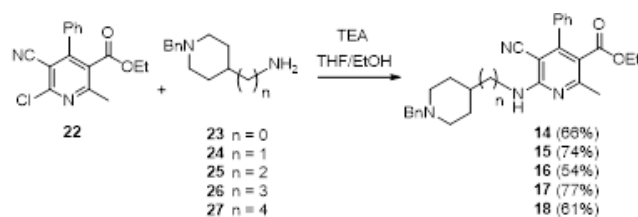
## 1 Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder (Bertram and Tanzi, 2008). Since the symptoms of AD were associated with an altered cholinergic function, research has focused on the basal forebrain cholinergic system (Wenk, 2003). In the context of our continued interest in the development of new multipotent drugs for the treatment of AD (Leon and Marco-Contelles, 2011) we have started a project targeted to the design and biological analysis of dual AChEIs endowed with additional properties. The syntheses and biological assessment of **pyridonepezils 14-18** (Figure 1), and the **quinolinodonepezils** derivatives **19-21** (Figure 2) are briefly reported.

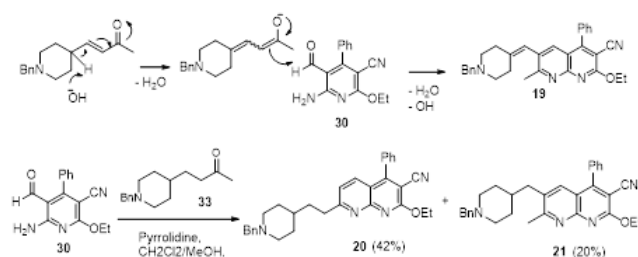
## 2 Methods

The syntheses of pyridonepezils **14-17** and quinolinodonepezils **19-21** have been carried out according to the schemes outlined in Figures 1 and 2, respectively (Silva et al., 2013).

Pharmacological assessment involved inhibition of EeAChE/eqBuChE, hAChE/hBuChE (Ellman's protocol) (Ellman et al., 1961), kinetic analysis of AChE inhibition (compounds **16** and **18**) (Silva et al., 2013),



**Figure 1:** Synthesis of pyridonepezils **14-18** (Silva et al., 2013).



**Figure 2:** Synthesis of pyridonepezils **19-21** (Silva et al., 2013).

propidium iodide assay (Rosenberry et al., 1999), effect on neuronal viability with MTT (Rosenberry et al., 1999; Lopes et al., 2009), neuroprotection against A $\beta$ -toxicity (Resende et al., 2008), the impact on the enhancement of AChE activity caused by A $\beta$  peptides (Melo et al., 2003), the effect of compounds in intracellular  $Ca^{2+}$  homeostasis dysregulation (Demuro et al., 2010), and molecular modelling studies (Silva et al., 2013).

## 3 Findings and argument

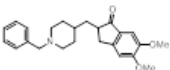
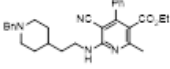
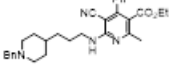
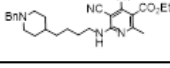
**Table 1:** Inhibition of EeAChE and eqBuChE by the most potent pyridonepezils (**16-18**)<sup>a</sup>. (Silva et al., 2013)

Compound	Structure	$IC_{50}$ AChE ( $\mu$ M)	$IC_{50}$ BuChE ( $\mu$ M)
Donepezil		0.0134(9)	0.84(5)
16		0.0167(7)	0.88(8)
17		0.019(2)	0.31(5)
18		0.030(4)	0.380(18)

<sup>a</sup> Data are expressed as the mean  $\pm$  SEM of at least three different experiments in quadruplicate.

Pyridonepezils **14-18** were found to be selective regarding the inhibition of EeAChE and hAChE.

**Table 2:** Inhibition of hAChE/hBuChE by the most potent pyridonepezils (**16-18**)<sup>a</sup>.

Compound	Structure	IC <sub>50</sub> hAChE (μM)	IC <sub>50</sub> hBuChE (μM)
Donepezil		0.016(1) <sup>b</sup>	8.2(2) <sup>c</sup>
<b>16</b>		0.31(4)	4.00(50)
<b>17</b>		0.320(37)	8.59(60)
<b>18</b>		0.25(2)	> 10

<sup>a</sup> Data are expressed as the mean ± SEM of at least three different experiments in quadruplicate;

<sup>b</sup> Notes: Human recombinant AChE

<sup>c</sup> Human serum BuChE (Silva et al., 2013).

IC<sub>50</sub> values were similar in a few cases (ranging from 0.25 to 4.57 μM). The most potent and selective *Ee*AChE inhibitor was ethyl 6-(2-(1-benzylpiperidin-4-yl)ethylamino)-5-cyano-2-methyl-4-phenylnicotinate (**16**) [IC<sub>50</sub> (*Ee*AChE)=0.0167(7) μM] (Table 1), which exhibits similar inhibitory potency as donepezil against hAChE (Table 2). The most potent and selective hAChE inhibitor was ethyl 6-(4-(1-benzylpiperidin-4-yl)butylamino)-5-cyano-2-methyl-4-phenylnicotinate (**18**) [IC<sub>50</sub> (hAChE)=0.25(2) μM] (Table 2). Kinetic analysis of the AChE inhibition by compounds **16** and **18** showed they were non-competitive inhibitors. Cell viability measured as MTT reduction showed that exposure of SH-SY5Y cells during 24 h with 5 μM of the pyridonepezils (**14-18**) did not significantly affect neuronal viability. Moreover, the decrease in cell viability caused by Aβ<sub>1-42</sub> was significantly prevented by compounds **17** (2.5 μM) and **18** (2.5 μM). In this study Aβ<sub>1-42</sub> peptide was also examined to enhance AChE activity by about 20%. All compounds were effective in preventing the enhancement of AChE activity induced by Aβ<sub>1-42</sub>. Pyridonepezils **17** and **18** decreased the activity by 50% and 30%, respectively; whereas compound **16** caused a reduction of about 12%. At 100 μM, compound **18**, which seems to inhibit AChE by a non-competitive mechanism, showed a slight but significant ability to displace propidium iodide 2 μM from the PAS of AChE (12(3)% over control). It was observed that compounds **17** and **18** expressively prevented the Ca<sup>2+</sup> influx evoked by KCl (50 mM) depolarization, suggesting that this set of compounds can act as antagonists of VSCC. Compounds **16** and **18** were found to adopt different orientations and conformations inside the active-site

gorges of hAChE and hBuChE. Compound **16** showed a binding geometry in *Ee*AChE very similar to the one displayed by the compound in hAChE. The structural and energetic features of the ligand-AChE complex compared to the ligand-BuChE complex account for a higher affinity of the ligands toward AChE. For ligands **16** and **18** the energy of the bioactive conformation in BuChE is higher than that in AChE. This energy difference is much higher for compound **18**, which may explain its selectivity.

## 4 Conclusions

The present data indicate that compounds **17** and **18** are attractive multipotent molecules acting in different key pharmacological targets. Thus, they may accomplish a potential disease-modifying role in the treatment of AD.

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## Chronic L-DOPA alters the activity of monoamine oxidase in vivo

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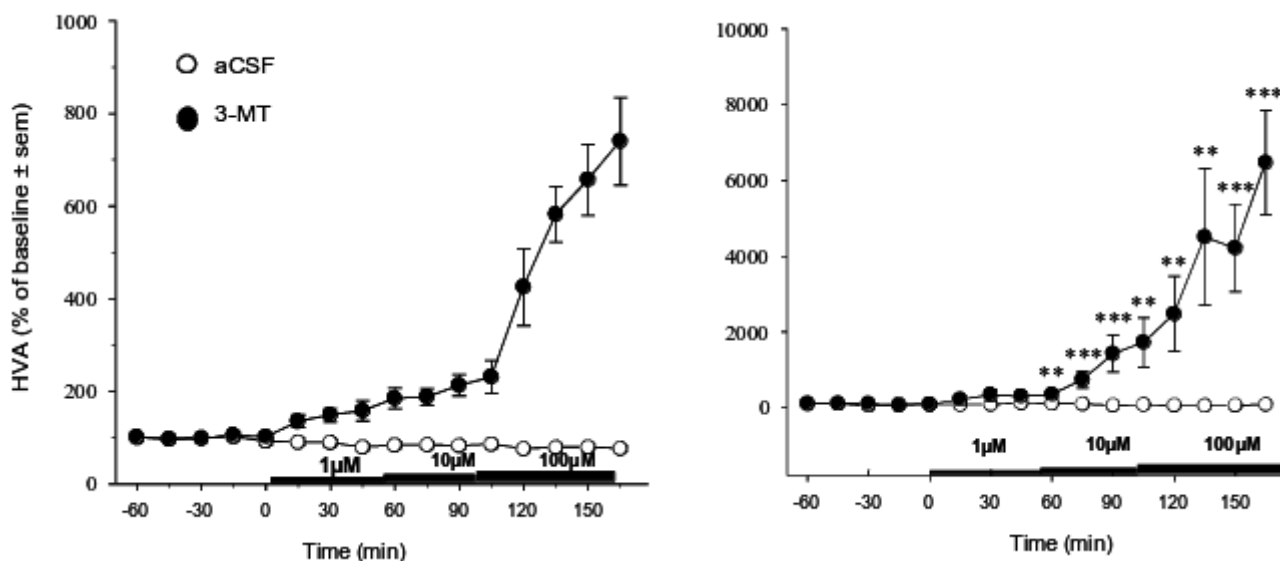
**Abstract.** We have hypothesised that the function of monoamine oxidase (MAO), normally using three tyrosines to correctly place the substrate in the catalytic site, should be diminished by the substitution of tyrosine by exogenous L-DOPA, the main treatment of Parkinson's disease. We have studied the activity of MAO in vivo after chronic treatment of L-DOPA in naïve or hemiparkinsonian rats. To study MAO activity, increasing concentrations of 3-methoxytyramine (3-MT) were applied by reverse intracerebral microdialysis in the striatum and the cortex of rats and the product of 3-MT via MAO, homovanillic acid (HVA) was collected and analyzed using HPLC. We show that the ability of 3-MT to enhance HVA dialysate content in a concentration-dependent fashion was lowered by the chronic treatment with L-DOPA in naïve (50 mg/kg) or 6-hydroxydopamine (6-OHDA) rats (3 or 50 mg/kg). The effect of 3-MT was not affected by peripheral administration of the inhibitor of MAOB F2MPA. The chronic treatment with L-DOPA lowers the function of MAO, presumably MAOA. The research will continue with members of the action CM1103 using appropriate and new pharmacological compounds, namely IMAOA.

**Keywords** Parkinson's disease – monoamine oxidase – in vivo intracerebral microdialysis – Dopamine and homovanillic acid extracellular levels – 3-methoxytyramine.

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## 1 Introduction

Exogenous L-DOPA is a very efficient medication in Parkinson's disease, but its efficacy is impaired after several years of treatment by numerous side effects including dyskinesia. The presumed role of neo-synthesized DA itself, produced from the decarboxylation of L-DOPA, is not very clear in L-DOPA-induced dyskinesia in both rats and monkeys (Navailles et al., 2011; Porras et al., 2014). This is in part related to the main role of serotonergic neurons in releasing neo-synthesized



**Figure 1:** Effect of the local infusion of 1, 10 and 100  $\mu\text{M}$  (each 1-h) 3-MT on the extracellular levels of HVA in the striatum and the prefrontal cortex. Baseline has been calculated from the mean raw data of the 3 fractions preceding the application of 3-HT. \* refers to Student's t-test ( $p < 0.01$  or  $0.001$ ).

dopamine in the whole brain (Navailles et al., 2011, 2014). This suggests that other mechanisms exist apart from the excessive DA released in L-DOPA-induced dyskinesia. Monoamine oxidase (MAO) A and B are involved in the intracellular catabolism of monoamines. Within the COST action CM1103, several works have been conducted to show that the function of MAO relies on three tyrosines correctly placing the substrate in the catalytic site (Repič et al., 2014). We have postulated here that exogenous L-DOPA would be incorporated in MAO by substituting tyrosine residue (Chan et al., 2012), thereby diminishing the activity of the enzyme *in vivo*.

## 2 Methods

To study the activity of MAO *in vivo* after chronic L-DOPA treatment, we have applied increasing concentrations of 3-methoxytyramine (3-MT), a poorly, biologically active compound that is a substrate of MAO, by reverse intracerebral microdialysis in the striatum and the prefrontal cortex (PFC) of isoflurane-anesthetized rats, simultaneously (Navailles et al., 2013). We have measured dialysate content of homovanillic acid (HVA), the product of 3-MT via MAO, using high pressure liquid chromatography coupled to electrochemical detection (HPLC-EC). The aCSF perfusion (2  $\mu\text{L}/\text{min}$ ) lasted throughout the whole experiment and dialysate samples were collected every 15 minutes. Each concentration of 3-MT (1, 10 and 100  $\mu\text{M}$ ) has subsequently been applied for 1-h. Chronic treatment consisted of daily intraperi-

toneal administration for 15 days of exogenous L-DOPA methyl ester (free base) at 50 mg/kg in naïve rats and 3 or 50 mg/kg in 6-hydroxydopamine-lesioned (6-OHDA) rats. Stereotaxic coordinates for probe implantation and the experiments, including histological examine, lesion of DA neurons and post-mortem evaluation using HPLC-EC of the extend and the selectivity of the DA lesions performed with 6-OHDA, have been performed according to recently published papers (Navailles et al., 2011, 2013, 2014).

## 3 Findings and argument

HVA dialysate content in the striatum and the PFC were, respectively: 1630(15) and 8.0(3) pg/30 $\mu\text{l}$ . Local infusion of 3-MT enhanced HVA dialysate content in a concentration-dependent manner in both brain regions in naïve rats (Figure 1). This effect is maintained in 6-OHDA-lesioned rats in which basal extracellular levels were dropped to 160.0(77) and 3.5(18) pg/30 $\mu\text{l}$ , in the striatum and the PFC, respectively. Chronic treatment with exogenous L-DOPA (50 mg/kg) in naïve rats reduced the ability of 3-MT to enhance HVA in the striatum.

In 6-OHDA-lesioned rats, the chronic exposure to L-DOPA at both 3 and 50 mg/kg reduced the ability of 3-MT to produce HVA in both the striatum and the cortex. The rise in HVA extracellular levels was reduced by 70 % in the striatum in both 3 and 50 mg/kg L-DOPA-treated rats but only for the 10  $\mu\text{M}$  concentration of 3-MT ( $p < 0.01$ , Fisher PLSD after significant

ANOVA). The rise in HVA extracellular levels was reduced by about 80 % in the PFC for the whole concentration response of 3-MT ( $p < 0.001$ ).

MAOB was probably not involved in these effects as the intraperitoneal administration (1 mg/kg) of the selective MAOB inhibitor F2MPA (Di Giovanni et al., 2014), did not alter 3-MT-stimulated HVA production in either naïve or 6-OHDA-lesioned rats.

## 4 Conclusion

We have built a new in vivo approach for the function of MAO using local application of 3-MT. This allowed us to show that the chronic treatment with L-DOPA lowers the function of MAO, presumably MAOA, in both naïve and 6-OHDA rats. These findings are compatible with the incorporation of exogenous L-DOPA into proteins (Chan et al., 2012), though they still do not constitute any proof. The research will continue with appropriate pharmacological tools, namely IMAOA.

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## Continuous determination of hROS during microdialysis

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**Abstract.** A procedure has been developed for the direct determination of hROS formation by monitoring the conversion of terephthalic acid to the highly fluorescent 2-hydroxy terephthalate by direct flow fluorimetry. The method should allow the rapid assessment of hROS formation evoked by a variety of neurotoxins and other compounds in microdialysis experiments *in vivo*.

**Keywords** kainate – microdialysis – terephthalic acid – 2-hydroxy terephthalate

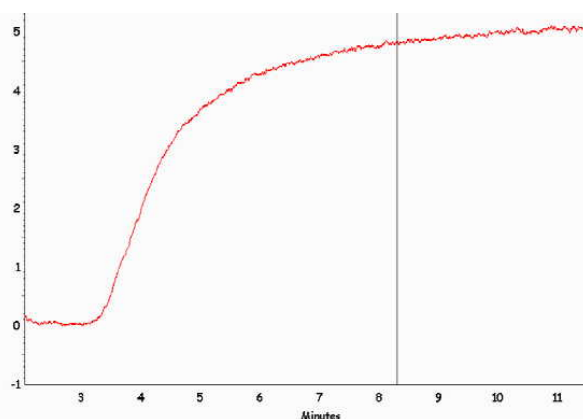
## 1 Introduction

Reactive oxygen species (ROS), which include hydrogen peroxide and superoxide, are more reactive than O<sub>2</sub> itself, but relatively stable and are involved in variety of cell-signaling processes. These may be converted to, the toxic, highly reactive oxygen species (hROS) in the presence of transition metal ions, such as Fe(II). The hROS may exist as free hydroxyl radicals (HO·), as bound (“crypto”) radicals or as Fe(IV)–oxo (ferryl) species. Although it is still unclear whether oxidative stress is the primary initiating event, hROS production is considered to play a major role in the cycle of events that results in neurodegeneration (Andersen, 2004).

Direct measurement of hROS is difficult, owing to their very short lifetime and high reactivity. We have previously shown that hROS may be detected by a reaction with terephthalic acid (TA<sup>2-</sup>) to form the fluorescent product 2-hydroxy terephthalate (OH-TA). The procedure involved inclusion of 250 μM TA<sup>2-</sup> in the artificial CSF microdialysis perfusing mixture and subsequent determination of OH-TA fluorescence in fractions of the microdialysate, collected at 20 min intervals, with excitation and emission wavelengths set to 340 and 455 nm (Freinbichler et al., 2008). This assay has been

shown to be highly sensitive and specific for hROS. Furthermore, TA<sup>2-</sup> administered during microdialysis appears to be non-toxic and does not interfere with evoked release of the neurotransmitters glutamate, aspartate and taurine. (Freinbichler et al., n.d.). The excitotoxin kainate has been shown to result in the release of each of these transmitters with concomitant hROS formation when administered directly into the striatum through a microdialysis probe (Freinbichler et al., 2008). Although the quantity of extracellular hROS detected appeared to be proportional to the administered kainate concentration, in the range 250-1000 μM, no temporal difference between neurotransmitter and hROS release could be detected.

Although the collection of microdialysate fractions was necessary for amino-acid determination, which involved post-collection reaction with o-phthalaldehyde followed by separation of the derivatives by hplc before fluorescence detection (Bianchi et al., 1999), such a procedure should not be necessary for hROS determination. The present work was designed to develop a continuous “on-line” procedure that could be used for the direct determination outlining extracellular hROS formation and levels in response to neurotoxic and other stimulants during microdialysis experiments. It was hoped such a ‘real-time’ procedure might provide a simpler and cheaper alternative with possibly improved time resolution. The apparatus involved the direct passage of the microdialysis effluent through a silica fluorimeter flow cell, with a T-piece splitter allowing a portion to flow into Eppendorf-tube fractions for subsequent neurotransmitter determinations, if required.



**Figure 1:** Fluorescence of a standard solution of 100 μM OH-TA applied through the on-line detection system ( $\lambda_{ex}$  340 nm &  $\lambda_{em}$  435 nm).

## 2 Findings and argument

The response of the system to directly administered OH TA is shown in Figure 1. The fluorescence detected

was proportional to the OHTA concentration. Recovery of OH-TA was estimated to be > 94% and the detection limit was 1.18 nM. Administration of the kainate (1 mM) through the microdialysis probe was shown to evoke the release of taurine with concomitant formation of hROS, validating the suitability of this on-line procedure for hROS determination.

### 3 Conclusion

Thus, this direct system provides a simple and convenient procedure for assessing extracellular hROS levels evoked by neurotoxins and other compounds in microdialysis experiments *in vivo*.

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## Serotonin<sub>2C</sub> receptor stimulation inhibits dopamine transmission in the nucleus accumbens independently of dopamine release: studies with cocaine

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### 1 Introduction

The serotonin<sub>2C</sub> receptor (5-HT<sub>2C</sub>R), in keeping with its ability to control the mesoaccumbens dopamine (DA) pathway, plays a key role in mediating the behavioral and neurochemical effects of drugs of abuse (Filip et al., 2012). Studies assessing the influence of 5-HT<sub>2C</sub>R agonists on cocaine-induced responses have suggested that 5-HT<sub>2C</sub>Rs can modulate mesoaccumbens DA pathway activity independently of accumbal DA release, by controlling DA transmission in the nucleus accumbens (NAc) (Navailles et al., 2004, 2008).

### 2 Methods

Combining neurochemical and molecular approaches in male Sprague Dawley rats, we assessed this hypothesis by studying the ability of the 5-HT<sub>2C</sub>R agonist Ro 60-0175 to modulate cocaine-induced changes of mesoaccumbens DA pathway activity, at both pre- and post-synaptic levels. First, we assessed the effect of Ro 60-0175 on cocaine-induced accumbal DA outflow measured by intracerebral microdialysis in freely moving animals. Then, we evaluated possible changes of post-synaptic neuronal activity in the NAc, by assessing the effect of Ro 60-0175 on cocaine-induced changes of c-Fos immunoreactivity and phosphorylation states at threonine 34 and 75 residues of the DA and c-AMP regulated phosphoproteins Mr 32kDa (DARPP-32). The effect of Ro 60-0175 on cocaine-induced DARPP-32 phosphorylation was further studied in animals pretreated with the selective 5-HT<sub>2C</sub>R antagonist SB 242084.

### 3 Findings and argument

We found that the intraperitoneal (i.p.) administration of 1 mg/kg Ro 60-0175 had no effect on cocaine (15 mg/kg, i.p.)-induced DA outflow in the shell and it increased in the core subregion of the NAc. Also, Ro 60-0175 inhibited cocaine-induced increase in c-Fos immunoreactivity in both subregions of the NAc. Finally, Ro 60-0175 inhibited cocaine-induced phosphorylation of the DA and c-AMP regulated phosphoprotein of Mr 32kDa (DARPP-32) at threonine residues in the NAc core, this effect being reversed by the selective 5-HT<sub>2C</sub> antagonist SB 242084 (0.5 mg/kg, i.p.).

### 4 conclusion

Altogether, these findings demonstrate that 5-HT<sub>2C</sub>Rs are able to modulate mesoaccumbens DA pathway activity at post-synaptic level, by specifically controlling DA signaling in the NAc core subregion. This interaction, in keeping with the tight relationship between locomotor activity and NAc DA function, could participate in the inhibitory control of cocaine-induced locomotor activity.

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## Effects of the agonist RO60-0175 and the antagonist SB242084 of 5-HT<sub>2C</sub> Receptors in the development of Maximal Dentate Activation in the Hippocampus of anesthetized rats

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**Abstract.** Substantial evidence indicates that 5-HT<sub>2C</sub> receptors are involved in the control of neuronal network excitability and in seizure pathophysiology. Here, we have addressed the relatively unexplored relationship between temporal lobe epilepsy (TLE), the most frequent type of intractable epilepsy, and 5-HT<sub>2C</sub>Rs. In the present study, we investigated this issue using a model of partial complex (limbic) seizures in urethane-anesthetized rat, based on the phenomenon of maximal dentate activation (MDA) using 5-HT<sub>2C</sub> compounds, electrophysiology, immunohistochemistry and western blotting techniques. The 5-HT<sub>2C</sub> agonists mCPP (1 mg/kg, i.p.) and lorcaserin (3 mg/kg, i.p.), but not RO60-0175 (1-3 mg/kg i.p.), were antiepileptogenic reducing the MDA response duration. The selective 5-HT<sub>2C</sub> antagonist SB242084 (2 mg/kg, i.p.) unveiled antiepileptogenic effects of RO60-0175 (3 mg/kg, i.p.) but did not alter those induced by mCPP and lorcaserin. Compared to control rats, electrically stimulated rats showed an increase in glutamic acid decarboxylase levels and a heterogeneous decrease in 5-HT<sub>2C</sub> immunoreactivity in different hippocampal areas. In our animal model of TLE, mCPP and lorcaserin were anticonvulsant; likely acting on receptor subtypes other than 5-HT<sub>2C</sub>. Epileptogenesis induced early adaptive changes and reorganisation in the 5-HT<sub>2C</sub> and GABA systems.

**Keywords** Temporal lobe epilepsy – dentate gyrus – serotonin receptors – memory – depression – serotonergic<sub>2C</sub> drugs – GABA



## 1 Introduction

The serotonin (5-hydroxytryptamine; 5-HT) 2C receptor (5-HT<sub>2C</sub>R) subtype is one of the most studied members of the serotonin receptor family that holds up to 14 subtypes (Di Giovanni et al., 2011; Higgins et al., 2013; De Deurwaerdère et al., 2013; Hoyer et al., 2002). This is not surprising, considering that it is widely expressed within the central nervous system (CNS), and is thought to play a major role in 5-HT regulation of a plethora of behaviours. Despite the importance of the 5-HT<sub>2C</sub>R, our understanding of its complex signal transduction properties remains incomplete. This is due to its distinctive regulatory properties, such as constitutive activity and RNA-editing *in vivo* and especially the scarcity of subtype-selective drugs (Di Giovanni et al., 2006; Navailles, Lagière, Guthrie and Deurwaerdère, 2013). Nevertheless, 5-HT<sub>2C</sub>R has been shown by experimental and clinical observation to represent a possible therapeutic target for the development of drugs for a range of CNS disorders such as schizophrenia, depression, drug abuse, eating disorders and Parkinson's disease to name but a few (Di Giovanni et al., 2011; Crunelli and Di Giovanni, 2014; Navailles, Lagière, Guthrie and Deurwaerdère, 2013; Di Giovanni et al., 2006). Since activation of 5-HT<sub>2C</sub>R suppresses neural network hyperexcitability in different brain areas (Jakus and Bagdy, 2011; Isaac, 2005; Crunelli and Di Giovanni, 2014) it might play a similar role in the hippocampus. This hypothesis is corroborated by the high 5-HT<sub>2C</sub>R mRNA and protein hippocampal expression (Pompeiano et al., 1994; Abramowski et al., 1995) with immunoreactivity for the 5-HT<sub>2C</sub>R receptor, widely located in the polymorphic cell layer of the dentate gyrus (DG), in the pyramidal cell layer of hippocampus proper (CA1, CA2, and CA3 fields), in the mossy fibers of CA3 and in the subiculum (Li et al., 2004; Clemett et al., 2000). Moreover, 5-HT<sub>2C</sub>R knock out (KO) mice show a selective impairment of DG plasticity *in vitro*, spatial learning impairment and emergence neophobia (Tecott et al., 1998). Consistently, 5-HT<sub>2C</sub>R activation decreases theta oscillations (Sörman et al., 2011) implying that 5-HT<sub>2C</sub>R antagonists might have therapeutic significance in psychiatric or neurological disorders associated with impaired cognitive functions and epilepsy. 5-HT<sub>2C</sub>R KO mice are extremely susceptible to audiogenic seizures (Brennan et al., 1997), and prone to spontaneous death from seizures (Tecott et al., 1995). Furthermore, an upregulation of 5-HT<sub>2C</sub>R with an increase in hippocampal gene expression and inositol triphosphate content and associated depressive mood behavioural changes have recently been shown in pilocarpine-induced temporal lobe epilepsy (TLE) in rats (Krishnakumar et al., 2009).

Despite these compelling data, research on the role of 5-HT<sub>2C</sub>R in TLE, the most frequent type of intractable epilepsy, has been relatively scarce and lead to conflicting results (Jakus and Bagdy, 2011; Bagdy et al., 2007).

In the present study, we used a model of partial complex (limbic) seizures based on the phenomenon of maximal dentate activation (MDA) recorded in the DG, induced by repetitive electrical stimulation of the perforant path (PP) in anesthetized rats (Orban et al., 2013; Stringer and Lothman, 1990). To answer the question of a possible involvement of 5-HT<sub>2C</sub>R in TLE, we evaluated the anticonvulsant properties of a 5-HT<sub>2C</sub>R agonist RO60-0175 and the selective 5-HT<sub>2C</sub>R antagonist SB 242084 using the MDA animal model.

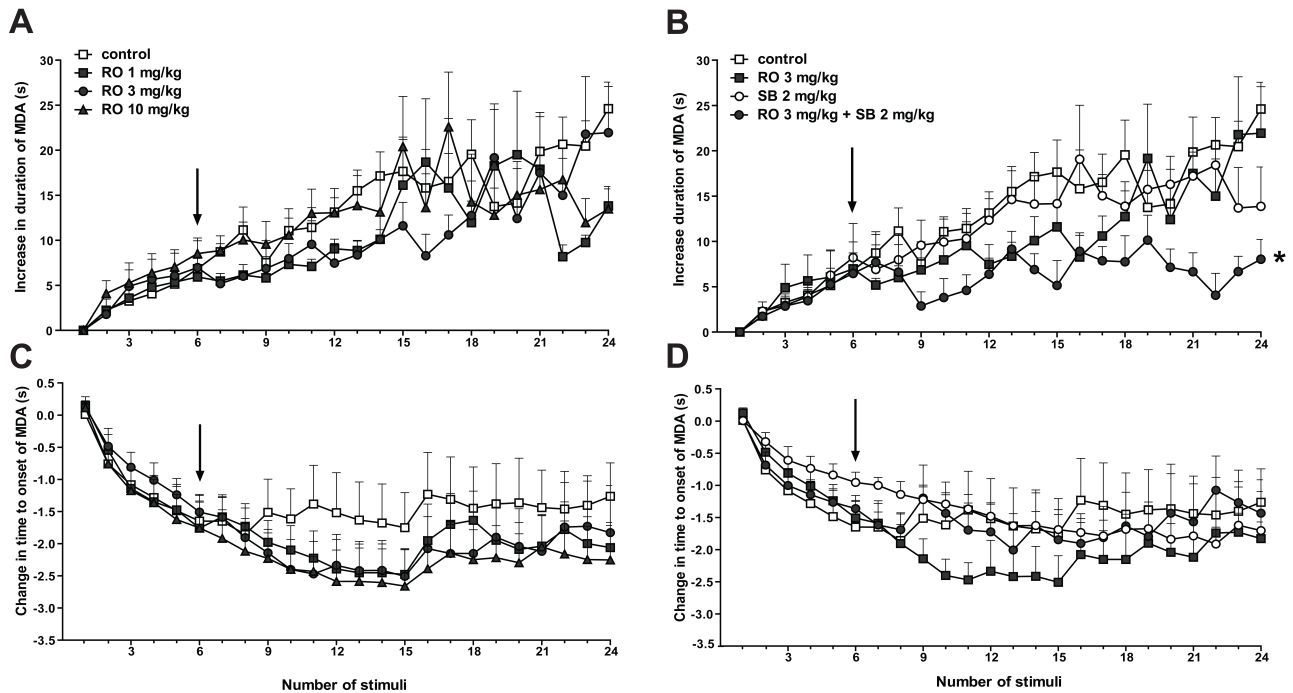
## 2 Methods

### 2.1 Maximal dentate activation

The induction of the MDA was started when normal DG excitability was revealed, 30 minutes or more following surgery. This was assessed by paired pulse stimulation with two different inter-pulse intervals (i.e. 25 and 150 msec), capable of inducing fast inhibition and excitation, respectively (Orban et al., 2013; Di Giovanni et al., 2014). MDA was characterized electrophysiologically according to published criteria (Stringer et al., 1989; Orban et al., 2013). Stimulus trains of 10 s (pulses of 0.3 ms duration, at 20 Hz) were delivered through the PP electrode at an initial intensity of 100  $\mu$ A. If MDA was not elicited, the stimulus intensity was increased in 50  $\mu$ A steps and redelivered every 2.5 minutes until MDA was induced. Threshold was reached at 350(100)  $\mu$ A, and stimulus intensity was further increased by 100  $\mu$ A. For each stimulus, the duration of MDA, time-to-onset and after discharge (AD) were measured as shown in Figure 1C.

Repeated trains inducing-seizure were delivered every 10 min for 4 h (total of 24 stimulus trains). As shown in Figure 1D, the latency to MDA onset was measured from stimulus onset to the point of PS appearance with half of the maximal amplitude (Orban et al., 2013).

After the AD began to lengthen, either drug or vehicle was administered, after six stimulus train. In the vehicle group, the duration of MDA increases and the time to onset gradually decreases (Stringer and Lothman, 1990; Orban et al., 2013). In order to make comparisons across animals, the measured durations of MDA and time to onset were 'normalized' by subtracting their duration in response to the first stimulus from the duration in response to each subsequent stimulus train. Thus, for individual stimulus trains after the first, a change in duration (or time to onset) was calculated. In this way, data from separate animals were averaged and comparisons across groups of animals were made (Stringer and



**Figure 1:** Effect of RO60-0175 (RO) on the parameters of the maximal dentate activation (MDA). The duration and time to onset of MDA were measured for each stimulus train. These values were then normalized, averaged and plotted ( $\pm$  SEM) against stimulus number. Drugs were administered i.p. at the arrows. The open square line indicates the mean values from the vehicle control animals ( $n = 9$ ). The effect of RO at 1 mg/kg ( $n = 5$ , filled squares), 3 mg/kg ( $n = 7$ , filled circles) and 10 mg/kg ( $n = 6$ , filled triangles) on the increase in duration of MDA (A) and on the change in the time to onset of MDA (C). The effect of SB242084 2 mg/kg alone ( $n = 5$ , empty circles) or combined with RO 3 mg/kg ( $n = 7$ , filled circles), on the increase in duration of MDA (B) and on the change in the time to onset of MDA (D). One-way ANOVA for repeated measures followed by Fisher's PLSD post-hoc test; \*  $p < 0.05$ . vs vehicle group.

Lothman, 1990; Orban et al., 2013).

### 3 Findings and argument

#### 3.1 Effect of RO60-0175 on MDA parameters and role of 5-HT<sub>2C</sub> receptors

Despite a trend toward a decrease after 1 or 3 mg/kg, RO60-0175 did not significantly alter the duration of MDA (Figure 1A). Statistical analysis revealed a significant reduction of the MDA elongation by co-treatment with SB 242084 (2 mg/kg, i.p.) and RO60-0175 (3 mg/kg). However, SB 242084 was without effect by itself (Figure 1B). Despite a trend toward inhibition, RO60-0175 (1, 3, 10 mg/kg  $n = 6$ ) did not alter the onset of the MDA (Figure 1C). Pre-treatment with the selective 5-HT<sub>2C</sub> antagonist SB242084 (2 mg/kg, i.p,  $n = 7$ ), without effect by itself, did not reveal any interaction with 3 mg/kg RO60-0175 on the time to onset of MDA (Figure 1D). Conversely, RO60-0175 (1, 3, 10 mg/kg,  $n = 6$  for each dose), SB242084 (2 mg/kg,  $n = 7$ ) and co-treatment with 3 mg/kg RO60-0175 and SB242084 did not effected the onset of the MDA (Figure 1C,D).

Effect of RO60-0175 (RO) on the parameters of the maximal dentate activation (MDA). The duration and time to onset of MDA were measured for each stimulus train. These values were then normalized, averaged and plotted ( $\pm$  SEM) against stimulus number. Drugs were administered i.p. at the arrows. The open square line indicates the mean values from the vehicle control animals ( $n = 9$ ). The effect of RO at 1 mg/kg ( $n = 5$ , filled squares), 3 mg/kg ( $n = 7$ , filled circles) and 10 mg/kg ( $n = 6$ , filled triangles) on the increase in duration of MDA (A) and on the change in the time to onset of MDA (C). The effect of SB242084 2 mg/kg alone ( $n = 5$ , empty circles) or combined with RO 3 mg/kg ( $n = 7$ , filled circles), on the increase in duration of MDA (B) and on the change in the time to onset of MDA (D). One-way ANOVA for repeated measures followed by Fisher's PLSD post-hoc test; \*  $p < 0.05$ . vs vehicle group.

### 4 Conclusion

It is interesting to note that that RO60-0175 was devoid of any significant antiepileptic effects over a wide range of doses (1-10 mg/kg). This regimen has

been previously shown to be efficient on various electrophysiological, biochemical and behavioural experiments (Beyeler et al., 2010; Di Matteo et al., 2004; Invernizzi et al., 2007; Navailles, Lagièrre, Le Moine and De Deurwaerdère, 2013; Di Giovanni et al., 2008; Di Matteo et al., 2008) strongly suggesting that 5-HT<sub>2</sub>CR stimulation is not involved in the control of MDA elongation. The presence of the 5-HT<sub>2</sub>C antagonist SB242084 unmasked a decrease in MDA response highlighting the anti-epileptic properties of RO60-0175. The mere involvement of 5-HT<sub>2</sub>CR in the electrophysiological feature of MDA has been also confirmed on the time to onset of MDA. The latency to onset of MDA can be used as a gauge of seizure threshold (anticonvulsant) and the duration of MDA as a measure of processes that terminate seizure activity in the limbic system and its decrease has been considered to be antiepileptogenic (White, 2002). The anticonvulsant and the antiepileptogenic processes likely involve different mechanisms (Löscher, 2012) and in accordance RO60-0175 did not affect the onset of the MDA.

Overall, these data suggest that MDA does not respond to phasic stimulation of 5-HT<sub>2</sub>CR. Moreover, our data show also that MDA response is under a poor tonic influence exerted by 5-HT<sub>2</sub>CR. Indeed, the selective 5-HT<sub>2</sub>CR antagonist SB242084 (2 mg/kg, i.p.) did not induce any proconvulsant effects seen such as elongation of the MDA and AD in rats or reduction of the MDA latency. Thus, in contrast to the situation reported in 5-HT<sub>2</sub>C KO mice (Tecott et al., 1995), the lack of a 5-HT<sub>2</sub>CR tonic control on epilepsy is in agreement with data from generalised epilepsy models (Bagdy et al., 2007). It has been previously reported that SB242084 or the other selective antagonist SB243213 were unable to reduce seizure threshold in adult rodents (Jakus and Bagdy, 2011). Although a constitutive activity of 5-HT<sub>2</sub>CR could have hardly been unmasked with SB242084 (De Deurwaerdère, 2004), previous data have reported that the prototypical inverse agonist SB206553 did not alter on its own seizure threshold (Upton et al., 1998). Thus, it appears that endogenous 5-HT<sub>2</sub>CR tone exerts a poor influence on the general activity.

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## Neuroinflammation in alzheimer's disease

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**Abstract.** Alzheimer's disease (AD) is a complex, multifactorial and progressive neurodegenerative disorder. Recent studies suggest that neuroinflammation plays an important role in neurodegenerative processes. Microglia and astrocytes are key brain neuroglial cells that regulate two opposite i.e. protective and harmful effects of inflammation on neurodegeneration. In normal aging and in early stage AD, microglia have a neuroprotective role in which they contribute to the clearance of amyloid-beta (Ab) aggregates and neurofibrillary tangles, and stimulation of anti-inflammatory cytokines. The age-related changes and progressive accumulation of AD specific pathological elements induce chronic activation of microglia in an attempt to remove these pathological structures. Activated microglia release pro-inflammatory cytokines (IL-1, IL-6, TNF- $\alpha$ ) and other neurotoxic proteins that further stimulate inflammatory processes and contribute to neuronal dysfunction and neurodegeneration. The studies in a transgenic animal model of AD suggest that astrocytic atrophy and astrogliosis are also associated with development of AD. At the later stages of disease, astrocytes become activated and contribute to the neuroinflammatory component of neurodegeneration. In summary, recent data suggest that neuroinflammatory processes and altered function of the brain innate immune cells represent important risk factor for the development and progression of AD. Targeting neuroinflammation and the immune system might be an attractive new approach in the treatment of AD.

**Keywords** Alzheimer's disease – inflammation – gliosis – anti-inflammatory compounds – NSAIDs

### 1 Introduction

Alzheimer's disease (AD) is a complex, multifactorial and progressive neurodegenerative disorder. The main symptoms are progressive decline in cognitive functions (memory, learning and executive functions) due to the progressive loss of neurons in hippocampus, entorhinal cortex and basal forebrain. Although there are several hypotheses that try to explain the development of AD, its etiology is still unclear. The main pathological hallmarks of AD are extracellular senile plaques (accumula-

tion of toxic amyloid-beta peptide; Ab) and neurofibrillary tangles (abnormal hyperphosphorylation of protein tau inside neurons). Recent studies suggest that inflammation and hypertrophy of glial cells (gliosis) play an important role in neurodegenerative processes (Weiner and Frenkel, 2006). Microglia and astrocytes are key brain glial cells that regulate two opposite i.e. protective and harmful effects of inflammation on neurodegeneration.

### 2 Findings and argument

Microglia are brain innate macrophages/phagocytes that remove and clear fragments of damaged or dead cells (Solito and Sastre, 2012). In normal aging and early stage AD activated microglia have a neuroprotective role in which they contribute to phagocytosis, clearance and degradation of Ab aggregates, neurofibrillary tangles and stimulation of anti-inflammatory cytokines (IL-4, IL-10) release in an attempt to remove these "foreign" structures from the brain.

The age-related changes and progressive accumulation of AD specific pathological elements like insoluble Ab aggregates induces chronic activation of microglia and transformation of neuroprotective microglia to neurotoxic cells. Activated microglia release pro-inflammatory cytokines (IL-1, IL-6, TNF- $\alpha$ ) and other neurotoxic proteins that further stimulate inflammatory processes and contribute to neuronal dysfunction and neurodegeneration (Guillot-Sestier and Town, 2013). Recent neuroimaging study (Zimmer et al., 2014) has confirmed the activation of microglia in AD.

Astrocytes are a component of the tripartite synapse that mediates connectivity between neuronal and non-neuronal cells and regulates extracellular homeostasis of ions and neurotransmitters. The perisynaptic astrocytes remove the majority of the excitatory neurotransmitter glutamate from extracellular space and prevent its neurotoxic effect. The studies in a transgenic animal model of AD suggest that astrocytic atrophy and astrogliosis are associated with development of AD. At the later stages of disease, astrocytes become activated and contribute to neurodegeneration (Heneka et al., 2010).

Although cytokines represent the important component of neuroinflammation in AD, the analysis of genotype and allele frequencies of IL-1, IL-6 and TNF- $\alpha$  gene polymorphisms did not show significant difference between patients with AD and healthy controls.

### 3 Neuroinflammation as a target of treatment

Despite remarkable progress in understanding the molecular mechanisms during development and progression of AD there is no effective medication that can stop

the progress of the disease. New data suggests that components of the inflammatory process could be the new targets for treatment of AD. So far treatment options include anti-inflammatory food (“healthy food”) and non-steroidal anti-inflammatory drugs (NSAIDs). Among evaluated natural compounds are ginkgo biloba extract, resveratrol, vitamins and omega3-fatty acids. Although ginkgo biloba extract prevents formation of Ab fibrils in neuroblastoma cell line in vitro (Luo et al., 2002), its antioxidative, anti-amyloidogenic and anti-apoptotic effects were not confirmed in longitudinal study in subjects with mild cognitive impairment (Vellas et al., 2012). Epidemiological studies have shown lower incidence of AD in patients with inflammatory diseases (arthritis, rheumatism) and pain that have been long-term treated with NSAIDs. The mechanisms of this protective effect of NSAIDs on cognition is associated with their inhibitory effects on cyclooxygenase (COX) activity and consequently on glial activation. Additionally NSAIDs stimulate non-toxic amyloidogenic pathway of APP metabolism and reduce formation of Ab by inhibition of  $\beta$ - and  $\gamma$ -secretase activity (Weggen et al., 2007). Although, NSAIDs may have protective effects, clinical trials did not confirm beneficial effects of NSAIDs in patients with AD. The key point is that NSAIDs most likely modulate activation of microglia and reduce the detrimental effects of inflammation in a very early, pre-clinical stage of neurodegeneration.

## 4 Conclusion

In summary, recent data suggests that neuroinflammatory processes and altered function of the brain innate immune cells represent an important etiological risk factor for the development and progression of AD. However it is still unclear if the inflammation is the cause, contributor or secondary event in AD. Targeting neuroinflammation and the immune system might be an attractive new approach in the treatment of AD. Since neuroinflammatory processes start very early and before appearance of AD symptoms, timing is important for the success of an anti-inflammatory/preventive treatment with NSAIDs.

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# Combined depletion of Monoamines in Parkinson's disease

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**Abstract.** Parkinson's disease is a neurological disorder characterized by motor and also by non-motor symptoms, which are under-studied and therefore not well supported therapeutically. We investigated the role of combined depletions of dopamine, norepinephrine and serotonin in the manifestation of Parkinsonian-like motor and non-motor deficits in the rat and also their impact on the behavioral effects of deep brain stimulation of the subthalamic nucleus. In a unilateral 6-OHDA rat model of the disease, we show that NA or DA depletion significantly decreased locomotor activity and that anxiety-like states required DA depletion plus the depletion of norepinephrine and serotonin. Anhedonia and "depressive-like" behavior emerged only from the combined depletion of all three monoamines. In the bilateral model, we show that dopamine depletion alone induced locomotor deficits associated with anxiety and mild "depressive-like" behavior and that combined depletions of the three monoamines dramatically exacerbated "depressive-like" behavior. This bilateral model can be considered as a model of advanced stage Parkinson's disease in the rat expressing motor deficits as well as severe mood disorders.

**Keywords** Parkinson's disease – motor and non-motor symptoms – monoamines – Deep Brain Stimulation.

## 1 Introduction

Parkinson's disease is characterized by the manifestation of motor symptoms mainly attributed to the degeneration of dopamine neurons in the pars compacta of substantia nigra. Furthermore, despite the focus on motor deficits, Parkinson's disease is also characterized by the manifestation of non-motor symptoms, such as anxiety and depression, for which the mechanisms are still not elucidated. In addition to dopamine cell degeneration, Parkinson's disease is a multi-system disorder characterized also by the loss of norepinephrine neurons of the locus coeruleus (for review, Delaville et al. (2011))

and serotonin cells of the dorsal raphe (Kish, 2003) suggesting that norepinephrine and serotonin depletions are other landmarks of the disease. We focused on the respective role of combined depletions of the monoaminergic systems using the unilateral and also the bilateral 6-OHDA rat model of Parkinson's disease.

## 2 Methods

We performed selective depletions of dopamine, norepinephrine and serotonin and the behavioral effects of different depletions were investigated using the open field for locomotor activity, the elevated plus maze for anxiety and the forced swim test for "depressive-like" behavior.

## 3 Findings and argument

One of the main findings is that norepinephrine depletion alone induced severe motor deficits that resembled those reported after 6-OHDA-induced dopamine cell lesion suggesting that norepinephrine, like dopamine, is essential in the control of motor behavior. In the unilateral dopamine depleted animals, the motor deficits were not potentiated by additional depletions of norepinephrine and/or serotonin. However, the manifestation of anxiety and "depressive-like" behavior were due to the combined monoamine depletions (Delaville et al., 2012). In the bilateral rat model, we showed that dopamine depletion alone induced locomotor deficits associated with anxiety and mild "depressive-like" behavior and that combined depletions of the three monoamines dramatically exacerbated "depressive-like" behavior. In addition, we show that deep brain stimulation of the subthalamic nucleus reversed locomotor deficits and anxiety behavior in animals with bilateral dopamine depletion alone. However, the additional depletion of norepinephrine and/or serotonin may interfere with these improvements.

## 4 Conclusion

Results of these studies highlight the important role played by monoaminergic system depletions in the pathophysiology of Parkinson's disease and propose new rat models with motor and non-motor disabilities. They provide evidence that combined depletion of monoamines may alter the efficacy of subthalamic stimulation used in the therapy of the disease.

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## Vesicular monoamine transporter and the effects of its inhibition in portacaval shunted rats

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**Abstract.** Large amounts of histamine are formed in the brain and gastric mucosa of portacaval shunted rats. Histamine removal from cytoplasm and sequestration of the amine into storage vesicles within the cell is accomplished by vesicular monoamine transporter VMAT2. To determine whether the brain level of VMAT2 is altered in PCS rats and what the effects of VMAT inhibition are, immunohistochemistry and 3H dihydrotetrabenazine (DTBZ) binding studies were done and in vivo reserpine was administered, respectively. Neither VMAT2 immunofluorescence, nor Kd and V max for VMAT2 differ between PCS and their sham operated control pairs, indicating a high capacity of this transporting system. On the other hand, the depletion of storage vesicles by the blockade of vesicular transporter was much more harmful for PCS rats.

**Keywords** histamine – portocaval shunt – immunohistochemistry – VMAT2 – reserpine

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## 1 Introduction

Portacaval shunt (PCS) drastically reduces participation of the liver in body metabolism. Among other effects this leads to profound hormonal alterations and intensive skeletal muscle protein breakdown, resulting in a changed blood amino acid profile and concentration with a decrease in branched chain amino acids and an increase in aromatic amino acids. Upon entering the brain, enhanced concentrations of aromatic amino acids, amine neurotransmitter precursors, causes excessive production of dopamine, serotonin and histamine. In the case of the two former, compensatory degradation takes place whereas large amounts of synthesized histamine are deposited in neurons. Increased histamine production also concerns gastric mucosa as well as the brain (Fogel et al., 2001). The vesicular monoamine transporter 2, VMAT2, an H<sup>+</sup>-ATPase an-



tiporter, acts to sequester cytosolic histamine in storage vesicles in neurons and endocrine cells (Erickson et al., 1995; Häkanson et al., 1976). In this study two questions were posed; 1) whether there is any modulation in the function of the transporter in the portacavally shunted rat brain and 2) what are the effects of VMAT inhibition (Pollard et al., 1973).

Binding studies with tritiated dihydrotetrabenazine and immunohistochemistry have been applied and reserpine used as a VMAT inhibitor. Results suggest no changes in the VMAT2 function after PCS. On the other hand, in animal treated with reserpine, much richer stores of histamine caused more pronounced injury in PCS rats.

## 2 Methods

Lewis rats portacavally shunted (end-to-side) according to Lee and Fisher (1961) and sham operated control pairs 2-11 months after surgery were used. Double fluorescent immunohistochemical techniques, employing rabbit-anti-HA (Anti-HA-19C; (Panula et al., 1990) and guinea pig anti VMAT2 (Euro-Diagnostica, Lund, Sweden) were used to disclose the localization and density of VMAT2 and histamine. The binding studies were performed using 3H dihydrotetrabenazine as a VMAT2 ligand (Teng et al., 1998). Reserpine was administered intraperitoneally in a single dose 5 mg/kg, and the rats' consumption and excretion were monitored daily for 1 to 5 days. Tissue samples were collected at autopsy after 1 day or 5 days following the reserpine administration. Both the cerebral cortex and hypothalamus were dissected, frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until assayed. The stomach was cut open and examined for peptic ulcers. The length of each lesion was measured under  $10\times$  magnification and summed per rat. The group mean  $\pm$ SD was calculated. The gastric mucosa was then scrapped off for histamine estimation (Fogel et al., 2001).

## 3 Findings and argument

Although histamine in the CNS was increased several fold following PCS, with regard to VMAT 2 no difference in either the distribution pattern or the intensity of immunostaining was noted between shunted and control rat brains. Likewise, there were no changes in DTBZ binding characteristics of the VMAT 2 between PCS rats and sham operated controls. Reserpine treatment evoked long lasting sedation. The rats neither ate nor drank and lost from 13% (sham) to 17% (PCS) of their initial body mass over 5 days. In the stomachs of these rats severe ulceration was found at autopsy, which was more extensive and deep in the reserpine-treated shunted rats

## 4 Conclusion

VMAT 2 capacity is beyond normal demands and may efficiently sequester even such excessive amounts of histamine as produced in hepatic encephalopathy (HE).

Reserpine treatment suggests that the drugs interfering with VMAT may have fatal consequences for HE patients.

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## An new animal model of anxiety

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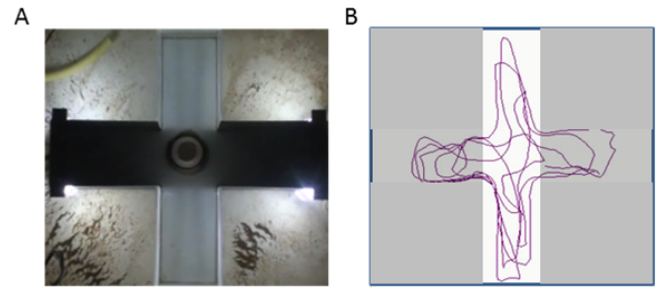
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**Abstract.** We cover, in this extended abstract, our recent work published in Science about anxiety in crayfish (Fossat, 2014). After exposure to stress, crayfish sustainably avoided the aversive illuminated arms of an aquatic plus-maze. This behavior was correlated with a significant increase in brain serotonin but not of dopamine and was abolished by the injection of the benzodiazepine anxiolytic chlordiazepoxide. Serotonin injection into unstressed crayfish induced avoidance; again, this effect was reversed by injection with chlordiazepoxide. Our results demonstrate that crayfish exhibit a form of anxiety similar to that described in vertebrates, suggesting the conservation of several underlying mechanisms during evolution. Analyses of this ancestral behavior in a simple model reveal a new route to understanding anxiety and may alter our conceptions of the emotional status of invertebrates.

**Keywords** Anxiety – Crayfish – behaviour – Serotonin and Dopamine

## 1 Introduction

Stress or danger (called stressors) provoke fear and generate immediate responses, such as escape, freezing or aggression. Stress can also lead to anxiety, a more complex state occurring when the stressor is absent or not clearly identified (Belzung and Philpott, 2007; D. C. Blanchard and R. J. Blanchard, 2008; Steimer, 2011). In humans and rodents, anxiety is experienced as an anticipatory fear that facilitates coping with unexpected situations and is revealed by a long-lasting behavioral adaptation intended to minimize threats, even in a different context and without the stressor (Belzung and Philpott, 2007; D. C. Blanchard and R. J. Blanchard, 2008; Steimer, 2011). Anxiety has been intensively studied in humans and rodents (Handley, 1995; López-Muñoz et al., 2011; Walters et al., 1981), but most animals are capable of perceiving danger and exhibit varying degrees of behavioral adaptation to stress (Belzung and Philpott, 2007). Though studies have described fear response after aversive conditioning in *Aplysia* or pessimistic bias after stress in bees (Walters et al., 1981; Bateson et al., 2011), the characteristics of anxiety have not been fully observed in a single invertebrate model. We show that stressed crayfish (*Procam-*



**Figure 1:** The dark/light plus maze. **A** The maze was cross-shaped with 4 arms (25 cm long and 10 cm wide each) and a middle zone (10×10 cm). 2 arms are illuminated and 2 are dark. **B** Unstressed crayfish spontaneously visit all arms as it is highlighted with a typical crayfish route.

*barus clarkii*) express context-independent anxiety-like behavior that can be promoted by 5-HT (Fossat et al., 2014).

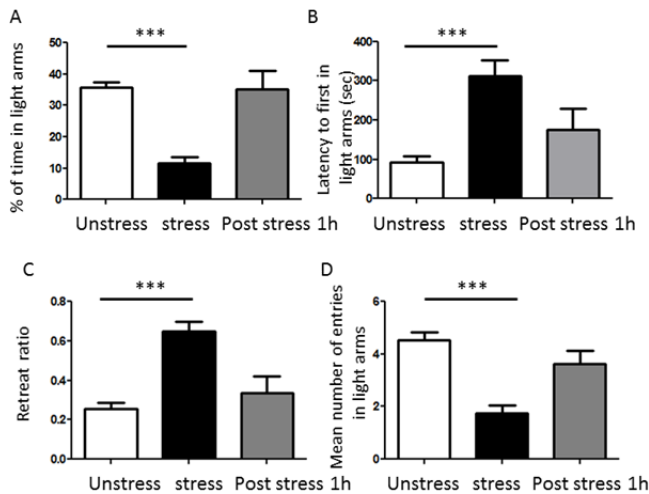
## 2 Methods

We used adult male crayfish, *Procambarus clarkii*. Crayfish were first submitted to an aversive stress by means of electric fields generated in a specific tank (see Fossat et al., 2014). Crayfish were then placed in the dark/light plus maze, cross-shaped maze divided in four arms (2 illuminated and 2 darks) (see Figure 1). The spontaneous exploratory behaviour of unstressed and stressed crayfish was recorded for 10 minutes and further analysed with the ethovision XT 8 software (Noldus, NL). We compared the behaviour related to the illuminated arms, namely the % of the total time in light arms, the mean number of entry in light arms, the latency of first in light arms and the retreat ratio (RR). RR is defined as the number of withdrawal towards illuminated arms divided by the total number of attempts. We then measured the brain bioamine levels by HPLC (see Fossat et al., 2014). Finally, we measured the effect of 5-HT injection on the crayfish exploratory behaviour. A quantity of 5 µg/g 5-HT was injected directly within the hemolymph, 15 minutes after behavioural test in the dark/light plus maze.

## 3 Findings and argument

The stress stimulation significantly modified the spontaneous exploratory behaviour of crayfish. While unstressed crayfish spontaneously explored the whole arms of the maze with a significant preference for the dark arms, stressed crayfish almost never visited the light arms (Figure 2). The consequence of the stress on the exploratory behaviour lasts for 30 minutes and then a normal behaviour is recovered after 1 hour.

We measured the bioamine levels in the crayfish brain and it appears that brain 5-HT is significantly higher



**Figure 2:** After stress crayfish spent less time in light arms, the latency to first entry is significantly increased, the retreat ratio is increased and the mean number of entries in light arm is decreased. All these behavioural changes disappeared 1 hour after stress.

in stressed as compared to unstressed crayfish while dopamine remained unmodified (5-HT: 118(20) pg/mg and 296.2(430),  $p < 0.01$ , Mann Whitney and dopamine 50.5(70) pg/mg and 65(11) pg/mg,  $p = 0.28$ , Mann whitney). Finally, 5HT injections mimick the stressful stimulation and trigger a strong aversion regarding illuminated arms.

## 4 Conclusion

Taken together, these results suggest that crayfish submitted to an important stress express an apprehension behaviour that limits their exploration of potentially aversive areas (here, the illuminated arms of the dark/light plus maze). This behaviour depends on 5-HT and lasts for minutes after stress and is remarkably similar to the anxiety-like behaviour measured in rodents.

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# Quinolylnitrones with neuroprotective activity for the treatment of cerebral ischemia

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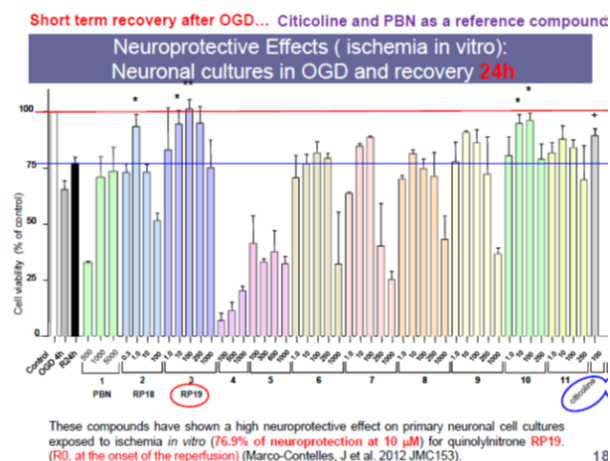
**Abstract.** We report the synthesis, theoretical calculations, the antioxidant, anti-inflammatory, neuroprotective properties, and the ability to cross the blood-brain barrier (BBB) of (Z)-a-aryl and heteroaryl-N-alkyl nitrones, as potential agents for stroke treatment. The majority of nitrones compete with DMSO for hydroxyl radicals, and most of them are potent lipoxygenase inhibitors. Cell viability-related (MTT assay) studies clearly showed that these nitrones give rise to significant neuroprotection. Particularly, nitrone RP19 which shows potent combined antioxidant and neuroprotective properties, and, therefore, can be considered as new lead compound for further development in specific tests for potential stroke treatment.

**Keywords** quinolylnitrones – neuroprotection – MTT – cerebral ischemia – RP19

## 1 Introduction

Cerebral Ischemia (CI) is one of the major causes of death, behind heart diseases and cancer, and is the leading cause of adult disability in developed countries. The large numbers of stroke patients and great economic expenses have imposed a great burden to society. Despite its great social impact, there is a lack of effective therapeutic treatment.

Occlusion of cerebral vessels by blood thrombus is the main cause of CI, which can be hemorrhagic or ischemic, can affect an area (focal ischemia) or whole brain (global ischemia), and can lead to a cascade of damaging biological events, including the release of excitatory amino acid, calcium influx, and neuroinflammation. There is increasing evidence showing that oxidative stress plays a key, major role in CI. This is why we have supported the possibility since the beginning of our project of a therapeutic approach based on antioxidant compounds able to trap reactive oxygenated species, such as hydroxyl radicals, superoxide, peroxynitrite, or peroxide. Thus, an agent that either lyses blood thrombus/inhibits platelet aggregation or scavenges free radicals, or a combination of these two effects, could be useful for the treatment of CI, and the reason



**Figure 1:** Effect of nitrones on primary neuronal cultures exposed to oxygen-glucose deprivation (OGD). Bar chart showing the percentage of cell viability at 24 h of recovery after 4 h OGD, either untreated (R24h) or treated with different concentrations ( $\mu$ M) of nitrones **12**, **12** (PBN), **16**, **19**, **20**, or citicoline. The value induced by OGD at 4 h is also indicated. Cell viability corresponding to control cells (1.497(50) AU) was considered as 100%. The values represent the average of three to five independent experiments; error bars representing the SEM. \*P < 0.05 versus R24h by Student's t test. Statistical significances below R24h value were not shown.

for the selection as our target molecules, nitrones, as a well-known organic compounds, which have evolved from classical agents able to trap free radicals to be intensively investigated as neuroprotective drugs for the potential treatment of cerebral ischemia (Floyd et al., 2008).

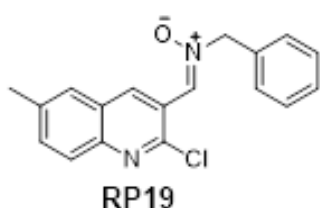
## 2 Methods, findings and argument

Cell viability assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) determination was performed to evaluate the potential neuroprotective effect on nitrones **1-11** against OGD. Exposure of neuronal cultures to 4 h OGD (OGD 4h) induced a significant decrease in cell viability (65.2%, p < 0.0001 versus 100% control, by one-sample t test), which was partially reversed after 24 h recovery (reperfusion) (R24h, 77.0%; p < 0.01 versus OGD 4h, Student's t test), but without reaching the 24 h control value (p < 0.0001 versus 100% control, one-sample t test) (Figure 1).

Nitrones **1-11** (ranged from 0.1  $\mu$ M to 1 mM) were added at the beginning of the reperfusion period to evaluate their potential neuroprotective effect. Cytidine-5'-diphosphocholine (citicoline or CDP-choline), a well-known neuroprotective agent, and PBN, described as protective against free radicals, were used as reference compounds to evaluate the neuroprotection on neuronal

cultures. Citicoline was assayed from 1  $\mu$ M to 1 mM, and we found a neuroprotective effect at 10 and 100  $\mu$ M (87.4 and 91.5 %, respectively, compared to 100 % control) (Figure 1). PBN did not induce neuroprotective effect in the range of assayed concentration (0.1-10 mM; Figure 2).

The addition of 100-250  $\mu$ M nitrone 1, 1  $\mu$ M nitrone 2, or 10-100  $\mu$ M nitrone 3 (= RP19, Figure 2) and 10, significantly increased cell viability during reperfusion, and returned near control values (100.3-95.7 %, 93.4 %, 94.4-101.2 %, and 94.9-96.1 %, respectively, compared to 100 % control) (Figure 1). The neuroprotection induced by nitrones 1-3 and 10 was compared with citicoline and PBN, these nitrones providing significantly higher neuroprotection than citicoline.



**Figure 2:** Chemical structure of RP19.

### 3 Conclusion

Based on these results, we conclude that quinolyl-nitron RP19 [(Z)-N-((2-chloro-6-methylquinolin-3-yl)methylene)-1-phenylmethanamine oxide] (Chioua et al., 2012; Figure 2) shows high neuroprotective activity, *in vitro* and *in vivo*, and can be considered a promising drug for the potential treatment of CI.

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## Aldo-keto reductase inhibitors and their potential in neurodegenerative disorders

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**Abstract.** Aldo-keto reductases belong to the recently indicated targets in neurodegenerative diseases due to the revealed correlation between neurodegenerative and type 2 diabetes mellitus related changes in the brain. As aldose reductase (ALR2) is one of the most promising targets in reducing glucose toxicity, ALR2 inhibitors could represent prospective compounds in developing the multi-target drug leads. We focused on a set of 20 indole derivatives with recently determined inhibition activities towards ALR2 and known selectivity factors as compared with aldehyde reductase (ALR1). We performed a molecular modeling study on the interaction of the indole compounds with monoamine oxidase A and B. The study was based on docking calculation and subsequent full optimization of the complexes. Experimental confirmation of human MAO-A and MAO-B inhibition activities of effective ALR2 inhibitors from the group of indole-1-acetic derivatives (**13** and **20**) is also presented. Compound **20** exhibited prospective properties as a multi-target lead for further design of bifunctional ALR2/MAO inhibitors.

**Keywords** Alzheimer's disease – type 2 diabetes mellitus – monoamine oxidase – indole-1-acetic acid derivatives – molecular modeling

## 1 Introduction

The multi-factorial character of neurodegenerative diseases has been commonly accepted as the basis for understanding their mechanisms and also the search for their efficient treatment. Alzheimer's disease and type 2 diabetes mellitus showed many common features (de

la Monte and Wands, 2008) connected mainly with malfunctions in glucose metabolism. They result in elevated sorbitol levels in the brain, mostly derived from glucose flux through the astrocyte polyol pathway (Regenold et al., 2004). After years of extensive study on design, synthesis, in vitro and in vivo testing of ALR2 inhibitors (ARI) (Štefek et al., 2008; Juskova et al., 2011), we performed a systematic search for other positive beneficial properties of ARI in profiling our compounds as multi-target drug leads (MTDLs).

Here we publish the results of in silico screening of 20 indole compounds (Figure 1) for monoamine oxidase inhibition. The compounds were recently tested for their inhibition activities towards ALR2 and ALR1 enzymes (Štefek et al., 2014). Two of the compounds (**13**, **20**) with higher affinities towards ALR2 and good predicted binding energies towards MAO-B were tested also for inhibition of human MAO enzymes. They both showed good MAO-B affinities and compound **20** also possessed considerable MAO-B/MAO-A selectivity.

## 2 Methods

The optimal conformers of the compounds were obtained by equilibrium conformer systematic search (MMFF94) in the program SPARTAN'08 (Wavefunction, Inc., Irvine, CA, 2009). For modeling the enzyme-ligand interaction, the PDB structure of MAO-A complexed with FAD and harmine was taken from Protein Data Bank (<http://www.rcsb.org>, structure 2z5x) and for MAO-B the complex with FAD and safinamide (structure 2v5z) was used. The local docking protocol of YASARA (Krieger et al., 2002) was applied. The first ten clusters were then searched for the minimum value of  $E_{\text{binding}}$  within the optimization protocol `em_run.mcr`.

Monoamine oxidase activities from recombinant human MAO A and MAO B (Sigma-Aldrich, Madrid, Spain) were measured using the Amplex UltraRed fluorometric coupled method according to the method described by Bautista-Aguilera et al. (2014).

## 3 Findings and argument

The set of ALR2 inhibitors was divided into three groups: A -with the highest ALR2 inhibition activity ( $IC_{50} < 1 \mu\text{M}$ ); B - compounds with moderate ALR2 activity ( $IC_{50} < 10 \mu\text{M}$ ) and C – compounds with poor ALR2 activity ( $IC_{50} > 10 \mu\text{M}$ ). Compound **13** from the first group showed favorable theoretical prediction of the binding energies towards MAO-A and MAO-B, which was confirmed by experimentation with human enzymes. Compound **20** showed high inhibition of MAO-B with good selectivity towards MAO-A. The group B (**1**, **9**, **10**, **11**, **14**, **15**, **16**, **18** and **19**) provided two compounds with promising MAO-A or MAO-B binding energies, namely **9** ( $E_{\text{binding}} = 315.6 \text{ kJ/mol}$ )

**Table 1:** Top: predicted binding energies for interaction with MAO-A and MAO-B for the most efficient ARIs ( $IC_{50} < 1 \mu\text{M}$ ); right side: experimental values of  $IC_{50}$  towards human MAO-A and MAO-B.

Compound	$E_{\text{binding}}$ (kJ/mol)		
	MAO-A	MAO-B	
A	13	326.1	343.7
	17	353.9	271.7
	20	135.7	588.1

Compound	$IC_{50}$		
	hMAO-A	hMAO-B	
A	13	7.1(165)	9.69(199)
	17	n.d.	n.d.
	20	> 800	3.20(57)

and **10** ( $E_{\text{binding}} = 322.8 \text{ kJ/mol}$ ).

Compound **20** provided the attractive interaction with the coenzyme FAD and important residue Ile199 in MAO-B model. This derivative is very similar to the compounds bearing propargyl amine scaffold already established as the potent MAO and cholinesterase inhibitors (Samadi et al., 2012).

## 4 Conclusion

Of the derivatives studied, 5-(benzyloxy)-1H-indole-1-yl] acetic acid (**20**) has been established to be the most prospective compound for the study and development of multi-target drugs for effective treatment of neurodegenerative diseases. Our next study will focus on further improvement of pharmacodynamic and pharmacokinetic properties of this structural motif.

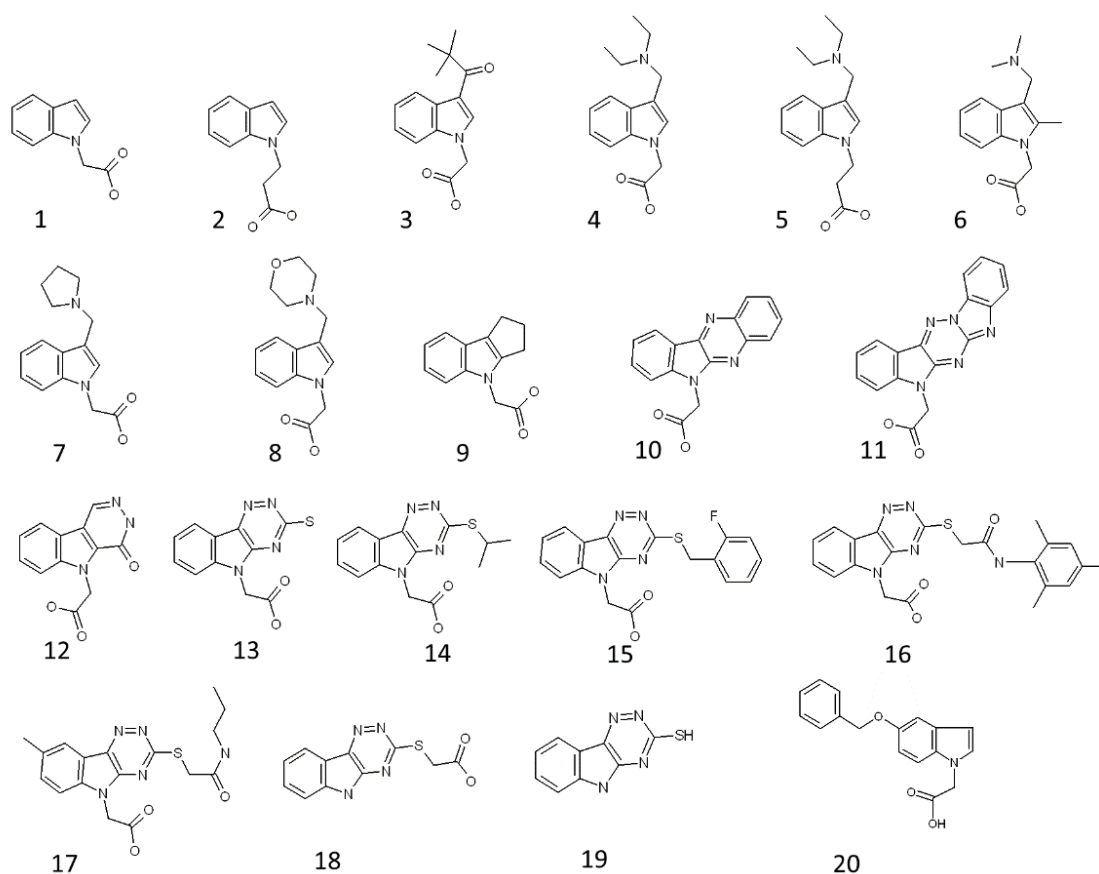


Figure 1: Structure of the indole derivatives

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## Pathogenesis and early diagnosis of Alzheimer's disease: involvement of the monoaminergic system

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**Abstract.** Pathogenesis of Alzheimer's disease (AD) is still not elucidated. There are several different views regarding the occurrence of two main features of AD: amyloid  $\beta$  (A $\beta$ ) and tau protein pathology. Generally, it is considered that the increase in the amount of A $\beta$  is an initiating event in AD (particularly in familial cases), while tau protein hyperphosphorylation and aggregation occur as a consequence. On the other hand, based on postmortally analysed brains Braak and Del Tredici documented that intraneuronal tau aggregation precedes diffuse plaque deposition, but A $\beta$  changes occur before increase of tau in cerebrospinal fluid (CSF). Some of the new hypotheses stressed the possibility that the first pathological changes in AD could occur in brainstem nuclei: noradrenergic locus coeruleus (LC), serotonergic dorsal raphe (DRN) and cholinergic nucleus basalis complex. Our recent study summarized all these findings into a unifying hypothesis: long projection fibres from brainstem nuclei release A $\beta$  that could induce neurofibrillary changes within vulnerable cortical glutamatergic pyramidal neurons. The involvement of monoaminergic nuclei in the pathogenesis of AD was further elaborated in an ongoing study showing that exposure to heavy metals (such as inorganic mercury) can lead to AD-like pathology in LC neurons that may spread to neighbouring DRN neurons. Since current biomarkers of AD detect the disease in symptomatic individuals (in whom neurodegeneration is already substantial), novel biomarkers are essential for early AD detection at preclinical stages. Encouraged by the finding of early monoaminergic nuclei degeneration in AD, many investigators tried to assess the diagnostic potential of monoamine metabolites in CSF. However, due to the differences between studies and methodological limitations, these biomarkers have not been proven as diagnostically useful. Although no individual CSF neurotransmitter proved to be specific for AD, usage of more sophisticated techniques could enable the development of several monoamine metabolites profile,



and possibly improve early AD detection (in addition to currently established core biomarkers).

**Keywords** Alzheimer's disease – pathogenesis – brainstem nuclei – dorsal raphe nucleus – locus coeruleus – early diagnosis – cerebrospinal fluid – monoamine metabolites

## 1 Involvement of the monoaminergic system in pathogenesis of alzheimer's disease

Two main pathological features of Alzheimer's disease (AD) are senile plaques (composed of amyloid  $\beta$ ; A $\beta$ ) and neurofibrillary tangles (composed of aggregated tau protein). It is considered that A $\beta$  pathology precedes tau pathology in cerebrospinal fluid (CSF) (Jembrek et al., 2013). However, based on a large series of postmortally analysed brains Braak et al. (2013) showed that intraneuronal tau aggregation precede diffuse A $\beta$  plaque deposition (Braak et al., 2013). New hypotheses emphasize that AD begins concomitantly in the transentorhinal cortex and brainstem nuclei, or even first in the brainstem nuclei (noradrenergic locus coeruleus - LC, serotonergic dorsal raphe - DRN, and cholinergic nucleus basalis complex - NBC) (Grinberg et al., 2009; for review see Šimić et al., 2014). We tried to summarize all of these findings into a unifying hypothesis where long projection fibres from brainstem nuclei release A $\beta$  that could induce neurofibrillary changes within cortical glutamatergic pyramidal neurons (Šimić et al., 2014). As being part of the default mode network, due to their constant activity these glutamatergic pyramidal neurons produce and release more A $\beta$  than elsewhere in the brain (e.g. within the basal ganglia or cerebellum). This leads to an increase in production, oligomerization, and aggregation of A $\beta$ , which initially compromise endosomal-lysosomal processing and mitochondrial metabolism thus activating caspases responsible for tau cleavage, as well as tau hyperphosphorylation, which is presumably caused by the released A $\beta$  oligomers (Šimić et al., 2014). The sequential cleavage of the tau protein leads to the formation of the tau protein fragment from the microtubule-binding repeat region. This fragment may start an autocatalytic process in which there is progressive accumulation of de novo truncated tau oligomers (Wischnik et al., 2014). Further fusion of oligomeric tau leads to formation of the core of the paired helical filaments – the main constituent of the neurofibrillary tangles, which have been neuropathologically confirmed to be an early event in AD (in the so-called “pre-tangle” stage). The fragmented tau is also able to propagate between neurons

trans-synaptically, ecto- and endosomally, which has recently been confirmed both in vivo and in vitro (de Calignon et al., 2012; Iba et al., 2013; Guo and Lee, 2013). Our previous study supported some of the aforementioned assumptions regarding the early involvement of the monoaminergic systems in the pathogenesis of AD. Namely, noncognitive, behavioral and psychological symptoms of dementia (such as disturbances in mood, emotion, and appetite, deficits in wake-sleep cycle, etc.) have been less considered as clinical criteria for diagnosis of AD. However, the occurrence of these symptoms indeed suggests the involvement of the serotonergic nuclei, which has been confirmed by at least three large retrospective clinical studies (for a review see Šimić et al., 2009. Recent study by Roh et al. (2012) suggested that plaque formation in the brain of transgenic mouse model (APP<sup>swe</sup>/PS1<sup>DE9</sup>) of AD causes the disturbance of sleep-wake cycle. Since lesions of the raphe nuclei cause insomnia and are directly associated with the sleep regulation, the pathology of raphe nuclei could be associated with A $\beta$  increase in two ways: through A $\beta$  release from raphe projection axons or through possible sleep deprivation that leads to inadequate clearance of A $\beta$  in AD (a vicious cycle where less sleep is causing more A $\beta$  production; in turn, more A $\beta$  accumulated leading to less sleep) (Roh et al., 2012). Recently, it has been demonstrated that even cognitively normal individuals with biomarker evidence of preclinical AD have worse quality of sleep than control individuals (Ju et al., 2013). Finally, the involvement of monoaminergic nuclei in the pathogenesis of AD was further elaborated in an ongoing study proving that exposure to heavy metals (particularly inorganic mercury) can lead to AD-like pathology in noradrenergic LC neurons, whereas these changes may also spread to neighbouring serotonergic raphe neurons. Since the cause of severe loss of LC neurons in AD is an early event, and these neurons are particularly prone to taking up circulating toxins, in this view it is highly likely that sporadic AD may be a predominantly environmental tauopathy combined with elements of individual genetic susceptibility levels responsible for additional diversity in time of onset, clinical picture and rate of pathological and clinical progression.

## 2 Monoamine metabolites in early diagnosis of alzheimer's disease

Current biomarkers of AD detect the disease in symptomatic individuals (in whom neurodegeneration is already substantial) (Babić et al., 2013, 2014), so novel biomarkers are essential for early AD detection at preclinical stages. Encouraged by the evidence of early monoaminergic nuclei degeneration in AD, many

investigators tried to assess diagnostic potential of monoamine metabolites in the CSF. The concentrations of monoamine metabolites homovanilic acid (HVA), dihydroxyphenylacetic acid and 5-hydroxyindoleacetic acid (5-HIAA) as well as monoamine synthesis cofactor bipterin were found to be significantly decreased in AD (Kawakatsu et al., 1990; Martignoni et al., 1991; Blennow et al., 1992; Parnetti et al., 1992; Sjögren et al., 1998). AD patients with severe forms of dementia demonstrated the most prominent decrease in the CSF levels of HVA and 5-HIAA, which may reflect a decreased turnover of dopamine and serotonin. However, unaffected (Parnetti et al., 1992; Sheline et al., 1998; Stuerenburg et al., 2004) or elevated (Zubenko et al., 1986; van der Cammen et al., 2006) levels of HVA and 5-HIAA were also reported in AD. Thus, the results of the studies on CSF monoamine metabolites in AD patients have been controversial. In addition to observed changes in the CSF levels of monoamine metabolites, findings of reduced CSF concentrations of neurotransmitters dopamine and serotonin, as well as serotonin precursor 5-hydroxytryptophan suggested a systemic damage of monoaminergic neurons in AD (Volicer et al., 1985). Regarding noradrenergic system, different studies reported increased (Tohgi et al., 1992), decreased (Sjögren et al., 1998) or unchanged (Blennow et al., 1992) levels of noradrenaline and noradrenaline metabolite 3-methoxy-4-hydroxyphenylglycol in CSF of AD patients.

### 3 Conclusion

In conclusion, due to the differences between studies and methodological limitations, monoamine metabolites have not been proven as diagnostically useful biomarkers for AD. However, although no individual CSF neurotransmitter proved to be specific for AD, usage of more sophisticated techniques could enable the development of several monoamine metabolites profile (Czech et al., 2012), and possibly improve AD detection with currently established (core) biomarkers.

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## Benzothiazoles - Scaffold of interest for CNS targeted drugs

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\*

**Abstract.** The novel benzothiazolyl molecules were synthesized and tested on human recombinant ABAD *in vitro*.

**Keywords** Alzheimer disease – mitochondria – ABAD modulator – synthesis – *in vitro*.

### 1 Introduction

Benzothiazole compounds represent heterocyclic systems comprising a benzene ring fused with a thiazole ring containing nitrogen and sulphur in its structure. Besides the presence of a benzothiazole core in naturally occurring molecules, synthesized compounds containing a benzothiazole moiety in their structure proved to be a significant class of potential therapeutics, as they exhibit biological effects such as antitumor, antibacterial, antitubercular, antiviral, anthelmintic, antidiabetic and many others. Apart from the aforementioned peripheral or microbial active sites, benzothiazole analogues are also biologically active compounds in the central nervous system, where some approved drugs containing benzothiazole moiety have already been identified and are used in the treatment of various neurological disorders.

Some benzothiazoles were found to be modulators of Amyloid-binding alcohol dehydrogenase (ABAD). ABAD is to date the most characterized A $\beta$ -binding intracellular protein. Direct interaction of this mitochondrial enzyme with A $\beta$  was confirmed by many different methods. A $\beta$  binding to ABAD triggers a series of events leading to mitochondrial dysfunction characteristic for Alzheimer disease. Thus this interaction may

represent a target for treatment strategy against AD (Muirhead et al., 2010).

### 2 Methods and Findings

The novel series of benzothiazolyl compounds were synthesized with structural modifications. Their purity and entity was checked by <sup>1</sup>H/<sup>13</sup>C NMR and HRMS techniques. The activity of prepared compounds was evaluated on human recombinant ABAD (hrABAD) *in vitro*.

The synthesis of novel compounds was established with yields of 30-90%. The novel compounds exhibited inhibition of hrABAD and some of them showed inhibition ability on low  $\mu$ M or nM scale *in vitro*. Among the prepared compounds, several structural motifs were identified within structure-activity relationship study to play crucial role in hrABAD inhibition (Guest under revision).

### 3 Conclusion

The inhibitors of hrABAD were successfully prepared and evaluated. The *in vitro* findings showed their ability to inhibit ABAD and thus for further research and development.

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## Neurobiology and neuropharmacology of PTSD

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**Abstract.** This review describes the neurobiology and neuropharmacology of post-traumatic stress disorder (PTSD) and discusses the molecular basis of PTSD. The interaction between various psychosocial, biological, environmental, genetic and epigenetic factors determine vulnerability or resilience to develop PTSD after exposure to a traumatic event. Etiology of PTSD is complex, and still not clear. Since not all subjects exposed to traumatic experience will develop PTSD, it is assumed that vulnerability factors include psychosocial, neuroanatomical, neurotransmitter and neuroendocrine changes. PTSD is frequently comorbid with other psychiatric disorders. The review highlights therapeutic strategies and non-response to current treatment options, and stresses the need for the development of new treatments of PTSD.

**Keywords** PTSD – genetic variants – neurotransmitter system – comorbidities – therapies

### 1 Review

Post-traumatic stress disorder (PTSD) is a common, prevalent, severe, disabling and debilitating mental disorder that develops in some, but not all people after exposure to an extreme traumatic event or events. According to the DSM-V criteria, PTSD is a trauma- and stressor-related disorder in which direct exposure to a traumatic experience or witnessing a psychologically stressful event leads to the specific cluster of symptoms: re-experiencing trauma, distressing recollections, dreams, flashbacks; numbing, persistent avoidance of stimuli that might be related or might recall traumatic memories; and hyper-arousal.

Prevalence of lifetime PTSD varies significantly in different countries, from 8% in US (Kilpatrick et al., 2013), to 18% in Croatia (Priebe et al., 2010), and to 25% in Bosnia and Hercegovina (Priebe et al., 2010). The risk of developing PTSD increases if risk factors (female gender, severity, duration and number of traumatic incidents, childhood abuse and neglect, lack of family and social support and existence of previous mental health issues) are present (Zoladz and Diamond, 2013). Half of the exposed victims show acute nature of the disorder but symptoms usually disappear after 3 months. After

progression into chronic PTSD, symptoms might reoccur after 12 months to 50 years (Zoladz and Diamond, 2013). The etiology of PTSD is still poorly understood. Since not all subjects exposed to a traumatic event develop PTSD, the extent to which individuals are vulnerable or resilient to trauma depends on a number of factors, primarily trauma related, psychosocial, biological, environmental, genetic and epigenetic factors, and the interaction between them (Domschke, 2012). Heritability ranges from 30-40% (Almli et al., 2014). Genetic variants in the serotonergic and dopaminergic systems, hypothalamic-pituitary-adrenal (HPA) axis, and other genes related to response to stress and fear are associated with PTSD (Almli et al., 2014). Serotonergic system is involved in the emotional responses and regulation of mood, dopamine has a role in attention, vigilance, sleep and arousal, HPA axis is associated with hyper-responsiveness to stress and emotion dysregulation, brain derived neurotrophic factor (BDNF) and apolipoprotein E are involved in dysregulation of stress, while genes controlling neuropeptide Y and GABA are associated with mechanisms of fear and anxiety. Recently, GWAS studies successfully identified and replicated variants in retinoid-related orphan receptor alpha (RORA) gene as potential genetic risk factors for PTSD, while other risk genes were not replicated, due to the fact that genetic loci contributing to risk of PTSD are numerous and comprised of many rare variants with small effect size, thus most studies are underpowered.

PTSD is frequently comorbid with depressive disorders, substance use disorders, and other anxiety disorders, and these comorbidities complicate the treatment, induce greater psychological distress, significant functional impairment and more frequent inpatient hospitalizations and diminish social functioning (Norman et al., 2012). Treatment strategies in PTSD are focused on reduction or elimination of symptoms, improvement of adaptive functioning, coming back to a safe and trustworthy state, restriction of the generalization of trauma and protection of a patient. Besides psychotherapy and psycho-education, pharmacological treatments that concentrate on the symptom reduction are selective serotonin reuptake inhibitors, anticonvulsants, atypical antipsychotics, alpha adrenergic antagonist, prazosin, and benzodiazepines that are not effective in PTSD symptom improvement but might reduce anxiety and improve sleep (Vieweg et al., 2006; Norman et al., 2012). Patients with PTSD are frequently non-responsive or modestly responsive to current treatment options (Ahearn et al., 2011), and because of the general refractoriness and high rates of comorbidities, there is a high priority for development of novel targeted treatments and interventions.

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## Theoretical and Pharmacological Study of Multitarget Compounds Against Neurological Diseases

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**Abstract.** Multi-target ligands, recently developed in our laboratories, are novel drug candidates able to interact with MAO-A and B; AChE and BuChE; or with HMT and H<sub>3</sub>R, as essential drug targets in the treatment of Alzheimer's disease, depression, obsessive disorders, and Parkinson's disease. Using the refined ChEMBL families and our validated cheminformatic approach of the protein target prediction we have identified the pharmaceutical target and off-targets associated with the 134 multipotent compounds able to interact with MAO-A and B; AChE and BuChE; or with HMT and H<sub>3</sub>R. High affinities for the top ranked off-targets of the selected ligands were confirmed by *in vitro* testing (5-HT<sub>1a</sub>R, 5-HT<sub>2a</sub>R) and 3D-QSAR(H<sub>3</sub>R/D<sub>1</sub>R/D<sub>2</sub>R/5-HT<sub>2a</sub>R) studies. Multi-target ligands with possible additional beneficial pharmacological activities were selected for further study.

**Keywords** multi-targeted ligands – circular fingerprints – off-target study – ChE – MAO

## 1 Introduction

Novel drug design has expanded beyond the one drug-one target theory. Polypharmacology is a new pharmacological concept for the study of drug action which can involve plural targets interacting with multiple targets to address disease in more effective and subtle ways. Therefore, development of multi-targeted ligands as novel drug candidates against neurological diseases was one of the main aims of our recent studies (Juárez-Jiménez et al., 2014; Bautista-Aguilera et al., 2014; Apelt et al., 2002).

## 2 Methods

Our recently developed PFClust clustering algorithm (Mavridis et al., 2013) was applied to all of the filtered ChEMBL families. A probabilistic method, the Parzen-Rosenblatt kernel density estimation method (Parzen, 1962), was applied to build a predictive model from the ChEMBL dataset. Circular fingerprint descriptors (Glem et al., 2006) were calculated for compounds used in the study and compared by Tanimoto similarity scores. The obtained Tanimoto similarity scores are then transformed into probabilities (pairwise p-values) using kernel (Gaussian) function.

The same cheminformatic methodology was used to

create a “predictor” model from the DrugBank database to determine the main pharmacological groups of the examined 134 multi-target ligands (Lowe et al., 2012).

## 3 Results

For the MAO/ChE inhibitor, compound 71/MBA-VEG8, the cheminformatic method has determined serotonin 5-HT<sub>1a</sub>R, 5-HT<sub>2a</sub>R, 5-HT<sub>2c</sub>R, 5-HT<sub>5a</sub>R, and D<sub>1</sub>R as possible off-targets (Figure 1).

The compound 71/MBA-VEG8 was further examined by the *in vitro* 5-HT<sub>2a</sub>R and 5-HT<sub>1a</sub>R binding assay. The binding study has confirmed relatively strong affinity of the 71/MBA-VEG8:  $K_i(5\text{-HT}_{2a}\text{R}) = 14.2\text{ nM}$  and  $K_i(5\text{-HT}_{1a}\text{R}) = 108\text{ nM}$  for the receptors. High affinities for the top ranked off-targets of the selected ligands were also confirmed by 3D-QSAR(H<sub>3</sub>R/D<sub>1</sub>R/D<sub>2</sub>R/5-HT<sub>2a</sub>R) studies. The developed workflow, composed of cheminformatic, 3D-QSAR and *in vitro* studies, represents a reliable methodology that can easily be used in initial phases of drug design process.

## 4 Conclusion

The identified novel off-targets for the examined ligands were confirmed by *in vitro* 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptor binding assay and by 3D-

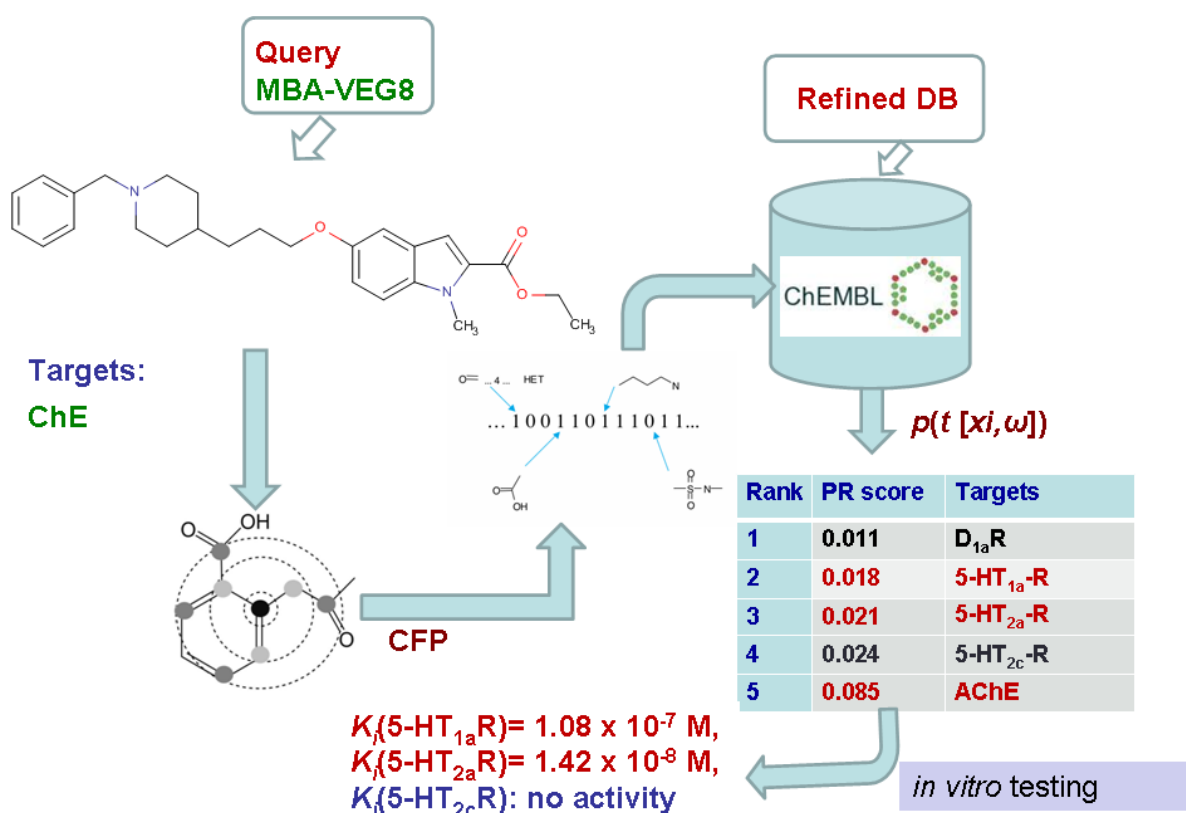


Figure 1: Target for a query compound (71/MBA-VEG8) retrieved from refined ChEMBL dataset.

QSAR(H<sub>3</sub>R/D<sub>1</sub>R/D<sub>2</sub>R/5-HT<sub>2a</sub>R) predictions. Several multi-target ligands were selected for further study, as compounds with possible additional beneficial pharmacological activities.

## Acknowledgement

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# Cis-cyclopropylamines as mechanism-based inhibitors of monoamine oxidases

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**Abstract.** Investigated for the therapy of depression and cancer, cyclopropylamines are known inhibitors of monoamine oxidases (MAO A and MAO B) and lysine-specific demethylases (LSD1). The antidepressant drug, tranylcypromine, which has a *trans* structure, inhibits all three enzymes. We investigated novel primary and secondary cyclopropylamines with an alkoxy group at the 2 position of the cyclopropyl ring, all with the less common *cis* relationship. Theoretical  $K_i$  values for both enantiomers were obtained by docking the compounds into MAO A or MAO B using Autodock but revealed very little difference between the enantiomers. Using a mixture of the enantiomers, the reversible inhibition of MAO A gave high  $IC_{50}$  values for most compounds. In contrast, the  $IC_{50}$  values were sub-micromolar for MAO B. Irreversible binding was similarly selective for MAO B. Spectral changes during the inactivation reaction, the stability of the adduct and its mass were consistent with an adduct to the flavin but rates of inactivation were slower than for tranylcypromine. The best inhibitor was *cis*-N-benzyl-2-methoxycyclopropylamine, with an  $IC_{50}$  of 5 nM for MAO B and 170 nM for MAO A after 30 min preincubation, but no activity on LSD1. This *cis*-cyclopropylamine is over 200-fold more effective than tranylcypromine so could be studied as a lead for selective inhibitors of MAO B that do not inhibit LSD1.

**Keywords** docking – enantiomers – flavin adduct – mechanism-based inhibitor – tranylcypromine

## 1 Introduction

Cyclopropylamine is a useful structural scaffold for the design of mechanism-based inhibitors and many structure-activity studies have appeared in the quest for lead compounds for the therapy of depres-

sion and cancer. Cyclopropylamines are known inhibitors of monoamine oxidases (MAO), cytochrome P450 (CYP) enzymes, and lysine-specific demethylases (LSD1) (Khan et al., 2013; Binda et al., 2010; Mimasu et al., 2010). The antidepressant drug, tranylcypromine (TCP), is a mechanism-based inactivator of monoamine oxidases (MAO) used in the treatment of depression and also an irreversible inhibitor of LSD1, one of the key demethylase enzymes in epigenetic gene regulation. Contrasting with TCP (a *trans* structure), we have investigated novel cyclopropylamines with the less common *cis* relationship to define their efficacy and selectivity.

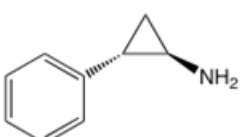
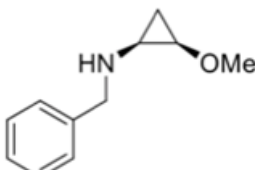
## 2 Methods

Activity for membrane-bound MAO (Sigma-Aldrich, UK) was determined in the coupled Amplex Red assay (Zhou & PanchukVoloshina, 1997). For the reversible inhibition,  $IC_{50}$  values in the presence of 2.5xKm substrate concentration (tyramine) with the enzyme added last. The  $IC_{50}$  values for the irreversible inactivation were determined from the activity remaining after 30 min of incubation of the enzyme and inhibitor in the presence of 1 mM tyramine. Data were analysed in Graphpad PRISM to give the parameter  $\pm$ sd from at least 20 points. The adduct was characterised by the spectral and mass change in MAO A (Esteban et al., 2014; Mitchell et al., 2001).

Molecular models of the *cis*-cyclopropylamine inhibitors were built and optimised using ArgusLab 4.0.1 on an Intel i7 HP Laptop, operating Windows 8 Home Premium. MAO A (PDB code: 2Z5X) and MAO B (PDB code: 2V5Z) protein structures were minimised using Accelrys 6.0 adopting a CHARMM force field and simulated annealing. Docking used AutoDock4 (Morris et al., 2009) and Vina coding scripts.

## 3 Findings and argument

Theoretical  $K_i$  values for both enantiomers of seven novel compounds obtained by docking the compounds into MAO A or MAO B using Autodock revealed very little difference between the enantiomers, so all experimental work used the mixture of enantiomers. The computed reversible  $K_i$  values ranged from 3  $\mu$ M to 2 mM with only a 2-fold difference between MAO A and B. In contrast, the experimental  $IC_{50}$  values revealed selectivity for MAO B with some of the compounds. The reversible inhibition of MAO A was poor and agreed with the predicted values but the experimental  $IC_{50}$  values for MAO B were all low micromolar and as good as or better than the standard drug, tranylcypromine. For the irreversible inhibition,  $IC_{50}$  values measured after 30 min pre-incubation of the enzyme and inhibitor are shown in Table 1. The 2-substituted *cis*-analog is less ef-

		
IC <sub>50</sub> (nM)	trans-cyclopropylamine ( <i>trans</i> )	<i>cis</i> -N-Benzyl-2-methoxycyclopropylamine
MAO A	175(68) nM	237(61) nM
MAO B	4.70(5) nM	73.5(49) nM
Selectivity for MAO B	104	3.2

**Table 1:** Efficacy and selectivity for MAO B for the best *cis*-cyclopropylamine compared to trans-cyclopropylamine

fective than trans-cyclopropylamine and less selective for MAO B. The slower rate of inactivation of MAO B by the *cis*-compound ( $0.104(5) \text{ min}^{-1}$  compared to  $0.263(5) \text{ min}^{-1}$  for trans-cyclopropylamine) may explain the lower efficacy on MAO B.

The irreversible inactivation was shown to be due to adduct formation by the spectral changes in MAO A, by the stability of the adduct to denaturation in urea and during pronase digestion to release the modified flavin co-factor, and by the mass increase.

## 4 Conclusion

*cis*-N-Benzyl-2-methoxycyclopropylamine (4) is a new irreversible MAOI with IC<sub>50</sub> 5 nM for MAO B, 170 nM for MAO A, and no activity on LSD1.

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## Evaluation of multiple amine oxidase inhibitory behaviour

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**Abstract.** Amine oxidases will be exposed to a variety of inhibitory compounds, in addition to specific drugs that may be administered for therapeutic purposes. Procedures for analysing the effects of multiple reversible inhibitors are presented in terms of their cumulative effects on enzyme activity.

**Keywords** inhibition – kinetics xenobiotics – monoamine oxidase – primary-amine oxidase

## 1 Introduction

The amine oxidases, monoamine oxidase (EC 1.4.3.4; MAO) and primary amine-oxidase (EC 1.4.3.21; SSAO), are sensitive to inhibition by a diverse range of drugs that have been developed for pharmacological and therapeutic uses (Tipton et al., 2011). In some cases it is a metabolite of the administered compound that is inhibitory. In addition a number of dietary components and xenobiotics can inhibit these enzymes (Benedetti Strolin and Tipton, 1999; Olivieri et al., 2011). In some cases, such compounds may actually act as competing substrates for the enzyme and, therefore, can be regarded as competitive inhibitors of the oxidation of the indigenous substrates. The equations describing reaction rates in the presence of two competing substrates are well known (McDonald and Tipton, 2003) and can be expanded to situations involving multiple substrates, each with defined kinetic parameters.

## 2 Analyses

Any evaluation of the effects of drugs or xenobiotics must take account of the totality of the inhibitory effects arising from all sources. The general equation for the inhibition of an enzyme by multiple reversible inhibitors may be written as:

$$\nu_{1\dots n} = \frac{V_{\max}}{\frac{K_m}{[S]} \left( 1 + \frac{[I_1]}{K_i} + \frac{[I_2]}{K_{i2}} + \frac{[I_3]}{K_{i3}} + \dots + \frac{[I_n]}{K_{in}} \right) + \left( 1 + \frac{[I_1]}{K'_i} + \frac{[I_2]}{K'_{i2}} + \frac{[I_3]}{K'_{i3}} + \dots + \frac{[I_n]}{K'_{in}} \right)}$$

where  $\nu_{1\dots n}$  is the velocity of the reaction in the presence of  $n$  inhibitors that bind to the enzyme in a mutually exclusive fashion, with no enzyme molecule being able to bind more than one of the inhibitors. The substrate concentration is represented by  $[S]$  and the inhibitor concentrations by  $[I_1] \dots [I_n]$ .  $V_{\max}$  is the maximum velocity. This equation may be written in condensed form as:

$$\nu_{1\dots n} = \frac{V_{\max}[S]}{K_m \left( 1 + \sum_{[I]=1}^n \frac{[I]}{K_i} + [S] \left( 1 + \sum_{[I]=1}^n \frac{[I]}{K'_i} \right) \right)}$$

or, in dimensionless terms, as

$$\frac{\nu_{1\dots n}}{[E]} = \frac{k_{\text{cat}}}{\alpha (1 + \beta_1 \dots \beta_n) + 1 (\gamma_1 \dots \gamma_n)}$$

where  $[E]$  is the enzyme concentration,  $\alpha = \frac{K_{extm}}{[S]}$ , the  $\beta$  values are the respective  $\frac{[I]}{K_i}$  values and  $\gamma$  values are those for  $\frac{[I]}{K'_i}$ .

For competitive inhibitors all the  $K'_i$  values will be equal to infinity so that the equation simplifies to

$$\nu_{1\dots n} = \frac{V_{\max}}{\frac{K_m}{[S]} \left( 1 + \frac{[I_1]}{K_i} + \frac{[I_2]}{K_{i2}} + \frac{[I_3]}{K_{i3}} + \dots + \frac{[I_n]}{K_{in}} \right)}$$

Whereas all the  $K_i$  values will be infinite for uncompetitive inhibition and both  $K_i$  and  $K'_i$  values will be finite for mixed inhibitors.

Clearly, it would be a simple matter to determine the overall effects of reversible inhibitors on the behaviour of an enzyme provided their concentrations and kinetic behaviour and parameters are known. It should also be noted that such effects may affect the rates at which irreversible mechanism-based inhibitors reduce enzyme activity. Unfortunately, such a rigorous analysis requires a great deal of experimental work and a simplified approach has been presented by Chou and Talaly (1977), which is independent of the type of inhibition but relies on the reaction rate at fixed substrate and inhibitor concentrations

$$\nu_{1\dots n} = \sum_{[I]=1}^n \nu_i - \frac{n-1}{\nu_0}$$

where  $\nu_i$  is the velocity observed in the presence of each individual inhibitor, and  $\nu_0$  is the velocity in the absence of inhibition. However, since the presence of inhibitor(s) is likely to affect the steady-state substrate levels and alterations in the concentrations of one, or more, of the inhibitors may vary with intake, many recalculation could be necessary.

### 3 Conclusion

The list of dietary components and drugs, which are taken for other purposes, that are known to inhibit one or more of the amine oxidases is quite extensive and include caffeine (MAO & SSAO), imidazolines, used for blood pressure control, (MAO); glucosamine (SSAO); L-lysine (SSAO); the anxiolytic hydroxyzine (MAO & SSAO); components of cigarette smoke (MAO) and products arising from alcohol metabolism (MAO) as well as the methionine arising from creatine metabolism, which acts as a competing SSAO substrate.

Thus a full assessment of the levels of inhibition of these enzymes *in vivo* would necessitate knowledge of an individual's drug and xenobiotic consumption, with consequent tissue levels. Unfortunately, these are generally unavailable.

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## Therapeutic approach to cerebrovascular diseases by MTDL containing propargyl moiety and an indole-substituted hydrazine using endothelial hSSAO/VAP-1 expressing cells as an experimental model of cerebral ischemia

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**Abstract.** The neurovascular unit, integrated by neurons, microglia and vascular cells maintains the brain homeostasis, but its functionality is altered and contributes to brain pathologies such as Alzheimer's disease (AD) or cerebral ischemia. Vascular adhesion protein 1 (VAP-1) is a pro-inflammatory vascular protein with semicarbazide-sensitive amine oxidase (SSAO) activity. This enzyme is overexpressed in AD cerebrovascular tissue, and elevated in plasma from AD and stroke patients, compromising the cerebrovascular function and therefore the neurovascular unit integrity. We hypothesize that the use of molecules able to interact with different cells types and molecules may enhance the beneficial effects for the treatment of these pathologies. Therefore we have assessed the effect of the indole-substituted hydrazine JL-72 and the MTDL compounds ASS234, DPH-4 and PF 9601N containing a propargyl group in an *in vitro* experimental model of ischemia using human SSAO/VAP-1-expressing endothelial cells. These molecules, with reported neuroprotective properties, also showed a protective effect on this model by SSAO/VAP-1-dependent (DPH-4) or independent (JL-72, ASS234 and PF 9601N) manner. These results suggest a potential use of these compounds for the vascular dysfunction associated to brain diseases and further design of new SSAO/VAP-1 inhibitors might be considered an appropriate therapeutic approach to vascular pathologies.

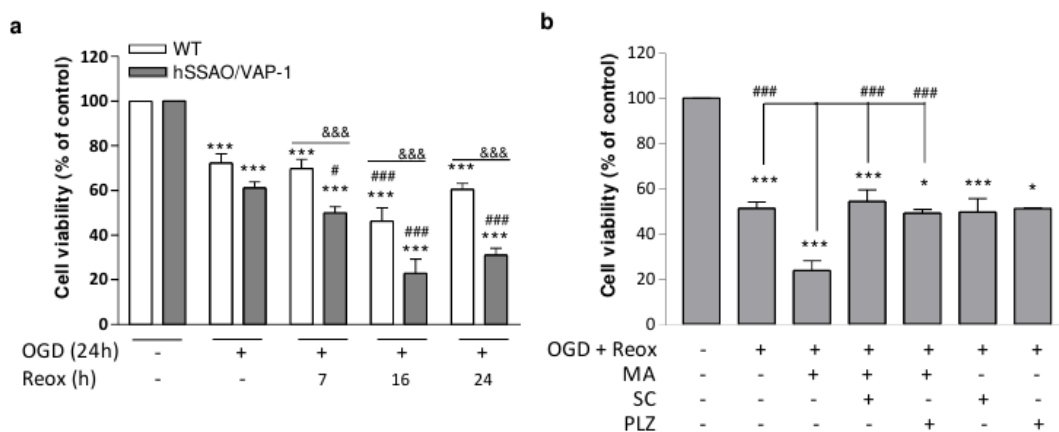
**Keywords** cerebral ischemia – Alzheimer's disease – neurovascular unit – MTDL – endothelial hSSAO/VAP-1

## 1 Introduction

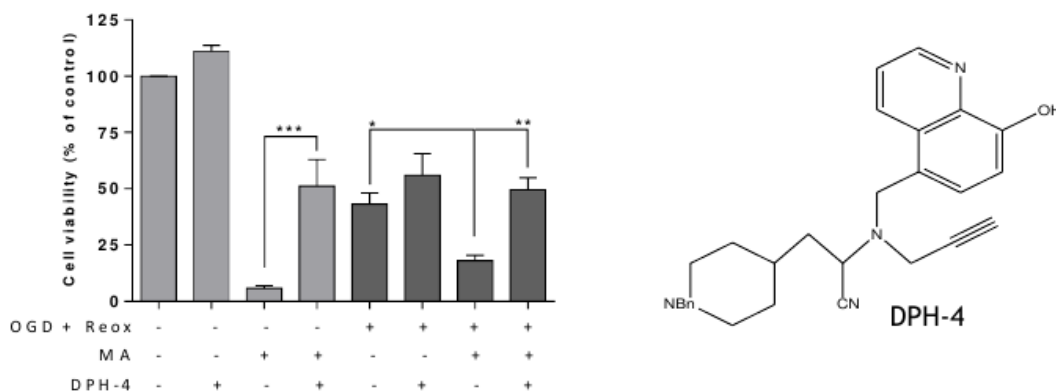
There is increasing evidence that cerebrovascular dysfunction plays a role in cognitive impairment related to Alzheimer's disease (AD). In recent years, the concept of "neurovascular unit" has emerged as a new paradigm for understanding the CNS pathologies, including stroke (Iadecola, 2004). Neurons, glia and vascular cells are closely interrelated and working in concert to maintain the homeostasis of cerebral microenvironment. The development of new agents able to interact with neuronal activity and vascular cells could be an appropriate therapeutic approach for both stroke and AD conditions.

Vascular adhesion protein 1 (VAP-1) is a pro-inflammatory vascular protein that mediates leukocyte recruitment through its semicarbazide-sensitive amine oxidase (SSAO) activity (EC 1.4.3.21) (Jalkanen and Salmi, 2008). This enzyme is over-expressed in human

cerebrovascular tissue affected by cerebral amyloid angiopathy (CAA), found in most AD patients (Ferrer et al., 2002) and it is also increased in plasma from severe AD patients (Hernández-Guillamon et al., 2005). Moreover, the soluble form of SSAO/VAP-1 present in plasma predicts the appearance of parenchymal hemorrhages after tissue plasminogen activator treatment in ischemic stroke condition and it is increased in hemorrhagic stroke patients as well (Hernández-Guillamon et al., 2010, 2012). In this concern, the beneficial effect of the indole-substituted hydrazine JL-72 (Esteban et al., 2013) and the multitarget-directed ligands (MTDL) ASS234, DPH-4 and PF 9601N has been already reported in experimental models of Parkinson's disease and AD (Bolea et al., 2013; Wang et al., 2014; Perez and Unzeta, 2003).



**Figure 1:** Time-course effect of oxygen and glucose deprivation (OGD) and reoxygenation (Reox) on WT or hSSAO/VAP-1 HUVECs cell viability (a). The effect of methylamine (MA, 1 mM) metabolism by SSAO was analyzed under 24h OGD with 7h Reox in SSAO/VAP-1-expressing cells (b). Semicarbazide (Sc, 1 mM) and phenelzine (PLZ, 100 nM) were used as SSAO inhibitors. Cell viability was determined by MTT reduction. Data represent the mean  $\pm$  SEM of three independent experiments. #,  $p < 0.05$ ; \*\*\*,  $p < 0.001$  by One-way ANOVA followed by the Newman-Keuls multiple comparison test.



**Figure 2:** hSSAO/VAP-1 HUVEC cells were subjected to OGD for 24h and then to reoxygenation for 7h in the presence of SSAO substrate methylamine (MA, 3 mM) and DPH-4 (1  $\mu$ M) (left). Controls of the same treatments were performed under normoxia conditions (Norm). Cell viability was determined by the MTT reduction. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  by one-way ANOVA and the Newman-Keuls multiple comparison test. Molecular structure of the DPH-4 (right).

## 2 Methods

Here, the protective effect of these molecules has been assessed in a new *in vitro* experimental model of ischemia developed in our group using human SSAO/VAP-1-transfected endothelial cells (Sun et al., 2014). Cells were subjected to oxygen and glucose deprivation (OGD) for 24 h in the presence of each compound. The cell viability was determined by the MTT reduction assay.

## 3 Findings and argument

The most suitable OGD and reoxygenation conditions selected were 24 h OGD with 7 h reoxygenation, conditions that rendered a 50% of cell death analysed by MTT (Figure 1a). When cells were incubated in the presence of methylamine, the specific SSAO/VAP-1 substrate, cell viability decreased significantly, confirming the involvement of the catalytic action of SSAO/VAP-1 in the endothelial cells damage. Furthermore, the cell death was prevented in the presence of semicarbazide or phenelzine (Figure 1b).

The protective effect of molecules JL-72, ASS234, PF 9601-N and DPH-4, were analysed in this experimental model. All of them were found to exhibit distinct inhibitory potencies towards the enzymes SSAO, MAO A, MAO B and AChE/BuChE (data not shown). In each case, a toxicity curve was previously performed in order to use non-toxic concentrations. Partial inhibition of the SSAO activity was only reached with DPH-4. However, a protective effect was observed with all of the molecules when cells were incubated in the presence of methylamine under the experimental ischemic conditions. The compounds JL-72, ASS234 and PF 9601-N, showed 50% protective effect in an independent SSAO/VAP-1-inhibitory manner, whereas DPH-4 exhibited the highest protection dependent on SSAO/VAP-1 inhibition (Figure 2).

Herein we report for the first time the involvement of SSAO/VAP-1 inhibition on the protective effect of a hydrazine and some propargyl-containing MTDLs on endothelial cells under ischemic conditions.

## 4 Conclusion

These results suggest a potential use of these compounds for vascular dysfunction associated to CAA-AD or stroke, which may also be applied for the design of new SSAO/VAP-1 inhibitors as an appropriate therapeutic approach to cerebrovascular pathologies.

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as new multifunctional metal-chelators, ChE and MAO inhibitors for the potential treatment of Alzheimer's disease. *Eur. J. Med. Chem.* 80, 543–561.

## Interaction of novel Monoamino Oxidase Inhibitor with Cytochrome P450

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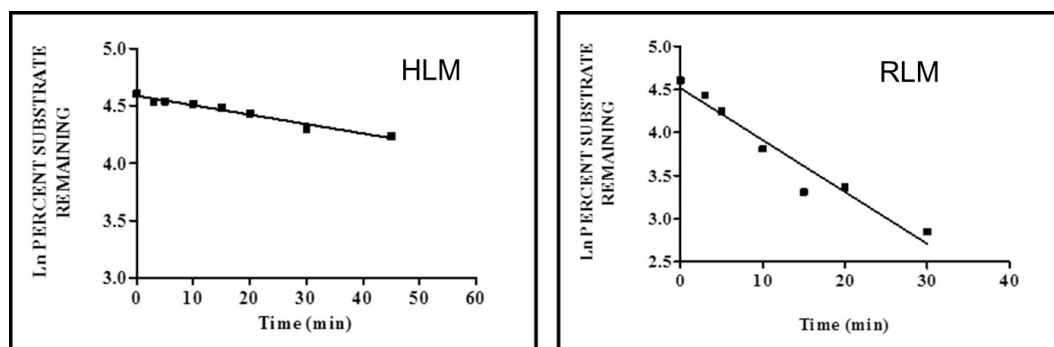
**Abstract.** The effects of monoamine oxidase inhibitors (MAOIs) towards cytochrome P-450 (CYP) dependent activity resulted an important aspect in the therapeutic use of these compounds. Several MAOIs present a propargylamino moiety. This chemical group confers them properties of irreversible inhibitors towards the MAO and could represent a potential molecular site to the formation of suicide substrates toward CYP. In fact CYP metabolism could give rise to the formation of an active electrophil binding site, resulting in an inhibition of CYPs, which could be responsible of a drug-drug interaction when compounds should be administer in a multiple therapy. For these reasons the metabolic features of a series of novel multitarget compounds, characterized by MAO and acetylcholine esterase (AChE) inhibiting properties were studied in human liver microsomes. The kinetic analysis showed that lead compound ASS234, is a poor substrate for human CYPs at variance to those observed in rat liver microsomes. These preliminary in vitro results indicate that ASS234 may have a suitable pharmacokinetic profile.

**Keywords** Drug Metabolism – Cytochrome P450 – Metabolic stability

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## 1 Introduction

In recent years, attention has been focused on the importance of studies on absorption, distribution, metabolism, excretion and toxicity (ADMET) of drug candidates. The goal of a successful drug discovery program is therefore not only the identification of a new molecule highly active and selective towards a suitable molecular target, but also the early assessment of



**Figure 1:** Kinetic parameters for the time-dependent metabolism of ASS234. Results are presented graphically: Ln of residual activity as a function of pre-incubation time. Curves were fitted by a straight-line equation for linear regression and  $r^2$  ranged between 0.99 and 0.93. HLM, human liver microsomes; RLM, rat liver microsomes.

its ADMET properties, in order to discard those compounds that are unlikely to be successful drug candidates. In this context, cytochrome P450 family (CYP) plays a crucial role both in metabolism and in the toxicity of a drug. CYP enzymes can be inhibited or induced by drugs, resulting in clinically significant drug-drug interactions that can cause unpredictable adverse reactions or therapeutic failures. For these reasons the metabolic features of a series of novel multitarget compounds (Bolea et al., 2011), characterized by MAO and acetylcholine esterase (AChE) inhibiting properties were studied in human and rat liver microsomes.

## 2 Methods

The incubation mixture (total volume 500  $\mu$ l) contained the following components (final concentration): TRIS-HCl, pH 7.6 (50 mM), MgCl<sub>2</sub> (25 mM), human or rat liver microsomes (0.5 mg protein / ml), NADPH (0.215 mM) and resorufine-derivative probe substrates, such as ETR (2  $\mu$ M), PTR (10  $\mu$ M) or BZR (5  $\mu$ M), and 50  $\mu$ M novel MOAIs (ASS 50, ASS 64, ASS 92 and ASS 234, see Samadi et al., 2011 according to D'Elia et al., 2009). In a second series of experiments the lead compound ASS234 (5  $\mu$ M) was incubated of liver microsomal proteins and the reactions were terminated at regular time intervals (0–60 min) by adding a double volume of acetonitrile. Samples were analyzed by HPLC-UV method. The intrinsic clearance ( $Cl_{intr}$ ) was calculated by the following equation

$$Cl_{intr} = k(\text{min} - 1) \times \frac{[V]}{[P]}$$

where  $k$  is the rate constant for the depletion of substrate,  $V$  is the volume of incubation in  $\mu$ l and  $P$  is the amount of microsomal proteins in the incubation medium in mg according to Baranczewski et al. (2006).

## 3 Findings and argument

All the compounds tested presented a significant inhibition of CYP-dependent metabolism of ETR, (CYP1A family, marker substrate), while the metabolism of other resorufine substrates was unaffected. Moreover, this effect resulted fully reversible and a competitive fashion. In spite the latter experimental evidences the metabolic stability of the lead compound ASS234 was studied.

As reported in figure 1, the plot of the natural logarithm of the percent of the compounds non metabolized versus time was linear, indicating that the substrate depletion by CYPs followed a monoexponential relationship. The calculated rate constant,  $k$ , ranged from 0.0083  $\text{min}^{-1}$  up to 0.0602  $\text{min}^{-1}$  for human liver microsomes and rat liver microsomes, respectively. The  $Cl_{intr}$  ranged from 1.7 up to 129.2 ( $\mu\text{l min}^{-1} \text{mg}^{-1}$ ) in human and rat liver, respectively. Furthermore the calculated  $t^{1/2}$  resulted 7.5 fold greater in human compared rat microsomal preparations, (83 min vs 11 min, respectively).

## 4 Conclusions

Richmond et al. (2010), classified the chemical compounds based on their  $Cl_{intr}$  value (expressed as  $\mu\text{l min}^{-1} \text{mg protein}^{-1}$ ), where, in rat,  $< 9$  = very low metabolic turnover;  $8 - 50$  = low clearance;  $50 - 150$  = moderate clearance;  $> 150$  = high clearance value. These data indicated that the studied compound, AASS 234, possess a double-acting, it presented a high metabolic stability in human, while an high clearance effect was observed in rat. The present results indicate that ASS234 may have a suitable pharmacokinetic profile in human.

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## Design of new compounds targeting cancer and CNS diseases

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**Abstract.** Alzheimer's disease (AD):

One of the strategies being pursued in the search for pharmacological therapies for AD is blockade of tau hyperphosphorylation by selective inhibitors of GSK-3 $\beta$ . Palinurin has emerged as a non-ATP-competitive inhibitor of GSK-3 $\beta$ . We have achieved the first enantioselective total synthesis of palinurin starting from commercially available furaldehyde and (R)-3-hydroxy-2-methylpropionate. The key steps of the synthesis include the use of a chiral pyrrolidine to create the chiral tetronic moiety, and Horner-Wadsworth-Emmons, Wittig and Wittig-Horner reactions to construct the alkene units.

Vitamin D field:

Next to its classical activities, 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> (**1**, calcitriol) has been shown to inhibit cellular proliferation, to induce cellular differentiation and to have numerous indirect effects on the immune system. However the therapeutical utility of **1** is hampered by the effective doses leading to calcemic side effects and this has stimulated the search for analogues having a relatively weak systemic effect on calcium metabolism while maintaining potent regulatory effects on cell differentiation and proliferation. Among the many new calcitriol analogs, worth mentioning those in which the C-21 methyl group was extended to form a second side-chain giving rise to new class of derivatives, known as Gemini. We developed a novel synthetic methodology for the preparation of Gemini Vitamin D<sub>3</sub> analogs, based on a sigmatropic rearrangement, giving access to a variety of Gemini analogs with potentially interesting biological properties.

Marine toxins:

Polycyclic ethers are the structural basis of many natural products such as the so-called marine ladder toxins, a family of red tide toxins with highly complex unusual molecular architecture, a series of fused cyclic ethers having regular trans-syn-trans stereochemistry. An efficient procedure for preparing enantiopure polycyclic ethers is reported. This method is based on the use of the "furan approach" we developed some years ago in our laboratories and which uses the oxidation of furan by singlet oxygen followed by and intramolecular Michael addition.

**Keywords** Alzheimer's disease – Vitamin D – Gemini analogs – Marine toxins

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## Homology modeling of the human dopamine transporter (hdat): the elucidation of the binding site

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**Abstract.** The dopamine transporter (DAT) is a sodium-coupled symporter, which is in charge of information transfer in neurons functioning in the nervous system. Disorders such as Parkinson's disease (PD), depression, bipolar disorder, attention deficit hyperactivity disorder (ADHD) and schizophrenia are implicated with the abnormal levels of dopamine. Therapeutics used to treat these diseases as well as many addictive drugs target DAT. Molecular modeling studies have become possible with the availability of the three dimensional (3D) structure of the DAT.

In this study the prospective 3D structures of the human DAT, which is predicted from primary amino acid sequence using homology modeling techniques, were used. We have determined the binding sites and relative binding affinities of several ligands with the predicted structures of DAT. These computationally obtained binding affinities and binding poses, correlate well with the reported experimental values.

**Keywords** Dopamine transporter (DAT) – homology modeling – docking

## 1 Introduction

The dopamine transporter (DAT) is a sodium-coupled symporter, which monitors the concentration of dopamine (DA) by re-uptaking the dopamine into presynaptic neurons in the brain (Huang and Zhan, 2007). The transport process of the DAT includes the translocation of the substrate dopamine (DA) and two sodium, one-chloride ions across the cell membrane (Krueger, 1990). The uptake is energetically coupled to transmembrane concentration gradient of  $\text{Na}^+$ , which is maintained by  $\text{Na}^+\text{K}^+$  ATPase (Hitri et al., 1994).

Many illnesses such as Parkinson's disease (PD), depression, bipolar disorder, schizophrenia and attention deficit hyperactivity disorder (ADHD) are implicated by the abnormal levels of DA (Vaughan and Foster, 2013). Therapeutics used to treat these diseases as well as many addictive drugs target DAT.

The initial structural study related to DAT was the x-ray crystal structure determination for a bacterial homolog of DAT, which was the bacterial leucine transporter ( $\text{LeuT}_{\text{Aa}}$ ) from sodium symporter (NSS) family (Yamashita et al., 2005). The early homology modeling study was the folding of rat DAT (rDAT) based on the  $\text{LeuT}_{\text{Aa}}$  as a template (Indarte et al., 2008). Based on both  $\text{LeuT}_{\text{Aa}}$  x-ray crystal structure and homology modeled rat DAT model, human DAT was folded using homology modeling methods.

## 2 Methods

The fully automatic model-building program, MODELLER, was utilized in our study. With the aim of satisfying constraints, MODELLER develops a hypothetical target structure based on a familiar template structure. First, these spatial constraints in the form of atom-atom distances and dihedral angles are extracted from the template and transferred into the target protein structure.

The alignment enables us to identify parallel residues between the target and the template. The optimization of the target model continues until a model that best satisfies the spatial constraints is obtained. MODELLER is most commonly used for homology modelling of protein's three-dimensional structure as the program deduces structure by satisfaction of spatial constraints (from Accelrys). The output of MODELLER is a tertiary structure of a protein that satisfies a set of constraints as well as possible (Martí-Renom et al., 2000). MODELLER protocol evaluates protein structure using the Probability Density Function (PDF) energy data and DOPE (Discrete Optimized Protein Energy) scoring function. Using Autodock (Morris et al., 1998) docking program several important model ligands were docked into the binding site of predicted structure of DAT and the binding pose and the relative binding affinities of these ligands were determined.

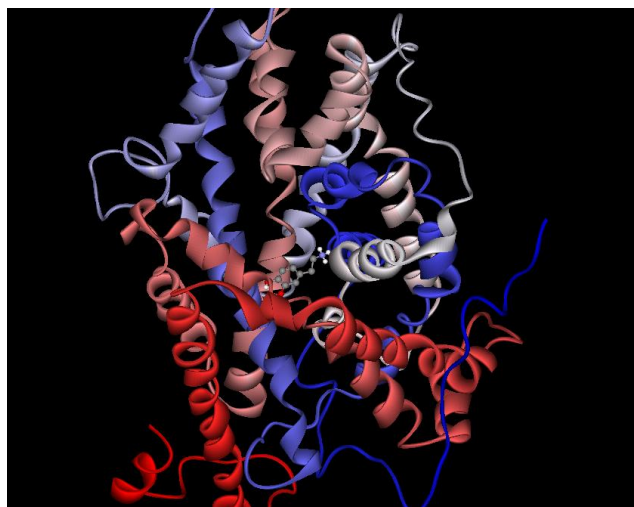
## 3 Results and discussion

The availability of three-dimensional structure of the DAT paved the way considerably to understand the binding mechanism of ligands into DAT. Table 1 shows the free energy of bindings (FEB) and inhibition constant values of the selected model compounds, which were docked by Autodock program.

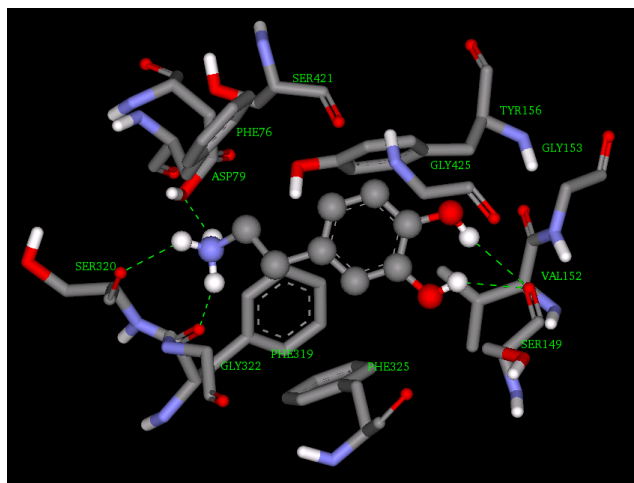
Reported experimental free energy of binding for the DAT-dopamine complex was  $-7.40$  kcal/mol (Dar et al., 2006). The score obtained from our computational study is  $-6.98$  kcal/mol. These two values are quite close confirming that computational value is in good agreement with the experimental values.

Figure 1 shows the 12 trans membrane structure of DAT (ribbon) and dopamine molecule (stick and ball).

Figure 2 shows the binding pose and interaction of dopamine substrate with the surrounding amino acid chains of DAT.



**Figure 1:** 12 trans membrane helices structure of DAT (ribbon) and dopamine molecule (stick and ball).



**Figure 2:** The binding pose and interaction of dopamine substrate with the surrounding amino acid chains of DAT.

Detailed rendering of the figures of hDAT-dopamine complex structure suggests that there is a common binding site for all the ligands buried deep in the transporter channel lined with the residues of PHE76, ASP79, PHE325, LEU322, GLY322, SER320, SER421, SER149, TYR156, VAL152, PHE319, and GLY425.

## 4 Conclusion

The binding sites and relative binding affinities of several ligands were determined with in the predicted structures of DAT. The computationally obtained binding affinities and binding poses within the predicted DAT

**Table 1:** Free energy of binding of selected model compounds and their inhibition values.

Compounds	FEB( $\Delta G$ ) kcal/mol	$K_i$ (mM)
Dopamine	-6.98	7.59
5-Hydroxydopamine	-6.37	21.36
6-Hydroxydopamine	-6.80	10.41
Tyramine	-6.64	13.56
Amphetamine_R	-7.09	6.32
Amphetamine_S	-7.19	5.37
Methamphetamine	-6.46	18.4
5-(2-aminoethyl)-1,3-diol	-6.80	10.42
4-amino-2-hydroxy-benzoate	-4.95	235.3
Cocaine	-7.54	2.88

structure are agreed with their experimental values. The obtained 3D structure of hDAT can be used in the future research studies to design more selective and more potent drug for hDAT.

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## Novel thiazole derivatives as dopamine D<sub>3</sub> receptor agonists with high selectivity and in vivo activity

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**Abstract.** L-DOPA is still the gold standard treatment for motor functions with dopamine substitution therapy in patients with Parkinson's disease. Dopamine receptor subtype agonists have great influence on therapeutic options as an ideal dopamine receptor agonist should fulfill the following criteria:

1. a physiological receptor profile with good anti-parkinsonian efficacy,
2. a good brain distribution,
3. oral bioavailability,
4. rapid onset,
5. long acting,
6. missing cross reactivity to off-targets and
7. no unwanted side-effects.

Although a small number of non-ergot derivatives are on the market, no single drug available fulfills all the criteria.

In a long-term development program we have changed the 2-aminothiazole motif of pramipexole as a prototypical catechol bioisosteric moiety by removing the aromatic amino functionality as described previously with etrabamine. This derivatisation maintained or improved affinity at dopamine D<sub>3</sub> receptor subtype, maintained agonist properties and simulated binding profile at dopamine D<sub>2</sub>-like receptor family.

Depending on the substitution pattern on the core pharmacophore element a series of highly affine and selective agonists have been developed. Selected com-

pounds were screened on unilateral 6-OHDA-lesioned rat model of Parkinson's disease and further selection on MTPP-treated marmoset model for their antiparkinsonian efficacy in comparison to L-DOPA, apomorphine and ropinerole.

At least two compounds simultaneously fulfilled all the criteria mentioned above and showed high drug potential due to the results of the initial preclinical toxicological screenings. Studies on functional signaling based on [<sup>35</sup>S]GTP-gamma-S shift and on ERK<sub>1/2</sub> phosphorylation showed significant differences and a good predictive factor for in vivo activities.

**Keywords** Parkinson's disease – D2 receptor – D3 receptor – biased signalling

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