Research objective 1 - Biological and pharmacological properties of new NMDAr inhibitors

Activity 1 - Neuropharmacology of potential medicaments with dual modulation of the cholinergic and glutamatergic system

The objective of this activity is the design, synthesis and biological testing of structurally different groups of compounds with a dual effect that can be utilised in research into neurodegenerative disorders. In particular it concerns the following individual activities:

- Using the method of organic synthesis, preparation and analysis of new dual compounds targeted at the CNS with appropriate physical-chemical properties. We envisage the preparation of three new ranges of these substances. The design of the substances shall be based on the simultaneously obtained data and a structural-activity evaluation thereof
- Screening *in vitro* evaluation of the prepared compounds, description of the mechanism of effect on NMDA receptors and acetylcholinesterase
- Subsequent *in vivo* evaluation of the most promising candidates. In addition to classic tests for potential medicaments (acute toxicity and bioavailability), the pharmacodynamics of the selected candidates will be studied .
- Evaluation of the relationships between biological activity and structure (SAR) and feedback for the design of new dually acting substances.

With regard to the numerous etiology of neurodegenerative disorders, in recent years research in the field of the development of pharmaceuticals has been focused on such structures that are capable of hitting a number of targets simultaneously. A combination of pharmaceuticals in clinical practice is not a new phenomenon. In particular MMT (multiple-medication therapy) is used, which is a combination of a number of medicaments used for a specific disorder with a different mechanism of effect, and "multiple - compound medication" (MCM), in which a number of different medicaments are incorporated into a single pharmaceutical form. From the perspective of the development of new pharmaceuticals, an interesting strategy is based on the assumption that a single compound would be capable of hitting a number of targets simultaneously. This concerns **multi-target-directed ligands** (MTDLs) . One of the most commonly used approaches in this area is the modification of the molecular structures of acetylcholinesterase (AchE) inhibitors, so as to provide supplementary biological properties suitable for the treatment of AD. In this case it shall concern a **combination of an acetylcholinesterase (AchE) inhibitor and an antagonist of the *N*-methyl-D-aspartate receptors (NMDAR).** Both groups of pharmaceuticals today represent standard treatment of AD. On the basis of the preliminary data (see below), new molecules will be designed and synthesised, influencing both targets simultaneously. These dually active substances shall thus be capable of simultaneously inhibiting AChE and also preventing an excess supply of calcium into the nerve cells. The combination of these effects is thus highly promising both from the perspective of basic research into new potential medicaments and with regard to their potential applicability .

The resolving team start out from the observations they have obtained in this area in recent years, in which they succeeded in preparing a derivate of the standard AChE inhibitor - tacrine (code indication K1395), which was designed in such a manner as to modulate both the aforementioned systems.

We developed a derivate based on a skeleton of tacrine, which is an effective AchE inhibitor, in *in vivo* conditions (table 1), both in the central and peripheral compartment, and selective and at the same

time a patent inhibitor on the GluNI/GluN2B receptors (table 2). The mechanism of binding to the GluN1/GluN2B receptors was examined by specific mutations in the N-terminus domains, and it was demonstrated that in addition to the region of the pore of the GluN1/GluN2B receptor specifically binds to the ifenprodil binding site . Our data therefore shows that this tacrine derivate inhibits the GluN1/GluN2B receptors in a manner which is not independent of tension, whilst on the GluN1/GluN2A receptors the compound binds only to the region of the pores in a tension-dependent manner. We envisage that this combined mechanism of effect could be a promising strategy for the treatment of neurodegenerative disorders such as Alzheimer's disease, vascular dementia, ischaemic damage to the CNS or comorbitidy of psychiatrie disorders, since it combines both current symmetrical approaches - choligernic stimulation and glutamatergic inhibition.

Table 1: Affinity to AChE and in vivo efficacy of substance KXXX

Wistar rat, i.p.	Dose*	% of AchE inhibition	
		Blood	Brain
K1395	10% LDS0 (16 mg/kg)	0	0
	20% LDS0 (32 mg/kg)	7.4	4 .2

*LDso = **160.4** (96.5 - 233.3) mg/kg, *i.p.*

Table 2: Antagonistic effect of KXXX on various GLU subunits

subunit	mV	ICSO	SEM	n	H	SEM
2A	-60	7.44	0.49	6	1.87	0.10
	-20	13.23	0.86	6	1.59	0.07
	40	26.27	1.26	S	1.74	0.06
2B	-60	1.66	0.14	14	1.06	0.0S
	-20	1.95	0.11	8	1.08	0.06
	40	2.01	0.14	10	1.12	0.09

The next objective will be effectiveness and behavioural evaluation of therapeutic effects of a lead candidate molecule in the animal models. The secondary objective will be assessment of the potential side-effects of a lead candidate molecule in intact animals.

We will study the effect of lead compound (analogues of K1395) in the model of bilateral hippocampal lesion induced by stereotaxic application of NMDA. This model represents an animal model of excitotoxicity-induced chronic neurodegeneration of principal neurons accompanied by severe neuroinflammation and subunit specific changes in NMDA and GABAA receptors. The outcomes of lesion and neuroprotective effect will be evaluated on the behavioral and histochemical level (Rambousek et al., 2016). Next we will focus on the analysis on the model of ischemie brin damage. We will use well-

established models of carotid artery ligation and cerebral artery occlusion using endotheline-1 (120 pmol) (Karhunen et al., 2005). The effect of the drugs on learning and memory will be measured by means of Morris water maze, radial maze, with the subsequent histochemical studies. The extent of brain damage will be assessed immunohitochemically similarly as in the first model. During the subsequent experiments we will focus on the study the effects og the drug in the behavioral models social defeat and tryptophane depletion (serving as a model of depression). Next we weill study the effect on the schizophrania-like behavior in the animal model. The schizophrenia-like behavior in rats will be induced by admiistration of dizocilpine (MK-801) at a dose of 0.1 mg/kg. The cognitive performance of rats will be tested in behavioral experiments such as open-field, Morris water maze and prepulse inhibition of startle response.

Last but not least to evaluate possible age-related changes in the behavioral and cognitive alterations of the rats, an ageing study will be performed. Animals will be tested in in the Open field, Passive avoidance step-through task and Morris Water Maze at the approximate age of S, 9 and 18 months.

Parallel work will be done to see potential side-effects of a lead compound administration to intact animals on cognitive and sensorimotor functions. To evaluate possible unexpected behavioural impacts will be tested in the battery of cognitive and motoric tasks. These tests will be grouped into functional catego ries. Firstly, we will study the effect on the reflexes and sensory domain . Furthermore, the general health and nonspecific effects will be assessed. Perception and locomotion will be evaluated using the Morris Water Maze Visible Plat form. This test will be applied to investigate the subject's vision, motivation for correct task performance, and locomotion functions . Locomotor functions will be further tested in the well-established Rotarod test and the Beam walking test. Locomotor activity in response to a novel environment also provides insight into the behavioral characteristics of individual mice. The Open Field task will be measured by means of EthoVision, as described [22].

lnformation processing and sensorimotor gating will be measured by the Prepulse lnhibition of Acoustic Startle Response (PPI) task. ln this task the brief presentation of a high intensity sound stimulus is preceded by a weak, non-startling stimulus (p repulse). ln intact mice, pairing of the two sounds results in a decreased startle reflex response. The PPI task will be performed as described [22]. Explorative activity can also be distorted as a consequence of altered glutamatergic signaling. Novel object recognition (NOR) is based on the premise that rodents will explore a novel object more than a familiar one, but only if they remember the familiar one. Therefore the results of this test will be evaluated in parallel with the memory and cognitive test results. The Morris water maze is a classic task for reference and working memory and will be performed as in Vales et al., 2006 [23].

Involved subjects

- University of Hradec Králové, Faculty of Science - Professor Kamil Kuča PhD
- NUDZ - Dr. Ondřej Soukup PhD, Dr. Karel Valeš PhD
- Institute of Physiology, Czech Academy of Sciences: Dr. Martin Horák PhD

Activity 2 - Biological properties of new dual molecules influencing excitability of the CNS

The objective of this activity is the design, synthesis and biological testing of structurally different groups of compounds with a dual effect that can be used in research into epileptic encephalopathies. ln contrast with activity 1, the design of the substances shall be based on in silico methods of molecular modelling (docking, virtual screening etc.). The course of the solution therefore includes especially these individua! ste ps:

- Research into the issue in question and design of structure with the aid of methods of molecular modelling
- Using the method of organic synthesis, preparation and analysis of new dual compounds targeted at the CNS with appropriate physical-chemical properties. We envisage the preparation of two new ranges of these substances.
- Screening *in vitro* evaluation of the prepared compounds, description of the mechanism of effect on NMDA and GABAa receptors
- Subsequent *in vivo* evaluation of the most promising candidates. In addition to classic tests for potential medicaments (acute toxicity and bioavailability), the pharmacodynamics of the selected candidates will be studied.
- Evaluation of the relationships between biological activity and structure (SAR) and feedback for the design of new dually acting substances.

Allosteric modulation of GABAA and NMDA/AMPA receptors represents therapeutically interesting bilogical target. GABAa and glutamate receptors are responsible for the excitation-inhibition balance of the brain. Results from preclinical and clinical studies support the potential efficacy of these compounds as a novel class of drugs for the treatment of epilepsy, depression, anxiety. They might be also devoid of psychotomimetic as well as cognitive side effects and they are predicted to have reduced abuse potential.

This activity also uses the theory of dual action of substances. In this case it concerns a design and synthesis of molecules combining the agonist effect on the GABAergic system, specifically GABAA receptors with an antagonistic effect toward the N-methyl-D-aspartate (NDMA) receptors, especially with regard to isoform containing an NR2B subunit. Modulation of the GABAA and NMDA or AMPA receptors represents a therapeutically attractive target, because the GABAA and glutamate receptors are responsible for the excitation inhibition balance of the brain, and can be used in the treatment of epileptic encephalopathies, affective disorders and a range of further neuropsychiatric disorders. The results of the preclinical and clinical trials support the potential effectiveness of these compounds as new classes of compounds for the treatment of epilepsy, depression or anxiety. In practice the combination of these properties is distinguished by felbamate, the contribution of which however is substantially reduced by its hepatotoxic properties and risk of occurrence of fatal aplastic anaemia. Further compounds (e.g. methaqualone) that modulate the receptors of glutamatergic and y-aminobutyric acid (GABA) are neurosteroids, which are endogenous regulators of brain excitability. Unfortunately only a limited number of compounds with this unique dual mechanism of effect are available in clinical practice (only felbamate), and newly prepared substances with this mechanism would be in high demand from the perspective of both basic and applied research.

Within the framework of the envisaged activity, a hybrid structure was designed in which we expect synergie modulation of the GABA and NMDA receptors. The structural aspects of such a compound are based on methaqualone, which increases the activity of GABA receptors with memantine, which acts as an NMDA receptor antagonist (Fig. 1).

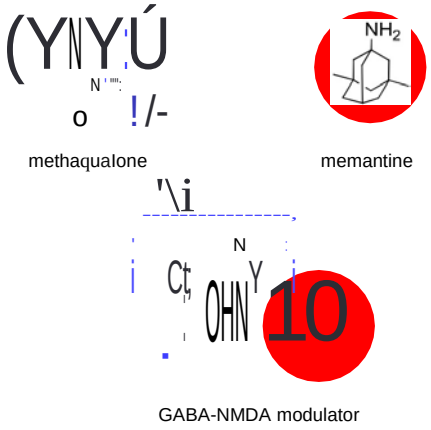

Fig. 1. Design of new modulator of GABA-NMDA receptors.

Epileptic encephalopathies is one example of aplication of these drugs. Patient suffering to this serious disease of the early infancy have very poor prognosis. At the moment there is no specific therapy for these syndromes as well as for their models.

Epileptic seizures will be elicited in two age groups - 12- and 25-day-old rats . These two age groups were chosen according to maturation of hematoencephalic barrier (mature in 25-day-old rats) and age-specific flexion convulsions induced by NMDA in 12- but not in 25-day-old animals. These flexion convulsions are generally accepted as a model of flexion seizures in infants.

Two convulsant drugs with a different mechanism of action will be used - pentetrazol (PTZ, an antagonist of GABAA receptors) in a dose of 100 mg/kg s.c. and N-methyl-D-aspartate (NMDA, a prototypical agonist at one type of ionotropic glutamate receptors). Doses of NMDA will be different in the two age groups dueto a marked developmental decrease of sensitivity to this drug.

Some antiepileptic drugs potentiating action of GABAA receptors are marginally effective against these seiz ures. We would like to study a treatment with drugs possessing positive action of GABAA receptors together with an antagonistic action on ionotropic glutamate receptors of NMDA and AMPA type. Both types of glutamate receptors change their subunit composition during postnatal maturation what might lead to age-specific anticonvulsant drugs.

Allosteric modulation of GABAA and NMDA/AMPA receptors represents therapeutically interesting biological target. GABAa and glutamate receptors are responsible for the excitation -inhibition balance of the brain. Results from preclinical and clinical studies support the potential efficacy of these compounds as a novel class of drugs for the treatment of epilepsy, depression, anxiety. They might be also devoid of psychotomimetic as well as cognitive side effects and they are predicted to have reduced abuse potential.

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