UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Sanja BOGIĆEVIĆ

REGULATION OF EXPRESSION OF ALTERNATIVELY SPLICED FORMS OF DOPAMINE D2 RECEPTORS WITH DOPAMINERGIC AGONISTS OF DOPAMINE D1 RECEPTORS IN RAT BRAIN

DOCTORAL DISSERTATION

Ljubljana, 2015

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DOCTORAL DISSERTATION

URAVNAVANJE IZRAŽANJA ALTERNATIVNIH mRNA DOPAMINSKIH RECEPTORJEV D2 Z AGONISTI DOPAMINSKIH RECEPTORJEV D1 V PODGANJIH MOŽGANIH

DOKTORSKA DISERTACIJA

Ljubljana, 2015

The doctoral dissertation is the completion of the PhD Study of Biological and Biotechnical Sciences and relates to the field of Genetics. Experiments and analyses were performed at the Brain Research Laboratory, Institute of Pathophysiology, Faculty of Medicine Ljubljana.

Based on the decision of the Senate of the Biotechnical Faculty number 33, dated 14/11/2012, the commission of PhD studies of the University of Ljubljana confirmed that the PhD candidate fulfilled the conditions to perform PhD studies in the PhD program of Biological and Biotechnical Sciences in the field of Genetics with the thesis: Regulation of expression of alternatively spliced forms of dopamine D2 receptors with dopaminergic agonists of dopamine D1 receptors in rat brains. Prof. Marko Živin, PhD was appointed the supervisor.

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Date of defence: 15.7.2015

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AB The drugs typically used to treat schizophrenia and Parkinson’s disease are D2 antagonists and agonists, respectively. Due to long-term treatment supersensitivity and D2 receptor up-regulation which is thought to be responsible for loss of efficacy of antipsychotics and the development of tardive dyskinesia in humans. Treatment of Parkinson’s disease with the dopamine precursor L-DOPA remains the most effective treatment. Unfortunately, L-DOPA, following a long-term administration, gradually loses its efficacy and eventually leads to severe motor complications. Dopamine depletion with reserpine also increases D2 receptor density and causes denervation hypersensitivity. LEK-8829 is a dopamine D2 receptor antagonist and dopamine D1 receptor agonist. We conclude that LEK-8829 retains its antipsychotic efficacy during prolonged treatment and possesses lower propensity for the induction of side effects as compared to typical antipsychotic haloperidol. Also LEK-8829 reduces dopaminergic supersensitivity and mRNA up-regulation. It is a promising antiparkinsonic because D1 receptors agonistic activity has been revealed by the robust psychomotor response that seems to be unabated by the concurrent blockade of dopamine D2 receptors and could have beneficial effects in the treatment of drug-induced psychosis with D2 agonists. Additionally, our data suggest that LEK-8829 is a D2 partial agonist due to its similar function with aripriprazole.

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AI Zdravila, ki se uporabljajo pri zdravljenju shizofrenije in Parkinsonove bolezni, so D2 antagonisti in D1 agonisti. Pri zdravljenju Parkinsonove bolezni je še vedno najbolj učinkovit dopaminski prekuzor L-DOPA, ki pa po dolgotrajnom jemanju izgublja učinkovitost in provzroča motorične motnje in tardivno diskinezijo. Izpraznjene zaloge lastnega dopamina provzročajo povečanje izražanja receptorjev D2 in denervacijsko preobčutljivost. LEK-8829 je antagonist dopaminskega receptorja D2 ter agonist receptorja D1. Sklepali smo, da LEK-8829 zaradi svoje lastnosti kot agonist D1 obdrži svojo antipsihotično delovanje po podaljšanom zdravljenju in provzroča manj neželenih učinkov kot haloperidol. LEK-8829 zmanjša dopaminergično preobčutljivost in povečano izražanje mRNK D2 receptorjev. Je tudi obetavan antiparkinsonik zaradi agonističnega delovanja prek receptorjev D1. LEK-8829 kaže močan psihomotorični odgovor in zdi se, da je v nezmanjšanem obsegu zaradi soočasnega zaviranja D2 receptorjev ter lahko ima korisne učinke pri zdravljenju z zdravili povzročene psihoze. Tudi rezultati kažejo, da je LEK-8829 delni agonist D2 zaradi svoje podobnosti z aripriprazolom.

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ABBREVATIONS

5HT	' 5 hydroxytrxptamine ' neurotransmitter
$5HT_{1A}$, $5HT_2$	subtypes of serotonin receptors
6-OHDA	6-hydroxydopamine
AMPH	amphetamine
ANOVA	analysis of variance
AP	alkaline phosphatase
BrEKT	bromoergocriptine
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CREB	cyclic AMP regulatory element-binding protein
CNS	central nervous system
COMT	catechol-O-methyl transferase
D1,D2	subtypes of dopamine receptors
D2L	long isoform of dopamine D2 receptor
D2S	short isoform of dopamine D2 receptor
DA	neurotransmitter dopamine
DARSS	Dopamine receptor supersensitivity
DARPP-32	cyclic AMP-regulated phosphoprotein, 32 kDa
DEPC	diethylprocarbonate
DMSO	dimethyl sulfoxide
DOPA	dihydroxyphenylalanine
DRD2	D2 receptor gene
DTT	Dithiothreitol
EP	entopeduncular nucleus
EPS	extrapyramidal syndrome
ESE	exon splicing enhancers
ESS	exon splicing silencers

GABA	Gamma amino butyric acid
GPe	Globus pallidus external
GPi	Globus pallidus internal
GPCRs	G protein-coupled receptors
HAL	haloperidol
ISE	introns-intronic splicing enhancers
ISS	introns-intronic splicing silencers
LEK-8829	9,10-didehydro-*N*methyl-(2-propynyl)-6-methyl-8-aminomethylergoline bimaleinate
MAO	monoamine oxidase
mRNA	messenger RNA
MSN	medium spiny neurons
NAcc	Nucleus accumbens
NE	norepinephrine
Nova 1	neuro oncology ventral antigen
PBS	phosphate-buffered saline
PD	Parkinson's disease
PFC	prefrontal cortex
ROD	'relative optical density'
SCH-23390	R-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SSC	saline sodium citrate
STN	subthalamic nucleus
TD	tardive dyskinesia
VMAT2	vesicular monoamine transporter 2

1 INTRODUCTION AND RESEARCH HYPOTHESIS

1.1 INTRODUCTION AND DEFINITION OF A SCIENTIFIC PROBLEM

Schizophrenic patients exhibit positive, negative and cognitive symptoms arising from an imbalance between the brain dopamine (DA) pathways mediating D2 and D1 receptor signaling (Yeganeh-Doost et al., 2011; Lewis and Lieberman, 2000). Subcortical increase of DA, leading to hyperstimulation of D2 receptors, would give rise to the positive symptoms, while a concomitant cortical deficit of DA, leading to hypostimulation of D1 receptors, would give rise to the negative and cognitive symptoms of shizophrenia.

Blockade of dopamine D2 receptors (Wang et al., 2000) is a prerequisite characteristic of all clinically effective antipsychotic drugs. A major difference between typical and atypical antipsychotic drugs is in the increased incidence of extrapyramidal adverse effects (EPS) by the typical vs. atypical (Niznik and Van Tol, 1992; Strange, 2001; Carlsson and Carlsson, 2006; Tarazi et al., 1997; Kapur and Remington, 2001). Extrapyramidal adverse effects (EPS) (Niznik and Van Tol, 1992; Tarazi et al., 1997; Kapur and Remington, 2001; Strange, 2001, Carlsson and Carlsson, 2006), characterized by Parkinsonism, akathisia, catalepsy, and, after long-term treatment, tardive dyskinesia (Kapur and Remington, 2001; Strange 2001). One of the most striking and consistent consequences of chronic antipsychotic exposure is supersensitivity D2 receptor (Meshul and Casey., 1989; Kostrzewa, 1995; Samaha et al., 2007) and increase in striatal D2 receptor density (Sirinathsinghji et al., 1994; Laruelle et al., 1992; Tarazi et al., 1997; Buckland et al., 1993; Hu et al., 1990; Meshul and Casey, 1989). This D2 receptor up-regulation is thought to be responsible for loss of efficacy of antipsychotics and the development of tardive dyskinesia (TD) in humans (Strange, 2001; Xu et al., 2002; Ginovart et al., 2009; Lewis and Lieberman, 2000; Hess et al., 1986; Jorgensen et al., 1994).

Parkinsonian patients exhibit deficits in motor function, which are attributed to the degeneration of dopaminergic neurons in the substantia nigra compacta (SNc) (Wang et al., 2000; Sirinathsinghji et al., 1994).

Treatment for Parkinson's disease is with the dopamine precursor L-DOPA and remains the most effective treatment. Unfortunately, L-DOPA, upon long-term administration, gradually loses its efficacy and eventually leads to severe motor complications, including dyskinesia (Gurevich and Gurevich, 2010). Dopamine depletion with reserpine also increases striatal D2 receptor density in the rat brain (Tarazi et al., 1997, Hu et al., 1990).

Long-term treatment with antipsychotic drugs arise striatal D2 receptor supersensitivity as well depletion with reserpine produces denervation hypersensitivity (increase responsiveness of D2 receptors) in the rat brain (Hu et al., 1990; LaHoste and Marshall, 1992).

It has already been described in the literature that striatal D2 receptor up-regulation and development of behavioral supersensetivity can be prevented by simultaneous stimulation of D1 receptors in the rat brain (Braun et al., 1997). A similar effect of D1 agonist treatment on behavioral supersensitivity and D2 receptor up-regulation induced by subchronic (21 day) haloperidol exposure has been reported by (Marin et al., 1993).

Previous observations suggest that combined D1 agonist, D2 antagonist treatment of psychotic disorders might be less likely to induce chronic extrapyramidal adverse effects, without significantly altering antipsychotic potential. This D1 agonist, D2 antagonist combination make it possible to selectively modify activity in direct and indirect striatal motor circuits, or to differentially regulate outflow through striatonigral and striatopallidal pathways. It would be a rationale design for pharmacotherapy for Parkinson's disease (Braun et al., 1997).

Research on the ergoline derivative LEK-8829 demonstrated an antagonist effects on dopamine D2 receptors and on serotonin 5-HT_2 and 5-HT_{1A} receptor subtypes. LEK-8829 was designed as a potential atypical antipsychotic drug (Krisch et al., 1994, 1996) and have additional D1 agonist properties (Zivin et al., 1996). Dopaminergic profile of the substance LEK-8829 was discovered by examining the short-term effects of LEK-8829 on behavior and gene expression in the striatum of experimental rats with normosensitive and hypersensitive dopamine receptors in the rat animal models for schizophrenia, parkinsonism, Huntington's disease and addiction with psychomotor stimulants (Zivin, 2010). Long-term effects of LEK-8829 on the animals' behavior and gene expression have not yet been investigated.

Recent studies have revealed that alternative D2 receptor pre-mRNA splicing is controlled by two splicing regulators, hnRNP M and Nova 1. HnRNP M directly binds to D2 receptor exon 6, and inhibits its inclusion thus favoring D2S mRNA production in a dose-dependent manner. Nova 1 blocks the effect of hnRNP M on D2S mRNA production via exon 6 binding. Nova 1 is known as a splicing factor which regulates the inclusion or exclusion of exons depending on the position of Nova binding relative to splice site (Ule et al., 2006). When Nova binds to the 3' region of the cassette exon, it promotes inclusion of this exon (Ule et al., 2006). Consistent with this, overexpression of Nova 1 dose-dependently increases D2 receptor exon 6 inclusion likely by blocking hnRNP M function (Park et al., 2011). On the other hand, in the literature importance expression of both dopamine receptors splicing forms in striatal neurons of the indirect pathway, as well as in the nigrostriatal dopamine neurons are many unknowns. There is also no data on the possible functional significance of the mechanisms regulating alternative splicing in pathophysiological conditions.

1.2 RESEARCH OBJECTIVES AND HYPOTHESES

The objectives of the research are to:

1) determine the impact on behavioral and neurochemical effect of subchronic treatment of LEK-8829;

2) establish whether D1 agonist reduce dopaminergic supersensitivity;

3) establish the D2 up-regulation-mediated influence on D2L and D2S isoforms;

4) establish whether D1 agonist properties of LEK-8829 may change expression levels of Nova 1.

Hypotheses:

- Subchronic treatment of experimental rats with LEK-8829 would cause less dopaminergic supersensitivity and up-regulation of dopamine D2 receptors in the striatum than chronic treatment with D2 antagonist haloperidol.
- Subchronic treatment and up-regulation of dopamine D2 receptors in the striatum would result in changes in relative splicing forms of D2S and D2L.
- Subchronic stimulation of dopamine D1 receptors (i.e. LEK-8829) in the striatum have resulted in a decreased expression of splicing factor Nova 1.

1.3 THE CONTRIBUTIONS TO SCIENCE

Results of the doctoral thesis will contribute to the understanding of the interaction between dopamine D1 and D2 receptors in the regulation of motor function. The results will contribute to a better understanding of regulation of gene expression of dopamine D2 receptors and their alternative splicing forms of D2S and D2L. I expect that research will show that the use of a dopamine D1 receptor agonist can reduce the negative effects of dopaminergic denervation hypersensitivity and supersensitisation of D2 receptors. This would allow the planning of new medicines and improve treatment strategies for schizophrenia and Parkinson's disease.

2 REVIEW OF THE LITERATURE

2.1 ERGOLINE DERIVATE LEK-8829

The ergoline derivative LEK-8829 (9,10-didehydro-N-methyl-(2-propynyl)-6-methyl-8-aminomethylergoline bimaleinate) is an antagonist on dopamine D2 receptors and on serotonin 5-HT_{2A} and 5-HT_{1A} receptors and was designed as a potential atypical antipsychotic drug (Krisch et al., 1994, 1996). In vitro experiments have shown that LEK-8829 stimulates adenylate cyclase and binds to dopamine D1 receptors with moderate affinity (Krisch et al., 1994). On the other hand, the drug has been tested in vivo for its effects on dopamine D1 receptors only in dopamine-depleted animals, using the rats with unilateral lesions of striatonigral neurons with 6-hydroxydopamine (6-OHDA). In this turning model, LEK-8829 induced a dose-dependent contralateral turning and the expression of c-*fos* mRNA in dopamine-depleted striatum that were both blocked by SCH-23390, a dopamine D1 receptor antagonist, but not by haloperidol (dopamine D2 receptor antagonist). It was therefore proposed that LEK-8829 may be an agonist on dopamine D1 receptors (Zivin et al., 1996). The interaction of LEK-8829 and bromocriptine has also been analyzed in a 6-OHDA model (Zivin et al., 1998). It was found that contralateral turning that was initiated with bromocriptine was not inhibited by the treatment with either LEK-8829 or SCH-23390, whereas the combined treatment with both drugs inhibited the turning. It was concluded that LEK-8829 has a dual action on the dopamine receptors in dopamine depleted striatum, as an agonist on dopamine D1 receptors and as an antagonist on dopamine D2 receptors (Zivin et al., 1998).

Further results have shown that LEK-8829 may be an agonist of dopamine D1 receptors and an antagonist at dopamine D2 receptors in dopamine-innervated striatum (Sprah et al., 1999). Glavan (2002) in their study clearly demonstrates that in hemi-parkinsonian rats D1 agonistic activity of LEK-8829 confers its anti-parkinsonian drug-like properties and modulates its neuroleptic drug-like properties, which are dependent on the blockade of dopamine D2 receptors. These findings imply that atypical antipsychotics with D1 intrinsic activity might have a reduced propensity for the induction of extrapyramidal syndrome (Glavan et al., 2002).

Although many questions regarding the beneficial mechanisms of LEK-8829 in parkinsonism, schizophrenia and drug-addiction remain to be addressed, it appears that agents with dual actions toward dopamine receptors may represent a new and potent drug class for the treatment of these disorders. LEK-8829 may be particularly useful whenever the treatment of Parkinson disease with D2 agonist drugs, is complicated by psychosis. Antiparkinsonic properties of LEK-8829 also suggest a lower propensity of this drug for the induction of EPS in the treatment of positive symptoms of schizophrenia. (Zivin, 2010)

2.2 BRAINSTEM AND RETICULAR FORMATION

Brainstem intrinsic systems are interconnected with virtually all parts of the central nervous system (CNS). The most important of these intrinsic systems is the reticular formation (Fig.1). The reticular formation consists of network of neurons deep in the tegmentum of the brainstem that extends thought the brainstem as well as the central core of the entire spinal cord. The vast majority of neurons in this network are inteneurons that have multiple efferent projections, resulting in literally trillions of synaptic contacts. The reticular formation can be subdivided into three functional components: a **lateral zone**, a **medial zone** and the sum of **neurotransmitter systems** that project to widespread areas of the CNS. In our focus will be dopaminergic (DA) systems beside norepinephrine (NE) and serotonin (5-HT) system (Krebs et al., 2012).

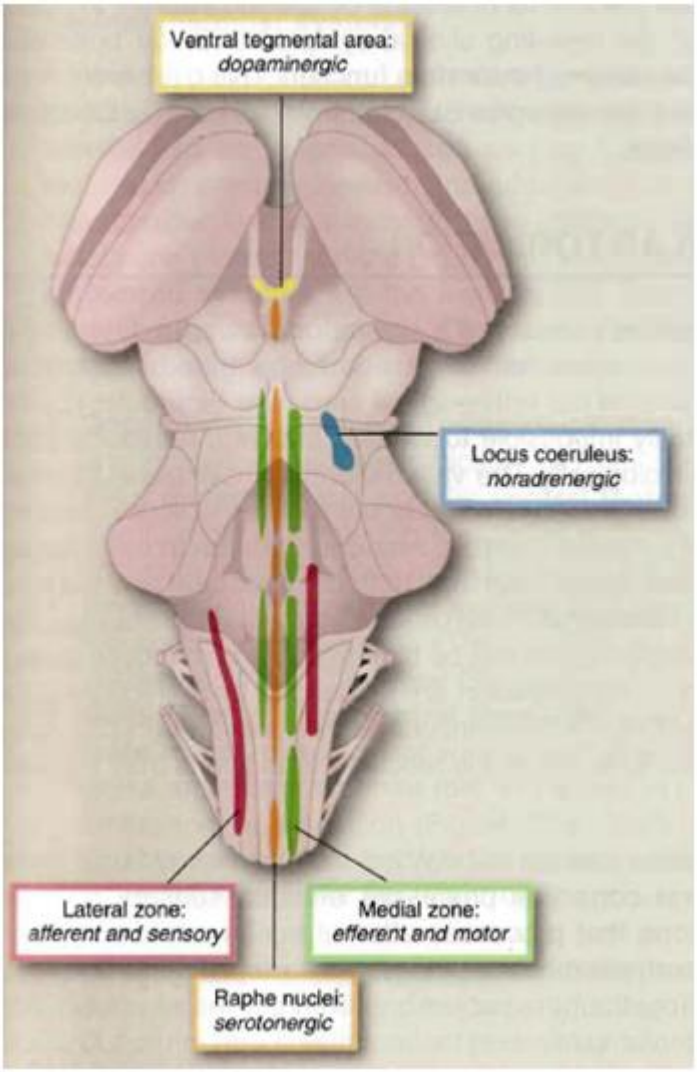

Figure 1: Overview of the reticular formation in the brainstem (Krebs et al., 2012).
Slika 1: Pregled mreže retikularnih nevronov v možganskem deblu (Krebs in sod., 2012).

Dopaminergic systems (Fig.2) Dopaminergic neurons in the brainstem are located in two anatomically and functionally distinct areas: **the substantia nigra** and the **ventral tegmental area** (VTA).

Dopaminergic systems are (Vallone et al., 2000):

Nigrostriatal system – dopaminergic cell bodies in the substantia nigra project to the caudate nucleus and putamen and play an important role in the control of movement.
Mesolimbic system - dopaminergic cell bodies in the ventral tegmental area project to the limbic system via the nucleus accumbens (NAcc).
Mesocortical system – dopaminergic cell bodies in the ventral tegmental area project to the frontal cortex.
Tuberoinfundibular system - transmits dopamine from the hypothalamus to the pituitary gland. This pathway influences the secretion of certain hormones, including prolactin.

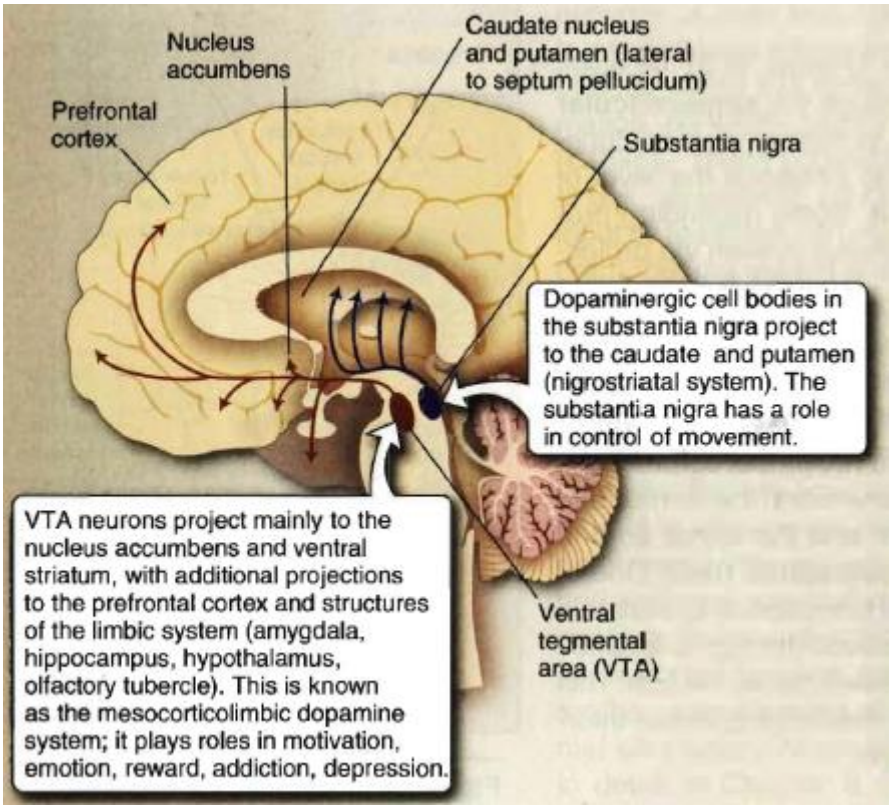

Figure 2: Dopaminergic projections from the ventral tegmental area and substantia nigra. (Krebs et al., 2012).
Slika 2: Dopaminergična pot iz ventralno-tegmentalnega področja in črne substance (Krebs in sod., 2012).

2.2.1 The basal ganglia

The basal ganglia are a group of interconnected subcortical structures involved in the control of motor, cognitive and limbic functions (Smith et al., 2009). They form a side loop to the descending motor pathways, and diseases affecting the basal ganglia lead to characteristic disturbances of voluntary movements and of muscle tone. The basal ganglia process information from large parts of the cerebral cortex before "answers" are sent back to the cortex (Brodal, 2010).

2.2.2 Striatum

The striatum is divided into dorsal (caudate nucleus and putamen, separated by the internal capsule) and ventral regions (nucleus accumbens and olfactory tubercle). Globus pallidus (external GPe and internal GPi), the subthalamic nucleus (STN), and the substantia nigra with its pars reticulata (SNr) and pars compacta (SNc) (Goole and Amighi, 2009). In most mammals, including rats, the homologue of GPi is entopeduncular nucleus (EP) (Albin et al., 1989).

Information from the cortex passes through the basal ganglia to the thalamus and then returns to the supplementary motor area of the cortex through the dopaminergic pathway. Under normal conditions, the striatum and the STN receive glutamatergic afferents from specific areas of the cerebral cortex or thalamus and transfer the information to the basal ganglia output nuclei, GPi and SNr (Fig.3). The projections between the striatum and GPi/SNr are divided into two separate pathways – direct connection and indirect projection – via the intercalated GPe and STN. Output from GPi/SNr goes to the motor thalamus which, in turn, projects back to the cerebral cortex and then to the striatum via the direct pathway (Gerfen, 1992).The striatum also receives a non negligible dopaminergic input directly from the SNc. This anatomic arrangement places the dopaminergic input in a position to regulate or gate the corticostriatal transmission. The D1 receptors are involved in the direct pathway, while the indirect pathway is mediated by the D2 receptors (Goole and Amighi, 2009; Gerfen et al., 1990; Alexander and Crutcher, 1990).

These pathways are thought to provide modulating antagonistic effects: direct pathway (Fig.4) activation may inhibit GPi/SNr activity, thereby disinhibiting thalamocortical interactions and stimulate movement. The indirect pathway (Fig.5) activation inhibits GPe which sends less inhibition to STN. STN became more active and they are able to stimulate GPi (via glutamate). Normal role of GPi is to inhibit the thalamus (via GABA). STN is more activate and stimulate GPi which became more active and inhibit thalamus better than they normally would. Therefore, it will be decrease excitatory flow from thalamus to motor cortex and as a result we have inhibition of unvoluntary movement (Galvan and Wichmann, 2008; Goole and Amighi, 2009).

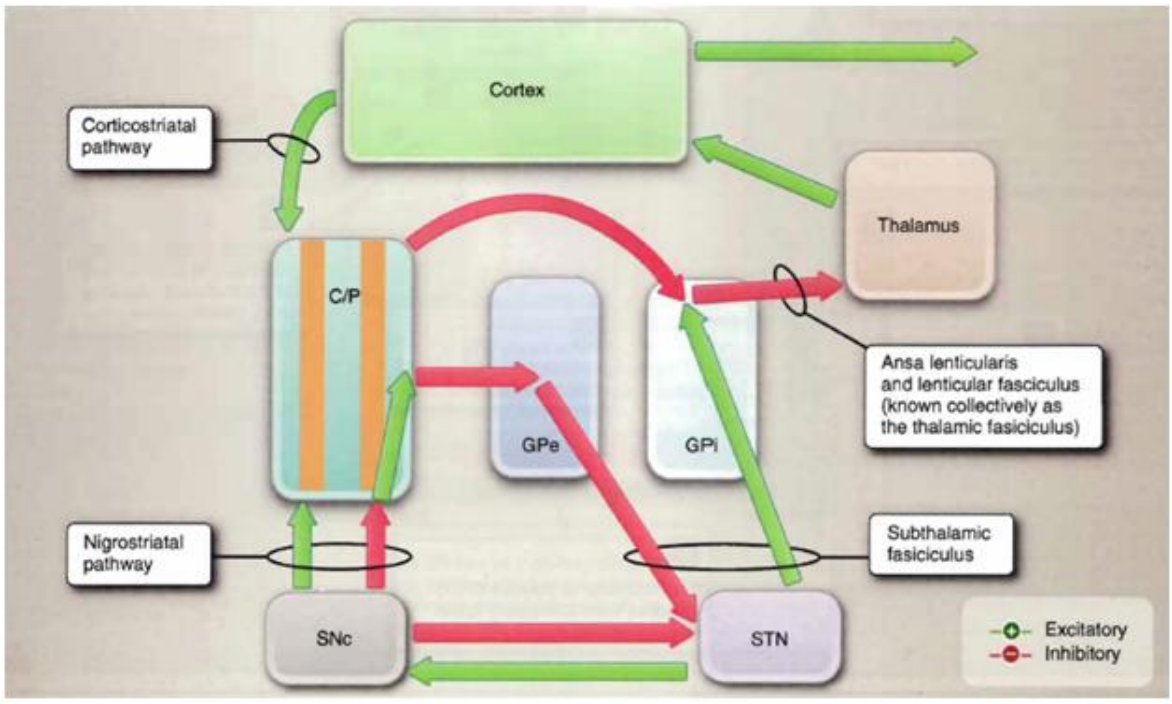

Figure 3: Direct and indirect pathways and the anatomical names of their connecting fibers. GPe - globus pallidus, external segment; GPi - globus pallidus, internal segment; C/P – caudate/putamen; STN – subthalamic nucleus; SNc – supstantia nigra pars compacta (Krebs et al., 2012).
Slika 3: Neposredna in posredna pot ter anatomska imena njihovih povezovalnih vlaken. GPe - globus pallidus, zunanji segment; GPi - globus pallidus, notranji segment; C/P – caudatus/putamen; STN – subtalamično jedro; SNc – črna substanca - pars compacta (Krebs in sod., 2012).

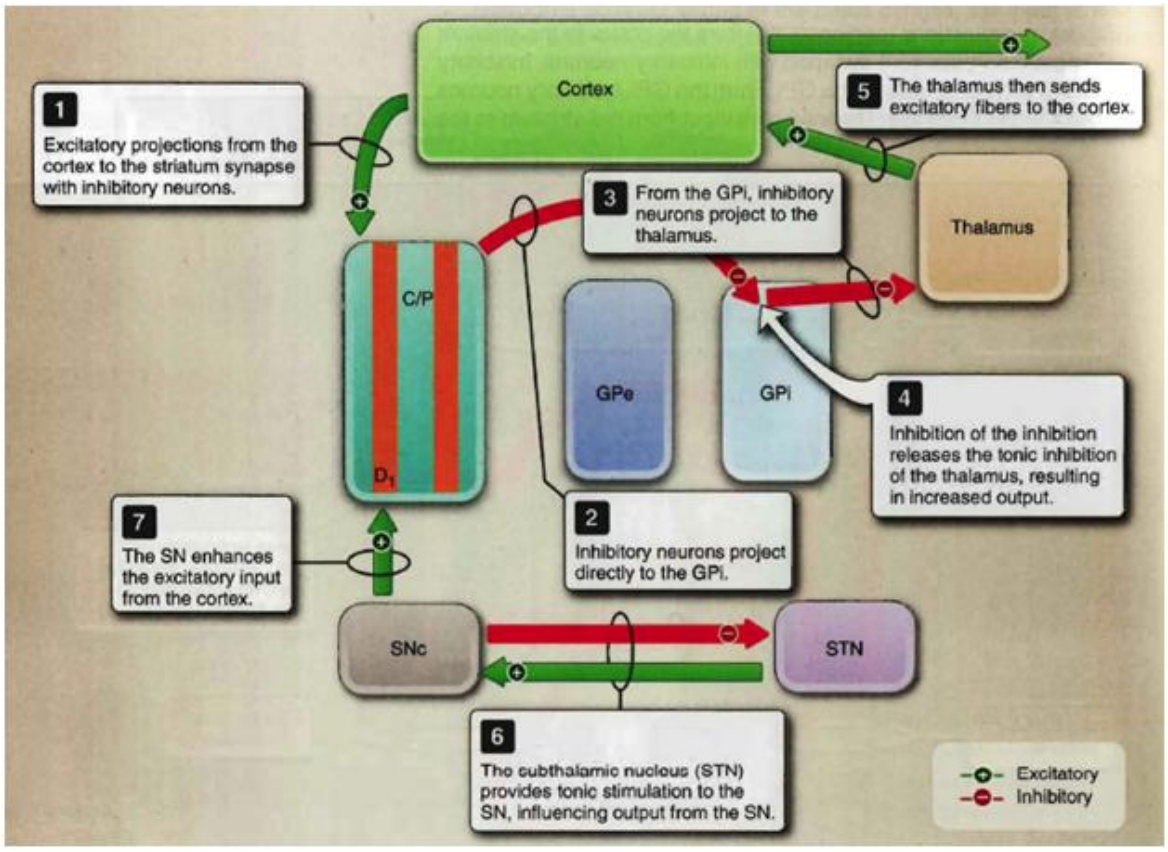

Figure 4: Wiring diagram for the direct pathway. GPe - globus pallidus, external segment; GPi – globus pallidus, internal segment; C/P – caudate/putamen; D1 – dopamine receptor type; SN – substantia nigra; SNc – supstantia nigra pars compacta (Krebs et al., 2012).
Slika 4: Načrt povezav za neposredno pot. GPe - globus pallidus, zunanji segment; GPi - globus pallidus, notranji segment; C/P – caudatus/putamen; D1 – tip dopaminskega receptorja; SN – črna substanca; SNc – črna substanca - pars compacta (Krebs in sod., 2012).

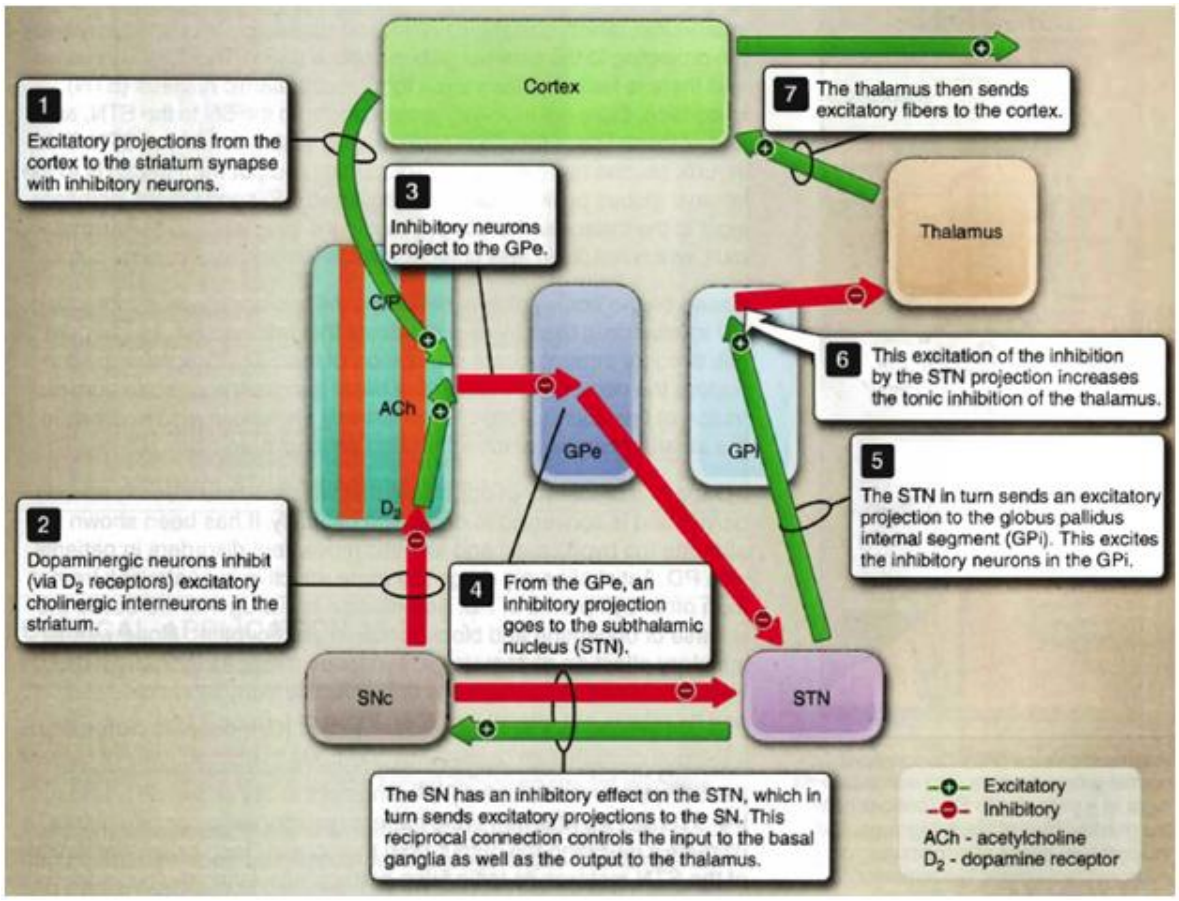

Figure 5: Wiring diagram for the indirect pathway GPe - globus pallidus, external segment; C/P – caudate/putamen; SNc – supstantia nigra pars compacta (Krebs et al., 2012).
Slika 5: Načrt povezav za posredno pot. GPe - globus pallidus, zunanji segment; C/P – caudatus/putamen; SNc – črna substanca pars compacta (Krebs in sod., 2012).

2.2.3 The substantia nigra

The substantia nigra and some of its connections have been mentioned several times, and we will also return to it when dealing with Parkinson's disease, in which the nigra plays a crucial role. A collective treatment of the main features of the substantia nigra may therefore be pertinent at this stage.

The substantia nigra can be divided anatomically into two parts, the **pars compacta** and the **pars reticulate.** The compacta is richer in cells than the reticulate and compacta neurons contain pigment (neuromelanin), which makes the nigra visible as a dark band in the cut human mesencephalon. The pars reticulata, located ventral to the compacta, is lighter. The dopaminergic nigrostriatal neurons are located in the pars compacta, whereas the GABAergic nigrothalamic neurons are located primarily in the pars reticulate.

The **efferent** connections of the **pars compacta** pass primarily to the striatum (with a smaller contingents to the subthalamic nucleus and some other nuclei). This is the largest

dopaminergic pathway in the brain, and nigra is the largest collection of dopamine containing neurons. **Pars reticulata** send GABAergic fibers to the thalamus (VA, MD).

The **afferent** fiber connections of the nigra arise in numerous cell groups, but quantitatively the most important input comes from the **striatum**. Even though most striatonigral fibers terminate in the pars reticulata, cells in the compacta can also be influenced because their long dendrites extend into the reticulata. GABA is the transmitter for the striatonigral fibers, exerting **inhibitory** effects on the cells in the nigra. **Excitatory** afferents to the nigra arise in the subthalamic nucleus (Brodal, 2010).

2.2.4 Medium spiny neurons

The medium spiny neurons (MSN) are the target of the dopamine innervation of the striatum, and comprise more than 90% of striatal neurons. These MSNs are, as their name suggests, of medium size and have radially projecting dendrites that are densely studded with dendritic spines. These projection neurons of the striatum use GABA as a transmitter, with two different peptide transmitters being co-localized to define two sets of MSNs. The first of these groups of MSNs project directly to the substantia nigra (SN), express the tachykinin peptide substance P, and express the D1 dopamine receptor. The other group of MSNs projects to the globus pallidus (neurons of which in turn project to the SN and motor thalamus), express the peptide leu-enkephalin as a co-transmitter with GABA, and express and are regulated by the D2 dopamine receptor (Deutch et al., 2007; Smith and Bolam, 1990; Gerfen, 1992b).

2.2.5 Striatal interneurons: cholinergic and GABAergic

The striatum has a complex intrinsic organization, even though the majority of neurons are "simple" projection neurons. For example, the axons of the medium spiny neurons give off recurrent collaterals in the striatum (Fig. 6A). Further, a number of different **interneurons** exist (i.e., their axonal arborizations remain within the striatum). One conspicuous kind of interneuron, which constitutes about 1% of all striatal neurons, has a large cell body and smooth dendrites and contains **acetylcholine** (Fig. 6A). These interneurons receive excitatory synaptic influences from the cerebral cortex and the intralaminar thalamic nuclei (glutamatergic), as well as inhibitory influences by the recurrent collaterals from GABAergic projection neurons. Acetylcholine acts via **muscarinic** receptors on striatal projection neurons with slow, modulatory effects. The overall effect of acetylcholine on the projection neurons is to ensure that they react with bursts of action potentials to excitatory inputs from the cerebral cortex: that is, the efficiency of signal transmission is enhanced. The cholinergic interneurons contribute in a yet unknown way to the symptoms in Parkinson's disease, since since muscarinic antagonists can improve the symptoms. (Brodal, 2010; Gerfen, 1992b).

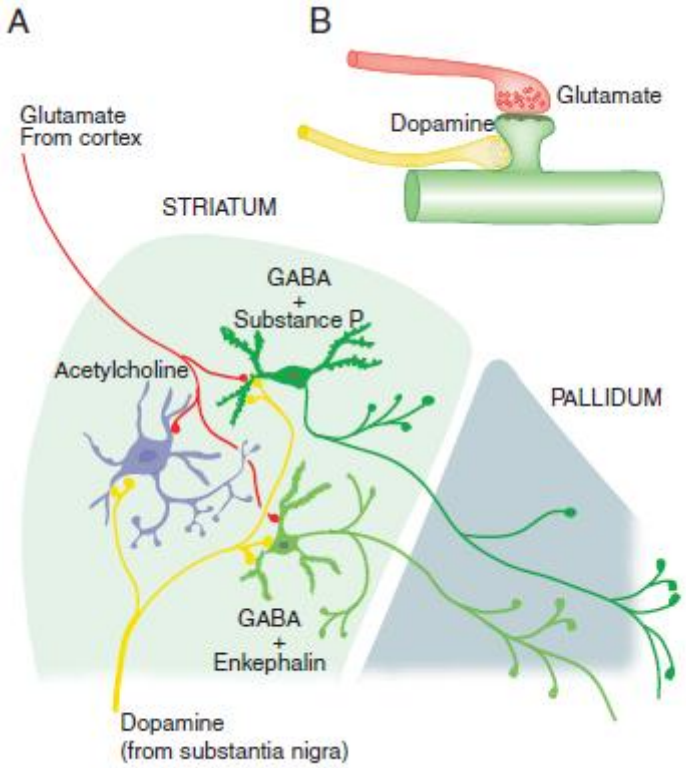

Figure 6: Three important kinds of neuron in the striatum, and the termination of dopaminergic fibers. **A:** The striatal projection neurons are relatively small and their dendrites have many spines (medium spiny neurons). All striatal projection neurons are GABAergic but two kinds can be distinguished based on their content of neuropeptides (substance P and enkephalin). Note the large, cholinergic interneurons. GABAergic interneurons are not shown. **B**: Dopaminergic synapses are often situated on spines that are contacted by glutamatergic nerve terminals from the cortex. Therefore, dopamine presumably acts via both specific synapses and volume transmission (Brodal, 2010).
Slika 6: Tri pomembne vrste nevronov v striatumu ter zaključki dopaminergičnih vlaken. **A:** Projekcije striatuma so majhne in njihovi dendriti imajo trnje. Vsi striatalni projekcijski nevroni so GABAergični in se lahko razlikujejo po vsebini nevropeptidov (snov P in enkefalin). Pozor na velike, holinergične internevrone. GABAergični internevroni niso prikazani. **B:** Dopaminergične sinapse se pogosto nahajajo na trnjih, ki so v stiku z glutamatergičnimi nevroni iz skorje. Torej, dopamin verjetno deluje preko specifičnih sinaps in volumskega prenosa (Brodal, 2010).

2.3 DOPAMINE

Dopamine serves as an important neurotransmitter in distinct CNS neurons. Dopamine signaling controls many physiological functions ranging from locomotion to hormone secretion and plays a critical role in addiction (De Mei et al., 2009). Because dopamine is involved in a variety of critical functions, it is not surprising that multiple human disorders have been related to dopaminergic dysfunctions. The most recognized dopamine-related disorder is Parkinson's disease (PD), which originates from a loss of striatal dopaminergic innervations in the brain (Fahn, 2005; Katzenschlager and Lees, 2002). The fact that almost all of the clinically effective antipsychotics block D2 dopamine receptors (Kapur

and Mamo, 2003), has provided a basis for the dopaminergic hypothesis of schizophrenia (Fudge and Emiliano, 2003; Beaulieu and Gainetdinov, 2011).

2.3.1 Biogenic amines

The **biogenic amines** constitute a subgroup of the small molecule transmitters. The group includes the **monoamines** norepinephrine, epinephrine, dopamine, and serotonin (one amine group) as well as **histamine** (two amine groups).

Norepinephrine, epinephrine, and dopamine are **catecholamines**. (Catecholamines: compounds consisting of a catechol group (benzene ring with two hydroxyl groups) with an attached amine group). Neurons that contain the monoamines norepinephrine, dopamine, serotonin, and histamine are said to be noradrenergic, dopaminergic, serotonergic, and histaminergic, respectively (the same terminology is used for the receptors corresponding to these transmitters). A common feature of the biogenic amines is that they are synthesized only in a small number of neurons, which, however, have widely branching axons. In this way, these few neurons ensure that the transmitters can act in most parts of the CNS. Like acetylcholine, the biogenic amines are to a large extent released from varicosities without typical synaptic contacts (Fig. 8); therefore, their effects are presumably mainly mediated by **volume transmission** and binding to extrasynaptic receptors.

Norepinephrine (noradrenaline), epinephrine (adrenaline), and dopamine are all synthesized from the amino acid tyrosine (Fig.7). Tyrosine is taken up from the bloodstream by active transport mechanisms and concentrated in the nervous tissue. The synthesis of catecholamines goes through several enzymatic steps. The first is the conversion of tyrosine to dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase (Fig.7) (Nagatsua and Sawadab, 2009; Kostrzewa et al., 2005), which appears to be the rate limiting for the synthesis of catecholamines under most conditions. The activity of tyrosine-hydroxylase is regulated by negative feedback from released catecholamines activating the presynaptic autoreceptors (other factors also influence the activity, however). DOPA is converted to dopamine by the enzyme aromatic amino acid decarboxylase (DOPA decarboxylase). The reaction is so rapid that very little DOPA can be detected in the brain normally. Therefore, the synthesis of dopamine can be increased by artificial supply of DOPA (in the form of levodopa), as done in Parkinson's disease in which parts of the brain have very low levels of dopamine. Dopamine itself does not pass the blood–brain barrier and therefore cannot be used therapeutically (Brodal, 2010).

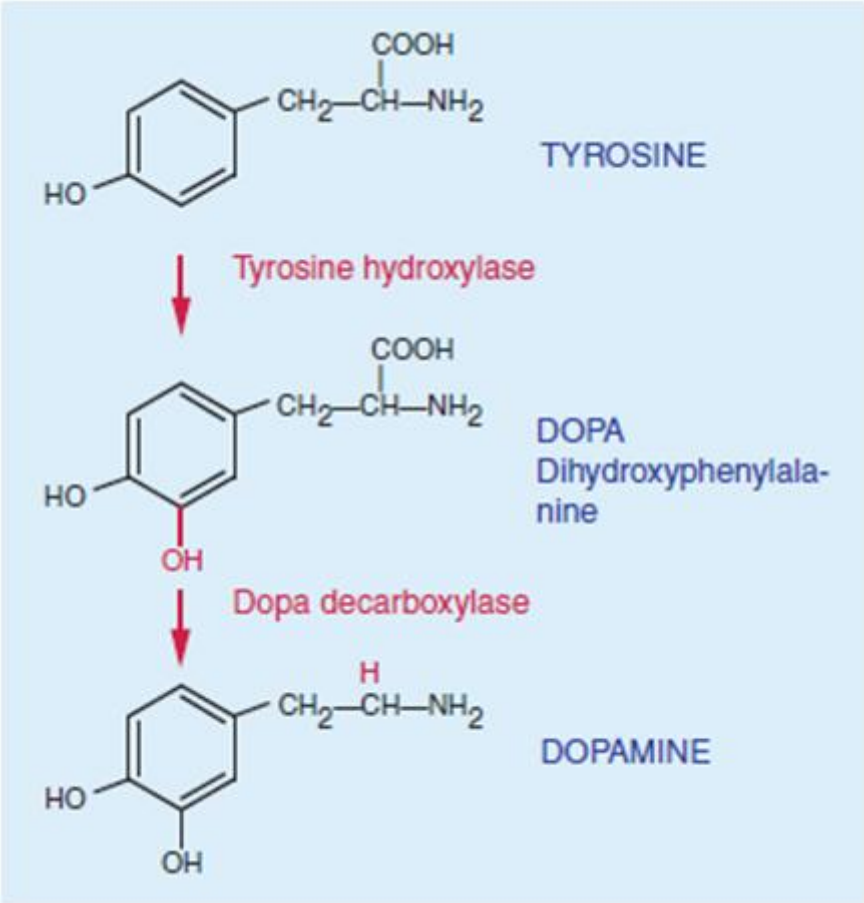

Figure 7: The catecholamines dopamine and the key enzymes in its synthesis (Brodal, 2010).
Slike 7: Kateholanini dopamin in ključni encimi njegove sinteze (Brodal, 2010).

Synthesized dopamine is finally sequestered into storage vesicles by vesicular monoamine transporter 2 (VMAT2). The vesicles play a dual role: they maintain a ready supply of dopamine at the nerve terminal that is available for release, and they mediate the process of release. When an action potential reaches the nerve terminal Ca^{2+} channels open, allowing an influx of the cation into the terminal; increased intracellular Ca^{2+} promotes the fusion of vesicles with neuronal membrane. The vesicles then discharge their soluble contents into extraneuronal space. Once in the synapse, dopamine binds to and activates dopamine receptors, which can be located either on postsynaptic target cells or on the membrane of the presynaptic dopamine-releasing cell itself (i.e. D2 short autoreceptors). After an action potential, the dopamine molecules quickly become unbound from their receptors (Brodal, 2010).

Transporter proteins in the membrane of nerve terminals end the transmitter action and control the extracellular concentration of monoamines. There are specific transporters for norepinephrine, dopamine, and serotonin - all belonging to the same protein family (called NAT, DAT, and SERT/5-HTT, respectively). The two catecholamine transporters have low selectivity, however, so that, for example, the dopamine transporter also can take up

norepinephrine, if it is present in the vicinity. After uptake into nerve terminals, the monoamines are partly transported back into vesicles, partly broken down by the enzyme **monoamine oxidase** (**MAO**) (Kuhar et al., 2006).

2.3.2 Release of neurotransmitters

Signal is conveyed from one neuron to the next by release of a **neurotransmitter** (transmitter). "Conventional" or "classical" neurotransmitters are small molecules, such as amino acids and amines. Another important group of signal substances, released at synapses, are peptide molecules, called neuropeptides. Although the "classical" transmitter is released and acts at receptors in a synapse, many transmitter receptors are found **extrasynaptically**, that is, without connection to a synapse (Fig 8). Indeed, many transmitters act both at synapses and extrasynaptically. The latter action is called **volume transmission**, and is obviously less precise than synaptic transmission. Many receptors are located presynaptically on nerve terminals. Some of them are **autoreceptors** (binding the transmitter released from the terminal) and others are **heteroreceptors** (binding other transmitters released from neurons in the vicinity). Many nerves terminals contain more than one transmitter; often a classical transmitter is **colocalized** with one or more neuropeptides (Brodal, 2010; Carlsson and Carlsson, 2006).

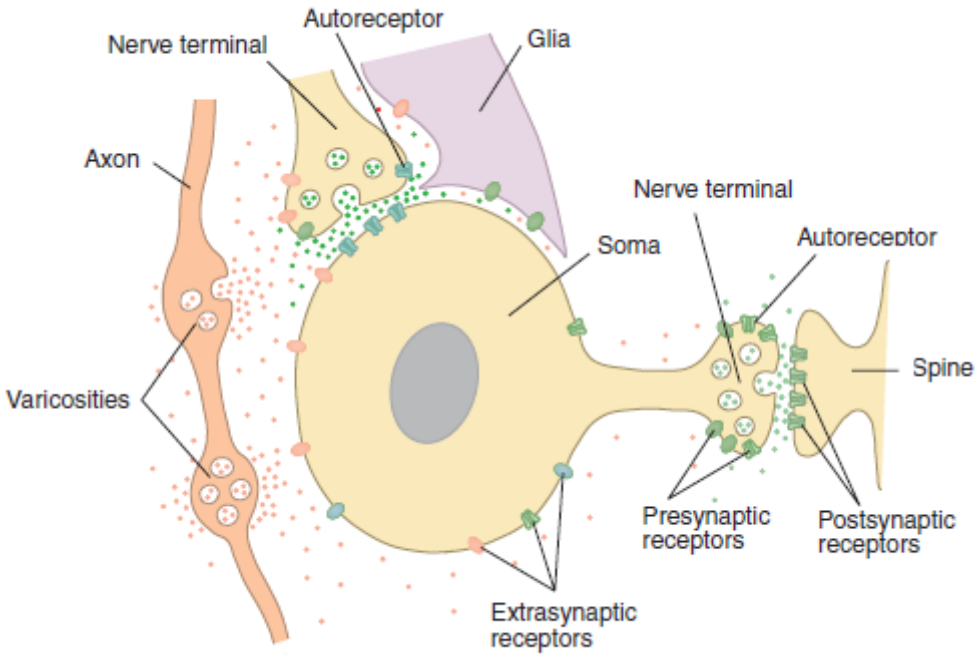

Figure 8: Extrasynaptic receptors and transmitter release outside synapses. Extrasynaptic receptors are localized both at the nerve terminals and on the somatic and dendritic surfaces of the neuron. Autoreceptors bind the transmitter released by the neuron itself. Note the release of transmitter from varicosities that do not form typical synaptic contacts (Brodal, 2010).
Slika 8: Zunajsinaptični receptorji in sproščanjeprenašalcev iz sinapse. Zunajsinaptični receptorji se nahajajo na končičiha in na telesu nevronov ter na površini. Na avtoreceptor se veže prenašalec, ki se sprosti iz samega nevrona. Sproščanje prenašalcev iz varikoz ne tvorijo tipične sinaptične stike (Brodal, 2010).

2.3.3 Receptors

The receptors are not static, immutable elements of the nervous system. We will discuss how changes in receptor density and activity may mediate **synaptic plasticity.** Drugs that interfere with transmitter actions often induce changes in the receptors (Brodal, 2010).

Receptor is most often used term to describe the target molecules through which soluble physiological mediators - hormones, neurotransmitters, inflammatory mediators, etc. - produce their effects (Rang, 2008). The tendency of a drug to bind to the receptors is governed by its **affinity**, whereas the tendency for it, once bound, to activate the receptor is denoted by its **efficacy.** Some compounds (known as full agonists) can produce a maximal response (the largest response that the tissue is capable of giving), whereas others (partial agonists) can produce only a submaximal response. The difference between full and partial agonists lies in the relationship between receptor occupancy and response (Rang, 2008). **Intrinsic activity** or **efficacy** refers to the relative ability of a drug – receptor complex to produce a maximum functional response.

2.3.4 Sensitization and desensitization

Chronic exposure to antagonists often results in increased responsiveness (i.e. greater than normal). This phenomenon commonly referred to as **supersensitivity or sensitization**. Dopamine receptor supersensitivity, as reflected by an enhanced response, sometimes accompanies receptor proliferation. However, dopamine receptor supersensitivity may occur in the absence of a change in the number of dopamine receptors (Kostrzewa, 1995).

Dopamine receptor supersensitivity (DARSS) often is invoked as a mechanism possibly underlying in psychiatric disorders. Dopamine (DA) receptors, experimentally, are prone to become supersensitive and to thus elicit abnormal behaviors when coupled with DA or a receptor agonist (Kostrzewa et al., 2011).

Heterologous sensitization

Whereas acute activation of Gαi/Gαo-coupled D2 receptors inhibits adenylate cyclase, prolonged activation leads to enhanced adenylate cyclase activity coupled D1 receptors (i.e. heterologous sensitization) (Watts and Neve, 2005).

Denervation supersensitivity of central dopamine receptors following destruction of dopamine neurons or dopamine depletion, is well known phenomenon (Hu et al., 1990).

Chronic exposure to agonists often results in diminished responsiveness. This phenomenon commonly referred to as **desensitization**. Redistribution (internalization) of protein receptors are occurred i.e. their separation of membrane.

2.3.5 Dopamine receptors

Dopamine exerts their main actions on metabotropic receptors. **Metabotropic receptors** are coupled indirectly (via intracellular second messengers) to ion channels. Their effects are therefore slower to start and longer lasting than effects mediated by ionotropic receptors. We also use the term **modulatory** of the synaptic effects of metabotropic receptors, because they adjust the excitability of the postsynaptic neuron so that it responds more or less vigorously to the precise effects of ionotropic receptors (Brodal, 2010).

2.3.6 Introduction

Dopamine receptors belong to the large family of heptahelical transmembrane spanning G protein-coupled receptors (GPCRs). Five dopamine receptor subtypes have been identified and are classified into two major groups, the D1-like (D1 and D5) and D2-like (D2, D3, D4) like receptors (Fig.9).

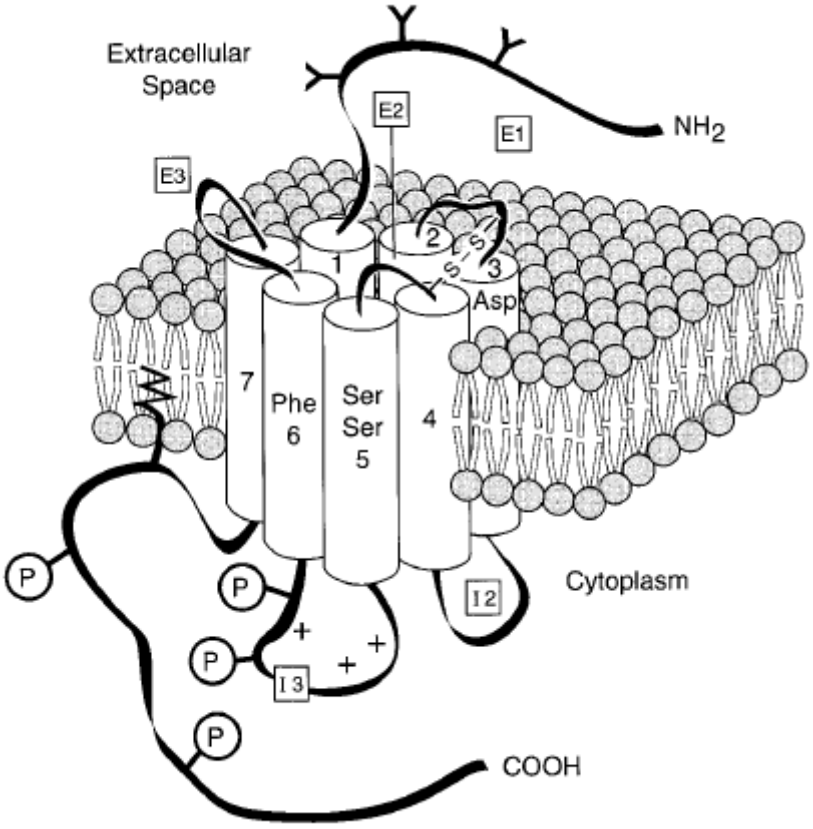

Figure 9: Dopamine receptor structure. Structural features of D1-like and D2-like receptors are represented. D2-like receptors are characterized by a shorter COOH-terminal tail and by a bigger 3rd intracellular loop. Residues involved in dopamine binding are highlighted in transmembrane domains. Potential phosphorylation sites are represented on 3rd intracellular loop (I3) and on COOH terminus. Potential glycosylation sites are represented on NH2 terminal. E1-E3, extracellular loops; 1–7, transmembrane domains; I2-I3, intracellular loops (Missale et al., 1998).
Slika 9: Struktura dopaminskih receptorjev. Strukturne značilnosti D1 in D2 receptorjev so prikazane na sliki. D2 receptori imajo krajši -COOH rep in večjo terciarno znotrajcelično zanko. Aminokislinski ostanki vključeni v vezavo dopamina so poudarjeni v transmembranskih domenah. Morebitna mesta fosforilacije so zastopana na terciarni znotrajcelični zanki (I3) in na -COOH koncu. Morebitna glikozilacijska mesta so zastopana na -NH_2 koncu. E1-E-3, izvencelična zanka; 1-7, transmembranske domene; I2-I3, znotrajcelična zanka (Missale et al., 1998).

2.3.7 Expression

Dopamine receptor subtypes are expressed differentially throughout the brain. D1 receptors are the most abundant, with mRNA transcripts found in the neostriatum, nucleus accumbens, and olfactory tubercle. Lower levels of D1 receptor mRNA is found in the cerebral cortex, hypothalamus, and thalamus (Meador-Woodruff et al., 1991). D2 receptors are the most abundant, with mRNA transcripts found in the neostriatum, nucleus accumbens, and olfactory tubercle, as well as the midbrain, including the substantia nigra and ventral tegmental area (Meador-Woodruff et al., 1991). D2 mRNA transcripts also are

found in the pituitary (Chronwall et al., 1994). Khan and colleagues (1998), using specific antibodies directed against the D2 splice variants, report that D2S is located predominantly in cell bodies and axons of dopaminergic neurons of the primate midbrain, whereas D2L is more strongly expressed by neurons of the striatum and nucleus accumbens that are targeted by dopaminergic neurons (Khan et al., 1998). Accordingly, in the primate brain, D2S and D2L are primarily localized to pre- and postsynaptic membranes, respectively.

2.3.8 Dopamine D2 long and D2 short isoforms

The two isoforms coexist in all brain tissue analyzed; however, the expression ratio of D2L versus D2S varies considerably from region to region (Neve et al., 1991). The differences in protein structure, expression pattern, and interaction with intracellular effectors suggest that the two D2 isoforms may have differential functions. In addition, the etiology of motor deficits and psychoses involves different brain regions, and the expression ratio of D2 isoforms differs in these regions. Thus, there is a possibility that D2L and D2S may play differential roles in defining the therapeutic ratio of antipsychotic and/or antiparkinsonian drugs (Wang et al., 2000). Analysis of mRNA of the two isoforms has shown that D2L is the most abundantly expressed (Vallone et al., 2000; Wang et al., 2000; Xu et al., 2002).
The lack of an isoform-selective pharmacological agent has hampered the progress toward understanding the specific function of D2L and D2S in the mammalian CNS (Wang et al., 2000).

Due to the lack of isoform-selective pharmacological agents, studies of differential roles of D2L and D2S in the brain currently rely mainly on genetic manipulation (Hranilovic et al., 2008). To contribute to understanding of functional roles of the two D2R isoforms in the mammalian brain, they have generated D2L receptor-deficient mice (D2L−/−) which only express functional D2S receptors at the level similar to that of the total D2R in wild-type (WT) mice (Wang et al., 2000). D2L−/− mice, in comparison to WT mice, displayed reduced level of locomotion in an open field, lower basal motor activity in their home cages, increased and enhanced stereotyped behavior (Wang et al., 2000; Fetsko et al., 2003). Moreover, mice lacking D2L were less sensitive to typical antipsychotics (Wang et al., 2000; Xu et al., 2002; Hranilovic et al., 2008). D2L−/− mice still express functional D2S isoform on the cell surface at a level similar to that of total D2R in WT mice, due to a compensatory increase in D2S expression in the mutants.

Buckland (1993) shows that mRNA levels of both isoforms of the D2 receptor are up-regulated by 32 day long administration of haloperidol (Buckland et al., 1993)

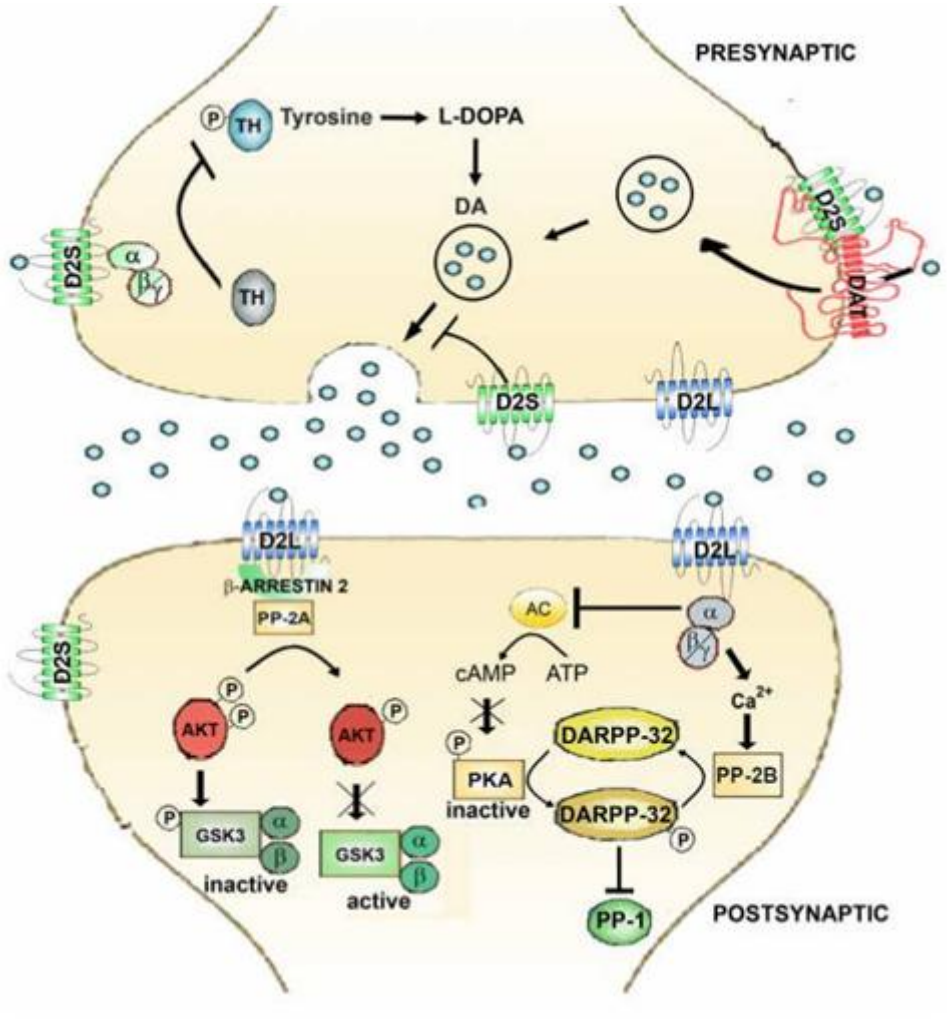

Figure 10: Pre- and postsynaptic signaling mediated by D2L and D2S (De Mei et al., 2009).
Slika 10: Pre- in post-sinaptično signaliziranje posredovano z D2L in D2S (DeMei in sod., 2009).

2.3.9 Dopamine receptor coupling to G proteins

Dopamine receptor signaling is mediated chiefly through heterotrimeric G proteins, which are comprised of an α, β, and γ subunit complex (McCudden et al., 2005). The G protein α subunit (Gα) binds guanine nucleotides and cycles between an *inactive* GDP-bound state and an *active* GTP-bound state (Oldham and Hamm, 2008). Upon agonist binding, receptors undergo a conformation change that activates Gα through the exchange of GDP for GTP, resulting in the dissociation of the constitutive dimeric G protein β and γ subunits (Gβγ). Gα-GTP and Gβγ directly modulate downstream targets of dopamine receptors, including second messengers and ion channels (Neve et al., 2004).

A distinguishing feature of D1-like and D2-like receptors is their differential coupling to heterotrimeric G proteins. D1-like receptors couple to Gαs and Gαolf, which activates adenylate cyclase (Brown and Makman, 1972; Kebabian et al., 1972). In the rodent

neostriatum, where expression of Gαolf is abundant and Gαs is relatively low, D1 receptor activation of adenylate cyclase is mediated by Gαolf (Zhuang et al., 2000; Corvol et al., 2001). In contrast, D2-like receptors couple to Gαi or Gαo, which inhibit adenylate cyclase (Ohara et al., 1988). Dopamine receptors couple to G proteins that modulate alternative effectors. For example, D1-like and D2-like receptor coupling to Gαq, which stimulates phospholipases and the consequent hydrolysis of phosphoinositides (Neve et al., 2004).

D1-like receptor stimulation of Gαs or Gαolf induces the activation of adenylate cyclase, an enzyme that catalyzes the conversion of ATP to cyclic AMP, and consequently the disinhibition of the catalytic subunit of cyclic AMP-dependent protein kinase (PKA). PKA in turn phosphorylates multiple downstream targets, including the cyclic AMP regulatory element-binding protein (CREB), the dopamine and cyclic AMP-regulated phosphoprotein, 32 kDa (DARPP-32), and various ion channels.

D1-like receptor-mediated phosphorylation of CREB by PKA leads to the translocation of CREB to the nucleus and CREB-dependent transcription of numerous genes, including the immediate early gene *c-fos* (Konradi et al., 1994). CREB-induced gene transcription is associated with synaptic plasticity and memory formation (Josselyn and Nguyen, 2005).

2.3.10 D2-like receptor regulation of adenylate cyclase

D2-like receptors inhibit adenylate cyclase through coupling to the Gαi and Gαo, thereby decreasing or preventing stimulation of cyclic AMP production (De Camilli et al., 1979; Stoof and Kebabian, 1981). Accordingly, D2-like receptors act oppositely to D1-like receptors in the regulation of substrates downstream of cyclic AMP, including PKA and DARPP-32. D2 receptor stimulation decreases DARPP-32 phosphorylation, inhibiting this phosphoprotein (Nishi et al., 2000). It would be expected that D2-like receptor stimulation also decreases CREB phosphorylation. However, in sagittal brain slices (including the neocortex, neostriatum, hippocampus, thalamus, and substantia nigra) the D2- like agonist quinpirole stimulates CREB phosphorylation, presumably through a cyclic AMP-independent mechanism (Yan et al., 1999).

D2S and D2L receptors are also thought to couple differently to G proteins, since the alternatively spliced exon lies in a region that mediates G protein interactions. Furthermore, D2S receptors more potently inhibit adenylate cyclase than D2L receptors (Montmayeur and Borrelli, 1991; Hayes et al., 1992). Taken together, D2S receptors may be more efficiently coupled to or interact with distinct subtypes of G proteins.
D1 and D2 receptor stimulate PLC and induce the release of calcium from intracellular stores through Gαq coupling (Rashid et al., 2007).

2.3.11 Arrestin-dependent signaling

Originally identified as a protein involved in receptor desensitization (Lohse et al., 1990) and then resensitization (Zhang et al., 1997), there is increasing evidence for a role of arrestin in GPCR signaling (Lefkowitz and Shenoy, 2005; Defea, 2008).
Arrestin-dependent signaling may involve the spatial redistribution of dopamine receptors from the plasma membrane into endosomal vesicles, facilitating the interaction of receptors with distinct cytosolic signaling proteins not available at the cell surface (Romanelli and Wood, 2008).
The dopamine D2 receptor signals by two independent pathways, the classical G-protein coupling (cAMP turnover) and the β-arrestin-mediated pathway. Previously, β-arrestins were thought to be involved only in desensitization and trafficking, but they also play an important role in signalling, and there is evidence that there could be bias in choosing either of these pathways by agonists thus leading to differences in their therapeutic or side-effect profile (Natesan et al., 2011).

2.3.12 D1/D2 receptor cooperativity – synergism

D1- and D2-like receptors act synergistically, to modulate dopamine-mediated behaviors, including psychiatric and motor disorders (Strange, 2001). Behaviorally, the requirement for activation of both receptors is reflected in the observation that administration of either D1 or D2 antagonists can prevent many dopamine-dependent responses. Depending on the efficacy of the agonist and the endogenous dopamine tone, coadministration of D1- and D2-like agonists may be required for the full development of some responses (Hu et al., 1990; LaHoste and Marshall, 1992; Ikemoto et al., 1997; Missale et al., 1998).
Therefore, functional synergism between D1 and D2 receptors is necessary for maintaining some normal physiological functions, and once the functional linkage between the two receptors is broken, disorders, such as schizophrenia, may develop (Yang et al., 2007).

2.3.13 Autoreceptors

Distinct pre-synaptic dopamine receptor subtypes, termed autoreceptors, regulate the tone of dopamine neurotransmission. Autoreceptors are expressed on the cell body and dendrites of dopaminergic midbrain neurons and modulate the rate of cell firing and impulse activity (Aghajanian and Bunney, 1977). It is well established that autoreceptors are D2-like receptors, as both agonists and antagonists that are selective for D2-like receptors act on dopamine neurons to cause inhibition and block dopamine-induced inhibition, respectively (Pinnock, 1983). Autoreceptors are also located on dopaminergic nerve terminals where they decrease dopamine synthesis, through inhibition of the rate-limiting enzyme tyrosine hydroxylase, and dopamine release (Kehr et al., 1972). D2-like autoreceptors also decrease dopaminergic activity by enhancing dopamine reuptake via the

dopamine transporter (Meiergerd et al., 1993; Wu et al., 2002). It is more accurate to interpret these data as demonstrating that the D2S receptor has the capability to function as the D2 autoreceptor (Carlsson and Carlsson, 2006).

2.3.14 Most drugs influence synaptic function

Most drugs acting on the nervous system do so by influencing synaptic transmission directly or indirectly, regardless of whether their aim is to alleviate disorders of mood, cognition, movements, memory, or behavior. The drugs may interfere presynaptically in the synthesis, release, degradation, or reuptake of transmitters or postsynaptically in the activity, numbers, or localization of receptors. Another important point is that storage, release, and uptake of transmitters, as well as the expression of receptors, is **dynamic processes**.

Finally, we should keep in mind that alteration of one transmitter's activity as a rule leads to alterations of other transmitters as well (Brodal, 2010).

2.4 SCHIZOPHRENIA

Schizophrenia is a most disabling psychiatric disorder characterized by a myriad of symptoms (Givovart and Kapur, 2010). Symptoms usually begin in late adolescence or early adulthood and are typically classified as positive symptoms with exaggeration of normal function, such as hallucinations, delusions, disorganized speech, and disorganized behavior. The negative symptoms comprise a diminution in mental functions, such as blunting, avolition, alogia, and anhedonia, and a deficit in social interaction (Yeganeh-Doost et al., 2011). Cognitive symptoms (disorganized thoughts, deficits in attention, working and verbal memory, social cognition, and executive function).

The course of schizophrenia is characterized by periods of symptom exacerbation (i.e. relapse) alternating with periods of relative remission, with a different pattern of exacerbation/remission episodes between the three psychopathology dimensions and between different individuals. Such findings have led several researchers to theorize that positive, negative, and cognitive symptoms reflect separate pathophysiological processes, though that still remains to be proven beyond doubt (Givovart and Kapur, 2010).

Schizophrenic patients exhibit positive, negative and cognitive symptoms which arise from an imbalance between the brain dopamine (DA) pathways mediating D2 and D1 receptor signaling (Yeganeh-Doost et al., 2011; Lewis and Lieberman, 2000). Subcortical increase of DA, leading to hyperstimulation of D2 receptors, would give rise to the positive symptoms, while a concomitant cortical deficit of DA, leading to hypostimulation of D1 receptors, would give rise to the negative and cognitive symptoms. There is indeed robust evidence that DA hypofunction and altered D1 receptor signaling within the prefrontal

cortex (PFC) play a central role in the induction of working memory deficits, suggesting that a reduced D1 receptor neurotransmission might cause cognitive impairments in schizophrenia (Perrault et al., 1997). Both hypofunctioning and hyperfunctioning DA systems thus likely coexist in schizophrenia, albeit in different brain regions (Wang et al., 2000).

The mesolimbic dopamine pathways are thought to have an important role in emotional behaviors, especially auditory hallucinations but also delusions and thought disorder (Fig.2). For more than 25 years, it has been observed that diseases or drugs that increase dopamine will enhance or produce positive psychotic symptoms, whereas drugs that decrease dopamine will decrease or block positive symptoms (Stahl, 2013).

The mesocortical dopamine pathway (Fig.2) projects to areas of the cerebral cortex, especially the limbic cortex. The role of the mesocortical dopamine pathway in mediating negative and/or cognitive symptoms of schizophrenia (Stahl, 2013).

The nigrostrial dopamine pathway (Fig.2) is a part of the extrapyramidal nervous system and controls motor movements. Deficiencies in dopamine in this pathway cause movement disorders, including Parkinson's disease, which is characterized by rigidity, akinesia or bradykinesia (i.e. lack of movement or slowing of movement), and tremor. Dopamine deficiency in the basal ganglia also can produce akathisia (a type of restlessness) and dystonia (twisting movements, especially of the face and neck) (Stahl 2013).

2.4.1 The Dopamine hypothesis

The “classical” dopamine hypothesis of schizophrenia proposed that subcortical hyperactivity of dopamine transmission in the brain is responsible for the positive symptoms of the illness (van Rossum, 1966; Fudge and Emiliano, 2003; Heinz and Schlagenhauf, 2010). This hypothesis was initially based on several lines of indirect evidence. First, exposure to dopamine enhancing drugs, such as amphetamine, induces psychosis in normal individuals and worsens psychotic symptoms in schizophrenia patients. Second, drugs alleviating psychotic symptoms of schizophrenia were suspected to act through blockade of central dopamine receptors (Kapur and Mamo, 2003; Lidow and Goldman-Rakic, 1997). Patients with schizophrenia, whether treated or untreated, are known to be supersensitive to dopamine-like compounds (Seeman, 2011; Kostrzewa et al., 2011).

2.5 CLASSIFICATION OF ANTIPSYCHOTIC DRUGS

Antipsychotic drugs exhibit possibly the most complex pharmacologic mechanisms of any drug class within the field of clinical psychopharmacology. The different antipsychotic agents, based upon their interactions with different neurotransmitter systems, such

interactions can often explain both the therapeutic actions and the side effects of various antipsychotic medications.

2.5.1 Typical Antipsychotics

Antipsychotic medications are the cornerstone treatments for reducing psychotic symptoms and relapse rates in schizophrenia. The first antipsychotic drug used for schizophrenia, chlorpromazine, was introduced in 1952 and, in the late 1950s, several other antipsychotics were subsequently introduced, including haloperidol, thioridazine, trifluoperazine, and loxapine. These first-generation antipsychotics, termed typical or conventional antipsychotics, are effective against the positive symptoms but have limited efficacy and may even exacerbate the negative and cognitive symptoms of schizophrenia. The therapeutic action of conventional antipsychotic drugs is due to blockade of D2 receptors specifically in the mesolimbic dopamine pathway (Fig.2). This has the effect of reducing the hyperactivity in this pathway that is postulated to cause the positive symptoms of psychosis. All conventional antipsychotics reduced positive psychotic symptoms if they are dosed to block a substantial number of D2 receptors there (Stahl, 2013). Moreover, some 30% of patients with schizophrenia show little or no response of their positive symptoms to typical antipsychotic therapy (Hellewell, 1999). Unfortunately, in order to block adequate numbers of D2 receptors in mesolimbic dopamine pathway to quell positive symptoms, one must simultaneously block the same number of D2 receptors throughout the brain, and this causes undesirable side effects (Stahl, 2013). Because of this, typical drugs are associated with a wide spectrum of side effects, including sedation, acute extrapyramidal symptoms (EPS), hyperprolactinemia and, although rarely, the neuromalignant syndrome. Acute EPS are dose-dependent and manifest as dystonia, akathisia, and pseudoparkinsonism (Levinson et al., 1990). The most worrisome form of EPS, tardive dyskinesia (TD), develops on long-term utilization with an incidence of about 5% a year and can be irreversible (Lieberman et al., 2005). These side effects are often severe and disabling and are a leading cause of patient noncompliance (Casey, 2006; Naber and Karow, 2001) which, in turn, leads to relapse (Dossenbach et al., 2005). Efforts to minimize EPS have revealed that lowering the dose indeed decreases side effects and still achieves therapeutic efficacy in many patients with schizophrenia (Geddes et al., 2000). However, lower doses also carry additional risk of relapse as the doses required for efficacy are only slightly lower than the doses that cause side effects (Dixon et al., 1995; Oosthuizen et al., 2001). Despite their clear benefits for the treatment of schizophrenia, the limited tolerability and narrow therapeutic index of typical antipsychotic (Fig. 11) drugs pointed out the need for better treatment options.

D2 receptors in the mesolimbic dopamine system are postulated to mediate not only the positive symptoms of schizophrenia, but also the normal reward system of the brain, and nucleus accumbens is widely considered to be the "pleasure center" of the brain. Thus, if

D2 receptors in the mesolimbic system are blocked, this may not only reduce positive symptoms of schizophrenia, but also block reward mechanisms, leaving patients apathic, anhedonic, lacking motivation, interest and joy from social interactions, a state very similar to that of negative symptoms of schizophrenia (Stahl, 2013).
Antipsychotics also block D2 receptors in the mesocortical dopamine pathway, where dopamine may already be deficient in schizophrenia. This can cause or worsen negative and cognitive symptoms even though there is only a low density of D2 receptors in cortex (Stahl, 2013).

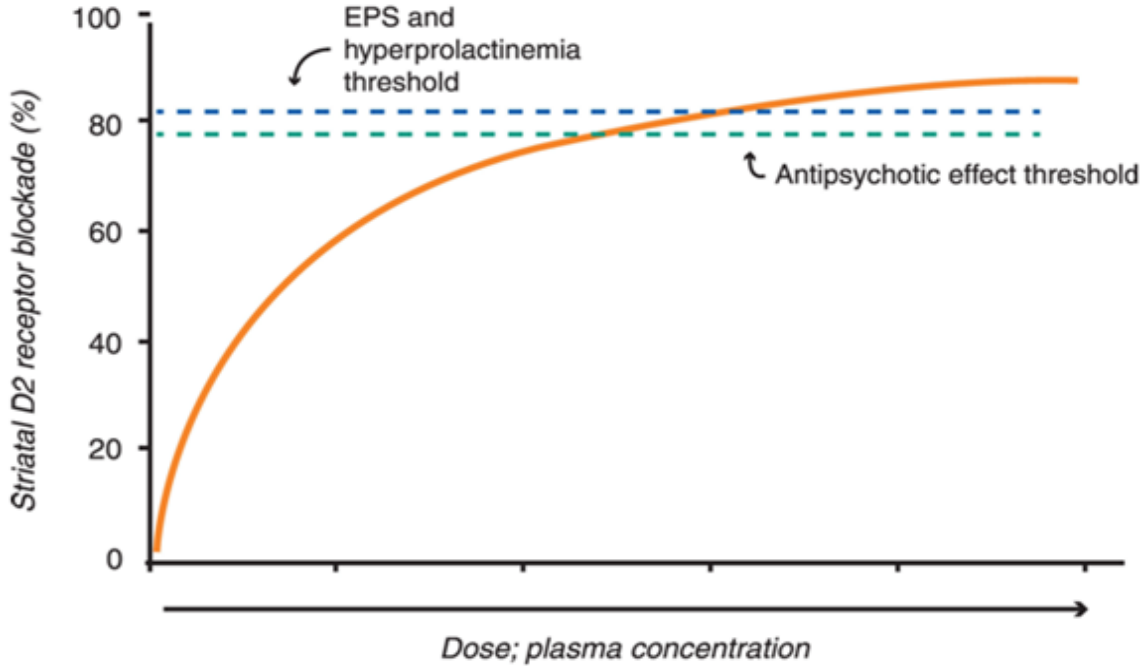

Figure 11: Hypothetical thresholds for conventional antipsychotic drug effect (Stahl, 2013).
Slika 11: Hipotetične mejne vrednosti učinkovitosti konvencionalnih antipsihotikov (Stahl, 2013).

When a substantial number of D2 receptors are blocked in the nigrostriatal DA pathway, this will produce various disorders of movement that can appear very much like those in Parkinson's disease. Since is nigrostrital pathway is part of the extrapyramidal nervous system, these motor side effects associated with blocking D2 receptors in this part of the brain are also called exprapyramidal symptoms or EPS.
Worse yet, if these D2 receptors in the nigrostriatal DA pathway are blocked chronically, they can produce a hyperkinetic movement disorder known as tardive dyskinesia. This movement disorder causes facial and tongue movements such as constant chewing, tong protrusions, facial grimacing, and also limb movements that can be quick, jerky or choreiform (dancing). Tardive dyskinesia is thus caused by long term administration of conventional antipsychotics and is thought to be mediated by changes, sometimes irreversible, in the D2 receptors of the nigrostriatal DA pathway. Specifically, these receptors are hypothesized to become supersensitive or to up-regulate (i.e. increase in

number), perhaps in a futile attempt to overcome drug-induced blockade of D2 receptors in striatum (Stahl, 2013). According to the CATIE report, the risk of TD may be correlated with a patient's age, duration of exposure to antipsychotics, exposure to conventional antipsychotics, use of anticholinergic medications, substance abuse, presence of EPS, and akathisia (Stip and Tourjman, 2010).

The use of conventional antipsychotic drugs presents a powerful dilemma. That is, there is no doubt that conventional antipsychotic medications exert dramatic therapeutic action upon positive symptoms of schizophrenia by blocking hyperactive dopamine neurons in the mesolimbic dopamine pathway, whereas blocking dopamine receptors in the remaining pathways may be harmful (Stahl, 2013).

2.5.2 Atypical Antipsychotics

Research to enhance the therapeutic benefits of antipsychotics while diminishing their side effects has led to the development of a new class of antipsychotic agents, the atypical antipsychotics. Clozapine was introduced in Europe in 1975 and marked a turning point in schizophrenia therapy. Clozapine appeared to be effective with a minimal incidence of EPS, thus challenging the dogma that antipsychotic efficacy required high levels of EPS (Hippius, 1999). Interestingly, this drug demonstrated benefits in cases of patient's refractory to typical antipsychotics and was found to be somewhat more effective against the negative and cognitive symptoms (Buchanan, 1995).

Atypical antipsychotics have the clinical profile of equal positive symptom antipsychotic action, but low extrapyramidal symptoms and less hyperprolactinemia compared to conventional antipsychotics. Thus, they are "atypical" from what is expected from classical, conventional, first-generation antipsychotics (Stahl, 2013).

Although atypical drugs improve positive symptoms with a similar efficacy (Rosenheck et al., 2003; Conley and Mahmoud, 2001), they differ from typical ones by their lower incidence of EPS and TD (Glick and Marder, 2005; Correll et al., 2004).

Rather than their efficacy against the positive symptoms, it is thus their side effect profile and efficacy against the negative and cognitive symptoms that are generally considered to differentiate typical and atypical antipsychotics. Clearly though, the newer atypical agents have emerged as a good long-term treatment option because of their wide therapeutic index and improved control of motor side effects (Fig.12).

While typical antipsychotic are usually preferential for D2-like receptors, atypical antipsychotic usually bind to a larger spectrum of receptor types. Clinical evidence also supports the importance of 5-HT_{2A} receptor blockade in the treatment of positive symptoms (Barnes et al., 2012). From a pharmacological perspective, the current atypical antipsychotic as a class are defined as serotonin-dopamine antagonists, with simultaneous serotonin 5-HT_{2A} receptor antagonism that accompanies D2 antagonism (Stahl, 2013).

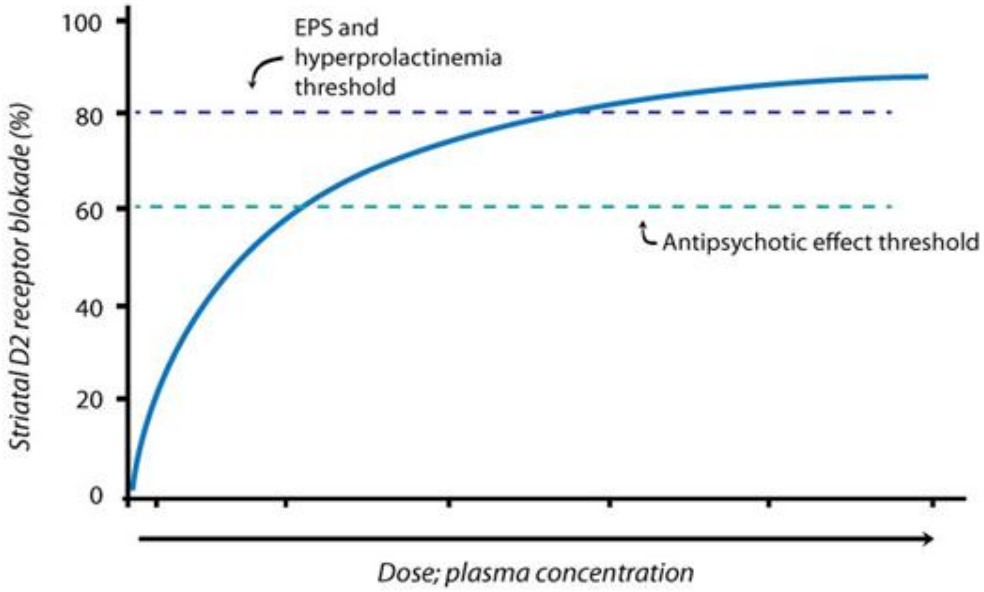

Figure 12: Hypothetical thresholds for atypical antipsychotic drug effect (Stahl, 2013).
Slika 12: Hipotetične mejne vrednosti učinkovitosti atipičnih antipsihotikov (Stahl, 2013).

All 5-HT_{2A} receptors are postsynaptic and located in many brain regions. 5-HT_{2A} receptors regulate dopamine release from nigrostriatal dopamine neurons. Serotonin neurons may innervate nigrostriatal dopamine neurons at the level of the dopamine neuronal cell bodies in the supstantia nigra and at the dopamine neuronal axon terminals in the striatum. This innervation may be either via a direct connection between the serotonin neuron and the dopamine neuron, or via an indirect connection with a GABA interneuron. 5-HT_{2A} receptor stimulation by serotonin blocks dopamine release in the striatum. On the other hand, 5-HT_{2A} receptor antagonism by an atypical antipsychotic at the same site stimulates downstream dopamine release in the striatum. Such release of dopamine in the striatum should mitigate EPS, which is why antipsychotic with 5-HT_{2A} antagonist properties are atypical (Stahl, 2013). The result of this increased dopamine release is that dopamine competes with D2 receptor antagonists in the striatum, and reduces the D2 receptor binding there below 80% to more like 60%, enough to eliminate extrapyramidal symptoms.

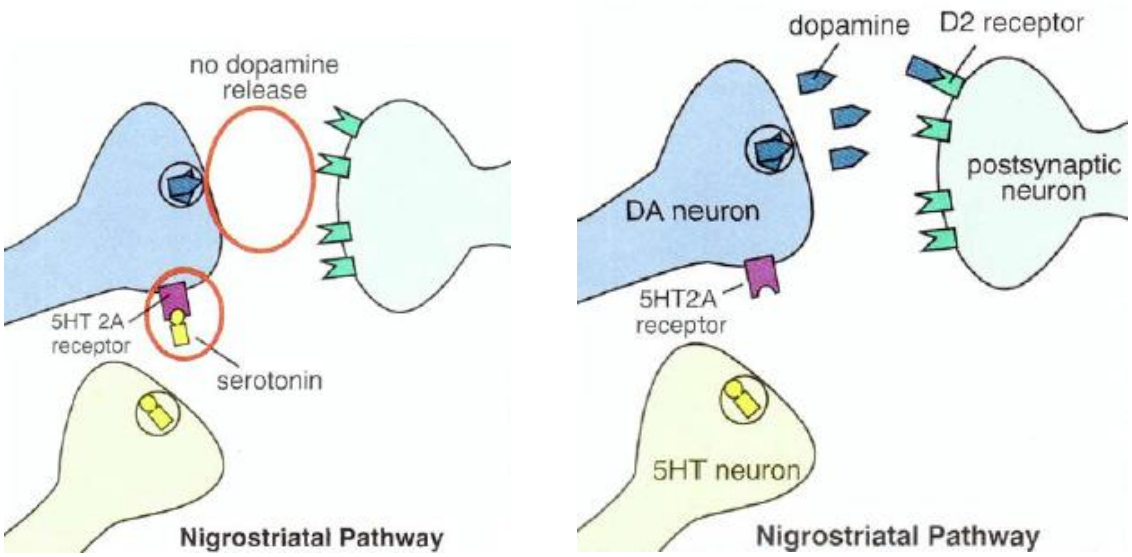

Figure 13:Serotonine and dopamine interactions in the nigrostriatal DA pathway (Stahl, 2013).
Slika 13: Serotoninsko in dopaminsko medsebojno delovanje v nigrostriatni DA poti (Stahl, 2013).

One of the most striking and consistent consequences of chronic antipsychotic exposure is an increase in striatal D2 receptor density (Sirinathsinghji et al., 1994; Laruelle et al., 1992; Tarazi et al., 1997; Buckland et al., 1993; Hu et al., 1990; Meshul and Casey, 1989). This D2 receptor up-regulation is thought to be responsible for loss of efficacy of antipsychotics and provokes unwanted extrapyramidal syndrome (EPS) (Strange, 2001; Xu et al., 2002; Ginovart et al., 2009; Lewis and Lieberman, 2000; Hess et al., 1986; Jorgensen et al., 1994).), characterized by parkinsonism, akathisia, catalepsy, and, after long-term treatment, tardive diskinesia (Kapur and Remington, 2001; Strange, 2001). Tardive dyskinesias are typically seen after several years of antipsychotic medication. Rarely, tardive diskinesia may supervene after only a few months of treatment. Tardive dyskinesia (TD) is a syndrome of involuntary movements that predominantly involve muscles in the tongue and face (Jorgensen et al., 1994). Due to long-term treatment arises supersensitivity D2 receptor (increase responsiveness of D2 receptors) (Meshul and Casey, 1989; Kostrzewa, 1995; Samaha et al., 2007). Moreover, even though most patients with schizophrenia are supersensitive to dopamine (Seeman, 2011).

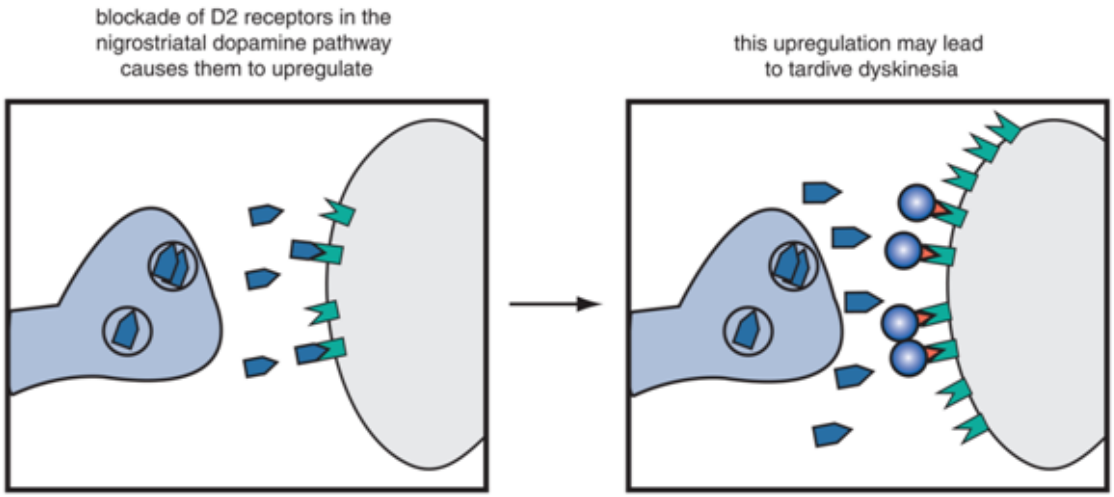

Figure 14:Serotonine and dopamine interactions in the nigrostriatal DA pathway (Stahl, 2013).
Slika 14: Serotoninsko in dopaminsko medsebojno delovanje v nigrostriatni DA poti (Stahl, 2013).

Aripriprazole is a new atypical antipsychotic with a pharmacological mechanism that distinguishes it from currently available antipsychotic agents. It acts as a potent partial dopamine D2 receptor agonist, a partial serotonin 5-HT_{1A} agonist and 5-HT_{2A} receptor antagonist. Partial D2 receptor agonism is believed to help stabilize dopaminergic neurotransmission in schizophrenia in the mesocortical and mesolimbic pathways. Partial agonist activity at the 5-HT_{1A} receptor may confer efficacy against negative and affective symptoms of schizophrenia, while antagonism at the 5-HT_{2A} receptor may contribute to efficacy against negative symptoms as well as reducing EPS liability.

These new atypical antipsychotics also have side effects which, due to the markedly different pharmacological profiles, range from weight gain, impaired glucose tolerance, hyperprolactinaemia with potential associated sexual dysfunction, to cardiotoxicity (Naber and Lambert, 2004).

Based on the clinical evidence, including data from short-term (4–8 weeks) and long-term (26–52 weeks) randomized, double-blind clinical trials, aripiprazole has been associated with improvements in positive, negative, cognitive, and affective symptoms of schizophrenia and schizoaffective disorder (Stip and Tourjman, 2010).

D1 and D2 receptors have important functional interactions. However, these interactions were abnormal in over half of the postmortem striata from schizophrenic patients, suggesting an impaired functional linkage between D1 and D2 receptors in schizophrenic patients (Goldman-Rakic et al., 2004). Therefore, the restoration of normal, functional linkage between D1 and D2 receptors may be an effective strategy for the treatment of schizophrenia. Before atypical antipsychotic were discovered, clinical doses of the available antipsychotic drugs primarily blocked D2 receptors (Seeman et al., 1984), but unfortunately they were less effective at PFC D1 receptors. As a result, a large proportion

of schizophrenic patients did not show adequate relief from their negative symptoms (Saha et al., 2005). However, even with the development of atypical antipsychotic, most of them display only very small D1 agonistic effects on the PFC D1 receptor. Therefore, there is an urgent need to develop an agent that exerts both D1 agonistic and D2 antagonistic effects, which should more effectively restore synergism between D1 and D2 receptors and alleviate both the negative and positive symptoms of schizophrenia. LEK-8829 possesses these characteristics and may serve as a novel, promising agent for schizophrenia therapeutics (Yang et al., 2007).

LEK-8829 acts through D1 receptors to increase adenylyl cyclase activity; subsequent signaling pathways regulated by adenylyl cyclase might be responsible for the physiological responses. By contrast, LEK-8829 inhibits both D2 autoreceptor-mediated feedback inhibition of dopamine containing neurons and D2 receptor-mediated effects on target non-dopamine-containing neurons (Jin et al., 2002).

2.6 ANIMAL MODELS OF SCHIZOPHRENIA

2.6.1 Preclinical evidence for an antipsychotic action at the D2 receptors

A number of animal models have been used to explore antipsychotic action and to investigate the mechanisms underlying the differences between the actions of typical and atypical drugs. Some models are used to predict antipsychotic efficacy (i.e. inhibition of amphetamine-induced hyperactivity, disruption of conditioned avoidance responding, and induction of c-fos expression in the shell region of the NAcc), while others are used to predict their motor side effect liability (i.e. induction of catalepsy, blockade of apomorphine-induced sniffing, and induction of c-fos expression in the dorsolateral striatum).

2.6.2 Antipsychotic effect on indirect DA agonist-induced behavior

Reversal of amphetamine-induced hyperactivity in rodents is one classical animal model used to screen antipsychotic drugs. Indirect DA agonists such as amphetamine or methylphenidate induce a strong increase in locomotor activity when injected into rodents. There is evidence that this increased locomotor activity is due to an increased DAergic activity in the mesolimbic system, mainly within the NAcc (Ginovart and Kapur, 2010). While lacking in face validity, blockade of amphetamine-induced hyperactivity is considered as one of the better preclinical predictors of antipsychotic activity. Accordingly, both typical and atypical antipsychotic drugs reduce the hyperactivity produced by amphetamine and this effect has been linked to D2 receptor blockade (Ellenbroek, 1993). Here again, the potency of antipsychotics to antagonize amphetamine-induced hyperlocomotion correlated with their affinity at D2 receptors (Bardin et al., 2007). The

similar action of typical and atypical antipsychotics in the amphetamine model is consistent with their similar blockade of D2 receptors and their similar antipsychotic effect in schizophrenia patients.

These models thus demonstrate in vivo antagonist activity of all antipsychotics at D2 receptors and suggest that a lower D2 action of atypical drugs in the nigrostriatal system may explain their lower propensity to cause EPS when compared to typical (Ginovart and Kapur, 2010).

2.6.3 Antipsychotic effect on catalepsy

The catalepsy test is a common and widely used preclinical screening test for the propensity of an antipsychotic drug to induce EPS in humans (Wadenberg, 1996). Antipsychotics such as haloperidol and risperidone, which have a dose-dependent propensity to induce EPS in humans, induce catalepsy in animals in a dose-dependent manner. In contrast, atypical drugs such as clozapine and aripiprazole, which significantly less produce EPS in humans do not produce catalepsy in animals (Natesan et al., 2006; Tang et al., 1997). The cataleptic behavior seems primarily to involve D2 antagonism in the DA nigrostriatal pathway that mediate extrapyramidal motor function and a direct relationship has been established between the blockade of striatal D2 receptors and catalepsy (Wadenberg et al., 2000).

2.7 PARKINSON'S DISEASE

Parkinson’s disease (PD) is an age-related movement disorder characterized by decreased levels of the neurotransmitter dopamine (DA) in the striatum of the brain, due to selective degeneration of the nigro-striatal DA neurons. In most instances PD appears to be sporadic, without any family history. While the etiology of PD has not been unequivocally determined, speculation has centered around complex mechanisms such as toxic environmental factors superimposed on susceptibility genes, oxidative stress caused by mitochondrial dysfunction, dysfunction of the ubiquitin proteasome system with resultant accumulation of misfolded proteins and endoplasmic reticulum stress, and formation of activated microglia accompanying an inflammatory process. These changes may lead to the programmed cell death (apoptosis or autophagy) of DA neurons (Nagatsua and Sawadab, 2009).

Dopamine loss in the striatum in Parkinson’s disease, the degeneration of dopaminergic SNc neurons and their projections to the striatum is a slowly evolving process that may take decades to develop. SNc projections to the putamen degenerate earlier than projections to associative or limbic portions of the striatum. Corresponding to this time course of degeneration, the motor symptoms and signs of Parkinson’s disease develop

before the non-motor signs. Recognizable motor or non-motor signs appear only after substantial degeneration of the nigrostriatal neurons (affecting at least 70 %, e.g. Bernheimer et al., 1973), testament to the remarkable compensatory capacity within the dopaminergic system, or in the circuits it modulates (Galvan and Wichmann, 2008).

The primary features of this disease include resting tremor, rigidity, bradykinesia or akinesia, postural instability and non-motor symptoms (sleep disturbances, depression, neuropsychiatric and cognitive deficits) (Ferguson, 2007; Gurevich and Gurevich, 2010).

In Parkinson's disease, the degeneration of nigrostriatal dopaminergic neurons reduces the dopaminergic modulation through reduced striatal dopamine levels. Reduced striatal dopamine results in less activation of striatal D1 receptors and D2 receptors affecting the direct and indirect pathways, respectively (Fig.11). Through decreased activation of D2 receptors, striatal inhibitory projections to the GPe become more active through reduced suppression. There is increased striatal inhibition of the GPe, subsequent disinhibition of the STN and therefore STN hyperactivity within the indirect pathway. Simultaneously, striatal projections to the GPi and SNpr become less active through decreased D1 receptors activation (Alexander and Crutcher, 1990). As a result of increased STN excitation and reduced striatal inhibition, there is increased activity of the GPi and SNr, and a subsequent increase in tonic inhibition of the thalamocortical projection neurons. Therefore, with PD, maladaptive modulation of tonic inhibition of the thalamus through the separate striatal neuron subpopulations and subsequent basal ganglia pathways (Albin et al., 1989) has a crucial impact on motor impulse control (MacDonald and Byblow, 2015).

Dopamine loss in the basal ganglia triggers prominent secondary morphological changes. One change that may have pathophysiologic significance is the reduction of the density of dendritic spines on MSNs, particularly in the putamen (Ingham et al., 1989; Zaja-Milatovic et al., 2005), which may greatly alter corticostriatal transmission. Recent studies have suggested that MSNs with D2 receptors (belonging to the indirect pathway) may be preferentially affected by the spine loss, and that the loss of spines may involve the dysregulation of calcium channels (Day et al., 2006).

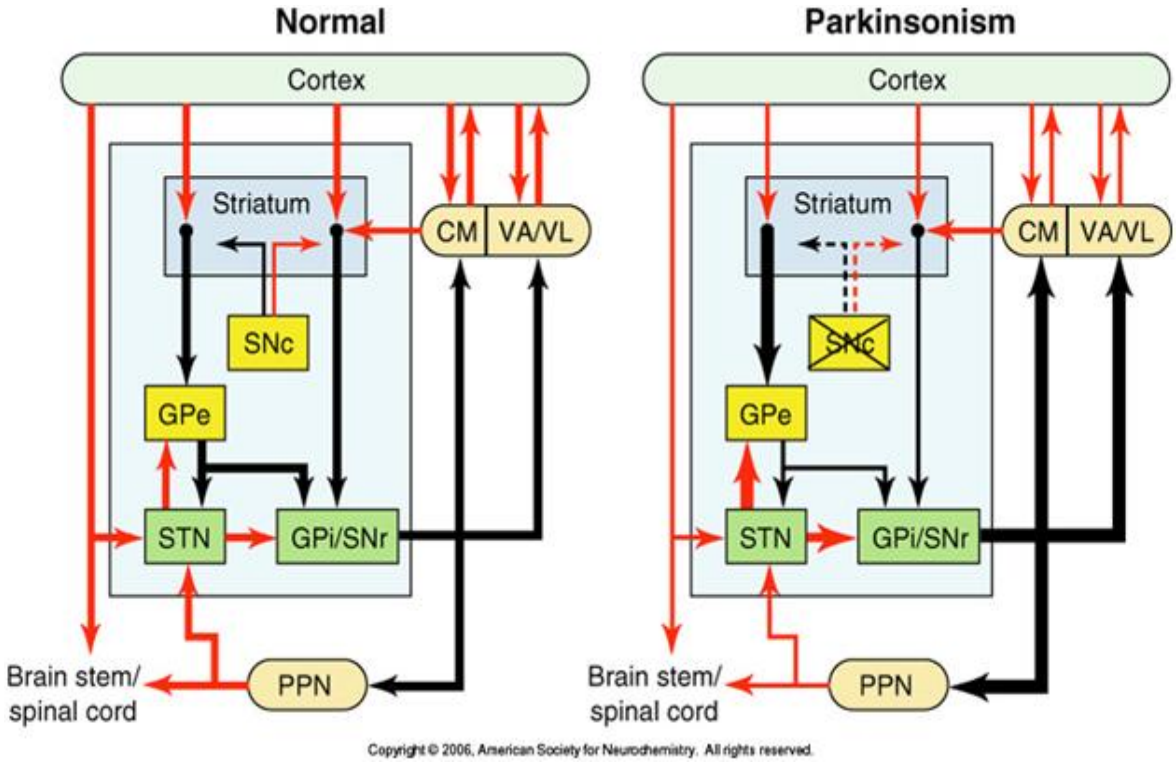

Figure 15: Simplified diagram demonstrating the anatomical connections within the basal ganglia circuitry, and changes in the activity of basal ganglia nuclei associated with development of parkinsonism (Siegel et al., 2006).
Slika 15: Poenostavljen diagram, ki predstavlja anatomske povezave znotraj povezav bazalnih ganglijev, ter spremembe v dejavnosti jedra bazalnih ganglijev v povezavi z razvojem parkinsonizma (Siegel in sod., 2006).

Dopamine depletion also triggers changes in the density and sensitivity of dopamine receptors. The mRNA expression for dopamine D2 receptors and binding sites in the striatum is increased in patients with Parkinson's disease and Parkinsonian animals (Aubert et al., 2005; Bezard et al., 2001; Bokobza et al., 1984; Creese et al., 1977; Guigoni et al., 2005). Changes (increases or decreases) in striatal D1-receptor binding were also reported in some studies (Buonamici et al., 1986; Marshall et al., 1989), but were not seen by other authors (Aubert et al., 2005; Bezard et al., 2001; Guigoni et al., 2005). Gerfen (1990) demonstrated that the expression of D1 receptor mRNA was specifically down-regulated in direct pathway MSNs, and that the expression of D2 receptor mRNA was up-regulated in indirect pathway MSNs (Gerfen et al., 1990; Galvan and Wichmann, 2008).

One of the most important implications of the enabling relationship between D1 and D2, DA receptors is its potential relevance for the treatment of Parkinson's disease (Hu et al., 1990).

2.7.1 Current treatment approaches

Drug therapy at correcting the neurochemical imbalances in the basal ganglia is the cornerstone of treatment for PD patients. However, to date, all therapies are symptomatic in nature; no drug has yet been discovered which impedes or reverse PD progression. In addition, all current drugs have a limit to their effectiveness; patients eventually become tolerant to their benefit and suffer from numerous debilitating side effects. Current drug therapies can be divided into several drug classes: levodopa (L-DOPA)/carbidopa, dopamine agonists, monoamine oxidase-B inhibitors, muscarinic antagonist s(Slusher, 1996).

2.7.2 Levodopa (L-DOPA)

PD being characterized by dopamine depletion, the first curative treatments were based on exogenous dopamine supply to restore dopaminergic transmission at striatal synapses. However, trials with oral dopamine failed because dopamine cannot cross the blood–brain barrier, leading to severe peripheral adverse events. Levodopa is a natural dopamine precursor that can cross the blood–brain barrier to reach the brain where it is converted into dopamine by peripheral decarboxylase and stored in vesicles in order to be progressively released onto postsynaptic receptors (Goole and Amighi, 2009).
The duration and dosage of levodopa therapy being known to be the major risk factors in the appearance of motor complications, numerous strategies are now used to prevent dyskinesia and the "on-off" effect: (1) delay of the need for levodopa, (2) reduction of the cumulative dose of levodopa, (3) avoidance of the pulsatile stimulation of dopamine receptors, and (4) neuroprotection to slow down disease progression (Goole and Amighi, 2009). The problems arising with long-term levodopa therapy are the dyskinesias that develop with treatment over months or years, presumably resulting from priming or supersensitization of D2 receptors (Kostrzewa et al., 2011).

With the progression of the disease, patients become very sensitive to rapid fluctuations in plasma levodopa concentrations. Dopaminergic terminals continue to degenerate and are no longer able to buffer the exogenous levodopa adequately. As a result, patients experience one or more periods during the day when the dose of levodopa wears off. Dopamine receptors are thus stimulated in a frequent abnormal and intermittent fashion, thereby developing an oscillating clinical response during chronic treatment of PD (Goole and Amighi, 2009).

Indeed, repeated dosing of levodopa leads to pulsatile stimulation of D1 and D2 receptors and subsequent sensitization, which is known to induce dyskinesia. These abnormal involuntary movements occur in 75 % of patients after about 6 years of levodopa therapy. In addition, nigrostriatal degeneration is also presumed to be an essential condition for the appearance of dyskinesia under levodopa medication. Therefore, motor fluctuations appear to be an all or nothing process, independent of the administrated dose. Among these motor

disturbances are choreatic movements, which are an expression of involuntary rhythmic contractions of the skeletal muscles, rigidity, which is an enhancement of the tonic stretch reflex and akinesia and bradykinesia, which are lack of movement and slowness of movement, respectively (Goole and Amighi, 2009).

Besides dyskinesias, the sudden return of parkinsonian symptoms during asymptomatic episodes can be observed in the latest stages of the treatment. As PD evolves, the brain loses its ability to regulate dopamine function as both storage and release become impaired. Consequently, a long duration response to a single levodopa dose is gradually replaced by a shortening interval of effect and a need for more drugs. This "on–off" effect comprises an "on" state when the patient has a good response from levodopa and an "off" period characterized by a sudden loss of benefit when the plasma level of the drug falls. Usually, a wearing off phenomenon can be defined to be present when an adequate dosage of levodopa does not last at least 4 h. However, in the late stages of the disease, the duration of the "on" response becomes shorter (Goole and Amighi, 2009).

Altering dopaminergic dosing and timing can abate dyskinesias, but usually impact the control of parkinsonism. Putative therapies to reduce the problem of dyskinesias could focus on the glutamatergic, GABAergic, α2 adrenergic, serotonergic ($5HT_{1A}$, $5HT_{2A}$), opioid, histamine H3, adenosine A_{2A} receptors, the monoamine transport or cannabinoid CB1 receptors systems. The only currently available drug with an evidence based recommendation on efficacy for dyskinesia is amantadine (Fabbrini et al., 2007).

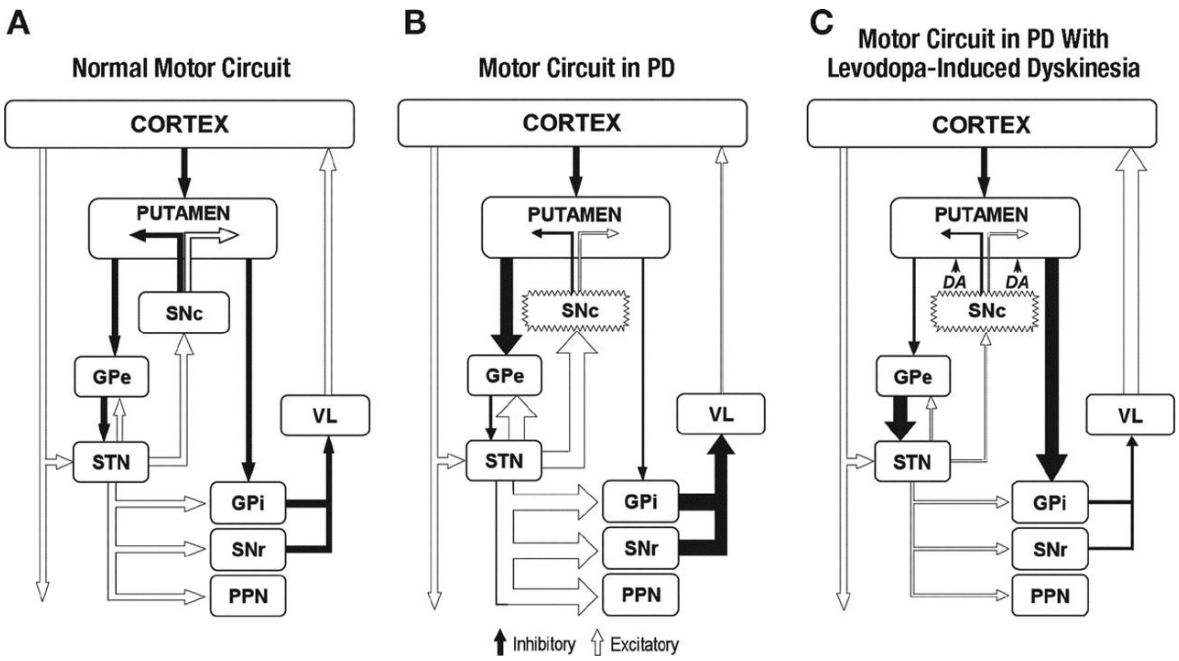

Figure 16: A lassic model of basal ganglia in a normal condition, and in Parkinsonian and dyskinetic conditions (Olanow et al., 2001).
Slika 16: Klasični model bazalnih ganglijev v normalnih pogojih, pri parkisonovi bolezni in diskineziji (Olanow in sod., 2001).

2.7.3 Non-DA neurons (e.g. 5-HT neurons)

It is significant that 5-HT neurons and other types of neurons can accumulate L-DOPA. If this occurs, L-DOPA will be converted via DOPA decarboxylase to DA and thereby become a 'false transmitter' in the 5-HT or other type of neuron. Exogenously administered L-DOPA is shown to enter 5-HT neurons to undergo conversion to DA which will be stored in vesicles in 5-HT neurons. This is so, because the VMAT in vesicles in 5-HT nerves is identical to the VMAT in vesicles in DA nerves. Accordingly, DA will displace some 5-HT, as indicated by reduced 5-HT content of striatal tissue following levodopa treatment of an animal model of PD (Kostrzewa et al., 2000).

In phenotypically different neurons in which DA is unable to be stored, DA might simply diffuse out of the nerve, independent of nerve activity.

Levodopa remains the most effective antiparkinsonian agent available to date. The major practical limitation to the use of L-DOPA is the risk of developing motor complications and considerable evidence suggests that these problems may be prevented or attenuated with the use of low doses and/or continuous delivery (Olanow, 2015).

Pharmacologically, dopamine agonists and levodopa differ in the spectrum of effects that they produce and clinically of course, levodopa is superior to dopamine agonists in efficacy (Jenner, 2008).

2.7.4 Dopamine receptor agonists

Dopamine agonists are drugs that directly stimulate dopamine receptors. It has long been known that these drugs have antiparkinsonian efficacy (Koller, 2002). These drugs have theoretical advantages over levodopa in terms of not requiring enzymatic activation, having longer duration of actions and causing fewer adverse effects since they offer receptor selectivity unlike levodopa. These drugs are useful as the initial choice for dopaminergic therapy as they delay the need for levodopa therapy and are associated with lower incidence of motor fluctuations and dyskinesias. They are also useful as an add on therapy to levodopa in patients who develop motor complications and allow for a reduction in levodopa doses. Adverse effect profile of dopamine receptor agonists includes hallucinations, confusion, nausea, postural hypotension, somnolence and an increased incidence of impulse control disorders including pathological gambling, shopping, eating and hypersexuality (Kakkar and Dahiya, 2015; Wolters et al., 1995; Olanow, 2002; Katzenschlager and Lees, 2002; Lipski et al., 2011; Schwartz and Sabetay, 2012; Calabresi et al., 2010; Olanow et al., 2006).

Dopamine agonists used in the treatments of PD have a longer half-life than levodopa. Assuming that plasma concentration predicts what occurs in the striatum, it is reasonable then to consider that a dopamine-like agent with a long plasma half-life will induce more

continuous stimulation of striatal dopamine receptors than a dopamine like agent with short plasma half-life. This might prevent adaptive changes that lead to the development of motor complications (Olanow et al., 2006).

All dopamine agonists have proven successful in the early stages of Parkinson's disease, but they are contraindicated in patients with cognitive impairment and psychiatric manifestations due to increased psychotoxicity (Schwartz and Sabetay, 2012).

Agonists are currently in use: D2 receptor agonist alone or in combination with levodopa. Bromocriptine became the first approved oral dopamine agonist and adjuvant therapy with levodopa. The duration of efficacy of bromocriptine combined with levodopa is approximately 2 years. The nonselective dopamine agonist apomorphine is also used in the management of resistant off periods. Dihydexidine was first reported in 1990 as a selective D1 agonist (Brewster et al., 1990; Lovenberg et al., 1989) and was subsequently shown to alleviate the symptoms of MPTP-induced parkinsonism in monkeys (Taylor et al., 1991). Although dihydrexidine has some affinity at D2 receptors (Ki=120 nM), its antiparkinsonian activity is not reversed when combined with D2 antagonist, suggesting that this effect is due to its D1 (Ki=12nM) receptor activity. Abbot also synthesized a selective D1 agonist A77636 which reverses PD symptoms in Parkinsonian marmosets (Kebabian et al., 1992). Blanchet (1993) compared the efficacy of D1 and D2 agonists in alleviating PD symptoms in MPTP – treated monkeys and reported that D1 agonists were as effective as D2 agonists and had fewer propensities to cause dyskinesias. These data support the hypothesis that the D1 receptor could play a crucial role in the treatment of PD. Rotigotine, mirapexine and ropinirol are in use.

2.7.5 The other drugs

The drugs also in use are selective monoamine oxidase B (MAO-B) retards the breakdown of dopamine in the striatum, thereby benefitting the PD patients, methyltransferase (COMT) inhibitors block peripheral degradation of levodopa leading to its increased half life and enhanced central bioavailability (Kakkar and Dahiya, 2015). With regard to non-dopaminergic systems, some therapeutic approaches are still at the developmental level while others are in different stages of clinical trials; these include adenosine A_{2A} antagonists (Shah and Hodgson, 2010), serotonin receptor agonists, and others (Schwartz and Sabetay, 2012). To support this is a fact that putative therapies to reduce the problem of dyskinesias could focus on the glutamatergic, GABAergic, α2 adrenergic, serotonergic ($5HT_{1A}$, $5HT_{2A}$), opioid, histamine H3, adenosine A2A receptors, the monoamine transport or cannabinoid CB1 receptors systems (Fabbrini et al., 2007).

2.8 ANIMAL MODELS OF PARKINSON'S DISEASE

Animal models are an essential tool to study human diseases, not only to enable a thorough investigation into the mechanisms involved in the pathogenesis of a disease but also to help in the development of therapeutic strategies. The discovery of profound dopamine depletion of basal ganglia in patients with Parkinson's disease and the development of antiparkinsonian drug therapy were largely based on animal models (Kaakkola and Teravainen, 1990).

2.8.1 The reserpine model

Systemic administration of reserpine depletes dopamine at the nerve terminals and induces a hypokinetic state in rodents. These movement deficits are due to loss of dopamine storage capacity in intracellular vesicles. Reserpine induced temporary changes and striatal reserpine administration does not induce morphologic changes in the dopamine neurons in supstantia nigra. Also, reserpine administration induces the release of other neurotransmitters that may not be directly implicated in PD. Nevertheless, this model has been used successfully to investigate the therapeutic effects of striatal dopamine replacement agents including L-DOPA and dopamine receptor agonists (Gossel et al., 1995). The predictive value of symptomatic drug testing in the reserpine model is imperfect, however, since some drugs that reverse reserpine-induced.

2.8.2 The 6-OHDA model

6-Hydroxydopamine (6-OHDA) represents one of the most common neurotoxins used in degeneration models of central catecholaminergic projections, including the nigrostriatal system. Inside neurons, 6-OHDA accumulates in the cytosol and induces cell death without apoptotic characteristics (Schober, 2004). Systemically administered 6-OHDA is unable to cross the blood-brain barrier. Thus, 6-OHDA has to be injected stereotactically into the substantia nigra. Following 6-OHDA injections, dopaminergic neurons start degenerating within 24 hours, and striatal dopamine is depleted 2 to 3 days later. The magnitude of the lesion is dependent on the amount of 6-OHDA injected, the site of injection and inherent differences in sensitivity between animal species. Usually 6-OHDA is injected in one hemisphere while the other hemisphere serves as an internal control. This unilateral 6-OHDA model is also known as the hemiparkinson model.

2.9 ALTERNATIVE SPLICING

Alternative splicing is highly abundant in brain relative to other, where it can influence neurophysiology through spatial and temporal alterations in proteins that comprise ion

channels and membrane bound receptors and are involved in neurotransmitter storage and release. In the nervous system, regulated alternative splicing allows the cell to ‘‘fine-tune’’ its protein composition in order to respond and adapt to different stimuli (Grabowski and Black, 2001; Licatalosi and Darnell, 2006).

Alternative splicing generates much of the enormous diversity needed in the proteins involved in forming specific synaptic connections and mediating synaptic transmission (Grabowski and Black, 2001). Importantly, an alternative splicing event may be regulated differently in a cell type and developmental stage specific manner (Licatalosi and Darnell, 2006). Disruption of normal splicing or splicing misregulation has been observed in a large number of diseases (Licatalosi and Darnell, 2006).

Pre-mRNA splicing is necessitated by the split nature of eukaryotic genes, in which the exons that will make up the mRNA product are interrupted by non-coding introns in the DNA and in the initial pre-mRNA transcript. Intron removal, and the concomitant joining of exons, is orchestrated by the spliceosome — a macromolecular ribonucleoprotein complex that assembles on the pre-mRNA in a series of complexes (E, A, B and C). Complex assembly is guided by consensus sequences at the ends of the introns. Conserved alternative exons are often flanked by more conserved intronic sequences than constitutive exons, and this has been used as an independent predictor of alternative splicing (Matlin et al., 2005). Certain exons are present in all messenger RNAs transcribed from a specific gene (constitutive exons). Other exons are only present in a subset of messenger RNAs transcribed from a specific gene (alternative exons). Alternative exons often have suboptimal splice sites and/or a suboptimal length when compared with constitutive exons (Caceres and Kornblihtt, 2002).

RNA splicing requires recognition of the nucleotide sequences at the boundaries of transcribed exons and introns (splice junctions). The nucleotides at the ends of introns are highly conserved: the vast majority of introns start with a GT (becoming GU in intronic RNA) and end with an AG (the **GT-AG rule**).

Although the conserved GT and AG dinucleotides are crucial for splicing, they are not sufficient to mark the limits of an intron. The nucleotide sequences that are immediately adjacent to them are also quite highly conserved, constituting splice junction consensus sequences (splice donor site on GT and splice acceptor site on AG). A third conserved intronic sequence that is also important in splicing is known as the **branch site** and is typically located no more than 40 nucleotides upstream of the introns 5’ terminal AG. Other exonic and intronic sequences can promote splicing (splice enhancer sequences) or inhibit it (splice silencer sequences), and mutation in these sequences can cause disease (Strachan and Read, 2011).

Pre-mRNA splicing takes place within the spliceosome, a large molecular complex composed of four small nuclear ribonucleoproteins (U1, U2, U4/U6 and U5 snRNPs) and

approximately 100 non snRNP splicing factors (Chen and Manley, 2009; Xiao and Lee, 2010; Graveley, 2001).

Five small nuclear RNAs form the core of the spliceosome. During processing, the pre-mRNA has extensive, specific interaction via base pairing with five small nuclear RNAs (U1, U2, U4, U5, and U6). Early in spliceosome assembly, U1 forms a base-pairing interaction with the 5′-splice site and U2 similarly base-pairs with the branch-point. Then a tri-snRNP complex containing U4, U5 and U6 associates with the forming spliceosome and U4 is removed from the complex. This allows U6 to replace U1 at the 5′ splice site and leads to a U6–U2 interaction that brings the 5′-splice site and the branch point close together, allowing for a transesterification step. By forming noncanonical interactions, U5 brings the two exons into close proximity and allows for the second step of splicing, when the two exons are joints. This shows that small RNAs play a crucial role in splicing and there is increasing evidence that some of the RNAs also play a role in catalysis of the splicing reaction (Tazi et al., 2009).

Proteins assembling on the pre-mRNA allow exon recognition by interaction with the core spliceosome. Since the information in splice sites is not sufficient for regulation, ribonuclear–protein complexes (RNPs) forming on the pre-mRNA help in the recognition of exons. The majority of splicing regulatory proteins in these complexes belongs to two major classes: hnRNPs and SR-proteins. **HnRNPs** are operationally defined as proteins binding to RNA. **SR-proteins** are characterized by serine and arginine-rich protein domain. These proteins contain RNA-binding and protein interaction domains. They bind with low specificity to accessible, mostly single-stranded parts of the pre-mRNA. To overcome the low RNA binding specificity, splicing regulatory proteins use their protein interaction domains to bind to each other. Another frequent strategy to overcome the low affinity between splicing factors and their recognition sequence is a repetitive arrangement of regulatory sequences (Tazi et al., 2009).

As already said, splicing is carried out by the spliceosome, a massive structure in which five small nuclear ribonucleoprotein particles (snRNPs) and a large number of auxiliary proteins cooperate to accurately recognize the splice sites and catalyse the two steps of the splicing reaction. Spliceosome assembly begins with the recognition of the 5′ splice site by the snRNP U1 and the binding of splicing factor 1 (SF1) to the branch point and of the U2 auxiliary factor (U2AF) heterodimer to the polypyrimidine tract and 3′ terminal AG (Fig.11). This assembly is ATP independent and results in the formation of the E complex, which is converted into the ATP-dependent, pre-spliceosomal A complex after the replacement of SF1 by the U2 snRNP at the branch point. Further recruitment of the U4/U6–U5 tri-snRNP complex leads to the formation of the B complex, which is converted into the catalytically active C complex after extensive conformational changes and remodeling i.e. U1 and U4 leave spliceosome. Splicing activators and repressors commonly function by influencing the formation of the E and A complexes early in spliceosome assembly (Matlin et al., 2005).

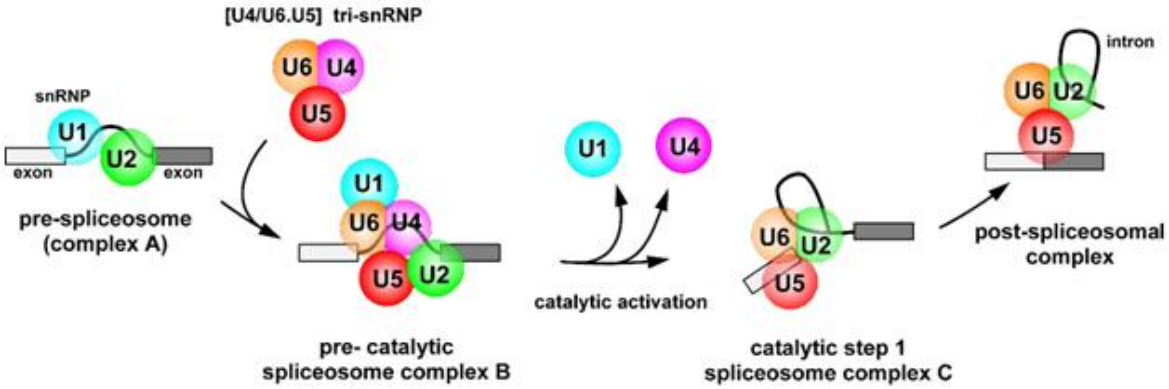

Figure 17: Spliceosome and steps of alternative splicing (Bessonov et al., 2008).
Slika 17: Spajalno telasce in koraki alternativnega zlepljanja (Bessonov in sod., 2008).

The spliceosome assembles onto each intron from a set of five small nuclear ribonucleoproteins (snRNPs U1, U2, U4–U6) and numerous accessory proteins that bind specifically to locations at or within the vicinity of the splice sites, and catalyzes the excision of the intron. While the 5' (donor) and 3' (acceptor) splice sites have well characterized consensus sequences that are recognized to play a major role in splicing, an increasing body of evidence reveals that previously unknown RNA elements located outside the splice signals, in exons and introns, contribute to the exon's inclusion or exclusion in the mature mRNA, in a network of interactions that appear to be centered on exons, rather than introns. These cis-regulatory elements can promote (splicing enhancers) or repress (splicing silencers) the inclusion of the exon in the mRNA through the activity of the bound regulatory proteins, and can be located in the exons -exonic splicing enhancers (ESEs) and silencers (ESS), or introns-intronic splicing enhancers (ISE) and silencers (ISS). They can act from both within the proximity of the exon, or from 300–1000 bp away. It is becoming increasingly evident that many exons, constitutive or alternative, and their surrounding introns harbor both silencing and enhancing elements, and that the exon's inclusion/exclusion is the result of competition between the two effects (Florea, 2006).

2.9.1 Mechanisms of splicing activation

Exonic enhancers are the binding sites of splicing activator proteins, the most studied activators being the SR (Ser/Arg) factors. The SR proteins are characterized by the presence of 1–2 RNA recognition motifs (RRM), and a C-terminal RS-domain enriched in arginine and serine residues (Arg/Ser). An RS-domain independent mechanism has also been hypothesized, in which the splicing factor binds to an ESE to antagonize the effect of a neighboring silencer (Florea, 2006).

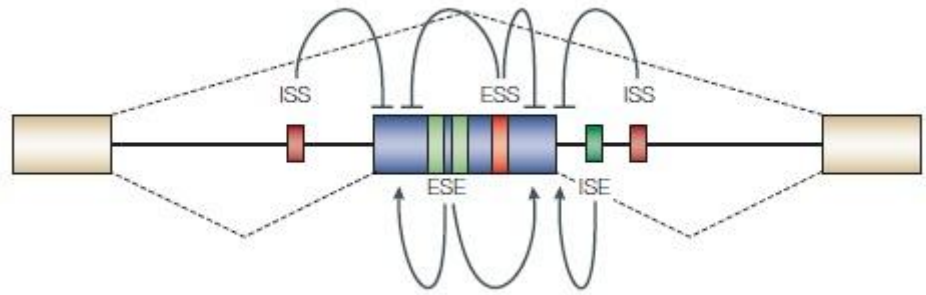

Figure 18: Elementary alternative splicing events and regulatory elements. In addition to the splice-site consensus sequences, a number of auxiliary elements can influence alternative splicing. These are categorized by their location and activity as exon splicing enhancers and silencers (ESEs and ESSs) and intron splicing enhancers and silencers (ISEs and ISSs). Enhancers can activate adjacent splice sites or antagonize silencers, whereas silencers can repress splice sites or enhancers. Exon inclusion or skipping is determined by the balance of these competing influences, which in turn might be determined by relative concentrations of the cognate RNA-binding activator and repressor proteins (Matlin et al., 2005).
Slika 18: Elementi alternativnega izrezovanja in njihova regulacija. Poleg osnovnih izrezovalnih zaporedij na alternativno izrezovanje vplivajo številna pomožna zaporedja. Ta se delijo glede na položaj in aktivnost kot eksonski ojačevalci (ESE) in utiševalci izrezovanja (ESS) ter intronski ojačevalci (ISE) in utiševalci izrezovanja (ISS). Ojačevalci lahko aktivirajo sosednja skupna izrezovalna mesta ali delujejo antagonistično na utiševalce, medtem ko lahko utiševalci zavirajo skupna izrezovalna mesta ali ojačevalce. Vključitev ali izključitev eksona je odvisna od ravnotežja med temi tekmujočimi si vplivi, ki so lahko določeni tudi z relativnimi koncentracijami aktivatorskih in represorskih RNA-vezivnih proteinov (Matlin in sod., 2005).

2.9.2 Mechanisms of splicing repression

Splicing silencers are thought to be binding sites of splicing repressor proteins. Splicing repression is effected largely through the activity of the hnRNP protein families. Of these, hnRNP I, also known as PTB, members of the hnRNP A/B families, are among the best characterized. Several mechanisms have been proposed for hnRNP-mediated silencing by interfering with the spliceosome assembly through cooperative binding of several inhibitory elements, by blocking neighboring ESEs, or by binding to duplicate intronic sequences on both sides of the exon to form a loop that renders the exon inaccessible to the spliceosome. Experimentally identified or validated ISS sequences were reviewed in (Florea, 2006).

Alternative splicing is important for generating functional diversity in the molecules controlling neuronal activity. Alternative splicing is also central to the development of the nervous system, the differentiation of neurons and the formation of their connection patterns. Molecules important for neural development whose activity is controlled in this way include transcription factors, cell adhesion molecules, exon guidance receptors and proteins involved in programmed cell death. Alternative splicing mechanisms are extremely well suited to produce large numbers of subtly different protein functions.

2.10 NOVA 1

Nova 1 is an important regulator of alternative splicing in the mammalian brain (Dredge et al., 2005). Nova 1 target genes are highly related in function. They were associated with the function of inhibitory synapses, postsynaptic and presynaptic structures, as well as signaling and protein synthesis, suggesting that a single splicing factor regulates isoform expression of different genes in inhibitory neurons. It is likely that other cell-type-specific splicing factors also control biologically coherent functions. Nova autoregulate its own expression by acting as a splicing repressor (Dredge et al., 2005).

The dopamine D2 receptor (D2R) is present *in vivo* in two isoforms, D2L and D2S, generated from the same gene by alternative pre-mRNA splicing. Each isoform has a specific role *in vivo*, underlining the importance of a strict control of its synthesis, yet the molecular mechanism modulating alternative D2R pre-mRNA splicing has not been completely elucidated (Park et al., 2011). By a mechanism of alternative splicing, the D2 receptor gene (DRD2) encodes two molecularly distinct isoforms, D2S and D2L. D2L differs from D2S by an additional 29 amino acids encoded by exon 6, inserted within the third cytoplasmic loop of the receptor, the region interacting with G proteins. This insertion likely accounts for a differential interaction of D2L and D2S with G proteins, activation of distinct downstream signaling pathways and function (Park et al., 2011). D2L acts mainly at postsynaptic sites and D2S serves presynaptic autoreceptor functions (Khan et al., 1998). The D2S isoform appears to be involved in regulation of prefronto-striatal synaptic plasticity associated with long-term potentiation, centrally implicated in the physiology of memory (Polydorides et al., 2000).

Alternative D2R pre-mRNA splicing is controlled by two splicing regulators, hnRNPM and neuro-oncological ventral antigen-1 (Nova 1). hnRNPM recognizes specific elements within D2R exon 6, and it enhances exon 6 excision during alternative pre-mRNA splicing. In contrast, Nova 1 antagonizes this effect via direct binding to both exons 6 and hnRNP M (Fig.14).
The identification and characterization of the factors binding to the *cis*-acting regulatory elements are important in unraveling mechanisms that modulate gene expression by affecting alternative pre-mRNA splicing. hnRNPM is a member of a family of RNA-binding proteins; it contains three RNA recognition motifs (RRMs). hnRNPs are known to be negative splicing regulators by directly antagonizing the recognition of splice sites or interfering with the binding of splicing regulators to splicing enhancer sequences (Park et al., 2011).

HnRNPM directly binds to D2R exon 6 and inhibits its inclusion, thus favoring D2S mRNA production in a dose dependent manner. This observation is consistent with previous studies implicating hnRNP M as a negative regulator of alternative pre-mRNA splicing.

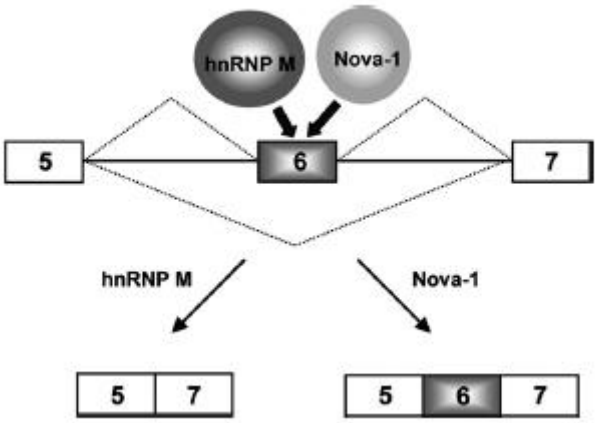

Figure 19: Schematic model of the molecular regulatory mechanisms for the alternative pre-mRNA splicing of D2R by Nova 1 and hnRNP M. Nova 1 and hnRNPM regulate alternative D2R pre-mRNA splicing antagonistically. Both Nova-1 and hnRNP M specifically interact with D2R exon 6, but hnRNP M inhibits the binding of Nova 1 to D2R exon 6. Nova 1 antagonizes the exon 6 exclusion activity of hnRNP M and enhances exon 6 inclusion (Park et al., 2011).
Slika 19: Shematski model molekularno regulatornih mehanizmov alternativnega izrezovanja D2R z Nova 1 in hnRNP M. Nova 1 in hnRNP M regulirata alternativno izrezovanje D2R pre-mRNA antagonistično. Nova 1 in hnRNP M specifično interagirata z D2R eksonom 6, pri čemer hnRNP M zavira vezavo Nova 1 za D2R ekson 6. Nova 1 deluje antagonistično na izključevanje eksona 6 posredovanega s hnRNP M ter tako povečuje vključevanje eksona 6 (Park in sod., 2011).

Although hnRNP M enhances D2S mRNA production, D2L mRNA is dominant in most brain regions. Thus, positive splicing regulators certainly exist that enhance D2R exon 6 inclusion during the D2R pre-mRNA splicing process. Recently, it was reported that PTBP1 (polypyrimidine tract-binding protein 1) enhances exon 6 inclusion (Sasabe et al., 2011), but there is no evidence that it binds to pre-mRNA of D2R directly.

Nova 1 directly binds to UCAU sequences within D2R exon 6 RNA (Fig. 13). Nova 1 is known as a splicing factor that regulates the inclusion or exclusion of exons depending on the position of Nova binding relative to splice sites (Ule et al., 2006). When Nova binds to the 3'-region of the cassette exon, it promotes inclusion of this exon (Ule et al., 2006). Consistent with this, overexpression of Nova 1 dose-dependently increases D2R exon 6 inclusion likely by blocking hnRNPM function (Fig.13). The antagonistic action of Nova 1 is very likely mediated by its binding to D2R exon 6. Thus two splicing proteins were identified that, by interacting with each other, contribute to the regulation of alternative D2R pre-mRNA splicing.

Several possible mechanisms might be evoked to explain how D2R pre-mRNA splicing can be regulated by an antagonistic interaction between hnRNPM and Nova 1 (Park et al., 2011), suggesting that hnRNP M and Nova 1 may bind to adjacent sequence within exon 6, rather than competing for the same site.

They favor the possibility that, by binding to exon 6, Nova 1 blocks the binding of hnRNP M to it. Indeed, disruption of Nova 1 binding to exon 6 decreases the inhibitory effect on hnRNPM activity during D2R pre-mRNA splicing. Nova 1 protein abolishes hnRNP M function and enhances D2L mRNA expression via RNA independent interaction with hnRNP M and/or specific binding to D2R RNA

Studies performed *in vitro* and most importantly on genetically engineered animals have shown that D2L and D2S signaling differentially affects physiological responses, and therefore, these isoforms do not have redundant functions. An altered D2S/D2L ratio has recently been found in schizophrenic patients (Bertolino et al., 2009) and importantly, most antipsychotics have D2R as a major target. Thus, control of alternative D2R pre-mRNA splicing might have major consequences on dopamine-mediated responses. However, the molecular regulatory mechanism of alternative D2R pre-mRNA splicing is not fully elucidated.

3 MATERIALS AND METHODS

3.1 ANIMALS

A total of 150 adult male Wistar rats of 270-300 g initial body weight were used in all experiments. The Wistar rats were obtained from the Medical Experimental Center (MEC, Ljubljana). The animals were allowed two weeks of adaptation to laboratory conditions before being used in experiments. The rats were housed four per cage under constant temperature and humidity, under 12 h light/dark cycles (on 8 am - off 8 pm). Food and water were continuously available *ad libitum* in home cages. The experiments were performed between 8 am and 2 pm.

The animals were handled according to the NIH Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Veterinary Administration of the Republic of Slovenia (34401-53/2012/7).

3.2 THE MOST IMPORTANT CHEMICALS AND THEIR SOLUTIONS

3.2.1 Chemicals used in behavioral experiments

- **9,10-didehydro-N-methyl-(2-propynyl)-6-methyl-8β-aminomethylergoline bimaleate** – dopamine D2 receptor antagonist and dopamine D1 agonist (LEK-8829; LEK, Ljubljana, Slovenia), was calculated as the free base and dissolved in 0.9 % saline.
- **haloperidol** – dopamine D2 receptor antagonist (Haldol ampoules, 5 mg/ml; Krka-Jenssen, Novo Mesto, Slovenia) was dissolved in 0.9% saline.
- **R-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride** – dopamine D1 receptor antagonist (SCH-23390; RBI, Natick, MA, USA) was dissolved in 0.9 % saline.
- **2-Br-α-ergocryptine mesylate** – dopamine D2 receptor agonist (bromoergocriptine; LEK, Ljubljana, Slovenia), was dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany), with the final solution being made up with 0.9 % saline and DMSO (2:1).
- **3,4,5-trimethoxybenzoyl methyl reserpate –** dopamine depletion (reserpine RBI, Natick, MA, USA) was dissolved in DMSO(Sigma Aldrich, Germany) and propylene glycol (1:1) (Kertus ABEE, Greece).
- **d-amphetamine sulphate** – indirect dopamine receptors agonist (amphetamine RBI, Natick, MA, USA) was dissolved in 0.9 % saline.

3.3 PREPARATION AND ADMINISTRATION OF THE SUBSTANCE

Solutions of the test compounds were prepared immediately before behavioral experiments. All the solutions were stored in dark bottles because some solutions are photo labile. Volume of the solution of the test compound has been 2 ml/kg body weight due to greater accuracy of injection. All the test compounds were administered subcutaneously (s.c.) in the dorsal area of the neck. These doses are deemed to substance with an additive salts with the exception of LEK-8829 which indicated doses apply to a free base.

3.4 BEHAVIORAL TEST PROCEDURES

3.4.1 Catalepsy test

Catalepsy test was performed using the method described by Krisch (Krisch et al., 1994). Catalepsy was assessed by the placement of the front limbs of the rats over a horizontal bar (a bar is elevated 11cm above the floor). Catalepsy was scored every 20 min for 260 min. The scoring started 20 min after the s.c. injection of the test compounds (LEK-8829 and haloperidol, 2 mg/kg and 0.2mg/kg respectively). The score was assigned on the basis of the duration of the cataleptic posture (until one forepaw touched the floor or the hind legs left the floor to climb onto the bar) as follows: score 1 - between 15 and 29 sec; score 2 - between 30 and 59 sec; and score 30 - 60 sec or more.

3.4.2 Spontaneous locomotor activity

Locomotor activity was measured in individual boxes 60cm x 40cm. Spontaneous locomotor activity was measured as distance moved for 20 min immediately after rats were placed in the boxes. These conditions produce high base-line levels of activity. Locomotor activity for individual rats was measured as distance moved in 20 min period using the video tracking system for automation of behavior experiments Noldus Ethovision Pro Version 3.0.

3.4.3 Amphetamine-induced locomotor activity

Rats were given s.c. injections of saline. After 20 min (spontaneous locomotor activity) they were given an injection of amphetamine (2 mg/kg). Locomotor activity for individual rats was measured as distance moved in the 120 min period using video tracking system for automation of behavior experiments Noldus Ethovision Pro Version 3.0.

3.4.4 Inhibition of amphetamine - induced locomotion with test compounds

Rats were given s.c. injections of test compounds (LEK-8829 and haloperidol 2 mg/kg and 0.2 mg/kg, respectively). After 20 min (spontaneous locomotor activity) they were given an injection of amphetamine (2 mg/kg). Locomotor activity for individual rats was measured as distance moved in the 120 min period using video tracking system for automation of behavior experiments Noldus Ethovision Pro Version 3.0.

3.4.5 Locomotor activity of reserpinized rats

Animals were treated three times, once in three days, with reserpine and every day twice a day (on 8 am and on 8 pm) with test compounds. The animals were recorded for 120 min after morning injections for nine days.

3.5 RECORDING WALKING TRIALS WITH VIDEO TRACKING SYSTEM

3.5.1 Noldus EthoVision

Noldus Ethovision Pro Version 3.0 (Noldus Information Technology, Wageningen, Netherlands) is an automated video tracking, motion analysis and behavior recognition system. It offers a wide range of video tracking options, extensive analysis of locomotory tracks and automatic behavior recognition.

An automated system in which a computer processes data originating from a video camera or a digital video file is referred to as a computer vision or imaging system. The technique by which an analog video image is translated into digital information, from which features are extracted and parameters are derived is called digital image processing. A video tracking system is special kind of computer vision system, designed to process moving video images.

3.5.2 Recording of walking trials

A video camera was deployed above individual boxes (60cm x 40cm) and four boxes were in the field of vision. The image from the video camera was recorded by VCR and later the images from the VCR were sent to the frame grabber in computer. The frame grabber is plug-in computer board, also called a digitizer or acquisition card, which forms the heart of a digital image processing system.

One video image from VCR is called a frame. The number of frames scanned in each second is called the frame rate. The frame rate differs according to which TV standard

camera uses. The American standard is 30 frames per second and the European standard is 25 frames per second. Sample rate is the sampling of a digitized video image or digital video file by an application program. The sample rate is the number of video images that is sampled per unit of time. I used the sample rate of 5 samples per second which was also recommended for rats in Noldus EthoVision manual.

I defined arenas which were the individual boxes 60cm x 40cm. An arena is an area within which one independent set of observations takes place. When arena module starts for the first time, EthoVision takes the image it gets from a camera or video recorder at the moment as the background image. The background is defined as everything in a scene which is not part of the object being tracked. After arenas were defined I did the calibration method because EthoVision measures the distance between two points in pixels. To convert these in real values (meters, inches) EthoVision must be calibrated.

The second thing that EthoVision has to do is to distinguish between the object being tracked and the background. **Object size** – the number of pixels composing the object. The object must be at least three pixels wide so that Ethovision can distinguish it from system noise. My objects were ten pixels wide. **Object position** – the x, y coordinates of the point mathematically in the center of the object (referred to as its center of gravity).

EthoVision prompts you to put the animals in the arenas and when you click OK the trial starts. Trial is one run of experimental data. During the trial each of the arenas being measured are recorded as a separate track file. The track file is one independent data set recorded from one arena during one trial. The data are from a series of tracks, each track being the x, y coordinates and the surface area of one object.

3.6 ANALYZING DATA

Each video frame of my moving animals converts into a series of numbers representing the x, y coordinates and the size of the animal. In order to make sense of this raw data EthoVison produces a series of parameters describing the behavior of the animal. These include simple calculations such as the speed, distance moved and direction of the object track, more complex parameters such the meander of the animal's track, parameters describing the interaction of pairs of objects and manually score behaviors like sniffing, aggression, sleeping, rearing. EthoVision produces an analysis report.

3.7 CATALEPSY TEST

3.7.1 Experiment 1

Six groups of eight animals were used. The animals (n= 48) were treated once daily for 21 days. First group were treated with 0.9 % saline. Second and third groups were treated with

LEK-8829 2 mg/kg and 0.2 mg/kg respectively. Fourth group were treated with 0.9 % saline. Fifth and sixth group were treated with haloperidol 2mg/kg and 0.2 mg/kg respectively. The catalepsy response of second, third, fifth and sixth group were measured on day 1 and day 21 of the experiment. On all other days the animals were put back into home cages after drug injection.

All animals were without test compounds, seven days after the last injection and catalepsy test. On 29th day of experiment the first group was divided and n= 4 injected saline; n= 4 injected LEK-8829 2 mg/kg. The second group was divided n= 4 injected saline; n= 4 injected LEK-8829 2 mg/kg. The third group was divided n= 4 injected saline; n= 4 injected LEK-8829 0.2 mg/kg. The fourth group was divided n= 4 injected saline; n= 4 injected haloperidol 2 mg/kg. The fifth group was divided n= 4 injected saline; n= 4 injected haloperidol 2 mg/kg. The sixth group was divided n= 4 injected saline; n= 4 injected haloperidol 0.2 mg/kg. Animals were euthanized 45 min after injection.

Table 1: Test compounds and doses according to groups. Treatment protocol.
Preglednica 1: Testne spojine in odmerki po skupinah. Protokol obravnavanja.

Saline		LEK-8829				Saline		haloperidol			
		2 mg/kg		0.2 mg/kg				2 mg/kg		0.2 mg/kg	
n=8		n=8		n=8		n=8		n=8		n=8	
1st group		2nd group		3rd group		4th group		5th group		6th group	
29th day of experiment. All groups were shared (n=4) and given the injections.											
saline	LEK 2mg/kg	Saline	LEK 2mg/kg	Saline	LEK 0.2mg/kg	Saline	hal 2mg/kg	Saline	hal 2mg/kg	Saline	hal 0.2mg/kg
Euthanized 45 min after injection.											

3.7.2 Experiment 2

Three groups of six animals were used. The animals (n= 18) were treated once daily for 21 days. First group were treated with 0.9 % saline. Second group were treated with LEK-8829 2 mg/kg. Third group were treated with haloperidol 0.2 mg/kg. The catalepsy responses were tested on day 1 and day 28 of the experiment. Seven days after the last injection we did the catalepsy test so between day 21 and day 28 the rats no received drug treatment. On all other days the animals were put back into home cages after drug injection. On 28th day after injection of test compounds I performed the catalepsy test and animals were euthanized 8 h after the injection of test compounds.

Table 2: Test compounds and doses according to groups.
Preglednica 2: Testne spojine in odmerki po skupinah.

Saline	LEK-8829	haloperidol
	2 mg/kg	0.2 mg/kg
n=6	n=6	n=6
1st group	2nd group	3rd group

3.8 STATISTICAL ANAYSIS

Data were analyzed using the SPSS computer program (SPSS 19.0 for Windows, Chicago, Illinois, USA).

The statistical significance between catalepsy scores was compared by using non-parametric statistical analysis. The Mann-Whitney U Test was used to test for the differences between LEK-8829 2mg/kg and haloperidol 0.2mg/kg on the first day. The Wilcoxon Signed Rank Test was used to test LEK-8829 2mg/kg and haloperidol 2 mg/kg, 0.2 mg/kg on the first and on the last day of the treatment.

3.9 AMPHETAMINE-INDUCED LOCOMOTOR ACTIVITY

3.9.1 Experiment 3

Six groups of eight animals were used. The animals (n= 48) were treated once daily for 21 days. First group were treated with 0.9 % saline. Second and third group were treated with LEK-8829 2 mg/kg and 0.2 mg/kg respectively. Fourth group were treated with 0.9 % saline. Fifth and sixth group were treated with haloperidol 2mg/kg and 0.2 mg/kg respectively.

On the first day of the experiment, the catalepsy test was performed 2 h following the injection of the test compounds. That was the 1st test. Two days after 1st test I performed the amphetamine-induced locomotion test (4th day of experiment). That was the 2nd test. Two days after 2nd test I performed another test, inhibition of amphetamine - induced locomotion with test compounds (7th day of experiment). That was the 3rd test.

The day after 3rd test a 21-day long treatment with test compounds was started. On the last day of the treatment, 2 h after injection I did the catalepsy test – the 1st test (28th day of experimenting). Two days after 1st test, I performed the amphetamine-induced locomotion test (31th day of experiment). That was the 2nd test after subchronic treatment. Two days after 2nd test, I performed the test of inhibition of amphetamine - induced locomotion with

test compounds (34th day of experiment). That was the 3rd test after subchronic treatment. The animals were euthanized 8 h after injection of the test compounds.

Table 3: Test compounds and doses according to groups. Groups were treated daily for 21 day.
Preglednica 3: Testne spojine in odmerki po skupinah. Skupine so bile zdravljene 21 dni.

Saline	LEK-8829		Saline	haloperidol	
	2 mg/kg	0.2 mg/kg		2 mg/kg	0.2 mg/kg
n=8	n=8	n=8	n=8	n=8	n=8
1st group	2nd group	3rd group	4th group	5th group	6th group

Table 4: 1st test performed before and after 21 day treatment.
Preglednica 4: Prvi test narejen pred in po 21. dnevnem zdravljenju.

1 TEST					
Catalepsy test, 2 h after injection					
Saline	LEK-8829		Saline	haloperidol	
	2 mg/kg	0.2 mg/kg		2 mg/kg	0.2 mg/kg
1st group	2nd group	3rd group	4th group	5th group	6th group

Table 5: 2nd test performed before and after 21 day treatment.
Preglednica 5: Drugi test narejen pred in po 21. dnevnem zdravljenju.

2 TEST					
Amphetamine-induced locomotion					
Injection of saline. 20 minuts after that injection of ampetamine (2 mg/kg)					
1st group	2nd group	3rd group	4th group	5th group	6th group

Table 6: 3rd test performed before and after 21 day treatment.

Preglednica 6: Tretji test narejen pred in po 21. dnevnem zdravljenju.

3 TEST					
Inhibition of amphetamine - induced locomotion with test compounds					
Injection of test compounds. 20 minuts after that injection of ampetamine (2 mg/kg)					
Saline + amphetamine (2mg/kg)	LEK-8829 (2mg/kg) + amphetamine (2mg/kg)	LEK-8829 (0.2mg/kg)+ amphetamine (2mg/kg)	Saline + amphetamine (2mg/kg)	haloperidol (2mg/kg) + amphetamine (2mg/kg)	haloperidol (0.2mg/kg)+ amphetamine (2mg/kg)
1st group	2nd group	3rd group	4th group	5th group	6th group

3.10 STATISTICAL ANAYSIS

Data were analyzed using the SPSS computer program (SPSS 19.0 for Windows, Chicago, Illinois, USA).

A paired Student *t*-test was used for the analysis of statistical significances between distance moved parameter on the first day and on the last day of the treatment for saline, LEK-8829 and haloperidol 2mg/kg and 0.2mg/kg. To evaluate if there was a significant difference in the effects of different treatments between different groups on the first day of the treatment was evaluated using one-way ANOVA followed with Scheffe's multiple comparison test. Statistical significance was set at $p \leq 0.05$ in all statistical analyses.

3.11 RESERPINE MODEL

3.11.1 Experiment 4

Five groups of animals were used. The animals (n= 36) were treated three times, once in three days day with reserpine and twice a day for 9 days with test compounds.

First group of twelve animals (n= 12) was treated three times, once in three days, with reserpine 10 mg/kg (1st, 4th and 7th day of the experiment) and every day twice a day (at 8 am and at 8 pm) with saline. After the last reserpine injection the animals were treated two more days with saline (8th and 9th day of the experiment). For nine days I was recording animals for 120 min after the morning injection. Next two days animals were without saline (10th and 11th day of the experiment). I performed the catalepsy test the day after (12th day of experiment). On the 13th day of the experiment I divided the first group into four groups of three animals (n= 3). Three animals got saline, three animals got LEK-8829 2mg/kg, three animals got bromoergocriptine 2 mg/kg and three animals got LEK-8829 2 mg/kg + SCH 23390 1 mg/kg. Locomotor activity for individual rats was measured as distance moved in a 240-min period using the video tracking system. The animals were euthanized 4 h after injection of the test compounds.

Second, third, fourth, fifth and sixth group consisted of six animals (n=6). I treated them three times, once in three days with reserpine 10 mg/kg (1st, 4th and 7th day of the experiment) and every day twice a day (at 8 am and at 8 pm) with LEK-8829 2 mg/kg and 0.2 mg/kg (2nd and 3rd group) bromoergocriptine 2 mg/kg (4th group) and LEK-8829 2 mg/kg + SCH 23390 1 mg/kg (5th group) (injection of LEK-8829 2 mg/kg and after 20min injection of SCH 23390 1 mg/kg). After the last reserpine injection the animals were treated for two more days with the test compounds, respectively (8th and 9th day of experiment). For nine days I was recording animals for 120 min after the morning injection. Next two days animals were without the test compounds (10th and 11th day of the

experiment). I performed the catalepsy test the day after (12th day of the experiment). The 13th day of the experiment I divided all groups into two groups of three animals (n= 3). Three animals got LEK-8829 2mg/kg, three animals got bromoergocriptine 2 mg/kg Locomotor activity for individual rats was measured as distance moved in a 240-min period using the video tracking system. The animals were euthanized 4 h after injection of the test compounds.

Table 7: Groups according to test compounds and doses. Protocol of the treatment.
Preglednica 7: Testne spojine in odmerki po skupinah. Protokol obravnavanja.

<table>
<tr><td colspan="2">Saline</td><td colspan="4">LEK-8829</td><td colspan="2">bromocriptine</td><td colspan="2">LEK-8829 + SCH 23390</td></tr>
<tr><td colspan="2"></td><td colspan="2">2 mg/kg</td><td colspan="2">0.2 mg/kg</td><td colspan="2">2 mg/kg</td><td>2 mg/kg</td><td>1 mg/kg</td></tr>
<tr><td colspan="2">n=12</td><td colspan="2">n=6</td><td colspan="2">n=6</td><td colspan="2">n=6</td><td colspan="2">n=6</td></tr>
<tr><td colspan="2">1st group</td><td colspan="2">2nd group</td><td colspan="2">3rd group</td><td colspan="2">4th group</td><td colspan="2">5th group</td></tr>
<tr><td colspan="10">13th day of experiment. All groups were divided (n=3) and given the injections.</td></tr>
<tr><td>saline</td><td>LEK 2mg/kg</td><td>LEK 2mg/kg</td><td>BrEKT 2 mg/kg</td><td>LEK 2mg/kg</td><td>BrEKT 2mg/kg</td><td>LEK 2mg/kg</td><td>BrEKT 2 mg/kg</td><td>LEK 2mg/kg</td><td>BrEKT 2 mg/kg</td></tr>
<tr><td>BrEKT 2mg/kg</td><td>LEK 2mg/kg +SCH 1mg/kg</td><td colspan="8"></td></tr>
<tr><td colspan="10">Euthanized 4h after injection.</td></tr>
</table>

3.12 STATISTICAL ANAYSIS

Data were analyzed using the SPSS computer program (SPSS 19.0 for Windows, Chicago, Illinois, USA).

One-way ANOVA followed by Scheffe's multiple-comparison test was conducted to evaluate if there was a significant difference in the effects of different treatments between different groups 1st to 13th day. Statistical significance was set at $p \leq 0.05$ in all statistical analyses.

3.13 PREPARATION OF BRAIN SLICES

Animals were put to sleep with CO_2. The brains were rapidly removed and quickly frozen on dry ice. Then they were wrapped in parafilm to prevent desiccation and transferred to a -80°C freezer until sections could be cut in a cryostat. The brains were transferred to a cryostat (Leica, Biosystems) and allowed to equilibrate to -20°C before cutting. Sections

were cut through the striatum (the area between the 1.7 mm and -2.6 mm distance from bregma) and substantia nigra (the area between the -4.8 and 5.8 mm distance from bregma), and then thaw-mounted onto poly-L-lysine coated slices (10 µm) and thaw-mounted onto parafilm (30µm), thoroughly air dried. Slices were prepared by soaking in the 0.01% poly-L-lysine (Sigma Aldrich, Germany) solution.

3.14 WESTERN BLOT

3.14.1 Sample preparation

Five striatum (left and right) were sliced from parafilm with scalpel or needle (cut in a cryostat on -20°C) and mixed with 100 µl lysis buffer (Sigma Cellysis buffer) and 2 µl protease inhibitor cocktail (1:50, Sigma Aldrich, Germany). Protease inhibitors must be included in lysis buffers to prevent degradation of proteins following the release of endogenous proteases during the process of cell lysis. Samples were on ice for 30 min with occasionally vortexing. For 15 min on 4°C and 10.000g, samples were centrifuged. Supernatant was taken in another tube and heated for 5 min on 95°C.

3.14.2 Determination of total protein concentration

When comparing the amount of protein from samples run in different lanes within the same gel or between gels, it is very important that the lanes have been loaded with same total amount of protein.

Several spectrophotometric methods are routinely used to determine the concentration of protein in solution. These include measurement of the intrinsic ultraviolet (UV) absorbance of the protein as well as methods based on a protein-dependent color changes, such as the classic copper-based Lowry assay, the Smith copper/bicinchoninic assay (BCA) and the Bradford dye assay. Although widely used, none of these procedures are particularly convenient.

The Bradford dye assay (Bradford, 1976) was used which is based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acidic conditions, the dye is most stable in its double protonated form (red). Upon binding to protein, however, it is most stable in an unprotinated form (blue).

BSA standard (TermoScientific, Rockford, Illinois, USA) was prepared from 1,5mg/ml – 0,125mg/ml and blank. In 96 well I pipette 250 µl Bradford (Sigma Aldrich, Germany) reagent and 5 µl standards and samples. After incubation of 10 min absorbance was read on 595 nm in Gene 5 program (Epoch, Biotec, Luzer, Switzerland). From the measured

absorbance of sample the concentration of protein was determined with extrapolating from calibration curve of standard (the absorbance of known concentration of BSA solutions).

3.14.3 Gel electrophoresis

XCell SureLock Mini-Cell (Invitrogen) system was used for electrophoresis by manufacture's instruction.

Electrophoresis is a commonly used method for separating proteins on the basis of size, shape and/or charge. Separation is based on the mobility of charged molecules in an electric field.

- Sample preparation: an appropriate amount of sample, 2,5 µl NuPage LDS Sample Buffer (4X) (Invitrogen), 1 µl NuPage Reducing agent (10X) and supplemented with an appropriate amount of deionized water to 10 µl. Reducing agent breaks any inter- and intra—chain disulfide bonds, linearizing polypeptides and disrupting quaternary and tertiary protein structures. Everything was done on ice. Prepared samples were heated on 95°C for 10 min for disrupting of secondary protein structures. After short spin samples were ready for loading to gel.
- Electrophoresis preparation: gel was placed to the electrophoresis and Running buffer (20X MEPS Running buffer, Invitrogen) 200 ml were added to upper chamber with 500 µl NuPage antioxidant. Lower chamber were filled with Running buffer 600 ml.

Gel was bought (NuPage 10% bis tris gel 1 mm, Invitrogen). Polyacrylamide gels are inert, crosslinked structures. The pore sizes in these gels are similar to the molecular radius of many proteins. Polyacrylamide is a thermostabile, transparent, strong and relatively chemically inert. Its versatility, however, lies in the fact that it can be prepare with a wide range of pore sizes. The pore size of a gel can be controlled by the user and is determined by the concentrations of both acrylamide monomer and bisacrylamide crosslinker. 10% bis tris gel in combination with MEPS Running buffer allows a greater distance between 40 kDa and 50 kDa where it was expected bands.

Comb was removed and wells were rinsed out with running buffer. Empty wells were loaded with 10 µl samples and 7 µl Spectra multicolor high range (Thermo scientific) molecular weight markers. Molecular weight markers are used to define the size of proteins run in a gel. Gel was run under 180 mV and 120 mA for 90 min.

3.14.4 Transfer

XCell Blot module (Invitrogen) was used for transfer manufacture's instruction. Gel, membrane, sponge and filter paper were fully immersed in the transfer buffer (NuPage Transfer buffer, Invitrogen) before making a sandwich and a current was applied in the direction of the gel to the membrane. The sandwich consisted of two sponges, lower filter

paper, gel, membrane, upper filter paper, two sponges. Nitrocellulose membrane (0,45µm, Invitrogen) was used because I had low background. The binding capacity of a membrane depends primarily on the pore size. The sandwich in XCell Blot module was immersed in the transfer buffer and put to the electrophoresis (XCell SureLock Mini-Cell). Lower chambers of electrophoresis were filled with 650 ml deionized cold water. The transfer was run under 30V and 220 mA for 90 min.

After electrotransfer it may be necessary to confirm that all the proteins in the gel have been completely eluted. This can be achieved by staining the gel. The membrane was washed for 5 min in deionized water after electrotransfer and rinsed for 5 min in Ponceau S (Sigma Aldrich, Germany) staining for verification of the protein transfer. Ponceau S was washed 3 times per 5 min in deionized water.

Block the membrane in blocking solution for 45 min at the room temperature. As non-specific binding of antibodies to the membrane is detrimental to the specificity and sensitivity of the assay, it is essential to "block" spaces not already occupied by proteins.

3.14.5 Antibody probing

Once protein samples are separated and transferred onto a membrane, the protein of interest is detected and localized using a specific antibody. Usually, Western blotting protocols utilize a non-labeled primary antibody directed against the target protein and a species-specific, labeled secondary antibody directed against the constant region of the primary antibody. The secondary antibody serves not only as a carrier of the label but is also a mechanism to amplify the emitted signals.

Membrane was incubated with primary antibody D2 receptor made in mouse 1:500 (Santa Cruiz, sc 5303) and GAPHD made in rabbit 1: 5000 (Santa Cruiz, sc 25778) 1h at room temperature and overnight at 4°C. Wash the membrane 3 times for 5min with Antibody wash (Western Breeze kit, Invitrogen) and 5min with deionized water. Incubate membrane with secondary antibody anti-rabbit with alkaline phosphatase (AP) conjugated (WesternBreeze kit, Invitrogen) for 45 min. Wash the membrane 3 times for 5min with Antibody wash (WesternBreeze kit, Invitrogen) and 5min with deionized water. Membrane was put on plastic bag and it was incubate 5 min with 1 ml Chemiluminescent substrate and 0,052 ml Chemiluminescent substrate enhancer. Membrane was covered with another clean transparency plastic to prepare a membrane sandwich for luminography. Expose an X-ray film (CP-BU x-ray film, Agfa, HeltCare NV, Mortsel, Belgium) to the membrane sandwich from several second to several minutes. Films were developed with developing machine (Curix 60, Agfa-Gevaert, Kista, Sweden).

3.15 IN SITU HYBRIDIZATION

In situ hybridization is technique used to detect mRNA, using a labeled nucleic acid probe of complementary sequence. In situ hybridization was performed by method develop in (Sirinathsinghji et al., 1994) laboratory. Synthetic oligonucleotide, 45 nucleotides long (45-mers) probes were used. Each oligonucleotide was designed to have CG content 50-60 %. Oligonucleotides with large numbers of AT residues will be less stable in forming DNA/RNA hybrids. However, if GC content is too high (> 65 %), this may cause non-specific binding since the thermal stability of the probes will be much greater (Wisden et al., 1991).

All solutions used prior to and during the hybridization step must be sterile and free of contaminating ribonucleases. Solutions were sterilized by treating with diethylprocarbonate (DEPC) 0.01 %. Gloves were worn at all stages prior to hybridization to avoid contaminating samples with human ribonuclease. After the hybridization step non-sterile conditions may be used.

3.15.1 Labeling of probes

Probes were labeled with addition of multiple residues of 5'-[α-^{35}S]dAMP to the 3' end of the molecule. In centrifuge tube it was pipetted a: 2,5 µl tailing buffer (5x Terminal Transferase Buffer, Promega); 1 µl oligonucleotide DNA probe (5ng/µl); 7 µl DEPC treated water; 1 µl ^{35}S (5'-[α-^{35}S]dAMP, 1250 Ci/mmol, Promega) 1 µl terminal deoxynucleotide transferase (20 U/µl, Promega). Mix gently by pipetting up and down. Incubate at 37°C for 1h. Reaction was stopped by adding 40 µl DEPC treated water.

Unincorporated nucleotides were separated from labeled probe by a spin column procedure using Sephadex G-50. Pipette the 50 µl of probe solution to the top of the pre-spin column and spin at 2000 r.p.m. for 2 minute and collecting the 50 µl probes in a sterile centrifuge tube. To eluate were added 2 µl 1M DTT to prevent cross-linking of sulphur residues. Probes were ready to use.

Scintillation solution and 2 µl of probe were transferred into scintillation vials for counting. Scintillation counter (LS 6500 Scintillation system, Beckman). The specific activities of the labeled probes ranged from 0.05-0.2 x10^6 cpm/ µl. A labeled probe was used immediately or stored at 4°C.

3.15.2 Fixation of slices

Slices thaw-mounted onto poly-L-lysine (Sigma Aldrich, Germany) coated slides were fixed for 5 min in 4 % phosphate-buffered paraformaldehyde. After that they were rinsed in phosphate-buffered saline (PBS) three changes of 2 min each and dehydrated for 5 min

in 70 % ethanol then 30 min in 100 % ethanol. Fixed slices were used immediately or stored in100 % ethanol at 4 °C.

3.15.3 Hybridization of labeled probes on slices

Labeled probes were diluted in hybridization buffers. It was used 1 µl of probe (from the 50 µl eluate from spin column) per 100 µl hybridization buffer. The probe was used at a concentration of 1pg/ µl. A quantity of 20µl of 1M DTT per 100 µl of hybridization buffer was added. Mixture was well vortex.

Sections were removed from 100 % ethanol and air-dried for 15-30 min. Hybridization buffers were applied to each slide covered with strip of parafilm and incubated in humidified containers at 42°C overnight.
Next day the cover slips were removed individually from the sections by immersing each slide in 1 x SSC. The racks of sections were transferred to a pre-warmed solution of 1 x SSC at 55°C in a gently agitating water bath for 1 h. After that, the racks were transferred through very brief (couple of seconds) series of rinse in 1xSSC, 0.1xSSC, 70 % ethanol and 100 % ethanol. The sections were allowed to air-dry. Dry sections were exposed to X-ray film (Kodak BioMax MR-1, Kodak).

3.15.4 Developing of x-ray film

After completing the exposition, the x-ray films were developed in the dark room. Firstly, the film was immersed in developer (Kodak GBX developer, Kodak) for 5 min, then very briefly rinsed in water and after that it was immersed in fixer (Kodak, fixer, Kodak) for 5 min. This was followed by another very brief rinse in water and air-drying.

3.15.5 Densitometric analysis

Films were laid on negatoscope (Northern light model B90) which transmitted diffuse light. Images were captured with black-with camera (CCD 72, MTI) and MCID program (MCID elite 6.0, Imaging Research). Individual striatum were marked with rectangle and then program was measured ROD. From those values were subtracted background.

Data were statistical analyzed (SPSS 19.0 for Windows, Chicago, Illinois, USA) with one-way ANOVA followed by Scheffe's multiple-comparison test with $p < 0.05$.

4 RESULTS

4.1 CATALEPSY ANALYSIS

4.1.1 Catalepsy test on 1st and 21st day (experiment 1)

We had 8 animals per group (see Table 1). The animals (n= 48) were treated once daily for 21 days. We wanted to see the consequences of the 21-day long subchronic treatment on the catalepsy score. The catalepsy test was performed on 1st and 21st day after the injection of the test compounds.

Haloperidol 2 mg/kg and 0.2 mg/kg produced significant catalepsy in rats. The onset of the response (measured in scores) was evident 40 min after administration haloperidol 2 mg/kg and 80 min after administration haloperidol 0.2 mg/kg on the 1st day and 40 min after administration haloperidol 0.2 mg/kg on the 21st day.

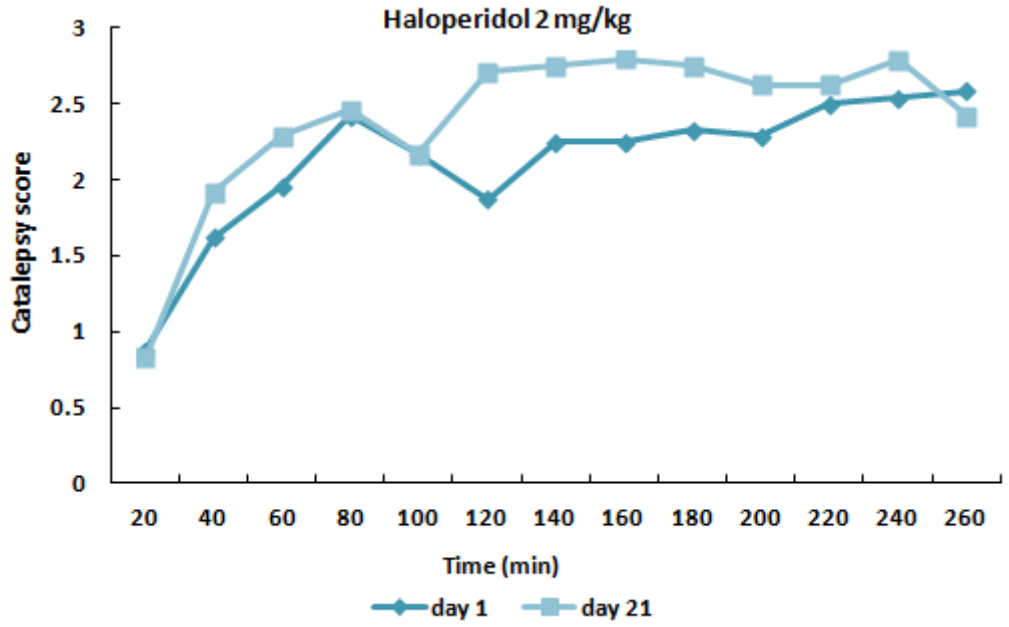

Figure 20: Haloperidol 2 mg/kg, on the 1st, the 21st day. Animals (n=8) got an injection and 20 min after it started the measuring of catalepsy scores. The onset of the response (measured in scores) was evident 40 min after administration. Score 1 and everything above that was considered to be catalepsy. This was also shown by the catalepsy test with saline (Fig.30).
Slika 20: Haloperidol 2 mg/kg 1. in 21. dan. Živali (n=8) so prejele injekcijo, po 20. minutah pa se je začela meriti katalepsija. Začetek odgovora (merjeno v dosežkih) je bil razviden po 40. minutah. Dosežak 1 in vse nad tem se obravnava kot katalepsija. To je tudi pokazal test katalepsije z fiziološko raztopino (Slika 30).

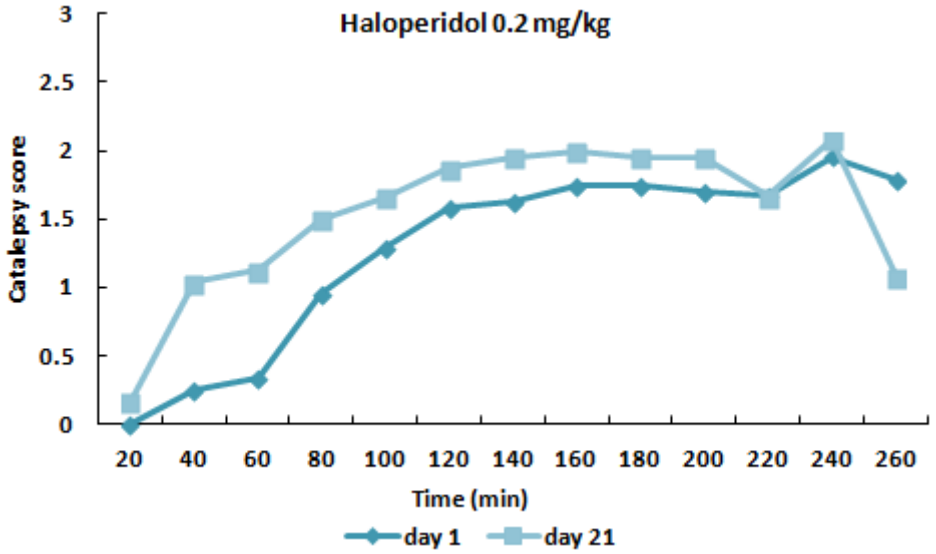

Figure 21: Haloperidol 2 mg/kg, on the 1st, the 21st day. Animals (n=8) got an injection and the measuring of catalepsy scores started 20 min later. The onset of the response (measured in scores) was evident 40 min after administration. Score 1 and everything above that is considered to be catalepsy. The catalepsy test with saline (Fig.30) showed the same.
Slika 21: Haloperidol 0,2 mg/kg 1. in 21. dan. Živali (n=8) so prejele injekcijo, po 20. minutah pa se je začela meriti katalepsija. Začetek odgovora (merjeno v dosežkih) je bil razviden po 40. minutah. Dosežak 1 in vse nad tem se obravnava kot katalepsija. To je tudi pokazal test katalepsije z fiziološko raztopino (Slika 30).

Haloperidol 2 mg/kg on the 1st and the 21st day shows more convincing catalepsy response than haloperidol 0.2 mg/kg which is in accordance with literature claims that haloperidol induces catalepsy in a dose dependent manner (Laruelle et al., 1992)

LEK-8829 2 mg/kg shows similar catalepsy curve as haloperidol 0.2mg/kg while LEK-8829 0.2 mg/kg does not show significant catalepsy. The curve is similar to the saline curve (Fig 23).

The onset of cataleptic effects occurred 100 min after administration LEK 8829 2 mg/kg on the 1st day and 20 min after administration on the 21st day. LEK-8829 2 mg/kg has maximal effect (measured in scores) on the 21st day (see Fig1).

LEK-8829 2 mg/kg shows similar catalepsy effect as haloperidol 0.2 mg/kg on the 1st day but LEK-8829 2 mg/kg on the 21st day shows a similar curve like haloperidol 2 mg/kg (1st and 21st day), probably because the LEK-8829 is still in circulation.

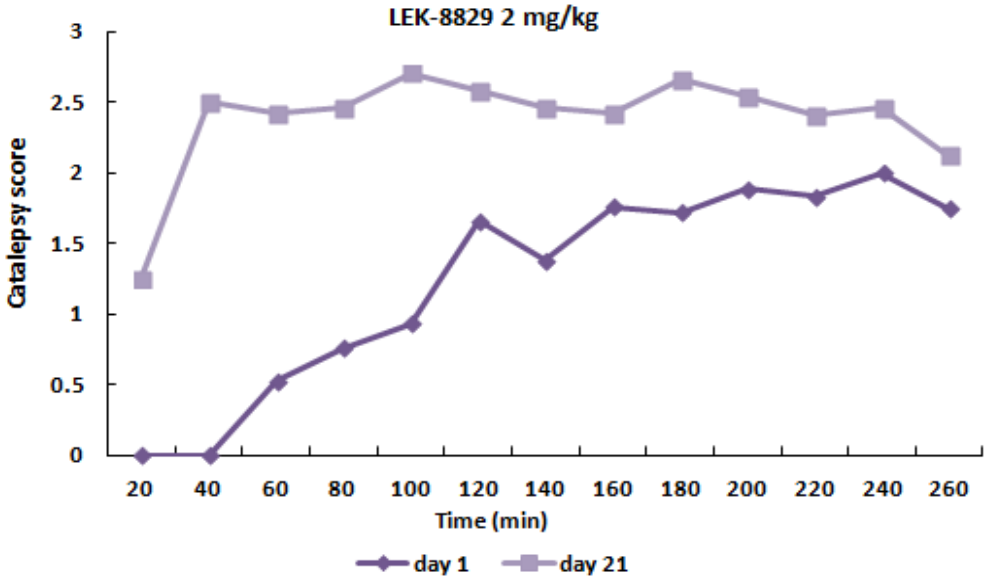

Figure 22: LEK-8829 2 mg/kg, on the 1st, the 21st day. Animals (n=8) got an injection and the measuring of catalepsy scores started 20 min later. The onset of the response (measured in scores) was evident 100 min after administration on the 1st day and 20 min after administration on the 21st day. Score 1 and everything above that is considered to be catalepsy. That is also shown by the catalepsy test with saline (Fig.30).
Slika 22: LEK-8829 2 mg/kg na 1. in 21. dan. Živali (n=8) so prejele injekcijo, po 20. minutah pa se je začela meriti katalepsija. Začetek odgovora (merjeno v dosežkih) je bil razviden po 40. minutah. Dosežak 1 in vse nad tem se obravnava kot katalepsija. To je tudi pokazal test katalepsije z fiziološko raztopino (Slika 30).

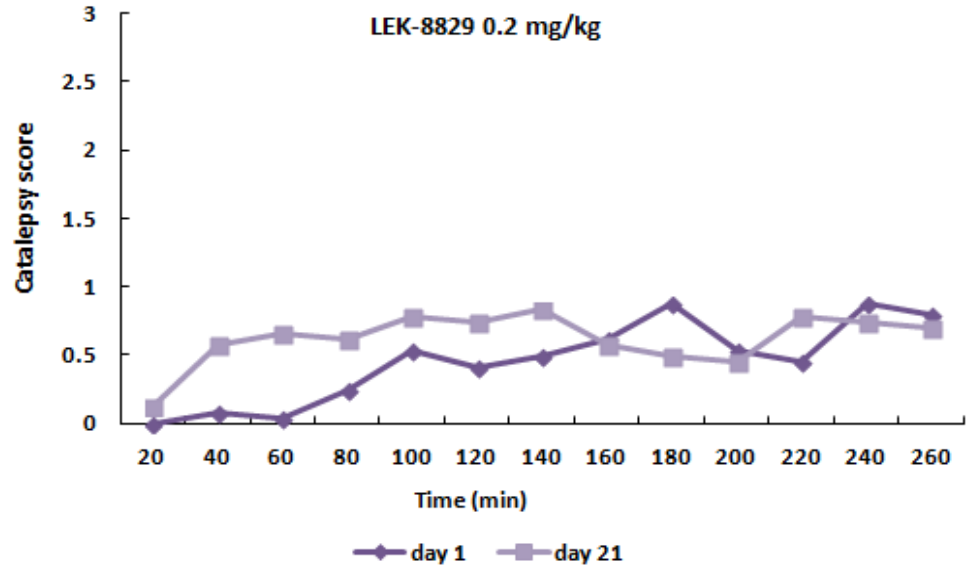

Figure 23: LEK-8829 0.2 mg/kg, on the 1st, the 21st day. Animals (n=8) got an injection and the measuring of catalepsy scores started 20 min later. This is a small dose and does not show catalepsy on the 1st day and the 21st day. The curve is similar to the saline curve (see Fig 30).
Slika 23: LEK-8829 0,2 mg/kg na 1. in 21. dan. Živali (n=8) so prejele injekcijo, po 20. minutah pa se je začela meriti katalepsija. Ta doza je majhna in ne pokaže katalepsije na 1. ali 21. dan. Krivulja je podobna krivulji z fiziološko raztopino (Slika 30).

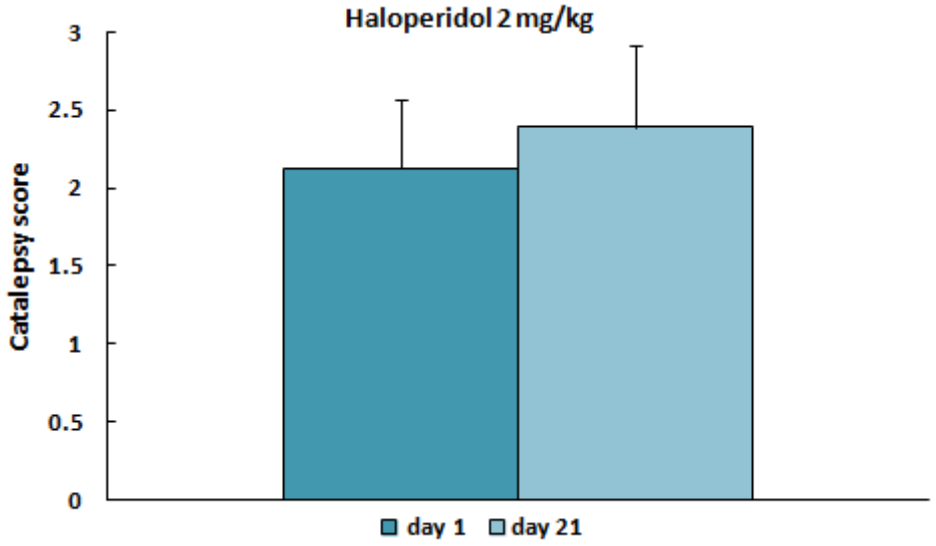

Figure 24**:** Haloperidol 2 mg/kg on the 1st and 21st day of the treatment: catalepsy scores are not significantly different. A Wilcoxon Signed Rank Test revealed no statistically significant difference in the catalepsy effect of haloperidol 2mg/kg. $p \leq 0.05$ was considered to be statistically significant.
Slika 24: Haloperidol 2 mg/kg na 1. in 21. dan: dosežki katalepsije se ne razlikujejo bistveno. Wilcoxon test ranga ni pokazal statistično pomembne razlike v učinku katalepsije haloperidola 2 mg/kg. $p \leq 0.05$ se šteje za statistično značilne.

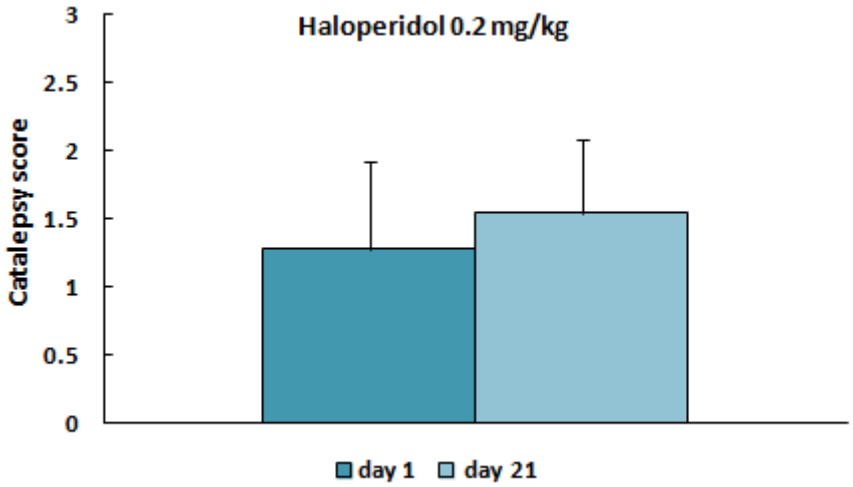

Figure 25: Haloperidol 0.2 mg/kg on the 1st and 21st day of the treatment: catalepsy scores are not significantly different A Wilcoxon Signed Rank Test revealed no statistically significant difference in the catalepsy effect of haloperidol 0.2 mg/kg. $p \leq 0.05$ was considered to be statistically significant.
Slika 25: Haloperidol 0,2 mg/kg na 1. in 21. dan: dosežki katalepsije se ne razlikujejo bistveno. Wilcoxon test ranga ni pokazal statistično pomembne razlike v učinku katalepsije haloperidola 0.2 mg/kg. $p \leq 0.05$ se šteje za statistično značilne.

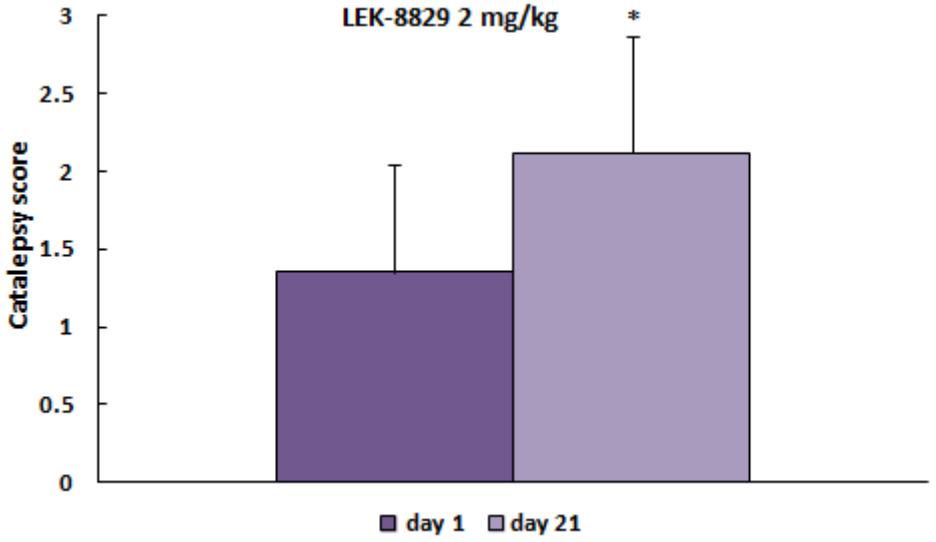

Figure 26: LEK-8829 2 mg/kg on the 1st and 21st day of the treatment: catalepsy scores are significantly different A Wilcoxon Signed Rank Test revealed a statistically significant increase of catalepsy effect on 21st day. $p \leq 0.05$ was considered to be statistically significant.
Slika 26: LEK-8829 2 mg/kg na 1. in 21. dan: * dosežki katalepsije se bistveno razlikujejo. Wilcoxon test ranga je pokazal statistično pomembne razlike v učinku katalepsije 21 dan. $p \leq 0.05$ se šteje za statistično značilne.

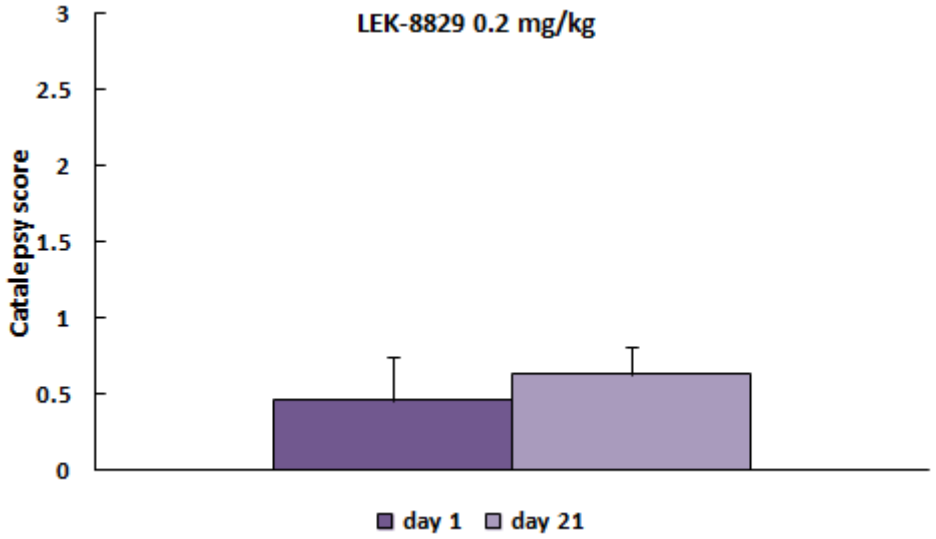

Figure 27: LEK-8829 0.2 mg/kg on the 1st and 21st day of the treatment: catalepsy scores are not significantly different A Wilcoxon Signed Rank Test revealed no statistically significant difference in the catalepsy effect of LEK-8829 0.2mg/kg the 1st and 21st day. $p \leq 0.05$ was considered to be statistically significant.
Slika 27: LEK-8829 0,2 mg/kg na 1. in 21. dan: dosežki katalepsije se ne razlikujejo bistveno. Wilcoxon test ranga ni pokazal statistično pomembne razlike v učinku katalepsije 21 dan. $p \leq 0.05$ se šteje za statistično značilne.

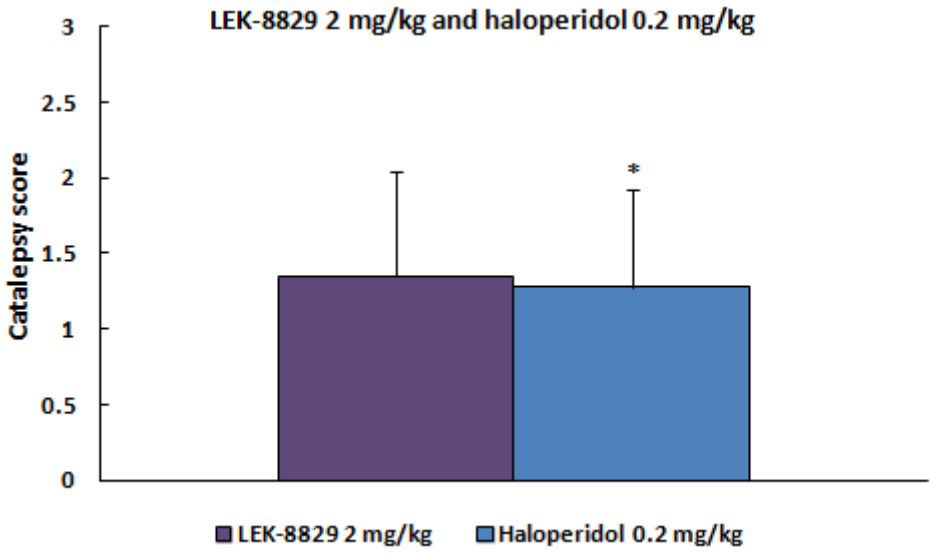

Figure 28: LEK-8829 2 mg/kg and haloperidol 0.2 mg/kg on the 1st day of the treatment: *catalepsy scores are not significantly different A Mann-Whitney U Test revealed no significant difference in the catalepsy effect of LEK-8829 2 mg/kg and haloperidol 0.2 mg/kg on the 1st day of the treatment. $p \leq 0.05$ was considered to be statistically significant.
Slika 28: LEK-8829 2 mg/kg in haloperidol 0,2 mg/kg na 1. in 21. dan: *dosežki katalepsije se ne razlikujejo bistveno. Mann-Whitney test ni pokazal statistično pomembne razlike v učinku katalepsije LEK-8829 2 mg/kg in haloperidol 0,2 mg/kg 1 dan. $p \leq 0.05$ se šteje za statistično značilne.

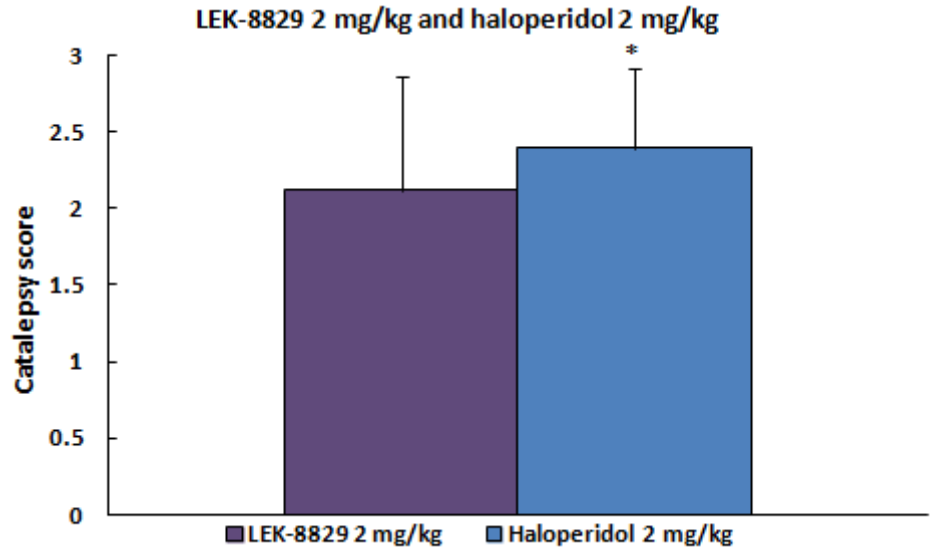

Figure 29: LEK-8829 2 mg/kg and haloperidol 2 mg/kg on the 21st day of the treatment: *catalepsy scores are not significantly different A Mann-Whitney U Test revealed no significant difference in the catalepsy effect of LEK-8829 2 mg/kg and haloperidol 2 mg/kg on the 21st day of the treatment. $p \leq 0.05$ was considered to be statistically significant.
Slika 29: LEK-8829 2 mg/kg in haloperidol 2 mg/kg 21. dan: *dosežki katalepsije se ne razlikujejo bistveno. Mann-Whitney test ni pokazal statistično pomembne razlike v učinku katalepsije LEK-8829 2 mg/kg in haloperidol 2 mg/kg 21. dan. $p \leq 0.05$ se šteje za statistično značilne.

A Wilcoxon Signed Rank Test revealed no statistically significant difference in the catalepsy effect of haloperidol 2mg/kg (Fig.19) and haloperidol 0.2mg/kg on the first and on the twenty first day of the treatment (Fig.20). A Wilcoxon Signed Rank Test revealed a statistically significant increase of catalepsy score on twenty first day of LEK-8829 2mg/kg (Fig.21). A Mann-Whitney U Test revealed no significant difference in the catalepsy effect of LEK-8829 2 mg/kg and haloperidol 0.2 mg/kg on the first day of the treatment (Fig.23). A Mann-Whitney U Test revealed no significant difference in the catalepsy effect of LEK-8829 2 mg/kg and haloperidol 2 mg/kg on the twenty first day of the treatment (Fig.24).

4.1.2 Catalepsy test on 1st and 28th day (experiment 2)

We had 6 animals per group (Table 2). Animals (n= 18) have been treated once daily for 21 days. The catalepsy responses were tested on 1st and 28th day of the experiment. Seven days after the last injection we do the catalepsy test so between day 21 and day 28 the rats no received drug treatment. On 28th day after injection of test compounds I performed the catalepsy test. In this experiment we waited 7 days after last injection because we want to drugs disappear from circulation and then performed the catalepsy test. On this way we are able to see effects of 21 day treatment on dopamine receptors in striatum. It was chosen 7 day drug free because the striatal up-regulation D2 receptors reaching the highest values (Vasconcelos et al., 2003). This time we include saline group because we noticed that rats could be learned the catalepsy in the same conditions and with saline, we wanted to include the basic-line of learning (Fig.33).

We consider score 1 and everything above that as catalepsy. That also shows the catalepsy test with saline (Fig.30).

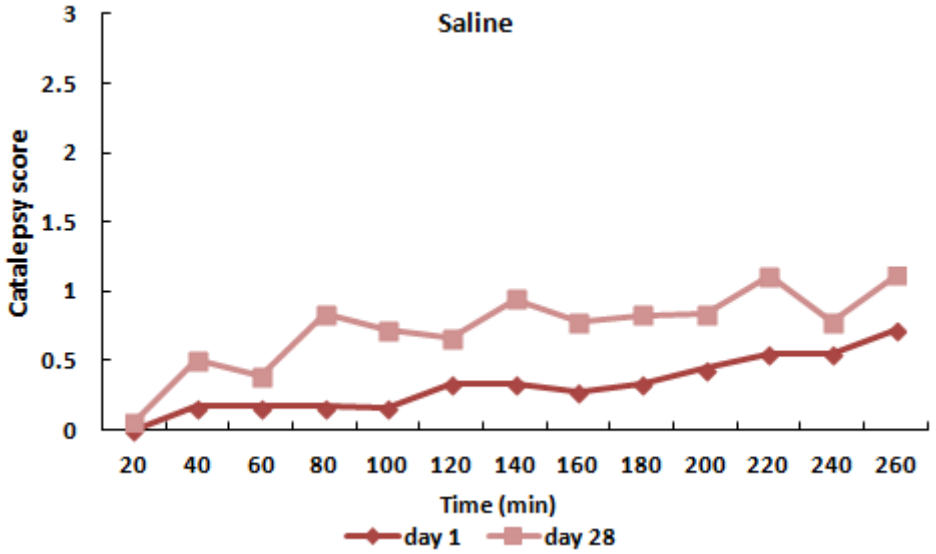

Figure 30: Saline on the 1st, the 28th day. Animals (n=6) got an injection and 20 min after were started measure of catalepsy scores. Score 1 and everything above that we consider like catalepsy.
Slika 30: Fiziološka rastopina 1. in 28. dan. Živali (n=8) so prejele injekcijo, po 20. minutah pa se je začela meriti katalepsija. Dosežek 1 in vse nad tem se obravnava kot katalepsija.

Effect of haloperidol 0.2 mg/kg on the 28th day was a slightly higher than on the 21st day. The onset of the response was evident 100 min after administration on the 1st day and 60 min after administration for the 28th day.

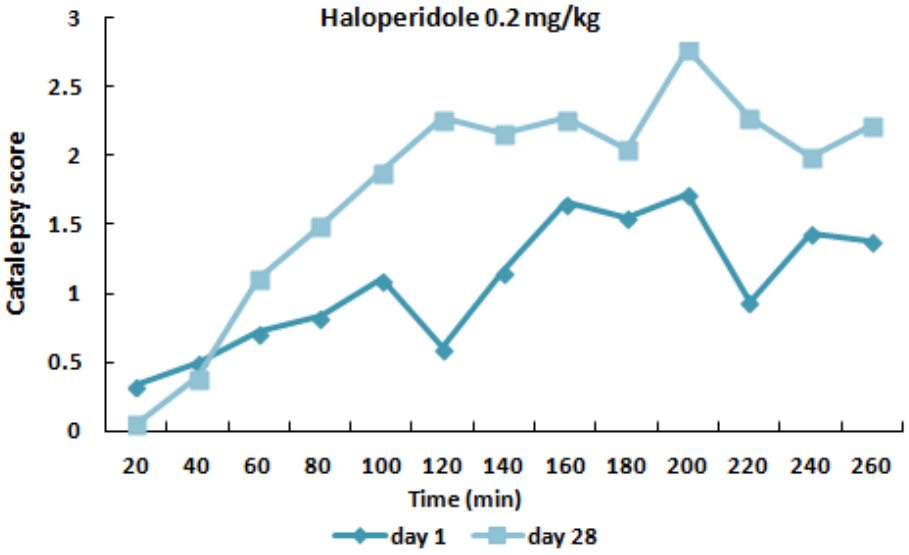

Figure 31: Haloperidol 0.2 mg/kg, on the 1st, the 28th day. Animals (n=6) got an injection and 20 min after were started measure of catalepsy scores. The onset of the response (measured in scores) was evident 100 min after administration on the 1st day and 60 min after administration on the 28th day.
Slika 31: Haloperidol 0,2 mg/kg 1. in 28. dan. Živali (n=8) so prejele injekcijo, po 20. minutah pa se je začela meriti katalepsija. Začetek odgovora (merjeno v dosežkih) je bil razviden po 100. minutah prvega dne ter 60. minutah po injekciji 28. dan.

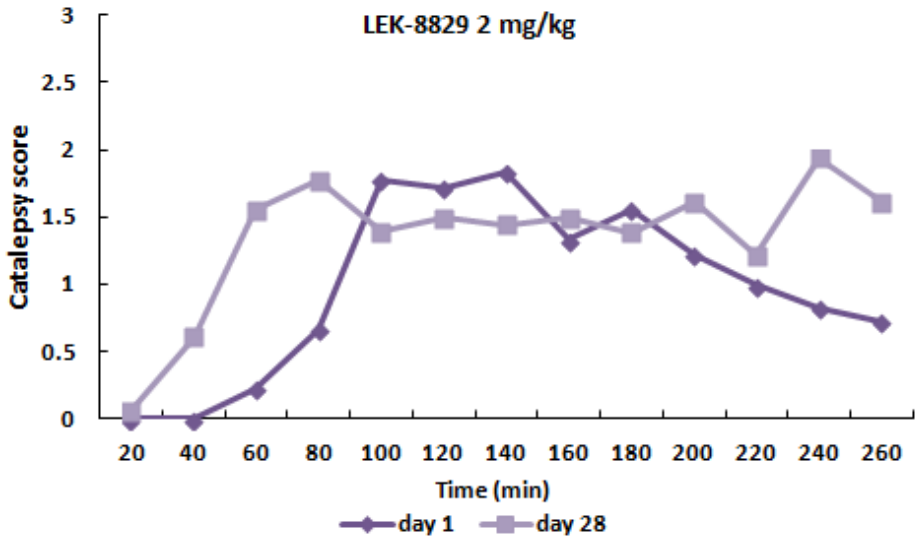

Figure 32: LEK-8829 2 mg/kg, on the 1st, the 28th day. Animals (n=6) got an injection and 20 min after were started measure of catalepsy scores. The onset of the response (measured in scores) was evident 90 min after administration on the 1st day and 50 min after administration on the 28th day.
Slika 32: LEK-8829 2 mg/kg 1. in 28. dan. Živali (n=8) so prejele injekcijo, po 20. minutah pa se je začela meriti katalepsija. Začetek odgovora (merjeno v dosežkih) je bil razviden po 90. minutah prvega dne ter 50. minutah po injekciji 28. dan.

LEK-8829 show slight decrease on the 28th day from 21st day in experiment 1. The onset of the response was evident 90 min after administration on the 1st day and 50 min after administration on the 28th day.

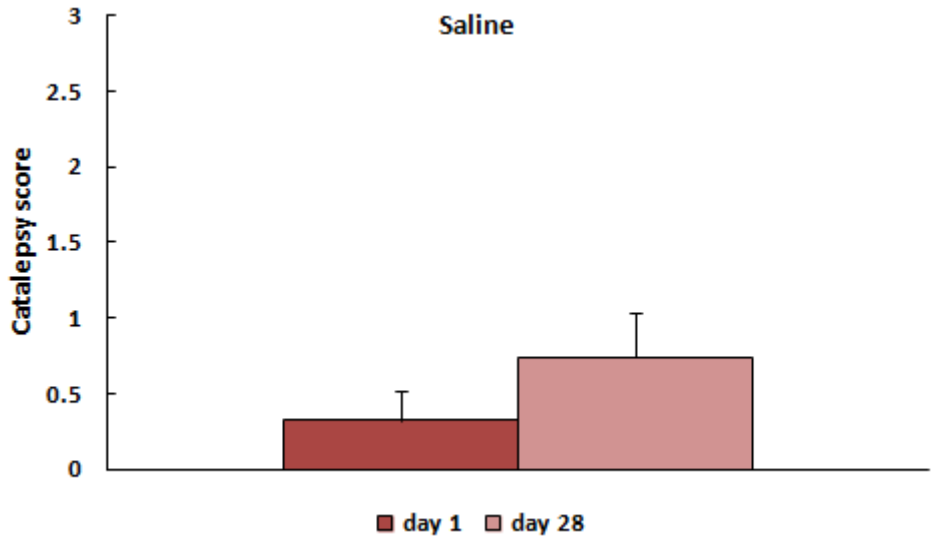

Figure 33: Saline on the 1st, the 28th day: catalepsy scores are not significantly different. A Wilcoxon Signed Rank Test revealed a statistically significant increase of catalepsy score on the 28th day. $p \leq 0.05$ was considered to be statistically significant.
Slika 33: Fiziološka rastopina 1. in 28. dan: dosežki katalepsije se ne razlikujejo bistveno. Wilcoxon test ranga ni pokazal statistično pomembnega povečanja v učinku katalepsije 28. dan. $p \leq 0.05$ se šteje za statistično značilne.

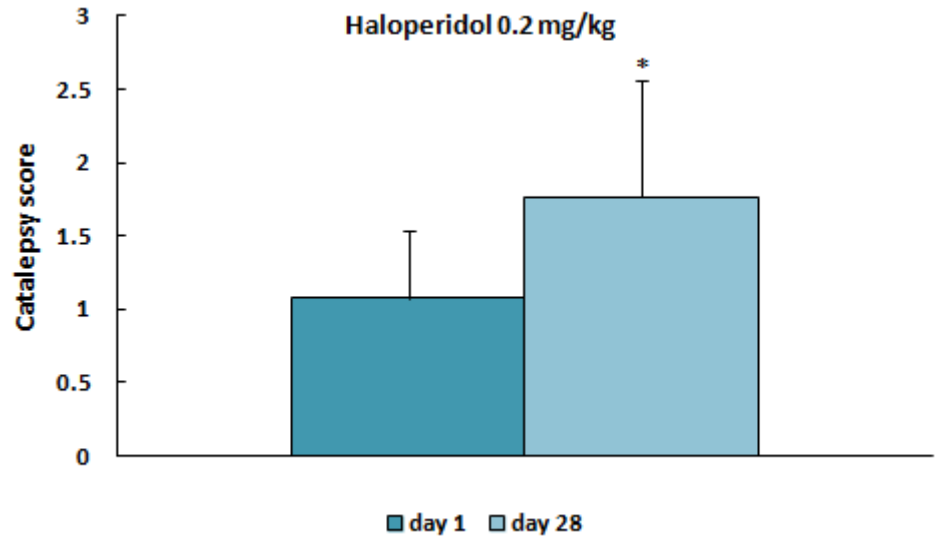

Figure 34: Haloperidol 0.2 mg/kg on the 1st and 28th day of the treatment: *catalepsy scores are significantly different A Wilcoxon Signed Rank Test revealed statistically significant difference in the catalepsy effect of haloperidol 0.2 mg/kg. $p \leq 0.05$ was considered to be statistically significant.
Slika 34: Haloperidol 0,2 mg/kg 1. in 28. dan: *dosežki katalepsije se bistveno razlikujejo. Wilcoxon test ranga je pokazal statistično pomembne razlike v učinku katalepsije za haloperidol 0,2 mg/kg. $p \leq 0.05$ se šteje za statistično značilne.

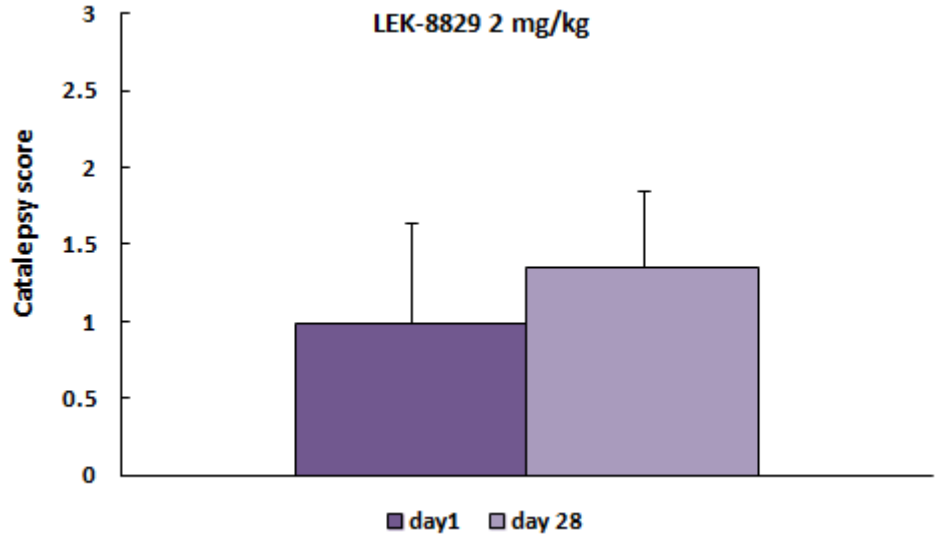

Figure 35: LEK-8829 2 mg/kg on the 1st and 28th day of the treatment: catalepsy scores are not significantly different A Wilcoxon Signed Rank Test revealed no statistically significant difference of catalepsy effect on 28th day. $p \leq 0.05$ was considered to be statistically significant.
Slika 35: LEK-8829 2 mg/kg 1. in 28. dan: dosežki katalepsije se ne razlikujejo bistveno. Wilcoxon test ranga ni pokazal statistično pomembne razlike v učinku katalepsije 28. dan. $p \leq 0.05$ se šteje za statistično značilen.

A Wilcoxon Signed Rank Test revealed no statistically significant difference in the catalepsy effect of LEK-8829 2mg/kg on the first and on the twenty eight day of the treatment. A Wilcoxon Signed Rank Test revealed a statistically significant increase of catalepsy score on 28th day of haloperidol 0.2 mg/kg and saline.

4.2 ANALYSIS OF AMPHETAMINE-INDUCED LOCOMOTOR ACTIVITY

4.2.1 Catalepsy analysis (1 test)

Six groups of eight animals were used. The animals (n= 48) were treated once daily for 21 days (Table 3). Three tests were performed before and after the 21-day treatment. The catalepsy test was 1st test (Table 4). It was noticed that rats could be learned the catalepsy in the same conditions and with saline it was confirmed (Fig.30). We wanted to confirmed that and on the 1st group (saline group) the catalepsy test was performed using the method described by Krisch (Krisch et al., 1994). Results were the same as for the saline group on 1st and 28th day of the treatment (Fig.30). On the 4th group (saline group) (Table 3) catalepsy test was performed 4 h and 8 h after the saline injection. Catalepsy scores were 0. The animals were not cataleptic.

Previous catalepsy tests (Fig.20, 21, 22, 30, 31, 32) showed catalepsy for all doses of test compounds. Based on that and on observation that rats could learn the catalepsy in the

same conditions it was decided to perform a catalepsy test 2 h after of the injection of the test compounds.

Before the 21-day treatment, the catalepsy scores for all other groups were around score 1, except for the group which received haloperidol 2 mg/kg, which had a slightly higher catalepsy score. After the 21-day treatment catalepsy scores for all other groups were around score 1, except for the group which received haloperidol 2 mg/kg and had a slightly higher catalepsy scores, even slightly higher on the 1st test before 21 day treatment.

4.2.2 Amphetamine-induced locomotion (2 test)

Six groups of eight animals were used. The animals (n= 48) were treated once daily for 21 days (Table 3). Three tests were performed before and after the 21-day treatment. Amphetamine-induced locomotion was 2st (Table 5).

Before the 21-day treatment locomotion (distance moved) induced by amphetamine was the same for all groups. One-way ANOVA followed by Scheffe's multiple-comparison test shows that groups are not significantly different (have the same efficiency).

After the 21-day treatment locomotion (distance moved) induced by amphetamine was the same for all groups. One-way ANOVA followed by Scheffe's multiple-comparison test shows that groups are not significantly different (have the same efficiency).

A Two-tailed paired Student's *t* test was conducted to evaluate the impact of the 21-day treatment on amphetamine-induced locomotor activity. Saline, LEK-8829 0.2 mg/kg and haloperidol 0.2mg/kg were not significantly different in the distance moved before and after the treatment. LEK-8829 2 mg/kg and haloperidol 2 mg/kg were significantly different in the distance moved before and after the treatment.

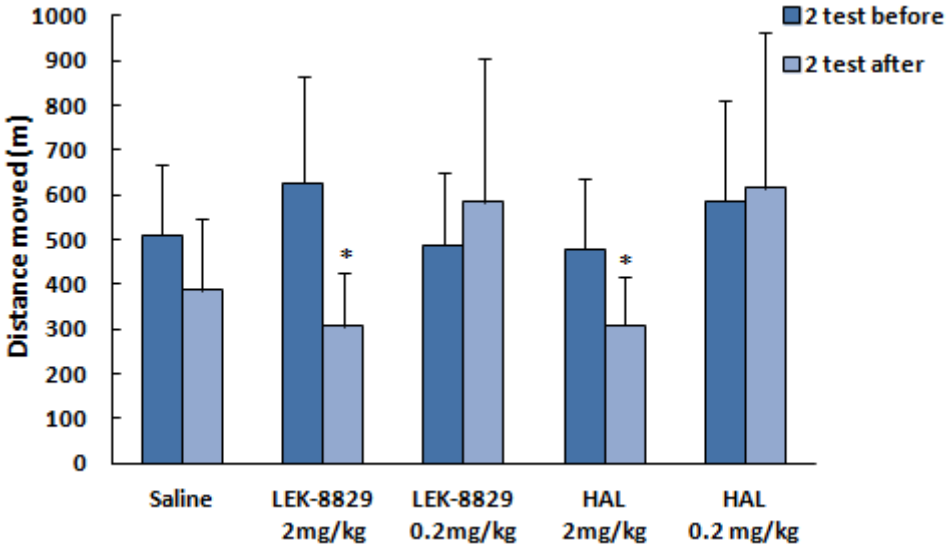

Fig 36: Amphetamine-induced locomotor activity with test compounds before and after the treatment.
* Significant difference as compared 2 test before 21 day treatment *vs.* 2 test after the 21 day treatment. (Two-tailed paired Student's *t*-test, $p \leq 0.05$ was considered to be statistically significant).
Slika 36: Z amfetaminom izzvana lokomotorna aktivnost z testnimi spojinami pred in po zdravljenju.
* Značilna razlika v primerjavi drugega testa prej in po 21-dnevnem zdravljenju. (parni Student *t-test* $p \leq 0.05$ se šteje za statistično značilen).

4.2.3 Inhibition of the amphetamine-induced locomotion with test compounds (experiment 3)

Six groups of eight animals were used. The animals (n= 48) were treated once daily for 21 days (Table 3). Three tests were performed before and after the 21-day treatment. Inhibition of amphetamine - induced locomotion with test compounds was 3rd test (Table 6).

One-way ANOVA followed by Scheffe's multiple-comparison test showed that before treatment saline and LEK 0.2 mg/kg were not significantly different (had the same efficiency) LEK-8829 2 mg/kg, haloperidol 2 mg/kg and haloperidol 0.2 mg/kg were not significantly different.

One-way ANOVA followed by Scheffe's multiple-comparison test showed that after the treatment saline and LEK 0.2 mg/kg were not significantly different (had the same efficiency). LEK-8829 2 mg/kg, haloperidol 2 mg/kg and haloperidol 0.2 mg/kg were not significantly different.

A Two-tailed paired Student's *t* test was conducted to evaluate the impact of the 21-day treatment on amphetamine-induced locomotor activity with haloperidol 2mg/kg and 0.2 mg/kg. There was a statistically significant increase in the distance moved from the first to the twenty-first day. This indicates that haloperidol 2 mg/kg and 0.2 mg/kg produces

behavioral supersensitivity which is in accordance with clinical observations and animal studies which show that a withdrawal of antipsychotic treatment reveals an increased psychomotor response to amphetamine (Samaha et al., 2007).

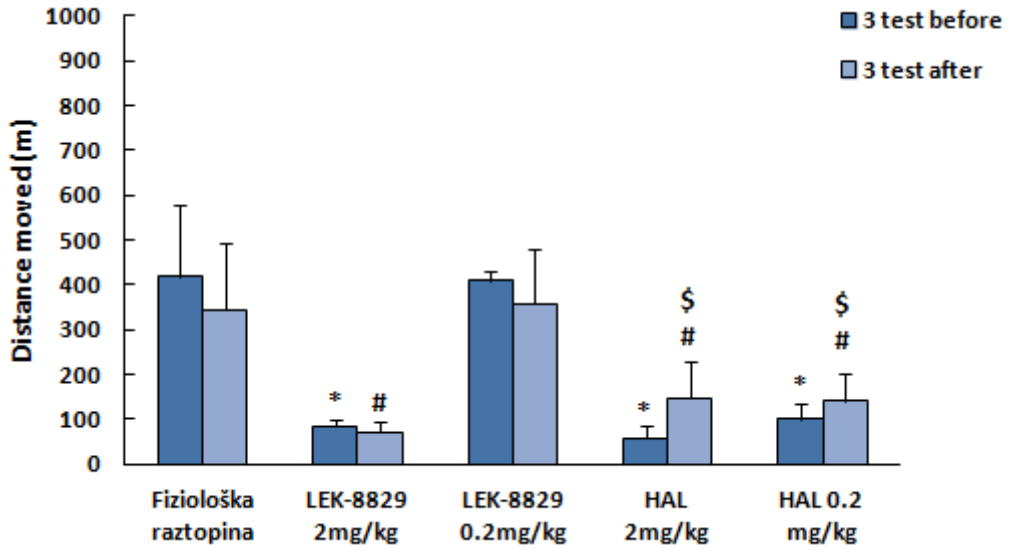

Figure 37: Inhibition of the amphetamine-induced locomotion with test compounds. * Significant difference as compared with saline group 3 test before the 21 day treatment period with the respective dose of the drug. # Significant difference as compared with saline group 3 test after the 21 day treatment period with the respective dose of drugs (One way ANOVA with Scheffe's *post hoc* analysis, $n = 8$, $p \leq 0.05$). **$** Significant difference as compared 3 test before the 21 day treatment *vs.* 3 test after the 21 day treatment. (Two-tailed paired Student's *t*,test, $p \leq 0.05$ was considered to be statistically significant).
Slika 37: Zaviranje z amfetaminom inducirane lokomotorne aktivnosti s testnimi spojinami. * Značilna razlika v primerjavi s fiziološko skupino tretjeg testa pred in po 21-dnevnom zdravljenju z ustreznim odmerkom zdravila. # Značilna razlika v primerjavi s fiziološko skupino tretjeg testa pred in po 21 dnevnom zdravljenju z ustreznim odmerkom zdravila (enosmerna ANOVA s Scheffe's test $n = 8$, $p \leq 0.05$). $ Značilna razlika v primerjavi tretjeg testa pred in po 21 dnevnim zdravljenjem. (parni Student t-test $p \leq 0.05$ se šteje za statistično pomembne).

A two-tailed paired Student's *t* test was used to verify the statistical significance before and after the treatment. Saline, LEK-8829 2 mg/kg and LEK-8829 0.2 mg/kg were not significantly different in the distance moved from the first to the twenty-first day i.e. LEK-8829 2 mg/kg and 0.2 mg/kg did not produce significant differences in the inhibition of the amphetamine-induced locomotion with the test compounds before and after the twenty-one-day treatment. LEK-8829 in both doses did not produce behavioral supersensitivity (increased behavioral responsiveness) after the treatment i.e. a decreased psychomotor response to amphetamine.

4.3 ANALYSIS OF RESERPINE MODEL

Five groups of animals were used. The animals (n= 36) were treated three times, once in three days with reserpine and twice a day for 9 days with test compounds (Table 7).

One-way ANOVA followed by Scheffe's multiple-comparison test was conducted to evaluate the impact of prolonged daily treatment with saline and test compounds from 1st to 13th day. There was a statistically significant increase in the distance moved with prolonged daily treatment with LEK-8829 2mg/kg.

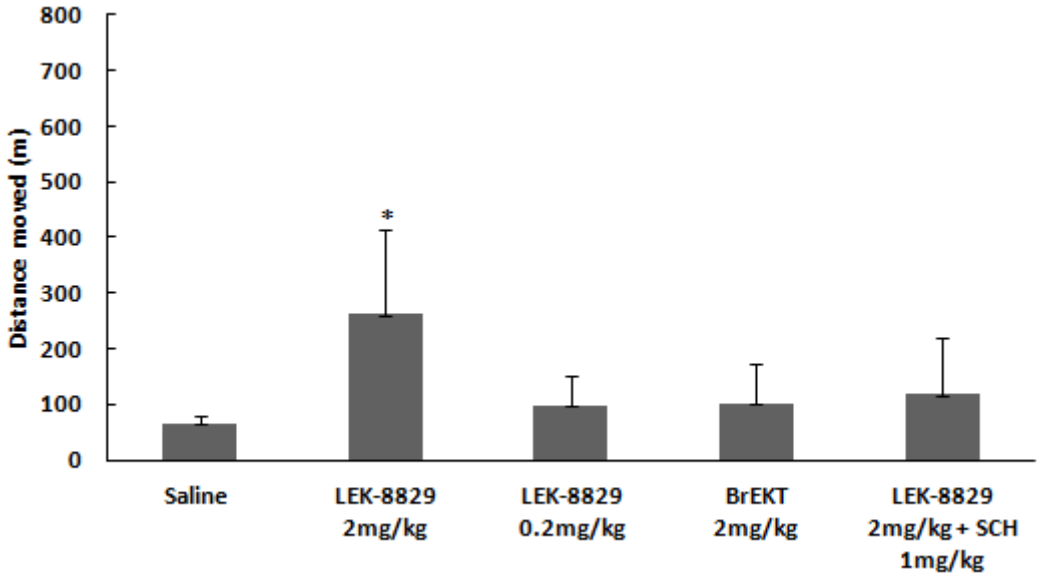

Figure 38: Comparison of prolonged daily treatment with saline and test compounds from 1st to 13th day. * Significant difference as compared with saline group. One way ANOVA with Scheffe's *post hoc* analysis, n = 6, saline n = 12, $p \leq 0.05$ was considered to be statistically significant.
Slika 38: Primerjava podaljšanega dnevnega zdravljenja s fiziološko raztopino in testnima spojinama. 0d 1. do 13. dne. * Značilna razlika v primerjavi s fiziološko skupino. Enosmerna ANOVA s Scheffe-jevim testom n = 6, fiziološka n = 12, $p \leq 0.05$ se šteje za statistično pomembne.

First group of twelve animals (n= 12) was treated three times, every three days, with reserpine 10 mg/kg (1st, 4th and 7th day of the experiment) and every day twice a day (at 8 am and at 8 pm) with saline. After the last reserpine injection the animals were treated with saline for two more days (8th and 9th day of experiment). For nine days I was recording the animals for 120 min after the morning injection. Next two days animals were without saline (10th and 11th day of the experiment). I performed the catalepsy test the day after (12th day of the experiment). On the 13th day of the experiment, I divided the first group into four groups of three animals (n= 3). Three animals got saline, three animals got LEK-8829 2mg/kg, three animals got bromoergocriptine 2 mg/kg and three animals got LEK-8829 2 mg/kg + SCH 23390 1 mg/kg (Table 7).

Catalepsy tests demonstrated that all animals were cataleptic which is in accordance with dopamine depletion. That means that reserpinization was successful.

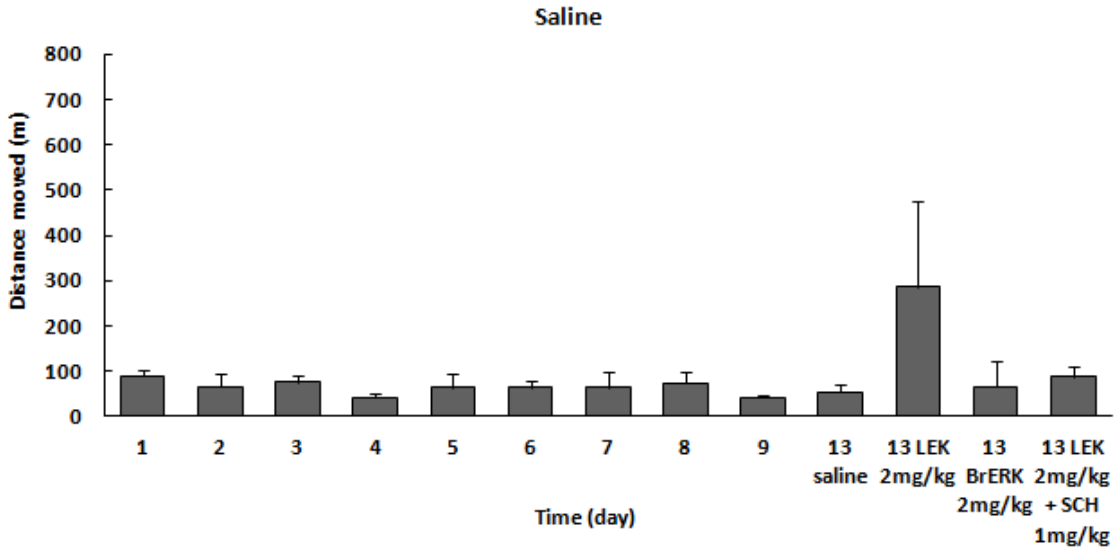

Figure 39: Daily treatment with saline demonstrated a minimal locomotor activity of reserpinized rats. Acute test doses on 13th day with saline (n = 3) demonstrated the same locomotor activity like before. Increase of locomotor activity with LEK 2mg/kg (n = 3) was observed on acute test dose on 13th day. Acute test doses on 13th day with BrEKT 2 mg/kg (n = 3) and LEK-8829 2mg/kg + SCH 1mg/kg (n = 3) demonstrated the same locomotor activity like saline on the 13th day.
Slika 39: Dnevno zdravljenje s fiziološko raztopino kaže, minimalno lokomotorno aktivnost rezerpiniranih podgan. Akutni test 13. dne s fiziološko raztopino (n = 3) pokaže enako lokomotorno aktivnost kot prej. Povečanje lokomotorne aktivnosti z LEK 2mg/kg (n = 3) je opaženo 13. dan z akutnim odmerkom. Akutni test odmerek 13. dan z BrEKT 2 mg/kg (n = 3) in LEK-8829 2mg/kg + SCH 1mg/kg (n = 3) pokaže enako lokomotorno aktivnost kot fiziološko raztopina na 13. dan.

The control group showed a minimal locomotor activity during all nine days of recording in accordance with the reserpine treatment. On 13th day three animals got an injection of saline, three animals got an injection of LEK-8829 2 mg/kg, three animals got an injection of bromokriptine 2 mg/kg and three animals got an injection of LEK 2 mg/kg + SCH 1 mg/kg respectively. After those injections only LEK-8829 2 mg/kg produced approximately a three-times increase of locomotor activity. Other test compounds exhibited almost the same locomotor activity as acute injection of saline, BrEKT 2 mg/kg and LEK-8829 2 mg/kg+SCH 1 mg/kg.

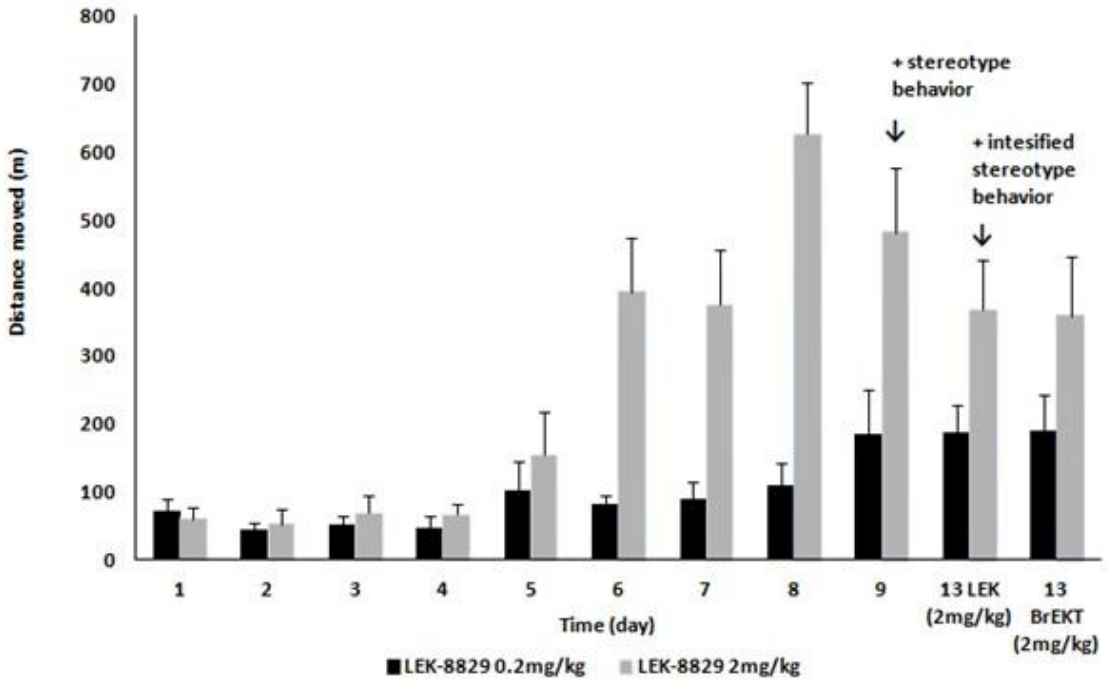

Figure 40: Comparison of daily treatment with LEK-8829 2mg/kg and 0.2mg/kg and acute test doses on 13th day. Increase of locomotor activity with LEK 2mg/kg was observed on 5th day, and day by day it was more intensive till 9th day when it was observed stereotype behavior. Locomotor activity was decrease on account of stereotype behavior. On 13th day (n = 3) with acute test dose of LEK 2mg/kg was observed more decrease locomotor activity on account of more intensified stereotype behavior. On 13th day (n = 3) with acute test dose of BrEKT 2mg/kg was observed the same rate of locomotor activity without stereotype behavior. Slightly increase of locomotor activity with LEK 0.2mg/kg was observed on 5th day, with substantial increase on 9th day. Stereotyped behavior was not observed. On 13th day with acute test dose of LEK 2mg/kg and BrEKT 2mg/kg was observed the same rate of locomotor activity.
Slika 40: Primerjava dnevnega zdravljenja z LEK-8829 2mg/kg and 0.2mg/kg in akutni test odmerjen 13. dan. Povečanje lokomotorne aktivnosti z LEK-8829 2 mg/kg je opažen 5. dan in je iz dneva v dan bolj intenziven do 9. dneva, ko je opaženo tudi stereotipno vedenje. Lokomotorna aktivnost se zmanjšuje na račun stereotipnega vedenja. Akutni test odmerka LEK 2mg/kg na 13. dan (n = 3) kaže zmanjšanje lokomotorne aktivnosti na račun stereotipnega vedenja. Akutni test odmerka LEK 2mg/kg na 13. dan (n = 3) kaže enako mero lokomotorne aktivnosti brez stereotipnega vedenja. Rahlo povečanje lokomotorne aktivnosti z LEK 0,2 mg/kg je opaženo 5. dne z znatnim povečjanjem 9. dne. Akutni test odmerka 13. dan z LEK 2mg/kg in BrEKT 2mg/kg kaže enako stopnjo lokomotorne aktivnosti.

The group treated with LEK 2mg/kg for 9 days showed strongly alleviated akinetic state but from the fifth day onwards, hyperlocomotion and stereotype behavior were perceived. On the fifth day, they exhibited an increase in locomotor activity and it lasted to the ninth day. The biggest locomotor activity was on the eight day when stereotype behavior was observed. On 13th day three animals got an injection of LEK 8829 2mg/kg, and three animals got injection of bromokriptine 2mg/kg. After those injections the animals exhibited the same locomotor activity but with different behavioral pattern. LEK-8829 produced an increase in locomotor activity and stereotypic behavior. Bromocriptine did not induce stereotypic behavior, only a modest locomotor activity. On the last day of the treatment (9th day of experiment) 6 animals out of 12 animals died. In accordance with

hyperlocomotion and stereotype behavior (by sniffing to the self-biting and self-mutilation) in half of the dead animals we concluded that 2mg/kg was an extremely high dose and may be toxic but should it kept in mind that our animals were in the group which was extremely sensitive to chronic reserpinization (Rosecrans., 1967)

The group treated for with LEK-8829 0.2mg/kg 9 days also exhibited an increase in locomotor activity but on 9th day the stereotypic behavior was missing. Effects were more moderate than in the case of a high dose and perhaps the dose was in the question. On 13th day three animals got an injection of LEK 8829 2mg/kg, and three animals got an injection of bromokriptine 2mg/kg. After those injections the animals exhibited the same locomotor activity but with different behavioral patterns.

Independent *t-test* was used to verify the statistical significance between LEK-8829 2 mg/kg and LEK-8829 0.2mg/kg. LEK-8829 2 mg/kg and LEK-8829 0.2mg/kg were significantly different.

The group treated for 9 day with Bromokriptine had a similar profile as LEK-8829 0.2mg/kg. On 13th day three animals got an injection of LEK-8829 2mg/kg, and three animals got injections of bromokriptine 2mg/kg. After those injections LEK-8829 2mg/kg produced a slight increase in locomotor activity and bromokrpitine markedly reduced locomotion.

The group treated for 9 day with LEK-8829 2mg/kg + SCH 1mg/kg showed the same profile as LEK-8829 2 mg/kg but with a decrease in values from LEK-8829 2 mg/kg (Fig.33) because 20 min after the injection of LEK-8829 2mg/kg, injection of SCH-23390 1 mg/kg prevented action of LEK-8829 2 mg /kg.

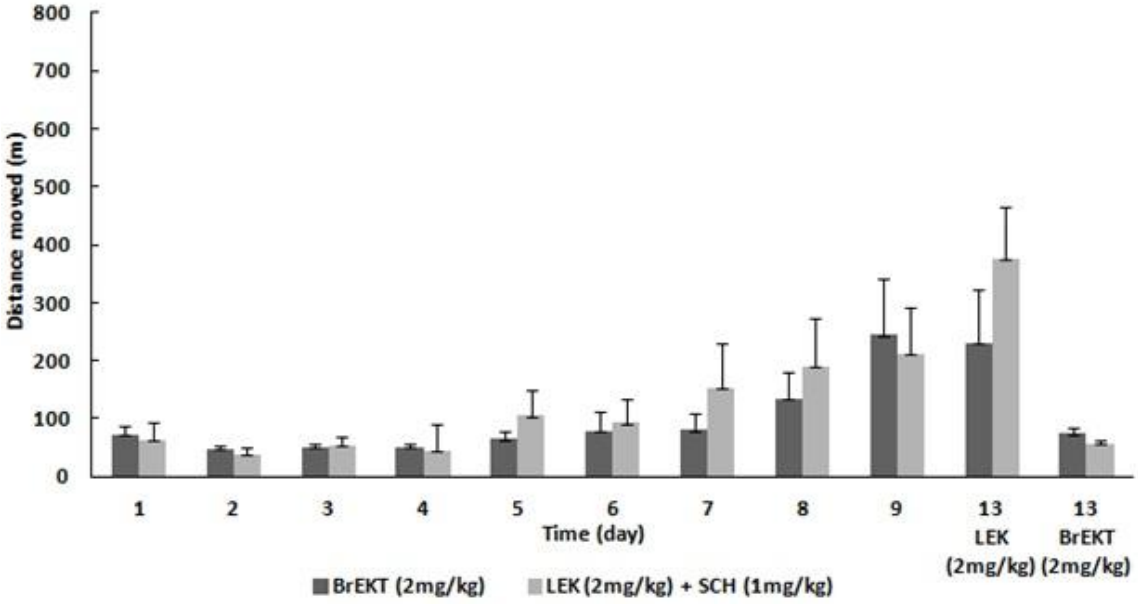

Figure 41: Comparison of daily treatment with bromoergocriptine (BrEKT) 2mg/kg and LEK -8829 2mg/kg + SCH 1mg/kg and acute test doses on 13th day. Increase of locomotor activity with BrEKT 2mg/kg was observed from 8th day. On 13th day (n = 3) with acute test dose of LEK 2mg/kg was observed the same rate of locomotor activity. On 13th day (n = 3) with acute test dose of BrEKT 2mg/kg was observed decrease of locomotor activity. Increase of locomotor activity with LEK 2mg/kg + SCH 1mg/kg was observed on 5th day, and day by day it was more intensive till 9th day. This increase was in account of first 20min locomotor activity with LEK-8829 2mg/kg. On 13th day (n = 3) with acute test dose of LEK-8829 2mg/kg was observed increase of locomotor activity. On 13th day (n = 3) with acute test dose of BrEKT 2mg/kg was observed decrease of locomotor activity.
Slika 41: Primerjava dnevnega zdravljenja z bromoergocriptine (BrEKT) 2 mg/kg in LEK -8829 2 mg/kg + SCH 1 mg/kg ter akutni test odmerka 13. dan. Povečjanje lokomotorne aktivnosti z BrEKT 2 mg/kg je opaženo od 8. dne naprej. Akutni test odmerka LEK-8829 2 mg/kg (n = 3) 13. dan, kaže isto stopnjo lokomotorne aktivnosti. Akutni test odmerka BrEKT 2mg/kg (n = 3) 13. dan, kaže zmanjšanje lokomotorne aktivnosti. Povečjanje lokomotorne aktivnosti z LEK-8829 2mg/kg + SCH 1mg/kg je opaženo 5. dne in je z vsakim dnem bolj intezivno do 9. dne. To povečjanje se pokaže na račun prvih 20 min lokomotorne aktivnosti z LEK-8829 2 mg/kg. Akutni test odmerki z LEK-8829 2 mg/kg 13. dan (n = 3) kaže povečjanje lokomotorne aktivnosti. Akutni test odmerki z BrEKT 2 mg/kg 13. dan (n = 3) kaže zmanjšanje lokomotorne aktivnosti.

Dopamine depletion leads to breakdown of D1/D2 synergism and increase of D2 receptors (LaHoste and Marshall, 1992). The increase in D2 receptors is both necessary and sufficient for the production of the enhanced behavioral supersensitivity to dopamine agonists (Hess et al., 1986).

It is very interesting that LEK-8829 D1 receptor agonist and D2 receptor antagonist demonstrate a maximum locomotor response (Fig.37). Prolonged 9-day treatment of LEK-8829 2 mg/kg and 0.2 mg/kg demonstrate an increase of locomotor activity in comparison with prolonged 9-day treatment with BrEKT 2 mg/kg. Acute test doses LEK-8829 2 mg/kg (D1 agonist and D2 antagonist) and BrEKT 2 mg/kg (D2 agonist) on the 13th day demonstrated higher supersensitivity with LEK-8829 2 mg/kg than BrEKT 2 mg/kg.

4.4 PROTEIN ANALYSIS OF D2 RECEPTORS

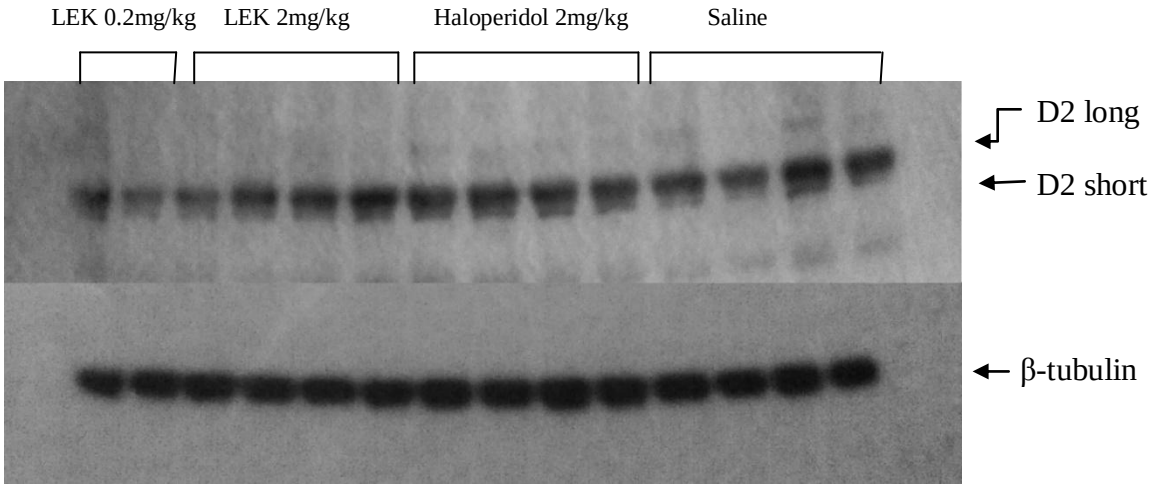

Figure 42: D2 receptor isoforms in striatum: D2 long 52kDa, D2 short 47kDa Housekeeping protein β-tubulin on 50 kDa.
Slika 42: D2 receptor izooblike: D2 dolga 52kDa, D2 kratka 47kDa. Protein β-tubulin na 50 kDa.

We are able to see D2 long and D2 short isoforms. No up-regulation can be detected due to different subchronic treatment. The first four samples is saline and the next four are treated with haloperidol 2 mg/kg, followed by four treated with LEK 2 mg/kg and the last two are treated with LEK 0.2mg/kg. We expect to see up-regulation in samples treated with haloperidol 2 mg/kg and lower lever of up-regulation in samples treated with LEK 2mg/kg and 0.2 mg/kg.

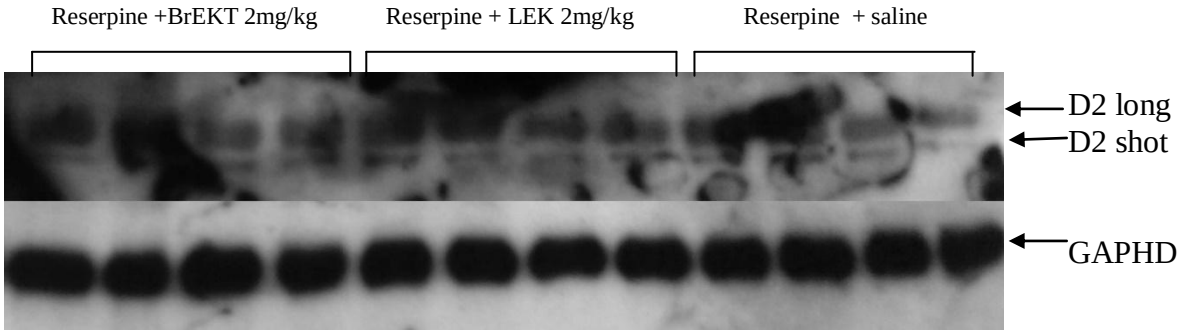

Figure 43: D2 receptor isoforms: D2 long and D2 short more separate. Housekeeping GAPHD on 37 kDa.
Slika 43: D2 receptorske izooblike: D2 dolga in D2 kratka izooblika bolj ločene. Protein GAPHD na 37 kDa.

Optimizing the western blot method we succeeded to separate D2 long and D2 short isoforms. No up-regulation can be detected due to different prolonged daily treatments. Samples are from the reserpine model. First four samples is reserpine treated 9 day with saline, the next four is reserpine treated 9 days with LEK 2 mg/kg and next four reserpine is treated 9 day with BrEKT 2 mg/kg. We expect to see up-regulation in all samples and

according to up-regulation of first for samples (reserpine + saline) to determine level of up-regulation of other samples.

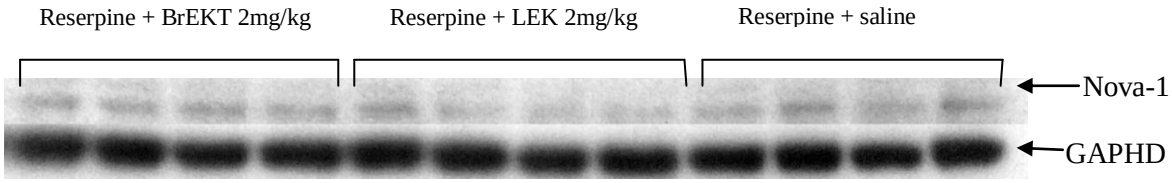

Figure 44: Nova-1 protein on 50kDa. Housekeeping GAPHD on 37 kDa.
Slika 44: Protein Nova-1 na 50kDa. Protein GAPDH na 37 kDa.

No up-regulation can be detected due to different prolonged daily treatments in the reserpine model. We expect if D2 long isoform of D2 receptor is up-regulated more than D2 short isoform also Nova 1 will be up-regulated. Samples are from the reserpine model. First four samples is reserpine treated 9 day with saline, the next four is reserpine treated 9 days with LEK 2 mg/kg and next four reserpine is treated 9 day with BrEKT 2 mg/kg. We expect no up-regulation Nova 1 in all samples because probably up-regulation of D2 receptor is on account of equal up-regulation of both isoforms.

It was extraordinary that we could not detect up-regulation of D2 receptor and/or D2 receptor isoforms; we suspected that primary mouse antibodies (D2 receptor and Nova 1) cross-reacted with the rats' IgG which are on 50 kDa. Around 50 kDa we have D2 receptor isoforms and Nova 1 on 50 kDa. To prove our suspicions we exposed only secondary antibodies (anti-mouse) without primary antibodies. We got bands and we confirmed cross-reaction between primary mouse antibodies and IgG in rat samples.

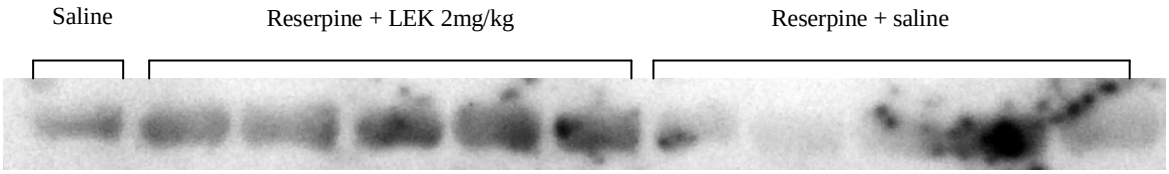

Figure 45: Membrane incubate only with secondary antibodies.
Slika 45: Membrana inkubirana le s sekundarnimi protitelesi.

4.5 mRNA ANAYSIS OF D2 RECEPTORS

The purpose of in situ hybridization was to detect changes in mRNA expression of D2 receptor, D2 long, D2 short isoforms and Nova 1 in striatum due to subchronic treatment with test compounds. Alternative D2 receptor pre-mRNA splicing is controlled by splicing regulator Nova 1.

The slices from experiment 3 (p.52) were analyzed here. The analysis comprised four animals from each group (saline, haloperidol 2 mg/kg, LEK-8829 2 mg/kg and haloperidol 0.2 mg/kg) and two slices per animal. One-way ANOVA followed by Scheffe's multiple-comparison test showed that the treated groups were not significantly different. The obvious tendency was noticed that prolonged daily treatment caused a higher up-regulation of mRNA D2 receptors in the group treated with haloperidol 2 mg/kg than in the group treated with LEK-8829 2 mg/kg.

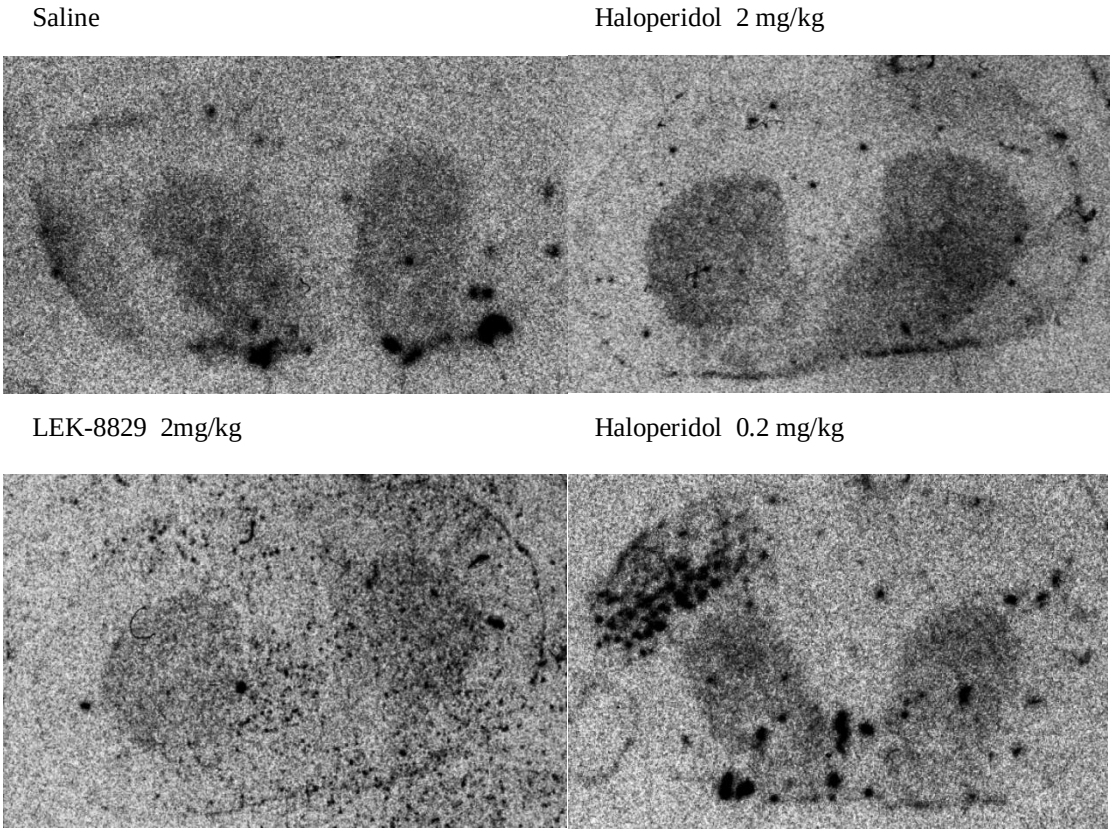

Figure 46: Black and white view of mRNA expression D2 receptors in the striatum.
Slika 46: Črno beli prikaz izražanja mRNA D2 receptorjev v področju striatuma.

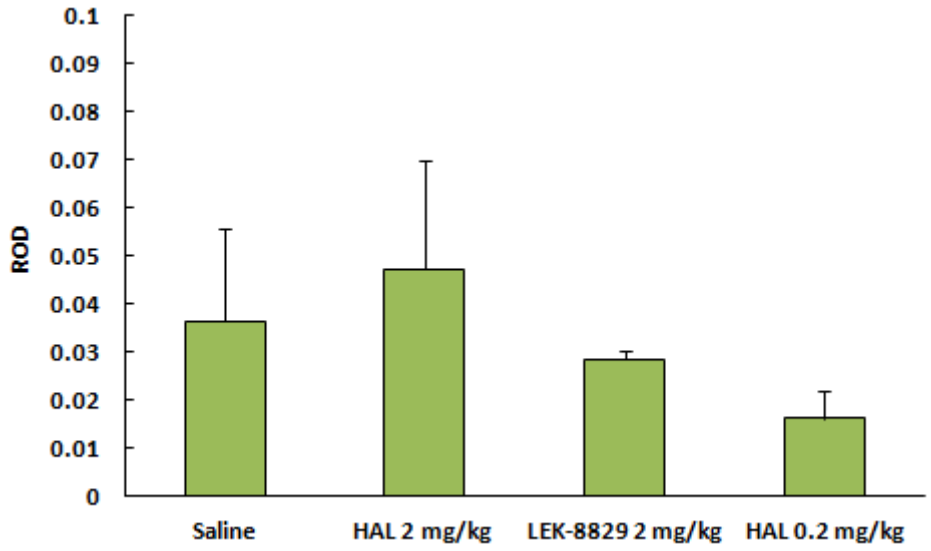

Figure 47: Expression of mRNA D2 receptors after prolonged daily treatment with test compounds. Groups are not significantly different. One way ANOVA with Scheffe's *post hoc* analysis, n = 4, $p \leq 0.05$ was considered to be statistically significant. The tendency is noticed that prolonged daily treatment causes a higher up-regulation of mRNA D2 receptors in the group treated with haloperidol 2 mg/kg than in the group treated with LEK-8829 2 mg/kg.

Slika 47: Izražanje D2 receptor mRNK po podaljšanem zdravljenju z testnimi snovmi. Enosmerna ANOVA s Scheffejevim testom (n = 4, $p \leq 0.05$) ni pokazala statističnih razlik med skupinami. Opaža se trend, da podaljšano zdravljenje provzroča večjo up-regulacijo mRNK D2 receptorjev, skupine tretirane z haloperidolom 2mg/kg, kot skupine tretirane z LEK-8829 2 mg/kg.

Here we analyzed slices from the 6-OHDA model. The 6-OHDA model has D2 receptor up-regulation and this was a preliminary analysis for verification D2 receptor and its isoforms. It included four animals for D2R, D2L and D2S; and two slices per animal. One-way ANOVA followed by Scheffe's multiple-comparison test showed that lesions and control D2R are significantly different and that lesion and control D2S are significantly different. One-way ANOVA followed by Scheffe's multiple-comparison test showed that lesion D2S and lesion D2L are significantly different.

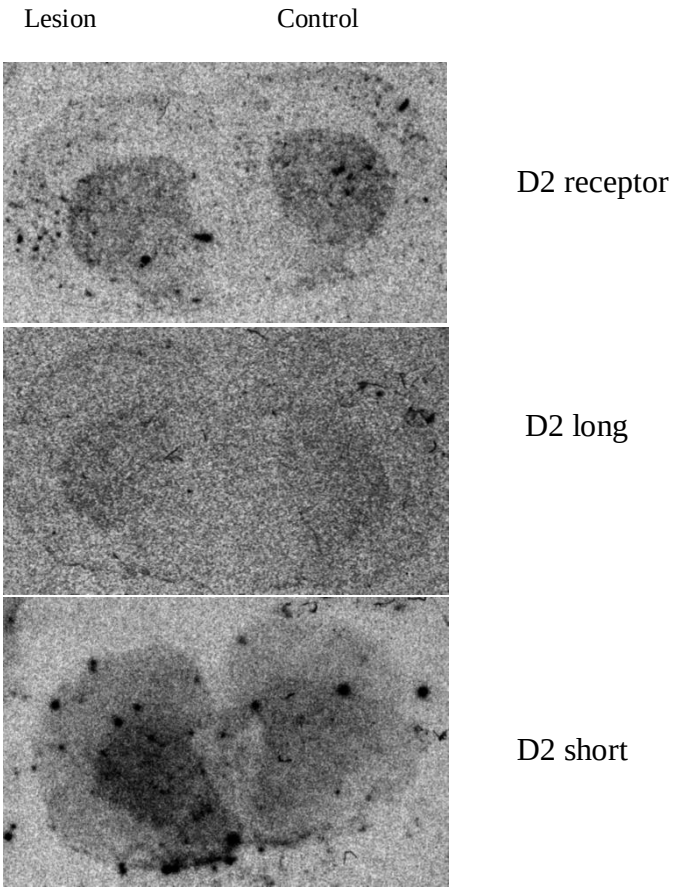

Figure 48: Black and white view of mRNA expression D2 receptors, D2L and D2S in striatum.
Slika 48: Črno beli prikaz izražanja mRNA D2 receptorjev, D2L in D2S v področju striatuma.

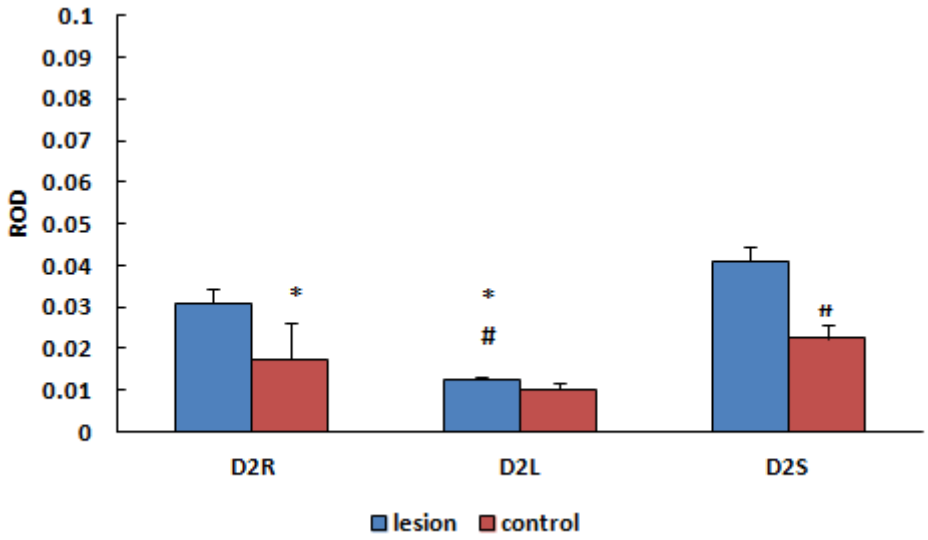

Figure 49: Expression of mRNA D2R, mRNA D2L and mRNA D2S isoforms. * Significantly different compare to lesion D2R. # Significantly different compare to lesion D2S. One way ANOVA with Scheffe's *post hoc* analysis, n = 4, $p \leq 0.05$ was considered to be statistically significant.

Slika 49: Izražanje mRNK D2R, mRNK D2L in mRNK D2S izooblik. * Značilna razlika v primerjavi s lezijo D2R. # Značilna razlika v primerjavi s lezijo D2S. Enosmerna ANOVA s Scheffejevim testom (n = 4, $p \leq 0.05$).

5 DISCUSSION

The dopamine D2 receptor system has been implicated in the modulation of motor activity, learning and memory, as well as in the pathophysiology of neuropsychiatric disorders such as Parkinson's disease (PD) and schizophrenia (Hranilovic et al., 2008).

It is already known that different periods of antipsychotics treatment and dopamine depletion with reserpine cause up-regulation dopamine D2 receptors. Also, reserpine caused a breakdown in D1/D2 synergism (Tarazi et al., 1997; LaHoste and Marshall, 1992).

Dopamine receptor supersensitivity (DARSS) is often invoked in long-term treatment with antipsychotics and the problems arising with long-term L-DOPA therapy are the dyskinesias presumably resulting from priming or supersensitization of dopamine D2 receptors. Dopamine receptors, experimentally, are prone to become supersensitive and to thus elicit abnormal behaviors when coupled with dopamine or a receptor agonist (Kostrzewa et al., 2011; Ushijima et al., 1995).

It has been already described in the literature that D2 receptor up-regulation (and development of behavioral supersensitivity) prevent by simultaneously stimulation of D1 receptors (Braun et al., 1997). A similar effect of D1 agonist treatment on behavioral supersensitivity and D2 receptor up-regulation induced by subchronic (21 day) haloperidol exposure has been reported (Marin et al., 1993).

The purpose of our experiments was to analyze whether LEK-8829 may reduce dopaminergic supersensitivity, determine the impact on behavioral and neurochemical effect of subchronic treatment of LEK-8829 and determine how D2 up-regulation influences D2L and D2S isoforms, and whether D1 agonist may change expression levels of Nova 1.

Effects of LEK-8829 were compared with haloperidol which is representative of the typical antipsychotics and on reserpine model for Parkinson's disease. LEK-8829 demonstrated an antagonist effects on dopamine D2 receptors and on serotonin 5-HT_{2A} and 5-HT_{1A} receptors and was designed as a potential atypical antipsychotic drug (Krisch et al., 1994, 1996). Zivin (1996) determine and D1 agonist properties. LEK-8829 2 mg/kg and haloperidol 0.2 mg/kg are equivalent doses and we choose them based on Krisch (1994) paper. The present work is result of subchronic treatment with LEK-8829, so far have made only acute treatments with LEK-8829 (Sprah et al., 1999, Zivin et al., 1996, Milivojevic et al., 2004, Glavan et al., 2002).

5.1 CATALEPSY ANALYSIS

Catalepsy is a complex phenomenon involving more than one neurotransmitter system and also dependent on the D2 receptor blockade or as a consequence of dopamine depletion. Catalepsy is characterized by akinesia, the maintenance of even abnormal body posture (Carlsson and Carlsson, 2006) and rigidity and is not a simple motor inactivation but rather an 'active immobility' response (Frank and Schmidt, 2003). When a normal animal is placed in an unusual posture, it will change its position within seconds. A cataleptic animal, on the other hand, will maintain this posture for a prolonged period of time (i.e. several minutes) (Sanberg et al., 1988). Catalepsy is an animal model for Parkinson's disease or for neuroleptic-induced parkinsonism in humans (Frank and Schmidt, 2003; Miyagi et al., 1996) and it is used as a standard preclinical test to predict antipsychotic activity and motor side effect liability (Xu et al., 2002; Wadenberg et al., 2001). The intensified catalepsy can be extinguished by repeated exposure to the test environment (Wadenberg et al., 2001; Schmidt and Beninger, 2006). Different results of catalepsy may be due to factors such as strain, behavioral conditioning, animal handling and catalepsy rating technique (Jorgensen et al., 1994; Vasconcelos et al., 2003) but in spite all of that catalepsy still remains a model for Parkinson's disease or for neuroleptic-induced parkinsonism in humans. A full catalepsy can be induced by blocking either D1 or D2 receptors (Frank and Schmidt, 2003). Coadministration of the D1 and D2 antagonists has a synergistic effect on catalepsy. Haloperidol is D2 antagonist. LEK-8829 is D1 agonist and antagonist on D2, 5-HT_{2A} and 5-HT_{1A} receptors.

5.1.1 Catalepsy test on 1st and 21st day (experiment 1)

Haloperidol 2 mg/kg on the 1st and the 21st day shows more convincing catalepsy response than haloperidol 0.2 mg/kg which is in accordance with literature that haloperidol induce catalepsy on dose dependent manner (Laruelle et al., 1992; Barnes et al., 1990). Haloperidol in both doses demonstrates almost the same cataleptic response on the 1st and the 21st day. In the literature is described tolerance to the cataleptic response after subchronic treatment (Marin et al., 1993; Ushijima et al., 1995; Ezrin-Waters and Seeman, 1977; Asper et al., 1973; Campbell and Baldessarini, 1981; Braun et al., 1997). The lack of tolerance to haloperidol-induced catalepsy that we saw here has been reported by some researches (Gyorgy et al., 1969; Moller Nielsen et al., 1974). A review by Barnes (1990) concluded that dose, drug administration schedule and behavioral test conditions all influence the evolution of catalepsy during chronic haloperidol treatment.

Seeman (1975, 1976) determine average dose of 0.5 mg/kg/day used for patients with schizophrenia (Seeman et al., 1975; Seeman et al., 1976).

LEK-8829 2 mg/kg shows similar catalepsy effect as haloperidol 0.2 mg/kg on the 1st day but LEK-8829 2 mg/kg on the 21st day shows a similar curve like haloperidol 2 mg/kg (1st and 21st day), probably because the LEK-8829 is still in circulation.
LEK-8829 0.2 mg/kg does not show significant catalepsy. The curve is similar to the saline curve (Fig 30).

5.1.2 Catalepsy test on 1st and 28th day (experiment 2)

Better view of the issue of cataleptic response, were when we performed the catalepsy test seven days after the last injection because then were no drug in circulation and all effects were due to responsiveness of receptors. It was chosen 7 day drug free because the striatal up-regulation D2 receptors reaching the highest values (Vasconcelos et al., 2003). Haloperidol 0.2 mg/kg produced slightly increased in catalepsy response on the 28th day than on the 21th day in experiment 1. LEK-8829 2 mg/kg show decrease in catalepsy response on the 28th day than on the 21th day in experiment 1 and onset of the cataleptic response on the 28th day is delayed. This delay is probably due to drugs are out of circulation. Since were no drugs in circulation and a lower dose of haloperidol 0.2 mg/kg still have increase in catalepsy response and therefore LEK-8829 is better antipsychotic than haloperidol. LEK-8829 will have least exstrapyramidal side effects than haloperidol.

Consequently we do catalepsy test on control group to check effect of repeated exposure to the test environment because we do the test for 260 min every 20 min and we have shown that animals were learning but that still is not catalepsy (Fig.30). We wanted to confirmed that and on the1st group (saline group) catalepsy test (Table 3) was performed by method described by Krisch (1994). Results were the same like saline group 1st and 28th day of the treatment (Fig.30). On the 4th group (saline group) (Table 3) catalepsy test was performed 4h and 8h after saline injection. Catalepsy scores were 0. Animals were not cataleptic.

Haloperidol 2 mg/kg and 0.2 mg/kg demonstrates almost the same cataleptic response (catalepsy scores are not significantly different) on the 1st and the 21st day i.e. experiment1. Haloperidol 0.2 mg/kg in experiment 2, produced slightly increased in catalepsy response (catalepsy scores are significantly different) from 1st to the 28th day. Due to change in D2 receptors we have behavioral supersensitivity.

LEK-8829 2 mg/kg demonstrates increase in cataleptic response (catalepsy scores are significantly different) from the 1st to the 21st day i.e. experiment 1 but in experiment 2 it demonstrates almost the same cataleptic response (catalepsy scores are not significantly different). Behavioral supersensitivity is no longer evident on the 28th day, probably due to D1 agonist effect.

Subchronic treatment of rats with haloperidol enhances sensitivity to the motor effects of dopamine agonists and concomitantly increases D2 receptor number. Subchronic treatment with haloperidol, significantly increased dopamine agonist induced stereotypy (Marin et

al., 1993). Marin (1993) show that the dopamine D1 receptor agonist SKF attenuated the haloperidol induced increase in apomorphine stereotypes and that this decrease was correlated with a decrease in D2 receptor up-regulation. Concurrent treatment with D2 antagonist and D1 agonist profoundly affect the emergence of behavioral supersensitivity, tolerance of catalepsy and increase in D2 receptor density (Braun et al., 1997).

5-HT_{2A} receptor antagonism can each diminish catalepsy caused by typical antipsychotics such as haloperidol (Lucas et al., 1997) so LEK-8829 also has antagonist activity at 5-HT_{2A} receptors which may contribute to its low risk for extrapyramidal symptoms (Nakai et al., 2003).

5.2 ANALYSIS OF AMPHETAMINE MODEL OF SCHIZOPHRENIA

We have confirmed the already known - that haloperidol decreases amphetamine-induced hyperactivity behavior (Meshul and Casey, 1989; Vasconcelos et al., 2003) and antipsychotics often lose efficacy during chronic continuous treatment (Samaha et al., 2007). Chronic antipsychotic treatment reveals a state of dopaminergic supersensitivity that is characterized by increased vulnerability to psychosis and to the psychomotor activating effects of dopamine agonists, respectively. In humans, this has been termed "neuroleptic-induced supersensitivity psychosis" (Samaha et al., 2007). Patients with schizophrenia, whether treated or untreated, are known to be supersensitive to dopamine-like compounds (Seeman, 2011). In our experiment, 2 mg/kg dose of haloperidol strongly blocked amphetamine-induced hyperactivity then dose 0.2 mg/kg, which is in accordance with the fact that haloperidol is dose dependent. After 21 days of treatment, haloperidol demonstrates loss of efficacy (an increase in responsiveness) but it is interesting that haloperidol 0.2 mg/kg causes a greater loss of efficacy (increase in responsiveness) than haloperidol 2 mg/kg (Fig.32). This is to be expected due to the increase density of dopamine D2 receptors, but two distinct types of dopamine supersensitivity exist: modest one associated with increased D2 density and a more profound one associated with breakdown in D1/D2 synergism and independent of D2 density (LaHoste and Marshall, 1992). It is known that dopamine receptor supersensitivity (as reflected by an enhanced response) sometimes accompanies receptor proliferation. However, dopamine receptor supersensitivity may occur in the absence of a change in the number of dopamine receptors (Kostrzewa, 1995). Continuous antipsychotic treatment and D2 receptor blockade induces neuro adaptations that lead to antipsychotic failure.

The issue is not that simple, but we confirmed with LEK-8829 the evidence that is important breakdown of D1/D2 synergism. LEK-8829 2 mg/kg decreases amphetamine-induced hyperactivity much stronger than LEK-8829 0.2 mg/kg (Fig.35). Neither one dose of LEK-8829 loss efficacy (increases responsiveness) after 21 days. Maybe this confirms the hypothesis (Braun et al., 1997) that effect of chronic D2 receptor blockade can be modified by concurrent stimulation of the D1 receptor.

When selective D2 antagonist is combined with the agonist selective for the D1 receptors, both D2 receptor proliferation and behavioral supersensitivity is completely blocked (Braun et al., 1997). We therefore speculate that in our experiments the intrinsic activity of LEK-8829 at dopamine D1 receptors may have prevented the up-regulation of dopamine D2 receptors, such as occurs after the prolonged blockade of dopamine D2 receptors with haloperidol.

We therefore assume, that the decreased efficacy of haloperidol for the inhibition of amphetamine-induced locomotor activity indicates dopamine D2 receptor up-regulation that has developed during the period of prolonged treatment with haloperidol, while the prolonged treatment with LEK-8829 did not have such effect probably due to absence of up-regulation. Moreover, our results also imply that the prolonged treatment with LEK-8829 in experimental animals does not result in the development of the functional breakdown of dopamine receptors.

However, LEK-8829 has proven to be better antipsychotic than haloperidol because it has fewer side effects (catalepsy test) and does not show behavioral supersensitivity (reduces amphetamine-induced hyperactivity and after 21 day treatment). However, the D1 receptor may be an important target for improving negative and cognitive function in schizophrenia (Ginovart and Kapur, 2010).

5.3 ANALYSIS OF RESERPINE MODEL OF PARKINSON'S DISEASE

Reserpine produced a much greater effect when administered subcutaneously (Rosecrans, 1967). Animals weighing 280-300 g were found extremely sensitive to chronic reserpinization but animals weighing 140-160 g tolerated reserpine the best (Rosecrans, 1967). Systemic reserpine administration depletes dopamine at the nerve terminals and induces a hypokinetic state in rodents. These movement deficits are due to loss of dopamine storage capacity in intracellular vesicles. Reserpine-induced changes are temporary and striatal reserpine administration does not induce changes in the dopamine neurons in supstantia nigra. Also, reserpine administration induces the release of other neurotransmitters that may not be directly implicated in Parkinson's disease (Betarbet et al., 2002). Dopamine depletion leads to breakdown of D1/D2 synergism and increase of D2 receptors followed by supersensitisation (LaHoste and Marshall, 1992). One of the most important implications of the enabling relationship between D1 and D2 dopamine receptors it is potential relevance for the treatment of Parkinson's disease. With the discovery that D1 receptor stimulation is required for certain behavioral effects of D2 agonists, it was suggested that mixed D1/D2 agonists or combinations of D1 and D2 agonists would be more effective in alleviating Parkinsonian symptoms. The finding that D1 receptor stimulation is no longer necessary for D2 mediated responses in the dopamine denervated striatum appears to argue against the need for simultaneous stimulation of D1 and D2 receptors in Parkinson's disease patients (Hu et al., 1990).

Nine days long treatments showed the following. During all nine days of recording the control group showed a minimal locomotor activity in accordance with reserpine treatment. LEK-8829 2 mg/kg, shows strongly alleviated akinetic state but from the fifth day onwards, hyperlocomotion and stereotype behavior were perceived. LEK-8829 0.2 mg/kg also exhibits an increase in locomotor activity but on 9th day and stereotypic behavior was missing. Bromokriptine had a similar profile as a LEK-8829 0.2 mg/kg. LEK-8829 2 mg/kg + SCH 1 mg/kg showed the same profile as LEK-8829 2 mg/kg but with decrease in values from LEK-8829 2 mg/kg in last 100 min (Fig.33). First 20 min show an increase in locomotor activity because injection of LEK-8829 2 mg/kg. After 20 min of increase locomotor activity injection of SCH-23390 1 mg/kg prevent action of LEK-8829 2 mg/kg. This confirms that an increase in locomotor activity is due to D1 receptor agonist properties. Catalepsy test demonstrated that all animals were cataleptic which is in accordance with dopamine depletion. That means that reserpinization were successful.

Acute test doses of LEK-8829 2 mg/kg and bromorekokriptine 2 mg/kg in all treated groups (Table 7) demonstrate greater increase in locomotor activity with LEK-8829 2 mg/kg than with bromorekokriptine 2 mg/kg. Bromocriptine (D2 agonist) show a smaller increase in locomotor activity than LEK-8829.

Dopamine D1/D2 receptor interactions are at the behavioral, pharmacological and biochemical level (Clark and White, 1987; Waddington, 1989; Waddington and O'Boyle, 1989; Niznik and Van Tol, 1992). Basically, these interactions are either antagonistic or synergistic. While some behaviors are predominately influenced by the activity of D1-like receptors, such as grooming and self-mutilation, D2 receptors predominantly influence locomotor activity. Synergistic actions of dopamine receptors have been observed in locomotion, stereotypy, grooming, electrophysiological responses, yawning and climbing (Niznik and Van Tol, 1992). Bromcriptine (D2 agonist) demonstrate a moderate locomotion with shorter duration in compare with LEK-8829. That means that bromocriptine (D2 agonist) just stimulates D2 receptor without repair of synergism. LEK-8829 somehow restored synergism D1/D2 receptors (Niznik and Van Tol, 1992).

Synergistic effects between D1 and D2 receptors, since stereotypes induced by administration of D1 and D2 agonists together are more intense than those produced by either agonist alone (Vallone et al., 2000). In accordance with this it is surprising that D1 agonist alone have intensified stereotypes. Probably these results may be explained by findings White (1988) indicate that D1 agonist induced effects are not abolished, even by near total dopamine depletion. Their behavioral and electrophysiological studies indicate that D1 receptor stimulation is necessary for the expression of postsynaptic dopamine receptor mediated functional responses. Therefore, alterations of D1 receptor activity may play important roles in the Parkinson's disease and schizophrenia as well as in their pharmacological treatment (White et al., 1988).

Furthermore, dihydexidine was first reported in 1990 as a selective D1 agonist and was subsequently shown to alleviate the symptoms of MPTP induced parkinsonism in monkeys

(Taylor et al., 1991). Although dihydrexidine has some affinity at D2 receptors (Ki=120 nM), its antiparkinsonian activity is not reversed when combined with D2 antagonist, suggesting that this effect is due to its D1 (Ki=12nM) receptor activity. Abbot also synthesized a selective D1 agonist A77636 which reverses PD symptoms in parkinsonian marmosets (Kebabian et al., 1992). Blanchet (1993) compared the efficacy of D1 and D2 agonists in alleviating PD symptoms in MPTP treated monkeys and reported that D1 agonists were as effective as D2 agonists and had fewer propensities to cause dyskinesias. These data support the hypothesis that the D1 receptor could play a crucial role in the treatment of PD.

Blanchet (1998) study for the first time the clinical effects of dihydexidine in humans. Data suggest that monotherapy with full dopamine D1 receptor agonist is able to provide antiparkinsonian efficacy, but does not improve the therapeutic window over levodopa in levodopa treated PD.

Dihydexidine enters the brain readily and is fully bioavailable by parenteral administration. However, it has poor oral bioavailability and a relatively short half-life of 1-2 hours, which limits its clinical use (Salmi et al., 2004).

All of this is shown that dopamine D1 receptor agonist properties may have great promise as novel therapeutic agents for treatment of Parkinson's disease and underscore the importance of dopamine D1 receptors in the maintenance of normal motor function. Also shown that development of behavioral supersensitivity (and D2 receptor up-regulation) prevent by simultaneously stimulation of D1 receptors (Braun et al., 1997; Marin et al., 1993).

Another one reasonable explanation exists for these results. Aripiprazole represents the first atypical antipsychotic that acts as a partial agonist at dopaminergic receptors. Like full receptor agonists, partial agonists have full receptor affinity but possess limited intrinsic activity. Partial agonism at dopaminergic neurons results in a decrease and an increase in dopaminergic mediated neurotransmission in areas of hyperdopaminegic and hypodopaminergic activity, respectively. Therefore, complete blockade of dopaminergic receptors does not occur. Instead, intrinsic activity possessed by aripiprazole maintains dopaminergic mediated neurotransmission, although at a less intensive magnitude (DeLeon et al., 2004).

Partial agonism has been shown to occur on both the postsynaptic D2 receptor and presynaptic dopamine autoreceptors. Two possible results of partial agonism at the postsynaptic D2 receptor are a reduced tendency for receptor up-regulation and development of EPS by avoiding complete receptor blockade. Partial agonism at the dopamine autoreceptor decreases dopamine synthesis, release, and subsequent dopaminergic neurotransmission. Multiple studies have confirmed antagonistic and agonistic activity with aripiprazole, depending on the presence of a hyperdopaminergic or

hypodopaminergic environment, respectively (Burris et al., 2002; Matsubayashi et al., 1999; DeLeon et al., 2004).

Antagonism at the 5-HT_{2A} receptor minimizes excessive dopaminergic blockade by increasing dopaminergic release. In the nigrostriatal and mesocortical dopamine pathways, this results in a lower rate of EPS and may contribute to the improvement of negative symptoms, respectively. In the mesolimbic pathway, antagonism at the 5-HT_{2A} receptor does not cause an increase in dopamine release sufficient to prevent antipsychotic activity, a phenomenon possibly explained by regional differences in 5-HT_{2A} autoreceptor density (DeLeon et al., 2004).

Aripiprazole monotherapy was generally well tolerated and efficacious in the treatment of schizophrenia. It was associated with improvements in the positive, negative and cognitive aspects of these disorders with a low risk for the EPS and TD. Aripiprazole's lack of D2 up-regulating effects relative to agents such as haloperidol has been reported to translate into a low incidence of treatment emergent TD (Stip and Tourjman, 2010).

Naber (2002) has showed that aripriprazole is markedly superior to haloperidol during long term therapy i.e. haloperidol loses efficacy during the prolonged treatment (Naber and Lambert, 2004).
Aripiprazole barely failed to elicit catalepsy, a typical behaviour which is largely documented for dopamine receptor blockers such as haloperidol. Very high doses of aripiprazole were required in order to evoke a significant cataleptic behavior (Koener et al., 2011).

In rats, the liability of most antipsychotics to induce catalepsy correlates with striatal receptor occupation (D2 receptor antagonism). Aripiprazole, on the other hand, fails to induce significant motor impairments even in doses that induce up to 95% of receptor occupation, probably because of its ability to maintain the receptors slightly activated. Interestingly, the same profile is observed in humans. For most antipsychotics, the therapeutic window occurs between 60% and 80% of striatal occupancy. Higher levels of receptor occupancy by these drugs lead to extrapyramidal side effects, while aripiprazole has a safer profile even though it occupies more than 90% of receptors. This partial agonistic effect of aripiprazole led to the hypothesis that this compound may act as a dopamine stabilizer. At somato-dentritic receptors, where the levels of dopamine are low, it may exert effects similar to an agonist, whilst at postsynaptic sites, where the levels of this neurotransmitter are high, it may occupy the receptors and reduce (but not abolish) their activation. Thus, this modulatory action of aripiprazole may explain why aripiprazole blocked the effect of amphetamine and cocaine without induce significant motor impairments (Leite et al., 2008).

Aripiprazole has an ability to stabilize the sensitivity to dopamine (Tadokoro, 2013).

The previously shown results with LEK-8829 are the same as the results with aripriprazole. LEK-8829 induced lower catalepsy than haloperidol. Also, LEK-8829 inhibited the

amphetamine induced locomotion without losing efficiency after a prolonged treatment. In accordance with results it is rational to assume that LEK-8829 is a D2 partial agonist.

Dopamine depletion with reserpine LEK-8829 demonstrates a maximum locomotor response. That is uncommon because it was suggested that mixed D1/D2 agonists or combination of D1 and D2 agonists would be more effective in alleviating Parkinsonian symptoms. Dopamine D1/D2 receptor interactions are at the behavioral, pharmacological and biochemical level. Basically, these interactions are either antagonistic or synergistic. While some behaviors are predominately influenced by the activity of D1 like receptors, such as grooming and self-mutilation, D2 receptors predominantly influence locomotor activity. Synergistic actions of dopamine receptors have been observed in locomotion, stereotypy, grooming, electrophysiological responses, yawning and climbing. Bromcriptine (D2 agonist) demonstrate a moderate locomotion with shorter duration in compare with LEK-8829. That means that bromocriptine (D2 agonist) just stimulates D2 receptor without repair of synergism. LEK-8829 restored synergism D1/D2 receptors because it is a D2 partial agonist. Synergistic effects between D1 and D2 receptors, since stereotypes induced by administration of D1 and D2 agonists together are more intense than those produced by either agonist alone indicate that in absence of dopamine LEK-8829 have D2 partial agonist properties. LEK-8829 2 mg/kg shows intensified stereotypes behaviour.

Our data suggest that LEK-8829 is a D2 partial agonist and due to its properties has a lower propensity to induce EPS, inhibited the amphetamine induced locomotion without losing efficiency after prolonged treatment, it is more effective in alleviating Parkinsonian symptoms than bromocriptine (D2 agonist) and stabilize the sensitivity to dopamine.

5.4 PROTEIN AND mRNA ANALYSIS OF DOPAMINE D2 RECEPTORS

The purpose of in situ hybridization was to detect changes in mRNA expression of D2 receptor, D2 long, D2 short isoforms and Nova 1 in striatum due to subchronic treatment with test compounds. Alternative D2 receptor pre-mRNA splicing is controlled by splicing regulator Nova 1.

Expression of mRNA D2 receptors after prolonged daily treatment with saline, haloperidol 2 mg/kg and 0.2 mg/kg; and LEK-8829 2 mg/kg show the obvious tendency of prolonged daily treatment to cause a higher up-regulation of mRNA D2 receptors in the group treated with haloperidol 2 mg/kg than in the group treated with LEK-8829 2 mg/kg. Haloperidol 0.2 mg/kg causes less up-regulation than haloperidol 2mg/kg because of a smaller dose (Fig.47).

We have confirmed the hypothesis that subchronic treatment of experimental rats with LEK-8829 causes less dopaminergic supersensitivity and up-regulation of dopamine D2 receptors in the striatum as a chronic treatment with D2 antagonist haloperidol.

The 6-OHDA model has D2 receptor up-regulation and this was a preliminary analysis for verification D2 receptor and its isoforms (Fig.49).

Results show up-regulation of D2 receptors (due to its internal control) and up-regulation of D2S isoform (due to its internal control). D2L does not show up-regulation due to its internal control.

It is surprising that results show an up-regulation of D2S isoform because Khan and colleagues (1998), report that D2S is located predominantly in cell bodies and exons of dopaminergic neurons of the primate midbrain, whereas D2L is more strongly expressed by neurons of the striatum and nucleus accumbens that are targeted by dopaminergic neuron. Accordingly, in the primate brain, D2S and D2L are primarily localized to pre- and postsynaptic membranes, respectively.

The dorsal striatum represents an ideal system in which to study dopaminergic transmission. In this region, dopamine D2 receptors are expressed both postsynaptically, on striatal medium spiny neurons, as well as presynaptically, on dopaminergic nerve terminals originating from the supstantia nigra pars compacta (Lindgren et al.,2003). In the 6-OHDA model, dopaminergic nerve terminals originating from the supstantia nigra pars compacta are destroyed and it is expected to have less D2S isoform.

Perhaps the explanation is in the specific involvement of the D2S isoform in the regulation of dopamine biosynthesis (Lindgren et al., 2003). Due to the deterioration of dopaminergic neurons from the supstantia nigra pars compacta leads to a lack of dopamine. Loss of dopamine less than 80% can be compensated due to increasing activity of remaining neurons. It is possible that this increasing activity of the remaining neurons leads to an increase in D2S isoform.

We showed that up-regulation of dopamine D2 receptors in the striatum have resulted in changes in relative splicing forms of D2S and D2L. D2S show up-regulation.

Further work on slices from subchronic treatment with test compounds will show an amount of up-regulation of dopamine D2 receptors in the striatum and changes in relative splicing forms of D2S and D2L. Also, on same slices we will check what it happening with splicing factor Nova 1.

6 SUMMARY (POVZETEK)

6.1 SUMMARY

Antipsychotics often lose efficacy during chronic continuous treatment. The loss of antipsychotic efficacy may be linked to an increase in D2 receptor number and sensitivity, e.g. such as occurrs after prolonged treatment with dopamine D2 receptor antagonist haloperidol. Consequently, the dopaminergic hypersensitivity that develops during the prolonged treatment with neuroleptic drugs often leads to undesired development of tardive dyskinesia.

LEK-8829 has D1 agonistic and D2 antagonistic properties in the nigrostriatal and mesocorticolimbic dopaminergic pathways. Here we speculate that such unusual pharmacological profile at dopamine receptors may confer a lower propensity for the development of dopaminergic hypersensitivity during prolonged treatment.

We have determined the impact on behavioral and neurochemical effect of subchronic treatment of LEK-8829, and the catalepsy analysis (experiment 1; catalepsy test on the 1st and on the 21st day) demonstrated that only LEK-8829 significantly increased its potential for the induction of catalepsy. However, when we tested the LEK-8829 treated animals after 7 days of drug-free period (experiment 2; catalepsy test on the 1st and on the 28th day), the latency to the onset of catalepsy was decreased, but the catalepsy score reverted to the level induced by 0.2 mg/kg of haloperidol in drug naive animals.

The analysis of amphetamine model of schizophrenia demonstrate, when tested in drug naive rats, that both LEK-8829 2 mg/kg and haloperidol 2 mg/kg almost completely inhibited the amphetamine-induced locomotor behavior in the open field. However, after prolonged treatment period with the respective drug, only LEK-8829 retained its inhibitory properties, while haloperidol mediated inhibition was significantly reduced. These results indicate that in comparison with neuroleptic haloperidol, prolonged treatment with LEK-8829 does not lose efficacy during subchronic continuous treatment and may have a lower propensity for the development of tardive dyskinesia.

In models of parkinsonism with prolonged dopamine depletion in the brain the intrinsic activity of LEK-8829 at dopamine D1 receptors has been revealed by the robust psychomotor response that seems to be unabated by the concurrent blockade of dopamine.

Our results indicate that D1 agonist propreties reduce dopaminergic supersensitivity and mRNA up-regulation of dopamine D2 receptors. Haloperidol causes higher up-regulation of dopamine D2 receptors than LEK-8829.

Results on the 6-OHDA model show up-regulation of D2 receptors (due to its internal contol) and up-regulation of D2S isoform (due to its internal contol). D2L does not show up-regulation due to its internal control. We showed that up-regulation of dopamine D2 receptors in the striatum has resulted in up-regulation of D2S isoform.

We were not able to analyse how subchronic treatment of dopamine D1 receptors and inhibition of D2 receptors (i.e. LEK-8829) in the striatum affect the expression of alternative splicing factors Nova 1.

In western blot analysis we were able to see D2 long and D2 short isoforms but we could not detect up-regulation of D2 receptor and/or D2 long and D2 short isoforms because cross-reaction between primary mouse antibodies and Ig G in rat samples (Fig.40).

To our surprise results indicate that LEK-8829 is a D2 patiral agonist and due to that properties have an ability to stabilize the sensitivity to dopamine. LEK-8829 is a D2 partial agonist and due to its propertis has a lower propensity to induce EPS, inhibited the amphetamine induced locomotion witouh losing efficency after prolonged treatment, it is more effective in alleviating Parkinsonian symptoms than bromocriptine (D2 agonist) and stabilize the sensitivity to dopamine.

6.2 POVZETEK

Pri nastanku simptomov shizofrenije in Parkinsonove bolezni, kot tudi pri zdravljenju in stranskih učinkih zdravljenja naštetih bolezni, ima pomembno vlogo spremenjena odzivnost dopaminoceptivnih nevronov v možganih.

Pri obeh omenjenih boleznih ima pomembno vlogo povečana aktivnost dopaminoceptivnih nevronov v tarčnih področjih dopaminskih nevronov. Le-ta je lahko posledica plastičnih sprememb, ki privedejo do povečane odzivnosti dopaminoceptivnih nevronov na endogeni dopamin, na primer zaradi povečanega izražanja genov dopaminskih receptorjev. Povečana dopaminergična odzivnost dopaminoceptivnih nevronov je najbrž vpletena v nastanek tardivne diskinezije, ki se lahko pojavi kot neželeni učinek pri uporabi antipsihotičnih in antiparkinsonskih zdravil.

Shizofrenija je huda duševna bolezen, ki jo spremljajo pozitivni simptomi (halucinacije, blodnje, zaznavno izkrivljenje…) in negativni simptomi (psihomotorična upočasnjenost, čustvena otopelost, pomanjkanje interesov in hotenja...). Po dopaminski hipotezi so pozitivni znaki te bolezni posledica povečanega sinaptičnega prenosa prek aktivacije subkortikalnih dopaminskih receptorjev podtipa D2, saj vsa klinično uveljavljena antipsihotična zdravila delujejo kot zaviralci dopaminskih receptorjev D2. V subkortikalnih limbičnih in motoričnih dopaminoceptivnih področjih (nukleus akumbens, striatum), ki so pomembna za nastanek pozitivnih znakov, so dopaminski receptorji D2 izraženi v približno enaki meri kot dopaminski receptorji D1.

Ublažitev pozitivnih znakov shizofrenije se ne pojavi že na začetku zdravljenja z antipsihotiki, temveč šele po dolgotrajnejšem jemanju, kar kaže na pomen možganske plastičnosti pri zdravljenju shizofrenije. Po drugi strani pa so po dopaminski hipotezi negativni znaki shizofrenije posledica zmanjšanega mezokortikalnega dopaminergičnega

sinaptičnega prenosa prek dopaminskih receptorjev D1, predvsem v čelni skorji (hipofrontalnost), ki so v tem področju sicer izraženi v večji meri kot dopaminski receptorji iz farmakološke skupine D2.

Pri Parkinsonovi bolezni, za katero je značilno postopno propadanje dopaminergičnih nigrostriatnih nevronov, nastane tako imenovana denervacijska dopaminergična hipersenzitivnost. Le-ta se pokaže kot pretiran motorični odziv na stimulacijo z antiparkinsonskimi zdravili (L-DOPA, apomorfin). Nastane zaradi povečanja dopaminergične odzivnosti dveh populacij dopaminoceptivnih nevronov v striatumu, ki izražajo bodisi dopaminske receptorje D1 ali D2, kot kompenzacijski odgovor na hudo in dolgotrajno pomanjkanje dopamina v striatumu.

Zato velja, da imajo spremembe dopaminergične odzivnosti v možganih pomembno vlogo pri obeh omenjenih boleznih.

Zdravljenje shizofrenije in parkinsonizma

Klasične antipsihotike iz zgodnjega obdobja zdravljenja shizofrenije, ki imajo zaradi relativno selektivnega zaviranja subkortikalnih dopaminskih receptorjev D2 tendenco povzročanja katalepsije in parkinsonskega sindroma, imenujemo nevroleptiki. Učinki zdravljenja shizofrenije se ne pojavijo takoj, temveč šele po dalj časa trajajočem zaviranju dopaminskih receptorjev D2, kar kaže, da so za ublažitev pozitivnih simptomov najprej potrebne plastične prilagoditve nekaterih možganskih področij na dolgotrajno zaviranje dopaminskih receptorjev D2 z dopaminskimi antagonisti. Na žalost pa dolgotrajno jemanje antipsihotičnih zdravil lahko povzroči pojav tardivne diskinezije, ki se kaže z nenadzorovanimi in pretiranimi gibi. Nastanek tardivne diskinezije povezujejo s povečanim izražanjem in/ali s povečano odzivnostjo striatalnih dopaminskih receptorjev, kot posledice dolgotrajnega zaviranja dopaminskega prenosa pri zdravljenju shizofrenije.

Katalepsija predstavlja živalski model za z nevroleptiki povzročen parkinsonizem pri ljudeh in se uporablja kot standardni predklinični preizkus za napoved antipsihotične aktivnosti ter odgovornosti za stranske učinke. Znano je, da antipsihotiki provzročajo katalepsijo pri glodalcih in imajo od odmerka odvisno nagnjenost k sprožanju EPS pri ljudeh. Antipsihotiki provzročajo katalepsijo zaradi zaviranja dopaminskih D2 receptorjev v bazalnih ganglijih. Katalepsijo se lahko sproži z zaviranjem dopaminskih D1 receptorjev ali dopaminskih D2 receptorjev. Sočasno dajanje dopaminskih D1 in D2 antagonistov ima sinergistični učinek na katalepsijo.

Velik problem pri zdravljenju shizofrenije z antipsihotičnimi zdravili, ki so trenutno v klinični uporabi, je tudi ta, da z zaviranjem dopaminskega prenosa v čelni skorji ne ublažijo oziroma lahko celo poslabšajo negativne simptome shizofrenije.

Zaradi omenjenih stranskih učinkov in neučinkovitosti glede zdravljenja negativnih simptomov razvijajo nove antipsihotične učinkovine, ki se jih je oprijelo ime atipični antipsihotiki. Razvoj atipičnih antipsihotikov je najprej temeljil na dopaminsko-

serotoninski hipotezi, pri čemer naj bi bila ob hkratnem zaviranju dopaminskih receptorjev D2 in serotoninskih receptorjev 5-HT_{2A}, ob enakem antipsihotičnem učinku (glede pozitivne simptomatike), manjša potreba po zaviranju dopaminskih receptorjev D2 kot pri zdravljenju z nevroleptiki. Vendar pa tudi omenjeno sočasno antagonistično delovanje atipičnih antipsihotikov na dopaminskih in serotoninskih receptorjih ni doprineslo k zdravljenju negativne simptomatike. Glede na že omenjeno prevlado dopaminskih receptorjev D1 v čelni skorji, ob zmanjšanem dopaminskem prenosu v tem področju pri bolnikih s shizofrenijo (hipofrontalnost), so nekateri raziskovalci predlagali, da bi spodbujanje dopaminskega prenosa v čelni skorji z dopaminskimi agonisti D1 morda lahko neposredno ublažilo negativne simptome. Po drugi strani pa bi dopaminski agonisti D1 s spodbujanjem kortiko-tegmentalnih piramidnih nevronov čelne skorje (le-ti v ventralnem tegmentumu sproščajo glutamat), s spodbujanjem GABAergičnih internevronov v tem področju, zavrli in tako ponovno uravnovesili delovanje deinhibiranih subkortikalnih dopaminergičnih nevronov, kar bi poleg negativnih hkrati ublažilo tudi pozitivne simptome shizofrenije.

LEK-8829 je snov z nenavadnim učinkom na dopaminskih receptorjih. Ta snov namreč po eni strani zavira delovanje dopaminskih receptorjev D2, po drugi strani pa spodbuja dopaminske receptorje D1. Ker so v striatumu dopaminski receptorji D1 in D2 ločeno izraženi na membranah različnih fenotipov dopaminoceptivnih nevronov t.i. neposredne poti (D1 receptorji) in posredne poti (D2 receptorji), pri čemer endogeni dopamin spodbuja delovanje nevronov, ki izražajo D1 receptorje in zavira delovanje nevronov, ki izražajo D2 receptorje, so postavili hipotezo, da v striatumu LEK-8829 spodbuja delovanje tako nevronov posredne kot neposredne poti. Raziskave učinkov LEK-8829 na vedenje in na izražanje genov v striatumu, ki so bile do sedaj opravljene na živalskih modelih za shizofrenijo, parkinsonizem ter na modelu sprožanja recidiva pri zasvojenosti s kokainom so pokazale učinke, ki se skladajo z opisano dopaminergično hipotezo glede receptorskih učinkov LEK-8829. Pri tem pa je potrebno omeniti, da LEK-8829 deluje zaviralno tudi na serotoninske receptorje 5-HT_{2A}, na katere se veže z večjo afiniteto kot na dopaminske receptorje D2, zaradi česar se uvršča med tako imenovane atipične antipsihotike, z delovanjem, ki je bolj podobno atipičnemu antipsihotiku klozapinu kot nevroleptiku haloperidolu. Za atipične antipsihotike nekoliko neobičajen dopaminergični profil LEK-8829 je bil odkrit s preučevanjem akutnih učinkov LEK-8829 na vedenje in na izražanje genov v striatumu poskusnih podgan z normosenzitivnimi in s hipersenzitivnimi dopaminskimi receptorji na modelnih poskusnih podganah za shizofrenijo, parkinsonizem, Huntingtonovo bolezen in za zasvojenost s psihomotoričnimi stimulansi. Kronični učinki LEK-8829 na vedenje živali in na izražanje genov v možganih modelnih poskusnih podgan pa do sedaj še niso bili raziskani.

Iz literature je znano, da je povečano dopaminergično odzivnost na dopaminske antagoniste D2 mogoče ublažiti s sočasnim tretiranjem z agonisti dopaminskih receptorjev D1. Zato predpostavljam, da bi s sočasnim antagonističnim delovanjem na dopaminskih

receptorjih D2 in agonističnim delovanjem na dopaminskih receptorjih D1, ob kroničnem tretiranju, LEK-8829 lahko zaviral nastanek dopaminergične hipersenzitivnosti in s tem neželene učinke pri zdravljenju shizofrenije, parkinsonizma. V doktorski nalogi sem zato raziskala nekatere vedenjske in nevrokemične učinke kroničnega tretiranja poskusnih podgan s potencialnim antipsihotičnim zdravilom LEK-8829, ki na dopaminskih receptorjih deluje kot D1 agonist/D2 antagonist. Osredotočila sem se na učinek LEK-8829 na gensko izražanje postsinaptičnih dopaminskih receptorjev D2 in njihovih spojitvenih oblik D2S in D2L v dorzalnem in ventralnem striatumu

Aripiprazole predstavlja prvi atipični antipsihotik, ki deluje kot delni agonist na dopaminergične receptorje. Kot agonist zasedenih receptorjev ima delni agonist polno receptorsko afiniteto vendar kljub temu omejeno aktivnost. Delni agonizem na dopaminergičnih neuronih privede do zmanjšanja ali zvišanja dopaminergične neurotransmisije na območjih povečane ali zmanjšane dopaminergične aktivnosti. Zato se popolna blokada dopaminergičnih receptorjev ne zgodi. Namesto tega ima aripiprazol aktivnost, ki ohranja dopaminergično neurotransmisijo, vendar z manj intenzivnim obsegom.

Dokazano je, da pride do delnega agonizma na postsinaptičnih D2 receptorjih in presinaptičnih dopaminskih avtoreceptorjih. Dva možna izhoda delnega agonizma na postsinaptičnih D2 receptorjih sta zmanjšana nagnjenost k povečjanju števila receptora in razvoj EPS z izogibanjem popolne blokade receptorjev. Delni agonizem na dopaminskih avtoreceptorjih zmanjšuje sintezo dopamina, njegovo sproščanje in kasnejšo dopaminergično neurotransmisijo. Več študij je potrdilo antagonistično in agonistično aktivnost aripiprazola, odvisno od količine dopamina.

Antagonizem na 5-HT_{2A} receptorijih zmanjšuje prekomerno dopaminergično blokado z večjim sproščanjem dopamina. Poteka v nigrostriatni in mesokortikalni poti in se kaže v nižji stopnji EPS in lahko prispeva k izboljšanju negativnih simptomov. V mesolimbični poti antagonizem na 5-HT_{2A} receptorijih ne povzroča povečjanja sproščanja dopamina, kar preprečiantipsihotične aktivnosti, pojav, ki ga lahko razložimo z regionalnimi razlikami v 5-HT_{2A} avtoreceptorski gostoti.

Zdravljenje z aripiprazolom pacienti na splošno dobro prenašajo, hkrati pa je zdravljenje shizofrenije s tem zdravilo učinkovito. To je povezano z izboljšanjem pozitivnih, negativnih in kognitivnih simptomov ter z nizkim tveganjem za EPS and TD. Aripiprazolu primanjkuje učinek na povečjanje števila D2 receptorjev, ki je prisoten pri haloperidolu, zato ima aripiprazol nizko pojavnost pri zdravljenju TD.

Naber (2002) kaže, da je aripriprazol precej boljši kot haloperidol pri dolgotrajnem zdravljenju, kjer haloperidol izgublja učinkovitost.Z aripiprazolom komaj uspemo, da izzvati katalepsijo, tipično vedenje zaradi blokade dopaminskih receptorjev, ki je v veliki meri dokumentirano za haloperidol. Le zelo visoki odmerki aripiprazola izzovejo znatno kataleptično vedenje.

Aripiprazole ima sposobnost da stabilizira občutljivost na dopamin.

Dopaminergično uravnavanje izražanja pre-mRNA in alternativnega zlepljanja eksonov dopaminskega receptorja D2

Znano je, da ima uporaba antagonistov dopaminskih receptorjev pri zdravljenju shizofrenije, kakor tudi uporaba agonistov dopaminskih receptorjev, ob hudem pomanjkanju endogenega dopamina pri Parkinsonovi bolezni, lahko za posledico povečano izražanje obeh oblik striatalnih dopaminskih receptorjev D2, ki nastajata z alternativnim izrezovanjem pre-mRNA z zapisom za dopaminski receptor D2 (pa tudi številnih drugih genov) in s temi spremembami povezano povečano dopaminergično odzivnost striatalnih nevronov. Kot je že omenjeno, bi omenjene receptorske spremembe lahko imele za posledico nastanek tardivne diskinezije pri zdravljenju shizofrenije in/ali Parkinsonove bolezni, pa tudi t.i dopaminergične senzitizacije, kot imenujemo povečano vedenjsko, elektrofiziološko in nevrokemično odzivnost.

V striatumu se dopaminski receptorji D2 nahajajo postsinaptično, na dendritih nevronih posredne poti ter na dendritih holinergičnih internevronih pa tudi kot presinaptični avtoreceptorji na končičih dopaminergičnih nevronov, ki v striatum projicirajo iz substance nigre kompakte (le-ti delujejo avtoregulatorno pri sproščanju dopamina iz dopaminergičnih končičev teh nevronov v striatumu). Pri tem je zanimivo, da sta v striatnih dopaminoceptivnih projekcijskih nevronih posredne poti, kakor tudi v dopaminergičnih nevronih, ki projicirajo v striatum, izraženi mRNA dveh oblik mRNA dopaminskih receptorjev D2, ki nastaneta z alternativnim izrezovanjem intronov pre-mRNA dopaminskih receptorjev D2. Vendar pa dobro znano inhibitorno delovanje dopamina na nevrone posredne poti povezujejo z vezavo dopamina na dopaminske receptorje D2L. Predel v D2L, ki ga kodira ekson 6 pre-mRNA dopaminskega receptorja (oblika D2S je brez tega inserta), namreč omogoča interakcijo tretje znotrajcelične zanke receptorja D2L z beljakovino Gαi2, ki deluje močno zaviralno na encim adenilat-ciklazo, pri obliki D2S pa je ta interakcija in zaviranje cAMP šibkejše, poleg tega pa določa tudi alternativni znotrajcelični promet in lokalizacijo na nevrolemi, npr. presinaptično lokalizacijo oblike D2S na dopaminskih nevronih, kjer ta oblika deluje kot inhibitorni avtoreceptor pri sproščanju dopamina v striatumu. Po drugi strani pa spodbujanje receptorjev D2L na dendritih postinaptičnih omenjenih nevronov z dopaminskimi agonisti ali z endogenim dopaminom zavira delovanje encima adenilat-ciklaze, zaviranje receptorjev D2L z antipsihotiki pa njegovo delovanje spodbuja.

Novejše raziskave so identificirale nekatere proteine, ki sodelujejo pri vključevanju oziroma izključevanju eksona 6 v mRNK D2L (Nova 1, hnRNP M, PTBP1). Tako so na primer pokazali, da je hnRNP M ključni dejavnik pri uravnavanju altrnativnega zlepljanja eksonov pre-mRNK D2 receptorjev. Njegova vezava na ekson 6 pre-mRNA dopaminskega receptorja D2 inhibira inkluzijo tega eksona v mRNK D2L, pri čemer dejavnik Nova 1, z vezavo na specifične sekvence pre-mRNA D2 receptorjev zavira delovanje hnRNP M in s

tem favorizira nastajanje daljše oblike tega receptorja, D2L. Nova 1 je dejavnik alternativnega zlepljanja eksonov, ki na splošno uravnava inkluzijo oziroma ekskluzijo eksonov glede na položaj vezavnega mesta Nova 1. Kadar se na primer Nova 1 veže na mesto 3' kasetnega eksona, to promovira inkluzijo tega kasetnega eksona. Na ta način je protein Nova1 v striatumu udeležen tudi pri uravnavanju alternativnega zlepljanja eksonov nekaterih drugih genov, ki jih tako kot dopaminske receptorje D2 izražajo dopaminoceptivni GABA-ergični nevroni posredne poti (GlyR-α2, K+ kanale –GIRK2), ki so tudi funkcionalno sklopljeni z inhibitornim delovanjem oblike dopaminskega receptorja D2L in dejavnik PTBP1 najbrž favorizira inkluzijo eksona 6 in s tem relativno večje nastajanje daljše oblike D2L. Po drugi strani pa je v literaturi glede pomena izražanja obeh spojitvenih oblik dopaminskega receptorja v striatalnih nevronih posredne poti, kot tudi v dopaminskih nigrostriatnih nevronih, še veliko neznank. Prav tako ni podatkov o morebitnem funkcionalnem pomenu mehanizmov uravnavanja alternativnega izrezovanja v patofizioloških razmerah.

Metode raziskovanja

V raziskavi sem uporabljala skupine odraslih poskusnih podgan (270 - 300 gr), samčkov linije Wistar. Vedenjski poskusi so bili: test katalepsije, spontana lokomotorna aktivnost v odprtem polju, z amfetaminom spodbujena lokomotorna aktivnost ter z testnimi snovmi zavrta z amfetaminom sprožena lokomotorna aktivnost. Živali sem posnela z Noldus Ethno Vision programom za spremljanje živali.

Test katalepsije

Test katalepsije sem naredila v dveh poskusih. Test katalepsije je izvajan z metodo po Krishu. Po injekciji testnih snovi vsakih 20 min je merjena katelepsija do 260 min. Na palico višine 11 cm nad tlemi se z prednjimi šapami dajo podgane. Ocena je podana na podlagi čas, v katerem podgana postavi sprednje noge na tla. Ocena 1: med 15 in 29 sec; ocena 2: med 30 in 59 sec; ter ocena 3: 60 sec ali več.

1) Poskus katalepsije: test katalepsije 1 in 21 dan, to je prvi in zadnji dan subhroničnega tretiranja z LEK-8829 2 mg/kg in 0,2 mg/kg ter z haloperidolom 2 mg/kg in 0,2 mg/kg. Skupine so bile sestavljene iz 8 podgan. Hotela sem preveriti ali LEK-8829 zmanjšuje toleranco do katalepsije po 21 dnevnem tretiranju. Rezultati so pokazali, da haloperidol 2 mg/kg povzroča večjo katalepsijo kot haloperidol 0,2 mg/kg, kar je v skladu z litetarurnimi podatki o od odmerka odvisne katalepsije (Slika 20, 21). LEK-8829 0,2 mg/kg ne povzroča katalepsije (ocena katalepsije je pod oceno 1) (Slika 23). LEK-8829 2 mg/kg na 1 dan testiranja provzroča enako katalepsijo kot haloperidol 0,2 mg/kg (merjeno v oceni), kar kaže na ekvivalentne doze (Slika 28). LEK-8829 2 mg/kg na 21 dan provzroča enako katalepsijo kot haloperidol 2 mg/kg (krivulje 1 in 21 dan so precej enake) (Slika 29). To pomeni da LEK-8829 v dozi 2 mg/kg provzroča zvišanje

katalepsije. Pri tem sta pomembni dve stvari: doza LEK 2 mg/kg je dokaj velika in verjetno ni terapevtska ter testne snovi so bile še vedno v obtoku tretiranih živali.

2) Poskus katalepsije: test katalepsije 1 in 28 dan. Tretiranje z fiziološko raztopino, z LEK-8829 2 mg/kg in s haloperidolom 0,2 mg/kg je potekalo od 1 do 21 dneva, 28 dan pa v krvnem obtoku testnih živali ni več bilo prisotnih testnih snovi. Skupine so bile sestavljene iz 6 podgan. Hotela sem preverit odzivnost receptorjev ko testnih snovi ni več v obtoku. Tokrat sem merila katalepsijo tudi fiziološki skupini in ugotovila, da se testne živali učijo katalepsije ter da je 28. dan ocena večja kot 1. dan, kljub temu pa sta obe krivulji pod oceno 1. Torej ne gre za pravo katalepsijo, kar je v saglasju z literaturo. Presenetljivo je, da je haloperidol 0,2 mg/kg na testu 28. dan povzročil večjo katalepsijo kot na testu 21. dan (1 poskus katalepsije) in Wilcoxon neparametrični statistični test je pokazal, da se je učinek katalepsije zvišal od 1. do 28. dneva. Medtem pa LEK-8829 2 mg/kg v nasprotju z prvim poskusom katalepsije oziroma testom katalepsije 21. dan ni pokazal razlik med krivuljama 1. in 28. dan.

To pomeni, da v prvem poskusu, kjer je bil LEK-8829 še vedno v krvnem obtoku, dobimo povečanje učinka katalepsije, medtem ko je katalepsija v drugem poskusu enaka kot na 1. dan, pri čemer LEK-8829 ni bil več v krvnem obtoku testnih živali. Manjša doza haloperidola 0,2 mg/kg, ko haloperidola ni več v obtoku, provzroča večji učinek. Zaradi tega sklepamo, da naj bi LEK-8829 po dolgotrajnem jamanju manj vplival na receptorje ter provzročil manj neželenih učinkov.

Statistična analiza rezultatov

Podatki so analizirani z SPSS programom (SPSS 19.0 for Windows, Chicago, Illinois, USA).

Katalepsija je analizirana z neparametričnim Wilcoxon testom nivoja, ki primerja iste skupine prej in potem in Mann-Whitney testom, ki primeja različne skupine.

Amfetaminski živalski model za shizofrenijo

Amfetaminski živalski model za shizofrenijo. Injiciranje testnih snovi je trajalo 21 dni. Skupine so bile sestavljene iz 8 podgan. Pred začetkom iniciranja in po končanem iniciranju je narejen test zaviranje z testnimi snovmi – z amfetaminom sprožene lokomotorne aktivnosti. Test izveden pri naivnih podganah je pokazal, da LEK 2 mg/kg in haloperidol 2 mg/kg popolnoma zavreta z amfetaminom sproženo lokomotorno aktivnost. Enak test po končanem 21. dnevnem injiciranju testnih snovi je pokazal, da le LEK-88829 obdrži zaviralni učinek na amfetamin, medtem ko se je zaviranje s haloperidolom bistveno zmanjšalo.

Rezultati primerjave subkroničnega tretiranja s haloperidolom in LEK-8829 kažejo, da LEK-8829 po subkroničnem tretiranju ne izgublja učinek in ima predvidoma manjšo nagnjenost k neželjenim učinkom ter razvoju tardivne diskinezije.

Statistična analiza rezultatov

Podatki so analizirani z SPSS programom (SPSS 19.0 for Windows, Chicago, Illinois, USA).

Zaviranje amfetaminske lokomocije s testnimi snovi je analizirano z dvo-smernim parnim testom t in analizo variance (ANOVA) s Scheffejevim testom.

Rezerpinski živalski model za Parkinsonovo bolezen

Rezerpinski živalski model za Parkinsonovo bolezen. Rezerpin so živali prejele 3-krat vsak 3. dan. Injiciranje testnih snovi je trajalo 9 dni, sledila sta še 2 dneva brez testnih snovi. Zadnji dan poskusa sem živali razdellila v skupine po 3 živali, ki so prejele LEK-8829 2 mg/kg ali bromoergokriptin 2 mg/kg. Skupine so bile sestavljene iz 6 podgan razen kontrolne skupine, ki je imela 12 živali. Testne snovi so bile: fiziološka raztopina, LEK-88209 2 mg/kg in 0,2 mg/kg, bromoerkokriptin 2 mg/kg, LEK-8829 2 mg/kg + SCH (D1 antagonist) 1 mg/kg.

Rezerpin ireverzibilno preprečuje vnos in shranjevanje monoaminov (dopamine, noradrenalin in serotonin) v sinaptični vezikel, saj zavira delovanje vezikularnega monoaminskega prenašalca (VMAT). Encim monoamin-oksidaza (MAO) znotraj živčne celice razgradi vse proste monoamine, kar provzroči, da se izpraznijo zaloge dopamina, noradrenalina in serotonina. Posledica pomanjkanja dopamina v striatumu privede do denervacijske dopaminergične preobčutljivosti ter razklopa D1 in D2 receptorjev. Pri tem selektivni agonisti, bodisi D1 ali D2, sprožijo psihomotorični odgovor kot stimulacijo lokomotorne aktivnosti in stereotipnega vedenja tudi v primeru ko so poskusne živali soočasno tretirane z antagonisti drugega tipa dopaminskih receptorjev.

Rezultati kažejo, da LEK-8829, ki deluje na izpraznjene zaloge dopamina v možganih, zaradi intrinzične aktivnosti na dopaminske D1 receptorje, sproži močan psihomotorni odgovor, ki se pojavi v nezmanjšanem obsegu sočasne blokade dopaminskih D2 receptorjev.

Pri podganah s prekinjenim dopaminskim prenosom, LEK-8829 v nasprotju z bromoergokriptinom, sproži značilno psihomotorično senzitizacijo posredovano z dopaminsko D1 receptorsko aktivnostjo. Ta psihomotorična senzitizacija se kaže v postopnem povečjanju lokomotornega in stereotipnega vedenja v odprtem polju, ki se lahko prepreči s SCH 23390 (dopaminski antagonist D1).

Sklepali smo, da podaljšano tretiranje z LEK-8829 lahko povečja stimulatorne učinke na vedenje posredovano z dopaminskimi receptorji D1.

Rezultati kažejo v prid hipotezi, da ima dopaminski D1 receptor lahko ključno vlogo v zdravljenju Parkinsonove bolezni in koristne učinke pri zdravljenju psihoz izzvanih z zdravljenjem z dopaminskimi agonisti D2.

Statistična analiza rezultatov

Podatki so analizirani s SPSS programom (SPSS 19.0 for Windows, Chicago, Illinois, USA).

Učinek testnih snovi na rezerpinizirane živali je analiziran z analizo varijance (ANOVA) s Scheffejevim testom.

Diskusija

Sistem dopaminskih receptorjev D2 je vpleten v spremembe gibalne aktivnosti, učenja in spomna, kot tudi v patofiziologijo nevropsihiatričnih motenj kot sta Parkinsonova bolezen (PD) in shizofrenija.

Iz raziskav je znano že, da različna obdobja zdravljenja z antipsihotiki in praznjenje dopaminskih zalog z rezerpinom povzroči povečanje števila dopminskih receptorjev D2. Prav tako pa rezerpin povzroči razklop sinergizma D1/D2.

Pri raziskavah preobčutljivosti dopaminskih receptorjev (DARSS) se pogosto sklicujejo na dolgotrajno zdravljenje z antipsihotiki. Težave, ki izhajajo iz dolgotrajnega zdravljenja z L-DOPA, so diskinezije, ki verjetno izvirajo iz preobčutljivosti dopaminskih receptorjev D2. Dopaminski receptorji poskusnih živaliso nagnjeni, da postanejo preobčutljivi, s čimer izzovejo nenormalno vedenje, ko jih uporabljamo skupaj z dopaminom ali z agonisti receptorjev.

V litereturi je opisano, da se povečanje števila receptorjev D2 (in razvoj vedenjske preobčutljivosti) prepreči z istočasnim stimuliranjem receptorjev D1. Podoben učinek na vedenjsko preobčutljivost in regulatorno povečanje receptorjev D2 kot ga ima zdravljenje z agonistom D1 je povzročena z subkroničnim (21 dni) dodajanjem haloperidola.

Namen naših poskusov je bil analizirati ali bi LEK-8829 zmanjšal dopaminergično preobčutljivost, ugotoviti vpliv na vedenjske in nevrokemične učinke subkroničnega zdravljenja z LEK-8829 ter ugotoviti kako regulatorno povečanje števila receptorjev D2 vpliva na izražanje izooblik D2L in D2S.

Učinek LEK-8829 je bil testiran na rezerpineskem modelu za Parkinsonovo bolezen. LEK-8829 je pokazal antagonistični učinek na dopaminske D2 in na serotoninske receptorje 5-HT_{2A} in 5-HT_{1A}. LEK-8829 2 mg/kg in haloperidol 0,2 mg/kg sta ekvivalentna odmerka, ki smo jih izbrali na temelju rezultatov iz literature Krisch (1994). Sedanje delo je rezultat subkroničnega zdravljenja z LEK-8829. Narejene so le akutne obravnave z LEK-8829.

Test katalepsije 1. in 21. dan

Haloperidol 2 mg/kg 1. in 21. dne pokaže bolj prepričljiv kataleptični odziv kot haloperidol 0,2 mg/kg, kar je v skladu z literaturo, da haloperidol povzroča katalepsijo odvisno od odmerka. Haloperidol v obeh odmerkih kaže skoraj enak kataleptični odgovor 1. in 21. dne. V literaturi je opisana toleranca do kataleptičnega odziva po subkroničnom zdravljenju. O pomanjkanju tolerance s haloperidolom povzročene katalepsije, ki smo jo

opazili pri naših poskusih, so poročali v nekaterih raziskavah. Pregled po Barnesu (1990) ugotavlja, da odmerek, urnik uporabe zdravil in vedenjski poizkusni pogoji vsi vplivajo na razvoj katalepsije med kroničnim zdravljenjem s haloperidolom. LEK-8829 2 mg/kg kaže podoben učinek katalepsije kot haloperidol 0,2 mg/kg 1. dne, med tem ko LEK-8829 2 mg/kg 21. dne kaže podoben učinek katalepsije kot haloperidol 2 mg/kg (1. in 21. dne). Učinek se verjetno pojavi zato, ker je LEK-8829 še vedno prisoten v krvnem obtoku.

LEK-8829 0,2 mg/kg ne kaže statistično značilne katalepsije. Krivulja je podobna krivulji s fiziološko raztopino (Slika 30).

Test katalepsije 1. in 28. dan

Boljši pogled o vprašanju kataleptičnega odziva smo dobili, ko smo izvedli test katalepsije 7 dni po zadnji injekciji, saj takrat zdravilo ni bilo več prisotno v obtoku. Vse učinke tako lahk pripišemo odzivnosti receptorjev. Izbralismo obdobje 7 dni brez zdravil, ko povečano izraženje rreceptorjev D2 v striatumu doseže največje vrednosti. Haloperidol 0,2 mg/kg daje nekoliko povečan kataleptični odgovor 28. dne kot 21. dne v eksperimentu 1. LEK-8829 2 mg/kg kaže zmanjšanje v kataleptičnem odgovoru 28. dne v primerjavi z 21-dnevnim poskusom v eksperimentu 1. Zato ker v primeru eksperimenta 2, ko zdravila 28. dan ni več v obtoku, dobimo zmanjšan kataleptični odgovor kot pri haloperidolu 0,2 mg/kg, je torej LEK-8829 bolj učinkovit antipsihotik kot haloperidol. Za LEK-8829 pričakujemo manj neželjenih učinkov kot za haloperidol.

Amfetaminski model za shizofrenijo

S poskusi smo potrdili že znano - da haloperidol zmanjšuje z amfetaminom provzročeno hiperaktivno vedenje ter da antipsihotiki pogosto zgubljajo učinkovitost med kroničnim neprekinjenim zdravljenjem. Kronično zdravljenje z antipsihotiki spreminja stanje dopaminergične preobčutljivosti, ki se kaže s povečano preobčutljivosti za psihoze in z vplivom dopaminskih agonistov na psihomotorno aktivacijo. Pri ljudeh to imenujemo "z nevroleptiki provzročena preobčutljivostna psihoza". Za bolnike s shizohrenijo je znano, da so preobčutljivi na dopaminu podobne snovi. V našem poskusu odmerek 2 mg/kg haloperidola močno zavira z ampfetaminom provzročeno hiperaktivnost v primerjavi z odmerkom 0,2 mg/kg, kar je v skladu z dejstvom, da je učinek haloperidol odvisen od odmerka. Po 21. dnevu zdravljenja haloperidol začne izgubljati učinkovitost (zmanjšanje odzivnosti). Prav tako pa haloperidol 0,2 mg/kg povzroča še večjo izgubo učinkovitosti (povečanje v odzivnosti) kot haloperidol 2 mg/kg (Slika 37). To lahko razložimo s povečanjem gostote dopaminskih D2 receptorjev, ki vodi v dopaminsko preobčutljivost, ki je povezana z razklopom sinergizma D1/D2. Znano je, da preobčutljivost dopaminskih receptorjev (kar se odraža z povečanim odzivom) včasih spremlja proliferacija receptorjev. Vendar se preobčutljivost dopaminskih receptorjev lahko zgodi v odsotnosti sprememb v številu dopaminskih receptorjev. Neprekinjeno zdravljenje z antipsihotiki in zaviranje D2 receptorjev povzroča nevroadaptacijo, ki vodijo k zmanjkani učinkovitosti antipsihotikov.

V poskusih smo potrdili, da je za LEK-8829 pomemben razklop sinergizma D1/D2. LEK-8829 2 mg/kg zmanjšuje z amfetaminom povzočeno hiperlokomocijo veliko močneje kot LEK-8829 0,2 mg/kg (Slika 35). Niti en odmerek LEK-8829 ne izgublja učinkovitosti (poveča odzivnost) po 21. dnevu. To potrjuje hipotezo (Braun et al., 1997), da se učinek kroničnega zaviranja receptorja D2 lahko spreminja s hkratno stimulacijo receptorja D1.

Ko je selektivni antagonist receptorjev D2 v kombinaciji z agonistom selektivnim za receptorje D1, je proliferacija in vedenjska preobčutljivost receptorja D2 popolnoma zavrta. Zato domnevamo, da je v naših poskusih intrinzična aktivnost LEK-8829 na dopaminskih receptorjih D1 sklopljena s povečanim izražanjem dopaminskih receptorjev D2, kot se dogaja po podaljšanem zaviranju dopaminskih receptorjev D2 s haloperidolom.

Naša predpostavka je, da je zmanjšanje učinkovitosti haloperidola za inhibicijo z ampfetaminom provzročene lokomotorne aktivnosti posledica povečanega izražanja dopaminskih receptorjev D2, ki se je razvili v času dolgotrajnega zdravljenja s haloperidolom. Medtem pa dolgotrajno zdravljenje z LEK-8829 ni imelo takšnega učinka verjetno zaradi odsotnosti regulatornega povečanja receptorskih molekul.. Naši rezultati tudi kažejo, da dolgotrajno zdravljenje poskusnih živali z LEK-8829, ne kaže razvoja funkcionalnega razklopa dopaminskih receptorjev. Tako se je izkazalo, da je LEK-8829 boljši antipsihotik kot haloperidol, saj ima manj neželjenih učinkov (test katalepsije) in ne kaže vedenjske preobčutljivosti (zmanjšuje z amfetaminom provzročene hiperaktivnosti in po 21. dnevnem zdravljenju). Sklepamo, da so receptorji D1 pomembna tarča za izboljšanje negativnih in kongnitivnih funkcij pri shizofreniji.

Model za Parkinsonovo bolezen

Prikazani rezultati z LEK-8829 so enaki kot rezultati s aripriprazolom. LEK-8829 provzroča manjšo katalepsijo kot haloperidol. Poleg tega LEK-8829 zavira z amfetaminom provzročeno lokomocijo brez izgube učinkovitosti pri dolgotrajnem zdravljenju. V skladu z rezultati je utemeljena domneva, da je LEK-8829 delni agonist receptorjev D2.

Pri izpraznjenih zalogah dopamina z rezerpinom LEK-8829 kaže, maksimalan lokomotorni odgovor. To je neobičajno, saj je bilo predlagano, da naj bi bila mešanica agonista D1/D2 ali kombinacija agonista D1 in D2 agonista bolj učinkovita pri lajšanju simptomov parkinsonizma. Medsebojno delovanje podtipov dopaminskih receptorjev D1/D2 poteka na na vedenjski, farmakološki in biokemični ravni. V osnovi ta medsebojno delujejo bodisi antagonistično ali sinergistično. Medtem ko na nekatera vedenja pretežno vpliva aktivnost receptorjev D1 (to so vzpostavljanje stikov in samouničenje), pa receptorji D2 pretežno vplivajo na lokomotorno aktivnost. Sinergistično delovanje dopaminskih receptorjev se opaža pri lokomociji, stereotipiji, vzpostavljanju stikov, elektrofiziološkem odgovoru, zehanju in plazenja. Bromkriptin (agonist receptorjev D2) kaže, zmerno lokomocijo s krajšim trajanjem v primerjavi z LEK-8829. To pomeni, da bromokriptin (agonist D2) stimulira le receptorje D2 brez pojava sinergizma. LEK-8829 obnavlja sinergizem receptorjev D1/D2, saj je delni agonist D2. Sinergistični učinki med receptorjema D1 in

D2, kot tudi stereotipije provzročene z dodajanjem D1 in D2 agonistov hkrati, so bolj intenzivni kot tisti, ki ga proizvajajo bodisi samo agonist. Kaže da ima v odsotnosti dopamina LEK-8829 lasnosti delnega agonista D2. LEK-8829 2 mg/kg kaže okrepljeno stereotipno vedenje.

Sklepi

LEK-8829 je bolj varen antipsihotik kot haloperidol saj povzroča manj EPS in ne kaže preobčutljivosti.

Naši rezultati kažejo, da agonist D1 zmanjšuje dopaminergično preobčutljivost in regulatorno povečanje števila dopaminskih receptorjev D2. Haloperidol provzroča povečano izražanje dopaminskih receptorjev D2 medtem ko ga LEK-8829 ne povzroča. *In situ* hibridizacija na 6-OHDA modelu pokaže, da se poveča izražanje izooblike D2S dopaminskih receptorjev D2.

Z metodo prenosa po Westernu nam je uspelo ločiti izoobliki D2L in D2S dopaminskih receptorjev D2, ni pa nam uspelo zagotovo potrditi povečano izražanje receptorjev D2 ter Nova 1, saj so primarna mišja protitelesa reagirala s podganjimi IgG, katerega velikost je okoli 50 kDa.

Na naše presenečenje rezultati kažejo, da je LEK-8829 delni agonist D2. Zaradi te lasnosti ima zmožnost, da stabilizira občutljivost na dopamin. LEK-8829 ima kot delni agonist D2 manjšo nagnjenost za povzročanje EPS, zavira z amfetaminom provzročeno lokomocijo brez izgubljanja učinkovitosti po dolgotrajnem zdravljenju, je bolj učinkovit pri lajšanju simptomov parkinsonizma kot bromokriptin (D2 agonist) ter ima stabilnejšo občutljivost na dopamin.

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