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Mgr. Branislav Krajčovič

Protective effect of pro-cognitive training during adolescence on neuronal coordination deficit in a pharmacological model of schizophrenia

Protektívny vplyv kognitívneho tréningu počas adolescencie na deficit neuronálnej koordinácie vo farmakologickom modeli schizofrénie

Diploma thesis

Supervisor: RNDr. Štěpán Kubík, PhD.
Institute of Physiology AS CR
Department of Neurophysiology of Memory

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PREHLÁSENIE

Prehlasujem, že som záverečnú prácu vypracoval samostatne na základe konzultácií so školiteľom a že som uviedol všetky použité informačné zdroje a literatúru. Táto práca ani jej podstatná časť nebola predložená k získaniu iného alebo rovnakého akademického titulu.

V Prahe dňa 13.8.2017 Mgr. Branislav Krajčovič

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ABSTRAKT

Schizofrénia je závažné neuropsychiatrické ochorenie charakterizované pozitívnymi, negatívnymi a kognitívnymi symptómami, ktoré narúšajú fungovanie jedinca v spoločnosti a predstavujú tak enormnú záťaž pre neho samotného, jeho blízkych aj spoločnosť. Napriek tomu, že kognitívny deficit tvorí integrálnu súčasť schizofrénie a je najsilnejším prediktorom pre fungovanie v spoločnosti, neexistuje preň žiadna zavedená liečba. Vyhýbanie sa skrytému miestu na plynulo rotujúcej aréne (Carousel) vyžaduje kognitívnu kontrolu a je animálnym modelom kognitívnej koordinácie informácií z disociovaných priestorových rámcov, ktorá je narušená v akútnom farmakologickom aj neurovývinovom modeli schizofrénie u potkana. Kognitívny tréning na rotujúcej aréne počas adolescencie výrazne zmierňuje kognitívny deficit počas dospelosti v neurovývinovom modeli schizofrénie u potkana a zlepšuje i neurálnu koordináciu (oscilácie v beta a gama pásme), ktorá je nevyhnutným predpokladom kognitívnych procesov.

Zisťovali sme, či kognitívny tréning počas adolescencie odstráni deficit neuronálnej koordinácie pozorovaný po akútnom systemickom podaní antagonistu NMDA receptorov MK-801 v dospelosti. Časť potkanov bola počas adolescencie trénovaná v úlohe Carousel, druhá časť podstúpila iba handling. Dospelé potkany boli dvakrát po 5 minút vystavené buď rovnakému (A/A) alebo dvom odlišným prostrediam (A/B) za účelom indukovania expresie časných génov (IEGs) v CA1 oblasti hipokampu. Následne sme mozgy analyzovali s využitím expresie časných génov *Arc* a *Homer 1a* (catFISH) k mapovaniu neuronálnych populácii aktívnych počas dvoch explorácií.

V súlade s predošlými výskumami sme pozorovali, že explorácia signifikantne zvyšuje, a MK-801 znižuje expresiu časných génov *Arc* a *Homer 1a*. Taktiež sme potvrdili predošlé výsledky ukazujúce u kontrolných zvierat zvýšenú podobnosť medzi CA1 populáciami neurónov aktivovaných pri opakovanej explorácii rovnakého prostredia (A/A), v porovnaní s podobnosťou neuronálnych populácii v A/B. Rozdiel v miere podobnosti neuronálnych populácii za podmienok A/A a A/B bol signifikantý, nedosahoval však takého výrazného rozdielu ako u iných autorov. Naša hlavná hypotéza bola, že podobnosť neuronálnych populácii aktívnych za A/B-typu explorácie u potkanov s MK-801 bude nižšia u trénovaných, v porovaní s netrénovanými potkanmi. Pozorovali sme viac ako dvojnásobné zníženie podobnosti neuronálnych populácii aktívnych za A/B podmienok u trénovaných potkanov oproti netrénovaným. Tento rozdiel však nedosiahol štatistickej významnosti, čo pripisujeme kombinácii nižšieho počtu použitých zvierat a nevyhovujúcich podmienok počas fázy indukovania expresie časných génov. Naše výsledky ale naznačujú, že kognitívny tréning v adolescencii môže pôsobiť protektívne na neuronálnu koordináciu v MK-801 animálnom modeli schizofrénie.

Kľúčové slová: kognitívna kontrola, kognitívna koordinácia, , neuronálna koordinácia, priestorová navigácia, rotujúca aréna, Carousel, animálny model, schizofrénia, nekompetitívny NMDA antagonista, MK-801, dizocilpin, časné gény (IEG), Arc, Homer1a, catFISH

ABSTRACT

Schizophrenia is a severe neuropsychiatric disorder characterized by positive, negative and cognitive symptoms with poor functional outcomes, placing an enormous burden on the individual, caregivers and society. Although deficits in cognition are an integral part of the disease and the best predictor of functional outcomes, there is as yet no established treatment addressing them. Avoidance of a hidden place on a continuously rotating arena (Carousel) requires cognitive control and is a rodent model of cognitive coordination of information from dissociated spatial frames, which is impaired in acute pharmacological and neurodevelopmental model of schizophrenia. Cognitive training on the Carousel during adolescence alleviates adult cognitive deficit in a neurodevelopmental model of schizophrenia and improves neural coordination (oscilations in the beta and gamma band), which is thought to be necessary for cognition.

We examined if cognitive training during adolescence eliminates the deficit in neuronal coordination observed in adult rats after acute systemic NMDA receptor antagonist MK-801 (0.15 mg/kg). During adolescence, rats were either trained in spatial avoidance on Carousel or merely handled. As adults, rats received two 5-min exploration sessions in the same (A/A) or in two distinct environments (A/B) to induce immediate-early gene (IEG) expression in hippocampal CA1 neurons. We analysed the brains using expression of IEGs *Arc* and *Homer 1a* (catFISH) to map neuronal populations (ensembles) activated during two exploratory sessions.

Consistent with previous research, exploration of a novel environment significantly increased and MK-801 decreased expression of IEGs *Arc* and *Homer 1a*. We also replicated the finding that in saline-treated animals, CA1 ensembles activated by exploring same environment twice (A/A) are more similar than ensembles activated by A/B. The difference in A/A to A/B similarity we observed was significant, but not as stark as reported by other authors, a difference that we ascribe to the suboptimal housing conditions of rats and noise during IEG-induction phase of the experiment. As to our main hypothesis, that similarity of A/B acitvated ensembles in MK-801-treated rats would be lower in trained animals, similarity was decreased more than two-fold in trained animals compared to non-trained controls. The difference, however, did not reach significance, which we ascribe to small number of subjects we used combined with suboptimal conditions during IEG-induction phase of the experiment. Overall, our results are suggestive of protective effect of adolescent cognitive training on neuronal coordination in acute systemic MK-801 rat model of schizophrenia.

Key words: cognitive control, cognitive coordination, neural coordination, spatial navigation, rotating arena, Carousel, animal model, schizophrenia, non-competitive NMDA antagonists, dizocilpine (MK-801), immediate-early genes (IEG), Arc, Homer1a, catFISH

LIST OF ABBREVIATIONS

A/A – animal explored twice the same environment
AAPA – active allothethic place avoidance task, a variant of the Carousel task
A/B – animal explored two different environments
AC – adenylate cyclase
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA – analysis of variance
AOBS – acousto-optical beam splitter
Arc – activity-regulated cytoskeleton-associated protein (gene)
Arc – activity-regulated cytoskeleton-associated protein (protein)
Arg3.1 – Arc, for activity-regulated cytoskeleton-associated protein
BC – basket cell
BDNF – brain-derived neurotrophic factor
BLA – basolateral amygdala
bp – base pair
CA – cornu Ammonis
cAMP – cyclic-adenosine monophosphate
Carousel – circular arena, apparatus for the place avoidance task
Carousel task – avoidance task on a circular arena
catFISH – cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization
CC – cage control animal
CFC – contextual fear conditioning
COX-2 – cyclooxygenase-2 (gene)
CY3 – cyanine dye 3
DAPI – 4',6-diamidino-2-phenylindole
DG – dentate gyrus
DISC1 – disrupted in schizophrenia 1 (gene)
Dizocilpine – see MK-801
DLPCF – dorsolateral prefrontal cortex
DMT – N,N-Dimethyltryptamine
EC – entorhinal cortex
EDTA – ethylenediaminetetraacetic acid
EEG – electroencephalogram
epoch – exploratory session in open field during catFISH bevioural protocol
ERK – extracellular signal–regulated kinases (MAPK, mitogen-activated protein kinases)
FF – firing field of a place cell
FISH – fluorescence *in situ* hybridization
FITC – fluorescein isothiocyanate
fMRI – functional magnetic resonance imaging
GABA – γ-aminobutyric acid
GABAR – γ-aminobutyric acid receptor
Gad1 – Glutamate decarboxylase, isoform 1 (gene)
Gad2 – Glutamate decarboxylase, isoform 1 (gene)
GAD_{65} – Glutamate decarboxylase, isoform 65 (protein)
GAD_{67} – Glutamate decarboxylase, isoform 67 (protein)
GAT1 – GABA transporter 1
GC – granule cell
GlyT1 – glycine transporter 1
GSH – glutathione, γ-L-Glutamyl-L-cysteinylglycine
H1a – *Homer1a*
Homer1a – gene
Homer1a – protein
HPA – hypothalamo-pituitary-adrenocortical axis
HRP – horseradish peroxidase
i.p. – intraperitoneal

IEG – immediate-early gene
INF – intranuclear foci
IR – infrared light
kb – kilobase
LED – light emmiting diode
LSD – lysergic acid diethylamide
LTD – long-term depression
LTP – long-term potentiation
MEG – magnetoencephalogram
mf – mossy fibers
mGluR – metabotropic glutamate receptor
MK-801 – (+)-5-methyl-10,11-dihydro-5*H*-dibenzocycloheptene-5,10-imine maleate; dizocilpine
MTL – medial temporal lobe
NAAG – N-acetyl-aspartyl-glutamate
NAC – N-acetylcysteine
Narp – neuropathy, ataxia, and retinitis pigmentosa (gene) NMDA – N-methyl-D-aspartate
NMDAR – N-methyl-D-aspartate receptor
NR – NMDA receptor subunit
NVHL – neonatal ventral hippocampus lesion model of schizophrenia
OCT – optimal cutting temperature medium
PAM – postitive allosteric modulator
PCIA – phenol-chlorophorm-isoamlyalcohol extraction
PCP – phencyclidine
PD – postnatal day
PET – positron emission tomography
PFC – prefrontal cortex
PKA – protein kinase A
PKC – protein kinase C
pp – perforant pathway
PPI – prepulse inhibition
PSD – postsynaptic density
PV – parvalbumine
PV+ – parvalbumine postive neuron
PVBC – parvalbumine postive basket cell
PVN – paraventricular nucleus of the hypothalamus (PVN)
RDH – right dorsal hippocampus
rm-ANOVA – repeated measure analysis of variance
RTF – regulatory transcription factor
SEM – standard error of the mean
SNP – single nucleotide polymorphism
SSC – saline sodium citrate
TBS – Tris-buffered saline
TBS-T – Tris-buffered saline with Tween
Tris – trisaminomethane
TSA – tyramide signal amplification
Tween 20 – polysorbate 20, polyethylene glycol sorbitan monooleate
UTR – untranslated region
WLL – white light laser

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THEORETICAL PART

1 INTRODUCTION

Schizophrenia is a severe neuropsychiatric disease of complex aetiology, which involves disruptions in multiple brain systems. Both genetic and environmental factors play important roles in development of the disease. Current theories of schizophrenia pathophysiology (MacDonald and Schulz, 2009) involve alterations in multiple neurotransmitter systems (glutamatergic (Coyle, 2006; Howes et al., 2015), GABAergic (Nakazawa et al., 2012), dopaminergic neurotransmission (Howes and Kapur, 2009)) that result from an interaction of genetic predispositions (Ripke et al., 2014), environmental risk factors, immune system (Khandaker et al., 2015; Meyer, 2013) and neural development (Fatemi and Folsom, 2009; Insel, 2010). Abnormalities are observed in brain structure (Shenton et al., 2001), connectivity and neural coordination including neural oscillations (Schmitt et al., 2011; Stephan et al., 2009). Dysfunctions in cognition (Barch and Ceaser, 2012) are especially relevant for functional outcomes (Bowie and Harvey, 2006; Millan et al., 2016). Although above-mentioned theories of schizophrenia differ in emphasis put on various explanatory factors, they are not mutually exclusive. This can be embraced as an upcoming synthesis of disparate findings. On the other, the lack of exclusivity can be seen as a shortcoming hindering progress. In context of strong inference, contrasting predictions of alternative theories should be generated (Cannon, 2009) to allow crucial experiments (Platt, 1964).

Glutamatergic theory of schizophrenia has become a prominent framework in the field (Coyle, 2006, 2012). The theory proposes that disruption of the glutamatergic system resulting from neurodevelopmental alterations, particularly of NMDA receptors on inhibitory interneurons, critically contributes to the pathophysiology of schizophrenia. Hypofunction of NMDA receptors on GABAergic interneurons may lead to disinhibition of pyramidal neurons, disrupt neural coordination, and result in cognitive deficit and poor functional outcomes with enormous personal, social, and economic burden. Figure 1 shows risk factors, relevant developmental processes and time-course of schizophrenia. This theory can be illustrated by a metaphor of hourglass. At the top is a broad range of genetic and environmental risk factors. These factors ultimately converge on NMDA receptors, which susequently dirupts dopaminergic system. Then the hourglass widens again as NMDAR hypofunction gives rise to other mal/adaptive changes such as structural alterations, changes in neural network activity, gene expression and diverse symptomatology diplayed in patients. The top and bottom of the hourglass is wide, representing great interindividual differences, while the hypofunction of the NMDARs is, presumably, common to all patients.

Our hypothesis, that adolescent pro-cognitive training has protective effect on neuronal coordination of hippocampal ensemble activity patterns in adulthood in an animal model of schizophrenia by acute administration of non-competitive NMDAR antagonist, is rooted in the glutamatergic theory. The experimental part of this thesis was motivated by studies of Kubík (2014), Lee (2012), Fenton (2008), Guzowski (1999) and Vazdarjanova (2002).

2 SCHIZOPHRENIA

2.1 Cognitive symptoms and functional outcomes

Schizophrenia is a severe mental illness characterized by positive (hallucinations, delusions, conceptual disorganization), negative (loss of motivation, reduced anticipatory pleasure, social withdrawal, reduced spontaneous speech, affective flattening, alogia) and cognitive symptoms (a broad spectrum of cognitive functions including attention, executive functions, working and episodic memory, processing speed and social cognition) (Barch, 2005; Barch and Ceaser, 2012; Green et al., 2015; Owen et al., 2016; Tandon et al., 2009). Epidemiological data (McGrath et al., 2008; Tandon et al., 2008) shows an average annual incidence of 15 per 100 000 (which, contrary to previous interpretations, shows fivefold variability, mainly depending on uffigurbanicity, economic status and latitude), average point prevalence of 4.5 per 1000, and lifetime morbidity risk of 0.7% with slightly higher (1.4) risk for males than females. Ratio for males to females is 1.4 to 1. Onset of the illness is usually in late adolescence or early adulthood (earlier in males) with the average age of onset being 25 (Shaner et al., 2007; Tandon et al., 2009). Life expectancy is decreased by 15 years on average (Crump et al., 2013; Laursen et al., 2014; McGrath et al., 2008) and the risk of dying from all causes is increased 2-3 fold (Brown et al., 2010; McGrath et al., 2008; Saha et al., 2007). Although risk of suicide is approximately 13-20 fold higher (Laursen et al., 2014; Reininghaus et al., 2015) compared to general population (lifetime risk of 5% in schizophrenia patients (Hor and Taylor, 2010)), most of the excessive mortality is due to physical illneses, such as ischemic heart disease and cancer. These serious comorbidities are often not diagnosed despite the fact that people with schizophrenia have twice as many health care contacts (Crump et al., 2013), showing that physical health of patients with mental disorders is often neglected.

Approximately 10-15 % of patients recover fully after their first episode and are able to lead relatively normal lives. More than 50 % experience intermittent but long-term psychiatric problems and around 15 % develop chronic symptomatology and disability (Millan et al., 2016; Owen et al., 2016; Walker et al., 2004). The severity of positive and negative symptoms fluctuate over time, while cognitive dysfunctions remain relatively stable (Bowie and Harvey, 2006). Quality of life is affected more in first episode patients than in chronic patients (Katschnig, 2000; Krajčovič, 2012; Priebe et al., 2000) and also significantly in their caregivers (Caqueo-Urízar et al., 2009; Foldemo et al., 2005). Deinstitutionalization (Krajčovič, 2010; Lamb and Bachrach, 2001) rendered families and nonprofit organizations the primary caregivers (Awad and Voruganti, 2008; Caqueo-Urízar and Gutiérrez-Maldonado, 2006; Jungbauer et al., 2004). Unemployment in people with schizophrenia reaches 80-90 % (Marwaha and Johnson, 2004; Owen et al., 2016) and socioeconomic and personal losses in the form of direct costs (expenditures associated with treatment), indirect costs (years of lost productivity), and intangible costs (lost potential and pain) are enormous (Chong et al., 2016; Knapp et al., 2004).

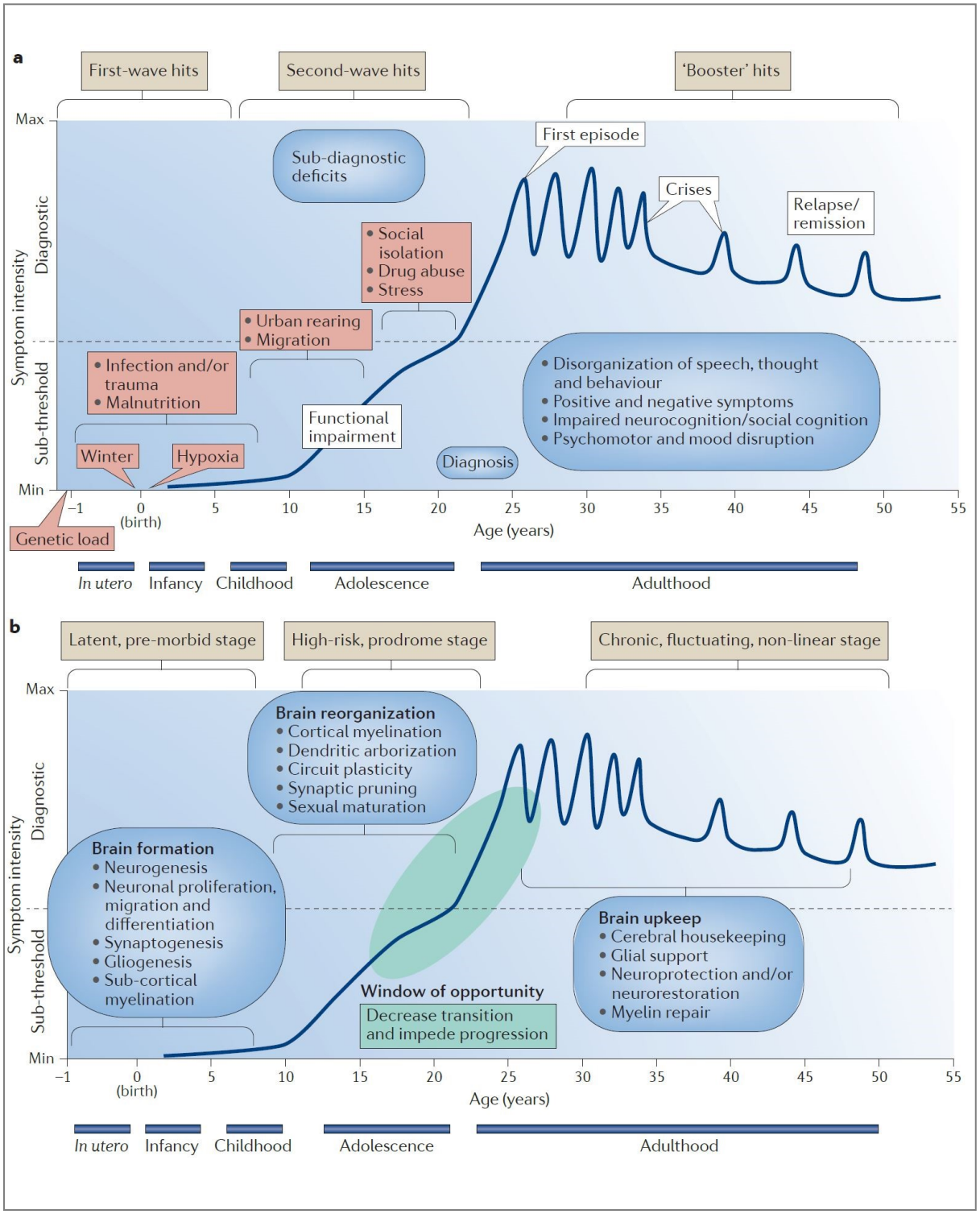

Figure 1. Onset and progression of schizophrenia in relation to risk factors and affected developmental processes
a: Risk factors and course of illness according to the intensity of symptoms.
b: Fundamental processes of brain development, maturation and upkeep in relation to the 'stages' of schizophrenia.
(from Millan et al., 2016)

Deficits in functional outcome are present in most people with schizophrenia (Keefe et al., 2005) and affect a wide range of life domains such as social functioning, employment, and independent living (Harvey et al., 2004), and therefore they represent a critical treatment target for patients, families, and society. Although the relationships among different symptoms, underlying pathologies, functional deficits, and real-world functional outcomes are complex, severity of cognitive deficits is the best predictor of functional outcome (Bowie and Harvey, 2006; Green, 1996, 2000, Green et al., 2004, 2015; Green, 2016; Lesh et al., 2011) .

Cognitive dysfunctions are at the core of schizophrenia (Frith, 1992; Kahn and Keefe, 2013; Minzenberg and Carter, 2012; Moskowitz and Heim, 2011; O'Carroll, 2000) because they are often present years before the first psychotic episode, they persist during phases of remission of psychosis, they are observed in most patients, they predict functional outcome, and they are also commonly found in less severe forms in unaffected relatives. The neurodevelopmental nature of schizophrenia (Catts et al., 2013; Fatemi and Folsom, 2009; Insel, 2010; Rapoport et al., 2012), signified by its onset in late adolescence, emphasize the importance of reliable early detection and intervention (Larson et al., 2010; Millan et al., 2016; Schultze-Lutter, 2009; Yung and McGorry, 1996). Although cognitive remediation and non-pharmacological therapies have beneficial effects on cognition (Medalia and Choi, 2009; Subramaniam et al., 2012; Wykes et al., 2011), there is as yet no established therapy for cognitive dysfunctions (Goff et al., 2011; Minzenberg and Carter, 2012). Therefore, the search for underlying mechanisms and effective treatments of cognitive deficits in schizophrenia is of utmost importance.

2.2 Cognitive control as a core cognitive deficit in schizophrenia

As already mentioned, a wide variety of cognitive functions is impaired in schizophrenia (Barch, 2005; Barch and Ceaser, 2012; Reichenberg and Harvey, 2007) including selective attention (Luck and Gold, 2008), working memory (Barch and Smith, 2008), episodic memory (Ranganath et al., 2008), processing and psychomotor speed, language production, reasoning, planning, comprehension and executive functions (Kerns et al., 2008). A parsimonious explanation of these diverse cognitive impairments is that they result, at least in part, from deficit in **cognitive control** (Barch and Ceaser, 2012; Lesh et al., 2011). Cognitive control is a high level cognitive process that integrates multiple processes (Ridderinkhof et al., 2004) and has been described as the ability to use information relevant to the current goal and ignore irrelevant and misleading information (Amer et al., 2016; Kelemen and Fenton, 2010; Lee et al., 2012; Miller and Cohen, 2001; O'Reilly et al., 2014). Alternatively, it has been defined as „the overarching ability to maintain the context for appropriate behavior in a given situation in the face of interference" (Lesh et al., 2011, p. 318). Context consists of current goals and relevant stimuli that could change on a moment to moment basis, therefore continuous updating is critical. Interference can be caused by irrelevant or ambiguous stimuli 'out of context' or by prepotent

habitual responses, which are not appropriate in the current context and interfere with the correct response selection. Impaired cognitive control would therefore lead to deficits in many diverse cognitive functions listed above and there is ample evidence thereof (Amer et al., 2016).

Cognitive control deficits are present in most schizophrenia patients (Kerns et al., 2008; Laurenson et al., 2015). One paradigmatic test of cognitive control (O'Reilly et al., 2014) is the **Stroop task** (Laurenson et al., 2015; MacLeod, 1991; Stroop, 1935), in which the subject is presented with a name of a colour that is written either in congruent or incongruent ink colour. The task is to name the colour of the ink, in which the word is written, and ignore the meaning of the word. If the ink is the same colour as is described by the word, then the situation is a 'low control' condition, requiring low level of cognitive control. If the ink colour is different than the word meaning, then the level of cognitive control necessary to name the ink colour is in the 'high control' condition. This is because the prepotent (and incorrect) response is to read the meaning of the word and ignore the colour in which it is written and this tendency has to be overridden by means of cognitive control processes (Lesh et al., 2011). Error rate in 'high control' condition is larger in patients with schizophrenia than in healthy control subjcts, whereas both groups perform equally in 'low control' condition (Perlstein et al., 1998). This pattern of impairment suggests it is caused by inability to attend to the task relevant stimulus dimesion (ink colour) (Barch et al., 2004) and therefore by reduced cognitive control. At the biological level, cognitive control relies on proper activation of the preforntal cortex (PFC) (Funahashi, 2001; Miller and Cohen, 2001) and especially its dorsolateral part (DLPCF) (MacDonald et al., 2000), which biases information processing and response selection in favour of the appropriate response. In line with the cognitive coordination account, patients with schizophrenia show reduced activation of DLPFC during tasks requiring cognitive control (Hill et al., 2004).

2.3 Animal model of cognitive coordination: Place Avoidance on the Carousel

It is possible to model cognitive control in animals by the use of **Place Avoidance on a Rotating Arena**, previously also known as Active Allothetic Place Avoidance (AAPA) (Stuchlik et al., 2013). Since the rotating arena is known as Carousel, we will sometime refer to the place avoidance on a rotating arena simply as the Carousel task. The apparatus consists of a circular (82 cm in diameter), continuously rotating (1 rpm) metallic arena equipped with transparent walls so that rats are able to see cues outside the arena. The room is softly lit with dispersed light. In the standard condition, a rat has to avoid an unmarked 60 degree sector, which is defined relative to the room-frame (Room+Arena–avoidance). If the rat enters this sector, it recieves a mild electric footshock via current-controlled circuit. The current (0.3 to 0.7 mA) is delivered via alligator clip connected to a subcutaneous electrode between the rats shoulders and grounded arena floor. The current is set individually to a level aversive enough to motivate avoidance, but not to cause freezing behaviour, which would interfere with place avoidance learning and could result in learned helplessness (Seligman and Beagley, 1975)

(which is a model of depressive behaviour, not cognitive control). The rats carry a 'backpack' with infrared (IR) LEDs and its trajectory is recorded by an overhead IR camera and passed to a behavioural software (iTrack, Biosignal Group, USA) which tracks animals position, delivers shocks and store data for off-line analysis. The number of entrances to the shock sector, maximum time of avoidance, latency to the first entrance, and proportion of time spent in the shock sector are used to evaluate the avoidance and total distance travelled by the animal is used assess general activity (locomotion). In the default version, the rat has access to the arena-frame stimuli (visual, olfactory, and tactile such as scent marks (Wallace et al., 2002), urine and feces) and also to distal visual cues in the room-frame. Another important set of cues are cues generated by self-motion (proprioception, vestibular signals and optic flow) used for idiothetic navigation. Navigation by external cues (visual, auditory, olfactory, tactile) is called allothetic. Rotation of the arena dissociates two independent frames of reference available for navigation, but only one is relevant for successful avoidance of the shock sector in the **Room+Arena–** (room-defined sector only) and **Arena+Room-** (arena-defined sector only) conditions with **high** demand for cognitive **control**. The rats have to attend to relevant and ignore irrelevant and potentially misleading information, maintain the context (goal and rules) of the task and overcome prepotent responses to avoid successfully. Conflict between cues from dissociated frames may be eliminated by selectively hiding cues from one frame (**Arena+** avoidance in darkness) or by stopping the rotation **(Room&Arena)+** in control conditions with **low** demand for cognitive **control**. In shorthand notation (Stuchlik et al., 2013), these conditions are named according to the availability of cues from either frame (**Room** and/or **Arena**) and their relevance (+ or –) for the desired avoidance. Possible conditions and respective notations are illustrated in Figure 2. This makes the Place Avoidance task analogous to the Stroop task used in humans (O'Reilly et al., 2014) in that it allows comparison of 'high' and 'low' cognitive control demands. Whereas hiding room cues in darkness is straightforward, hiding the arena cue is less trivial because they include proximal external cues as well as idiothetic information derived from self-motion. Substratal cues on the arena surface may be hidden by covering the arena with shallow (<1 cm) water in the **Room+** avoidance, but cues generated by self-motion cannot be hidden or eliminated. Therefore, Room+ avoidance is not a 'low control' condition in a strict sense, but rather a 'reduced' cognitive control demands somewhere between high and low control conditions. The highest demand for cognitive control is presented in the **Room+Arena+** double frame avoidance where animals have to avoid a two shock sectors, one in each frame. Foraging for randomly distributed food (cocoa puffs, Nesquik, Nestlé) may be used to motivate locomotion, prevent passivity, and facilitate comparisons between different versions of the task. Place avoidance in high cognitive control conditions is impaired in multiple established lesion (Lee et al., 2012), neurodevelopmental (O'Reilly et al., 2014) and pharmacological (Kubík et al., 2014) animal models of schizophrenia.

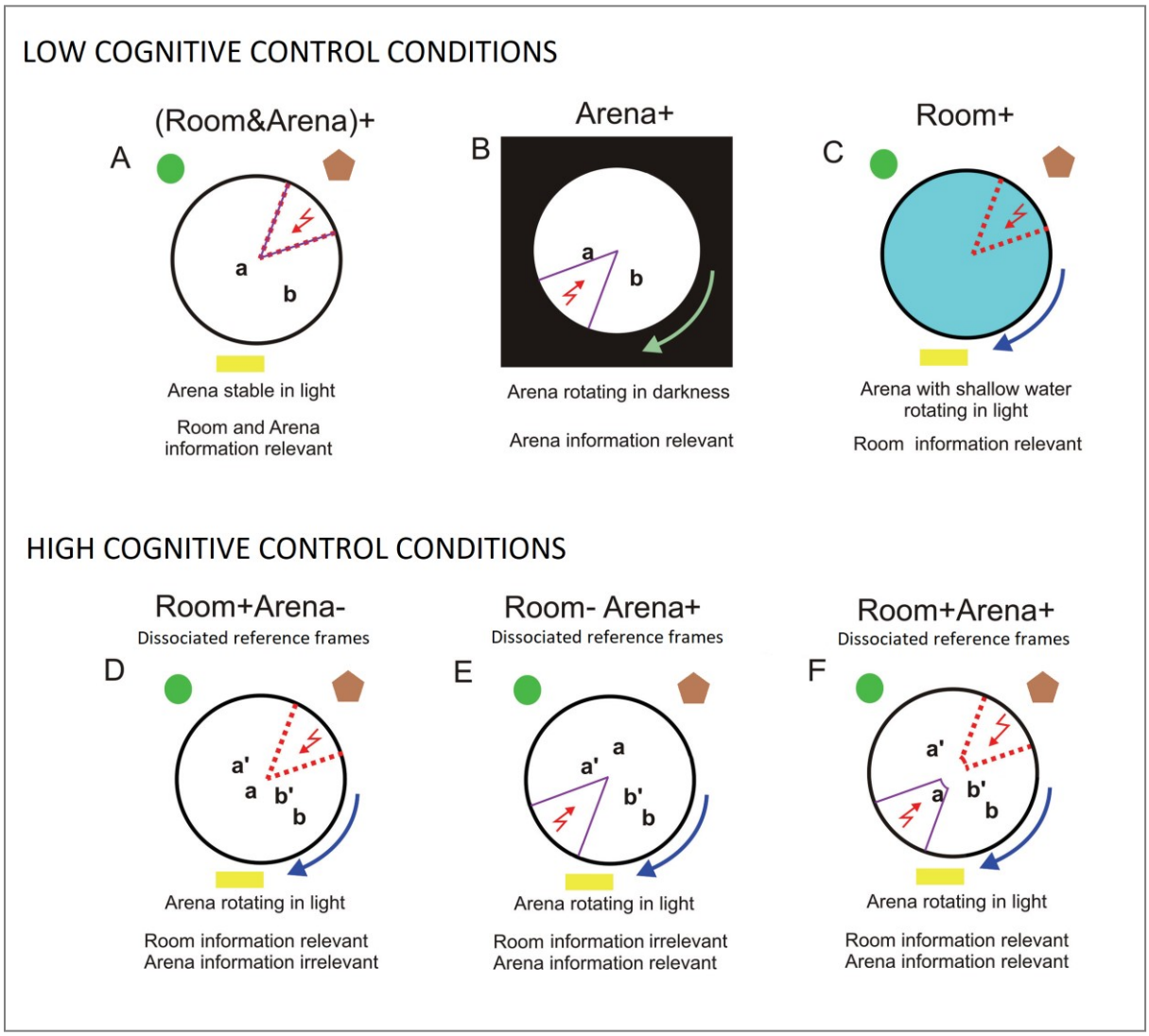

Figure 2. Variants of the place avoidance task.
Symbols outside the arena represent extramaze cues. Conditions A, B, C place low demands on cognitive control processes. Conditions D, E, F require high level of cognitive control. **A: (Room&Arena)+ avoidance.** Arena is not rotating, cues from both frames of reference are available and relevant and there is no conflict from between reference frames. Foraging is necessary to prevent passivity. **B: Arena+ avoidance.** Arena is rotating in darkness, no room cues are available and shock is defined in the arena-frame, therefore no information conflict is present. Forging is necessary to prevent passivity. **C: Room+ avoidance.** Arena is rotating in light but its surface is covered in shallow water (~1 cm), thus substratal cues are hidden and conflict between dissociated reference frames is attenuated. **D: Room+Arena– avoidance (active allothetic place avoidance - AAPA**). Arena is rotating in light, cues from both frames are available but only room cues predict the shock. Rats must ignore arena-bound cues and actively avoid being brought to the shock sector. Foraging is not required, but improves avoidance performance. **E: Room–Arena+ avoidance.** Similar to condition D but shock sector is defined in the aren-frame. Foraging eliminates solving the task by not moving. **F: Room+Arena+, two-frame (double) avoidance.** Two shock sectors are present, one defined in each frame. Both sectors are truncated from the center of the arena to provide opportunity for escape when rotation brings the two sectors together. Arena is rotating in light, both frames of reference are present and both are relevant. This is the most difficult version of the task. (modified from Stuchlik et al., 2013)

3 PATHOPHYSIOLOGY OF SCHIZOPHRENIA

3.1 Dopaminergic theory

Dopamine hypothesis of schizophrenia, one of the most enduring ideas in psychiatry (Howes and Kapur, 2009), was formulated during 1970's. In the first version, excessive dopaminergic (DA) transmission was proposed as a cause of schizophrenia. Supported by discoveries such that antipsychotic drugs (chlorpromazine and haloperidol) increase dopamine metabolism, that amphetamine (which can produce psychotic symptoms) elevates dopamine levels at synapses, and that reserpine (which shows antipsychotic properties) blocks reuptake of dopamine. But, most importantly, that effectiveness of antipsychotic drugs is directly related to dopamine receptor affinity (ibid.). The second, revised version, proposed that schizophrenia is due to dopaminergic hyperactivity in the nigro-striatal pathway and hypoactivity of dopaminergic projections from basal forebrain (ventral tegmental area – VTA) to the prefrontal cortex. Positive symptoms supposedly result from striatal hyperdopaminergia, and negative symptoms from prefrontal hypodopaminergia (Davis et al., 1991). The most recent, third version broadens its scope from the narrow focus on dopamine alone and includes environmental risk factors, genes and neural development (Howes and Kapur, 2009). Numerous factors presumably converge and cause striatal dopamine hyperfunction with subsequent psychosis. Instead of abnormal function of postsynaptic receptors, dysregulation at the presynaptic level is believed to be the proximal cause of hyperdopaminergic signaling. In contrast to previous version of the dopamine hypothesis, cognitive and negative symptoms are considered to result from dysregulation of multiple neurotransmitter systems. The authors conclude that "rather than being a hypothesis of schizophrenia – version III is more accurately a 'dopamine hypothesis of psychosis-in-schizophrenia'" (Howes and Kapur, 2009). Dopamine's function in schizophrenia might thus be related to psychosis in general, and less to the cognitive and negative symptoms that set schizophrenia apart from other psychoses.

The link between dopamine and reward has long been recognized but the precise nature of this relationship is still unclear. Three hypotheses attempt to explain dopamine's role in reward (Berridge, 2007): 'liking' (DA mediates hedonic impact of reward), learning (DA enhances associative links relevant for future rewards) and 'wanting' (DA mediates the pursuit of reward). Of these, 'wanting' or incentive salience gathers most evidential support (ibid.). An intriguing extension of the dopamine hypothesis (Howes and Kapur, 2009) is that the elevated release of dopamine in schizophrenia increases salience of otherwise neutral stimuli (illustrated in Figure 3). Hallucinations and delusions of psychosis might result from aberrant salience that is combined with pattern- and meaning-seeking and individual's cognitive and sociocultural schemas (ibid.). The state of aberrant salience makes it difficult for the individual to separate relevant from irrelevant or misleading stimuli (Grace, 2016), which is almost the definition of disrupted cognitive control seen in schizophrenia.

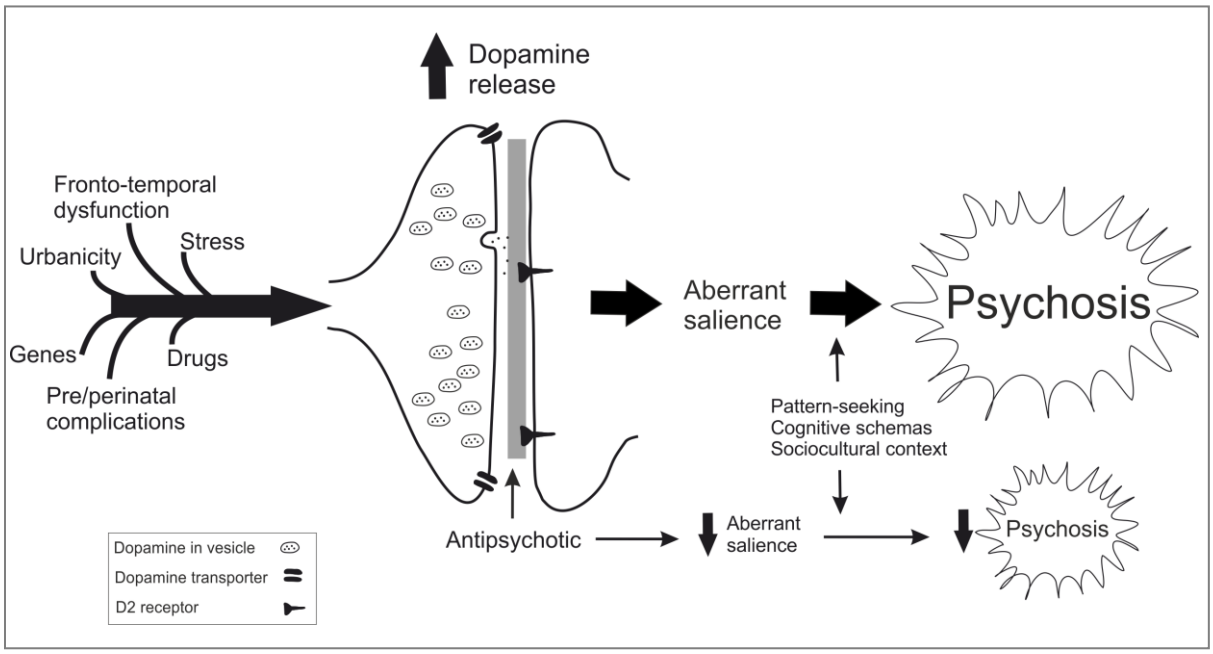

Figure 3. Dopamine hypothesis of psychosis in schizophrenia (version III).
Multiple factors combine and result in striatal dopamine dysregulation. Elevated dopamine levels increase the salience of stimuli and combined with individual's cognitive and sociocultural schemas result in psychosis seen in schizophrenia. The dysregulation of dopamine occurs mainly at the presynaptic level. Current antipsychotics act on the postsynaptic receptors (i.e. downstream of the primary site of dopaminergic dysregulation).
(adapted from Howes and Kapur, 2009)

3.2 Glutamatergic theory

Dopaminergic hypothesis of schizophrenia dominated the field for almost half a century (Coyle, 2012). However, substantial evidence from various lines of research has converged in support of the glutamatergic theory. Dysfunction or dysregulation of N-methyl D-aspartate (NMDA) receptor signaling has been called "the final common pathway to schizophrenia" (Kantrowitz and Javitt, 2010), because many diverse risk factors (genetic and pre-/peri-/postnatal) meet at this crossroad and exert their pathophysiological effects through NMDA receptor (NMDAR) hypofunction. Identification of NMDARs as the crucial node of schizophrenia pathophysiology has not only tied together disparate findings, which could not be previously explained, but also points the way to the development of new treatments (Moghaddam and Javitt, 2012), which are acutely needed, as the drug development has long stagnated (Abbott, 2010). Previous findings of dysregulated dopaminergic transmission can be integrated into the glutamatergic framework as a downstream effect of NMDAR hypofunction and disinhibition on dopaminergic system. This notion is supported by evidence showing that NMDAR hypofunction causes hyperdopaminergia in the ventral striatum and hypodopaminergia in the prefrontal cortex (Breier et al., 1998; Javitt et al., 2004; Smith et al., 1998; Stephan et al., 2009).

In general terms, the glutamate hypothesis of schizophrenia states that hypofunction of NMDARs on inhibitory GABAergic interneurons, caused by genetic and environmental factors during development, disrupts the excitation-inhibition balance and compromises neuronal coordination (Figure 4). NMDAR hypofunction can be modeled in animals by administration of noncompetitive NMDAR antagonists.

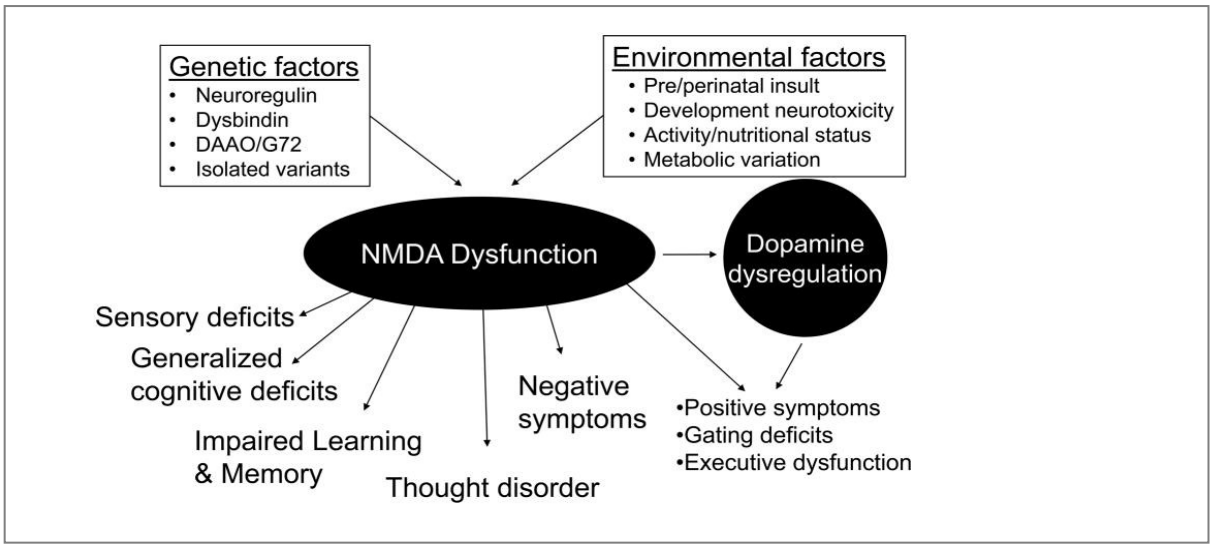

Figure 4. Role of NMDARs in schizophrenia pathophysiology.
Various genetic and environmental factors converge on NMDA receptors and disrupts their function. Subequently, NMDAR dysfunction gives rise to complex symptomatology either directly or via dysregulation of the dopamine system. (from Kantrowitz and Javitt, 2010)

3.3 NMDA receptors

Glutamate, the primary excitatory neurotransmitter in the mammalian brain, acts on two main types of glutamate receptors (GluRs), metabotropic and ionotropic. Ionotropic GluRs are excitatory ligand-gated ion channels divided into four classes depending on their ligand: GluA (AMPA), GluK (kainate), GluN (NMDA), and GluD (δ) receptors (Vyklicky et al., 2014). NMDA receptors are tetramers of three types of subunits, NR1-3. Functional NMDARs have two NR1 subunit and the remaining two subunits consist of NR2, NR3 or their combination. Four different isoforms of NR2 subunits have been reported, NR2A-D, and two isoforms of NR3, NR3A-B. NMDAR subunit composition switches from NR2B to NR2A and from NR3A to NR3B during maturation from juveniles to adults. This may be particularly relevant to typical schizophrenia onset between late adolescence and early adulthood.

NMDARs play a crucial role in synaptic plasticity (changes in synaptic efficacy) and brain development due to their unique properties, but they are also vulnerable to genetic and environmental perturbations (Gao and Snyder, 2013; Spear, 2000). NMDARs are unique in that 1) they are both ligand- and voltage-gated and 2) their permeability for Ca^{2+} ions. NMDAR activation requires binding of two molecules of glutamate (agonist), two molecules of glycine or D-serine (co-agonist) to the Gly/Ser-modulatory site, and a postsynaptic membrane depolarization. At the resting potential, NMDARs' ion channel lumen is blocked by Mg^{2+} ion(Nowak et al., 1984) and depolarization relieves

this blockage. When opened, cations may flow through the NMDAR channel, causing further depolarization. Importantly, NMDARs (unlike most AMPARs) are permeable to Ca^{2+} ions. Calcium ions are major regulators of intracellular signaling including that related to synaptic plasticity, long-term potentiation and depression (LTP and LTD) and memory formation. Activation of the NMDARs is critical for induction of (some forms of) LTP (Miyamoto, 2006), but not for its maintanence and this finding is in accord with the observation, that patients with schizophrenia show deficits in memory formation rather than retention (Kantrowitz and Javitt, 2010). Non-competitive antagonists of NMDAR such as phencylidine (PCP), ketamine, and dizocilpine (MK-801) bind to the PCP-site located inside ion channel which is only available when the channel is opened and therefore these antagonists act in a 'use-dependent' manner (Morris et al., 2005). This has important implications for preferential binding of NMDAR antagonists to NMDARs located on GABAergic interneurons and subsequent disinhibition of pyramidal cells, which I discuss later. MK-801 is a highly selective and very potent noncompetitive NMDAR antagonist (Wong et al., 1986) with deleterious effects on cognition (van der Staay et al., 2011).

The paradox that blocking excitatory receptors (NMDARs) increases excitation is explained by the following consideration. As stated previously, GABAergic interneurons receive excitatory input from pyramidal neurons they inhibit. Therefore, the inhibitory action of GABAergic interneurons is dependent on excitatory input they receive from pyramidal neurons. NMDARs contribute to postsynaptic depolarization of inhibitory interneurons to much larger extent than in pyramidal neurons (levels of AMPARs on PVBCs are low (Morris et al., 2005)). Hence, blocking NMDARs on inhibitory interneurons will cause disinhibition of pyramidal cells. However, since NMDARs are also present on pyramidal neurons, NMDAR antagonists could be expected to reduce their activity, which paradoxically increases. In GABAergic interneurons, NMDARs play greater role in basal synaptic transmission in than in excitatory (pyramidal) neurons. NMDARs on fast spiking GABAergic interneurons are more sensitive to NMDAR antagonists than NMDARs on pyramidal cells, approximately tenfold (Grunze et al., 1996). The reason for this sensitivity is that the depolarization threshold for action potential generation in GABAergic interneurons is lower than in pyramidal cells. Since NMDARs are voltage-gated, more depolarized membrane potential of GABAergic interneurons allow more NMDARs to open on GABAergic interneurons than on pyramidal cells (Moghaddam and Javitt, 2012). In addition, in fast-spiking GABAergic interneurons, NMDARs spent more time open resulting in their greater availability to noncompetitive NMDAR antagonists acting selectively on open channels in a 'use-dependent' manner. Therefore, in the presence of NMDAR antagonists, NMDARs on GABAergic interneurons are blocked to a greater degree than NMDARs on pyramidal cells, resulting in disinhibition of pyramidal cells. It is also possible that the subunit composition of NMDARs differs between pyramidal and GABAergic neurons, and so does their sensitivity to antagonists (Paoletti et al., 2013). Basic mechanism of pyramidal cell disinhibition upon application of MK-801 is illustrated in Figure 5.

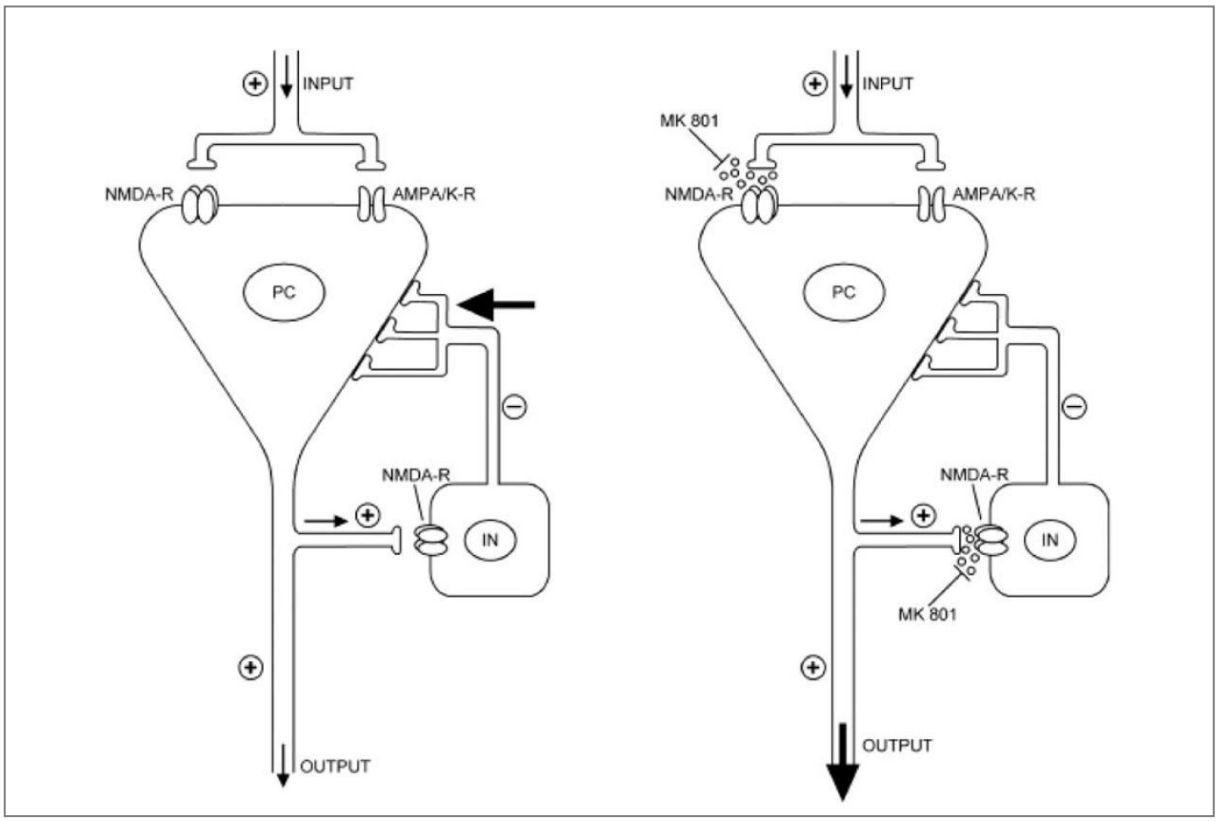

Figure 5. Model of pyramidal cell disinhibition in response to MK-801. The GABAergic interneuron (IN) receives excitatory input from the pyramidal cell (PC) and exerts an inhibitory control by feedback projections to the PC. In presence of the NMDA receptor antagonist MK-801, this local feedback inhibition is disrupted, whereas the excitatory input is sustained via nonNMDA (AMPA/Kainate) receptors, which do not respond to MK-801. Due to this imbalance the total excitatory output will be enhanced.
(from Rujescu et al., 2006)

Acute administration of NMDAR antagonists produces psychotic symptoms strongly resembling schizophrenia in healthy people and exacerbates symptoms in patients with schizophrenia. This is perhaps the strongest set of arguments for the glutamate hypothesis. Abuse of PCP causes symptoms very similar to schizophrenia, including negative and cognitive symptoms, that do not occur after psychostimulants acting on dopamine receptors (amphethamines) (Javitt, 1991) or hallucinogens acting on serotonine receptors (LSD) (Kantrowitz and Javitt, 2010). Administration of subanesthetic doses of ketamine to healthy volunteers induces full range of symptoms associated with schizophrenia (Krystal et al., 1994). Administration of ketamine to stabilized patients with schizophrenia leads to exacerbation of their unique positive symptomatology (Lahti et al., 1995, 2001), whereas stimulants or halluciogens (amphetamine, LSD, DMT) do not induce these individually specific symptoms (Bubeníková-Valešová et al., 2008).

3.4 Animal models of schizophrenia based on NMDAR hypofunction

NMDAR hypofunction mimics key features of schizophrenia also in animals. Jones (2011) sumarized number of changes that have translational relevance to core symptom domains of schizophrenia in animal models (Figure 6). NMDAR hypofunction animal models show many alterations in these domains. Therefore, models based on NMDAR hypofunction show phenomenological, constructive and predictive validity (Adell et al., 2012; Bubeníková-Valešová et al., 2008). Comparison of clinical symptoms of schizophrenia with schiophrenia-like behaviour in animls is shown in table 1.

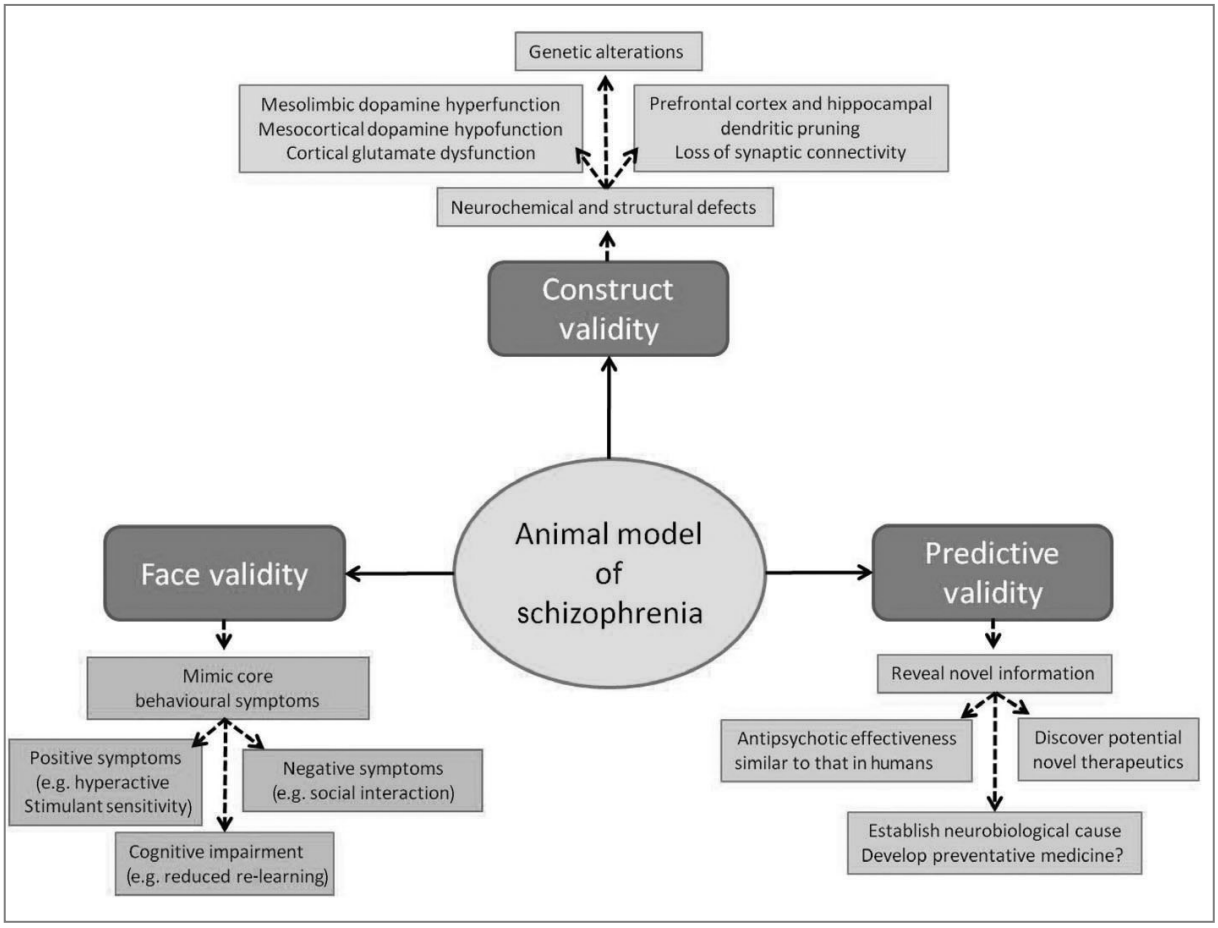

Figure 6. Validity of animal models of schizophrenia.
Schematic diagram of the key behavioural, neurochemical and structural changes expected to be present and to have translational relevance to the three core symptom domains of schizophrenia in an animal model of the disorder. (adapted from Jones et al., 2011)

Acute administration of NMDAR antagonists to animals resembles first-episode schizophrenia, while (sub)chronic administration leads to changes analogous to later phases of the illness (Adell et al., 2012; Morris et al., 2005). Acute administration increases glutamate and dopamine levels in the frontal cortex, decreases GABA release, causes hyperlocomotion (analogue of positive symptoms), deficits in information processing, working and spatial memory, executive control and social interactions, increases behavioural stereotypy and decreases prepulse inhibition of acoustic startle

response (PPI), which is a hallmark symptom of impaired sensorimotor gating (Bubeníková-Valešová et al., 2008). Deficits in PPI are also present in patients with schizophrenia (Kumari et al., 2000; Parwani et al., 2000). Metabolism in the PFC is increased in first-episode patients (Callicott et al., 2000; Manoach et al., 2000) as well as in healthy volunteers administered ketamine (Bubeníková-Valešová et al., 2008). This increased activity is inefficient and detrimental to the PFC-related cognitive functions. Acute administration of ketamine to volunteers increases the release of endogenous dopamine (Vollenweider et al., 2000).

Chronic administration of NMDAR antagonists to animals decreases glutamate and dopamine levels in the frontal cortex (Adell et al., 2012) and decreased metabolic activity in the PFC (Morris et al., 2005). This is in accord with hypofrontality observed in long-term schizophrenia (Hill et al., 2004). Changes in metabolic activity were also observed in the hippocampus, thalamus and auditory cortex, which is consistent with the presumed disruption of the corticolimbothalamic circuit in schizophrenia (which is regulated by GABAergic interneurons) (Morris et al., 2005). Chronic treatment with NMDAR antagonists impairs cognitive functions and social behaviour and increases stereotypy (Bubeníková-Valešová et al., 2008). In addition, various biochemical abnormalities occur after subchronic and chronic administration of NMDAR antagonists resembling those seen in patients, therefore lending constructive validity to the glutamate hypothesis. For example, levels of PV (Adell et al., 2012; Braun et al., 2007; Morris et al., 2005) and GAD_{67} mRNA (Adell et al., 2012) in GABAergic interneurons are decreased in specific areas implicated in schizophrenia. Chronic treatment with MK-801 also caused alterations of NR1 and NR2 subunits of NMDAR similar to those found in post-mortem samples from patients with schizophrenia (Gao et al., 2000; Rujescu et al., 2006). The number of PV-positive interneurons also is also decreased after prenatal administration of NMDAR antagonists (Abekawa et al., 2007), pointing to neurodevelopmental aetiology. Dystrophic dendrites with reduced synaptic spines are reliably found in schizophrenia patients (Coyle, 2012). *Disrupted-In-Schizophrenia 1* gene (*DISC1*) is involved in intracellular signaling, growth of neurites and neuronal migration (Morris et al., 2003, p. 1). Subchronic administration of MK-801 reduces spine density and levels of DISC1, suggesting that synaptic levels of DISC1 are regulated by NMDARs (Ramsey et al., 2011).

Genetic manipulations of NMDAR function also support the validity of models based on NMDAR dysfunction. For example, mutant mice with NMDAR hypofunction (NR1-knockdown mice with NMDARs levels reduced to 10 % of normal) show reduced complexity of dendrites and lower spine density (Ramsey et al., 2011). In another model (Belforte et al., 2010), selective postnatal ablation of approximately half of cortical and hippocampal GABAergic interneurons, mainly consisting of PV-positive cells, resulted in schizophrenia-like behavioural phenotype displaying various positive-, negative-, and cognitive-like symptoms that only emerged after adolescence. If NR1 subunit deletion was induced after adolescence, schizophrenia-like abnormalities did not occur. This latency period is very important as it points to the importance of NMDA-related neurodevelopmental

nature of schizophrenia. Importantly, reduced levels of GAD_{67} and PV were observed in GABAergic neurons with NR1 deletion, lending constructive validity to the model.

Other findings regarding NMDAR hypofunction in schizophrenia involve **kynurenic acid**, an endogenous NMDAR antagonist. Kynurenic acid acts preferentialy at the glycine-modulatory site of NMDARs and its levels are elevated in schizophrenia patients (Erhardt et al., 2007; Schwarcz et al., 2001). Additionaly, it has been reported that **antibodies** against NMDA receptors (antibody-mediated encephalitis) may in some patients cause symptomatology that is diagnosed as schizophrenia (i.e. cause schizophrenia) (Lennox et al., 2012).

Table 1. Comparison of clinical symptoms of schizophrenia with the schizophrenia-like behaviour induced by NMDA antagonist (modified version from Lipska and Weinberger, 2000).
(from Bubeníková-Valešová et al., 2008)

Clinical symptoms of schizophrenia	**Behavioural changes in animal model of schizophrenia**
Psychotic symptoms	An increase in locomotor activity
Stereotypic behaviours	A perseveration of behaviour (sniffing, face washing etc.)
Vulnerability to stress	Changes in locomotory activity or behaviour induced by stress
Information processing deficits	Deficit in prepulse inhibition of acoustic startle reaction or wave P50
Attentional deficits	Deficits in latent inhibition
Cognitive deficits	Impairment in spatial memory tests
Social withdrawal	Reduced contacts with unfamiliar partners

Importantly, **modulation of NMDAR function improves symptoms** in patients and schizophrenia-like behaviour in animal models, and therefore exhibits predictive validity. Improvement of NMDAR activity can be achieved in various ways. One target is the **glycine/D-serine modulatory site** of the NMDAR that can be stimulated either directly by glycine, serine, D-cycloserine (and probably sarcosine (Zhang et al., 2009)) or indirectly by sarcosine. Sarcosine si an inhibitor of **glycine transporter GlyT1** and thus reduces glycine reuptake to neurons and glia. These compounds significantly improved cognitive and negative symptoms in patients (Coyle, 2012; Hashimoto, 2010; Kantrowitz et al., 2010) and although their therapeutic use is limited for various reasons (poor blood-brain barrier penetration, NMDAR desenzitization or internalization and not sustained effects), these studies explore potential benefits of NMDAR enhancement in improving symptoms (Coyle, 2012). Another target is the **proton (redox)-sensitive modulatory site**, which can be influenced by substances that change local pH or redox state, such as polyamines or glutathion (**GSH**). GSH levels are low in unmedicated and first-episode patients (Do et al., 2000). The rate-limiting step in GSH synthesis is the availability of cysteine which, in turn, is synthesized from N-acetylcysteine (NAC). Administration of **NAC** to patients with schizophrenia alleviated their symptoms and improved global functioning (Berk et al., 2008).

Another approach focuses on **metabotropic glutamate receptors**, which are classified (Niswender and Conn, 2010) into group I (includes mGluR types 1 and 5, mainly postsynaptic and excitatory, coupled with G-protein of the G_q type), group II (mGluR 2 and 3, both pre- and postsynaptic, inhibitory, G_i/G_o-coupled) and group III (mGluR 4, 6, 7, 8, mainly presynaptic and inhibitiory, G_i/G_o-coupled). For example, **mGluR5** (group I) are postsynaptically located and their stimulation enhances activity of NMDARs (Hovelsø et al., 2012). Inhibition of mGluR5 by antagonists potentiates schizophrenia-like behaviour and impairs cognition in animal models induced by MK-801, whereas their stimulation by agonists normalizes behaviour and cognition (Hovelsø et al., 2012). Although mGluR5 are located both on GABAergic interneurons and pyramidal cells, the effects of mGluR5 agonists and antagonists suggest that their overall action is mediated preferentially by mGluR5 located on GABAergic interneurons. Therefore, further decrease of NMDAR activity by mGluR5 antagonists results in greater pyramidal cell disinhibition and potentiated schizophrenia-like behaviour. On the other hand, stimulation of mGluR5, which enhances NMDAR function, normalizes behaviour in MK-801 animal model (Hovelsø et al., 2012). Completely opposite effects of mGluR5 agonists/antagonists would be expected , if the overall effects were mediated by mGluR5 located on pyramidal cells, but this was not observed(ibid.).

Metabotropic **mGluR2/3** (group II) agonists and positive allosteric modulators (PAMs) are very promising for schizophrenia treatment. Acting presynaptically, mGluR2/3 agonists immediately and directly reduce glutamate release by inhibiting adenylate cyclase (AC) resulting in decrease of cyclic-adenosine monophosphate levels (cAMP), which affects various ion-channels directly or indirectly via protein kinase A (PKA). Reducing presynaptic glutamate release on excitatory neurons could reduce or reverse disinhibition of pyramidal cells and have antipsychotic effect (Li et al., 2015). Agonists of mGluR2/3 could be the first stand-alone antipsychotic agents acting primarily on glutamatergic, rather than dopaminergic system or be used as and adjuvant therapy for negative symptoms (Conn and Jones, 2009; Li et al., 2015). Various agonists and PAMs of mGluR2/3 have normalizing effect on schizophrenia-like behaviour in animal models (Hovelsø et al., 2012) and also on positive and negative symptoms in patients (phase II clinical trial) (Patil et al., 2007). Although phase III clinical trial with mGluR2/3 agonist LY2140023 (Adams et al., 2014) unexpectedly failed in 2012, this might have been due to suboptimal design of the study (Li et al., 2015). Agonists of mGluR2/3 also act postsynaptically, upregulating NMDA and AMPA receptors and enhancing postsynaptic excitatory signaling. Enhancement of NMDA and AMPA receptor function happens by activating various intracellular pathways that lead to NMDAR phosphorylation, increased exocytosis of NMDARs and increased expression of NMDA and AMPA receptors (for details see Li et al. 2015 (Li et al., 2015)). If upregulation of NMDA and AMPA receptors occurs in pyramidal neurons (as suspected), then it seems to be in conflict with the glutamate hypothesis. It is unclear how presynaptic decrease of glutamate release and postsynaptic enhancement of receptor function combines to produce antipsychotic effects of mGluR2/3 agonists. One possibility is that mGluR2/3 agonists reset signaling

between pyramidal neurons and set a new normal condition, alleviating pyramidal cell disinhibition (Li et al., 2015).

While antipsychotic effects of mGluR2/3 are probably mediated by mGluR2 and not by mGluR3 receptors (Fell et al., 2008; Woolley et al., 2008), **mGluR3** are also implicated in schizophrenia. N-acetyl-aspartyl-glutamate (**NAAG**) is an endogenous agonist of mGluR3 receptors (group II). Levels of NAAG are higher in patients (Jessen et al., 2013) and correlate with negative symptoms (Rowland et al., 2013). This observation may be explained by presynaptic localization of mGluR3, stimulation of which would lead to lower glutamate release from presynaptic axon terminals and reduced NMDAR activity on GABAergic interneurons.

Regarding group III mGluRs, single nucleotide polymorphisms (SNPs) related to **mGluR7** are associated with schizophrenia in humans and agonists of mGluR7 and **mGluR8** show beneficial effects in pharmacological animal model (Hovelsø et al., 2012) .

3.5 Neural coordination and rhythmic oscillations

Numerous findings support the idea that cognitive impairments in schizophrenia including cognitive control deficits result from disrupted **neural coordination** within and between neural networks (Cho et al., 2006; Fenton, 2015; Kelemen and Fenton, 2016; Lesh et al., 2011; Minzenberg et al., 2010). This may be true also for other neuropsychiatric disorders (including epilepsy, autism, Alzeheimer's and Parkinson's disease) (Palop et al., 2006; Uhlhaas and Singer, 2006). Such aberrant coordination of neural activity is referred to as 'dysconnection' (Stephan et al., 2009) (meaning both hyper- and hypo-functional connectivity), 'discoordination' (Fenton, 2015), 'hypersynchrony' (Kubík et al., 2014) or disrupted 'cognitive coordination' (Phillips and Silverstein, 2003) by different authors. In regard to the term 'cognitive coordination', as used by Phillips and Silverstein (Phillips and Silverstein, 2003), it might be misleading to use the term 'cognitive' coordination when one has neural processes in mind (as the authors do) rather than cognitive (psychological) ones (in which case the term cognitive coordination would be correct). Coordination of cognitive (psychological, mental) processes is not the same as the coordination of neural activity, and their confusion would constitute a category mistake (Ryle, 1949). This distinction stays true even if cognitive coordination results from neural coordination. Therefore, the term 'cognitive coordination' is used for psychological and behavioral phenomena, whereas 'neural coordination' refers to coordination of underlying neural processes throughout this thesis.

In general, proper neural coordination is the result of appropriate excitation and inhibition. This may occur at various scales, such as co-activation/inhibition of different brain areas, recruitment of a single brain region, activation or supression of activity in neuronal networks (ensembles) within a single structure, or even proper excitatory and inhibitory interactions within such network. The most apparent manifestation of neural coordination is synchronous neuronal activity observed as rhythmic oscillations at various frequency bands detected by electrophysiological recordings of extracellular

local field potentials. Neural activity in local networks is synchronized by high frequency oscillations, such as in the gamma (30-80 Hz). These fast oscillations are not a mere epiphenomenon but have a causal role in normal cognition (Cho et al., 2015; Yamamoto et al., 2014). Gamma-band oscillations are disrupted in patients with schizophrenia (Minzenberg et al., 2010; Uhlhaas and Singer, 2010) and their normalization improves cognitive functioning in animal models (Cho et al., 2015; Lee et al., 2014) and possibly also in patients (Farzan et al., 2012; Levkovitz et al., 2011). Slow oscillations in low frequency bands (such as theta) coordinate neuronal activity over long distances and interact with oscillations in the gamma-band (Moran and Hong, 2011) and they also affect cognition. Oscillations in the beta-band (12-30 Hz), which are intermediary between low- and low-frequency oscillations, can synchronize neural activity over long distances (when closer to 12 Hz) or in local networks (if closer to 30 Hz). Amplitude and synchronization of beta/gamma is disrupted in schizophrenia (Uhlhaas and Singer, 2015). Interestingly, improving neural synchrony might be sufficient to improve cognition even without necessarily treating the underlying cellular pathology, as has been shown in animal models of neurodegenerative diseases and recently also in a neurodevelopmental model of schizophrenia (Lee et al., 2014). Altered synchronization between (gamma) oscillations in different brain regions revealed abnormal functional connectivity in schizophrenia. Abnormal **functional connectivity** (neural discoordination or dysconection) between different brain regions in schizophrenia is well documented by PET, fMRI and EEG recording studies (Fornito et al., 2012; Lynall et al., 2010; Stephan et al., 2009).

3.5 GABAergic inhibitory interneurons

Gamma-band oscillations result from inhibitory action of **GABAergic** inhibitory interneurons on pyramidal neurons, which constrains and synchronizes their activity (Bartos et al., 2007; Wang and Buzsáki, 1996). Synchronized activity of pyramidal cells likely feeds back to the interneurons and supports synchronization of GABAergic interneurons (Mann et al., 2005). **GABAergic** inerneurons can be classified into many different categories along multiple dimensions (Markram et al., 2004; Nakazawa et al., 2012) such as morphology (e.g. unipolar, bipolar, horizontal, chandelier, basket cells), electrical properties (e.g. fast-spiking, low-treshold spiking), location of synaptic connection (e.g. soma, axon, dendritic shaft, distal dendrites), and gene expression (e.g. calretinin, parvalbumin, somatostatin, cholecystokinin). Considerable evidence suggests, that parvalbumin-expressing (**PV+**) interneurons, especially the basket cell type (**PVBC**), are the main type of inhibitory interneurons responsible for the **generation of gamma oscillations**, involved in normal cognition as well as in cognitive impairments in schizophrenia (Lewis et al., 2012; Nakazawa et al., 2012). PVBCs have the important ability to generate short-duration action potentials at high frequencies (fast-spiking type) that can be exactly phase-locked to gamma oscillations (Jonas et al., 2004). Moreover, PVBCs target the perisomatic region of pyramidal cells near the initial segment where action potentials are

generated, exert strong inhibition on pyramidal cells, and can rhythmically control their activity (Gonzalez-Burgos and Lewis, 2012). Optogenetic manipulations provided direct evidence of causal relationship between fast-spiking PV+ interneurons and gamma oscillaitons. Cardin et al. (2009) demonstrated that optogenetic activation of these fast-spiking interneurons selectively amplifies gamma oscillation, while light-driven activation of pyramidal neurons amplifies only oscillations at lower frequencies. In the same issue of the Nature magazine, Sohal et al. (2009) reported similar findings – that optogenetic stimulation of PV+ interneurons is sufficient for generating gamma-frequency rhythmicity and inhibiting PV+ interneurons abolishes gamma oscillations.

Many **abnormalities of GABAergic PVBCs** are found in schizophrenia. Concentration of GABA in the cortex of patients is reduced and these **reduced GABA levels** correlate with **worse cognitive performance** (Chen et al., 2014). This reduction in GABA levels is likely due to suppressed expression of glutamic acid decarboxylase (GAD), the rate-limiting enzyme in GABA synthesis. There are two important types of GAD, the 67-kDa isoform GAD_{67} (encoded by Gad1 gene) and the 65-kDa isoform GAD_{65} (encoded by Gad2). In mice, GAD_{67} is responsible for about 85% of GABA production (Asada et al., 1997; Nakazawa et al., 2012). In schizophrenia, single-nucleotide-polymorphism (SNP) in 5' UTR of Gad1 is present and leads to decreased expression of **GAD_{67}** in the PFC (Nakazawa et al., 2012). Low levels of GAD_{67} mRNA is one of the most consistently replicated pathology in schizophrenia (MacDonald and Schulz, 2009; Nakazawa et al., 2012). Furthermore, evidence supports the notion that deficit in GAD_{67} in schizophrenia is **specific to PVBCs** (Lewis et al., 2012). Levels of **parvalbumine** (PV), a calcium binding protein that buffers intracellular Ca^{2+}, are reduced in schizophrenia (Lewis et al., 2012). Reduced PV levels as well as decreased expression of GAD_{67} may result from reduced excitatory drive and lower influx of calcium ions due to hypofunctional NMDARs on PVBCs (Kantrowitz and Javitt, 2010). Expression of GABA membrane transporter **GAT1** responsible for GABA reuptake to presynaptic terminals is reduced in GABAergic interneurons of patients with schizophrenia, potentially reflecting a compensation for lower GABA levels (Lewis et al., 2012).

Reelin is a glycoprotein constitutively expressed and secreted by GABAergic interneurons. After secretion, reelin binds to integrin receptors located in postsynaptic density (PSD) of pyramidal neurons (probably acting as scaffold protein), stabilizing PSD and promoting synaptic plasticity in adults (Dong et al., 2003). Postsynaptic density is a network of proteins which anchors glutamate receptors and other postsynaptic membrane proteins to cytoskeleton and links them to downstream intracellular signaling pathways (Sala et al., 2003). Reelin increases phosphorylation of NR2A and NR2B subunits of NMDA receptors and facilitates entry of Ca^{2+} ions through NMDA receptors (Folsom and Fatemi, 2013). It also plays an important role during neuronal development (e.g. regulation of neuronal migration) (Folsom and Fatemi, 2013). Levels of reelin mRNA and protein are reduced in patients with schizophrenia by approximately 40% (Guidotti et al., 2000; Impagnatiello et al., 1998). Postmortem and experimental data suggest that reduced expression of reelin is due to

hypermethylation of its promoter (Grayson et al., 2005), but other mechanisms are also involved (Folsom and Fatemi, 2013). Reduction or loss of reelin in animals is associated with behavioral and cognitive deficits including reduced prepulse inhibiton (PPI) of acoustic startle response, a sensorimotor gating deficit typical for schizophrenia (Folsom and Fatemi, 2013; Kumari et al., 2000; Mena et al., 2016).

These abnormalities in inhibitory interneurons can result in reduced inhibitory signaling and disinhibition of pyramidal neurons, hypersynchrony, disrupted neural coordination, and impaired cognitive control in schizophrenia. In summary, so far we have seen that NMDA receptors play a crucial role in schizophrenia pathophysiology, that deficits in cognitive control can account for large part of the cognitive symptomatology seen in schizophrenia, that neuronal coordination (as exemplified by gamma oscillations) is necessary for proper cognitive functioning and that disruption of this coordination may be due to defficient PV+ GABAergic interneurons and resulting disinhibition of pyramidal neurons. Next we turn to neuronal coordination in the hippocampus.

4 NEURONAL COORDINATION IN THE HIPPOCAMPUS

Neuronal coordination can be seen in hippocampus as coordinated activation and deactivation of ensembles of place cells that represent environment, more specifically animal's location (in rats) or spatial view (in primates) (Rolls, 2016). Appropriate representations of space in the hippocampus are enabled by hippocampal anatomy and **connectivity** which give rise to network properties allowing coordinated encoding and recall of appropriate representations (Rolls, 2016).

4.1 Hippocampal subregions and circuitry

The hippocampus consists of dorsal and ventral blade of the dentate gyrus (DG), and areas cornu ammonis (CA1-CA4), illustrated in Figure 7. In addition, the hippocampal formation also includes subicular complex (subiculum, presubiculum, and parasubiculum) and sometimes also the main input region of the hippocampus, the entorhinal cortex (EC). These regions are connected within a "trisynaptic loop", which starts with perforant pathway projection from layer II of the EC to granule cells (GCs) in the DG and also to pyramidal neurons in area CA3. Next, dentate GCs project to CA3 pyramidal neurons via powerful mossy fibers. Pyramidal neurons in CA3 send Schaffer collaterals to CA1, in addition to recurrent collaterals and subcortical projection to lateral septum. Pyramidal neurons in CA1 also receive direct input from layer III of the EC via temporoammonic pathway and they project directly to EC layer V and areas in the subicular complex, major proximal output structures of the hippocampus. Figure 8. illustrates hippocampal circuitry, its major inputs and outputs. The hippocampus receives direct and indirect inputs from many cortical and subcortical areas via the EC as the main input/output hub of the hippocampus. EC possess reciprocal connections with many cortical and subcortical areas including the amygdala and the orbitofrontal cortex, structures implicated in reward-related processing. GCs of the DG receive divergent projection from the EC (GCs are far more numerous than EC projection neurons) and give rise to sparse but strong projection to CA3 and hilus via mossy fibers (mf) equipped with potentially ‘detonator’ synapses, capable of reliably depolarizing the postsynaptic neuron. It is estimated that any GC contacts only approximately 50 CA3 neurons. Together with sparse activity of dentate GCs, these properties predispose DG to operate as a competitive network supporting ‘pattern separation’, where similar inputs are recoded onto less similar (overlapping) outputs. An essential property of area CA3 is the network of recurrent collaterals projecting to other CA3 pyramidal neurons, which confer autoassociative properties to the CA3 network.

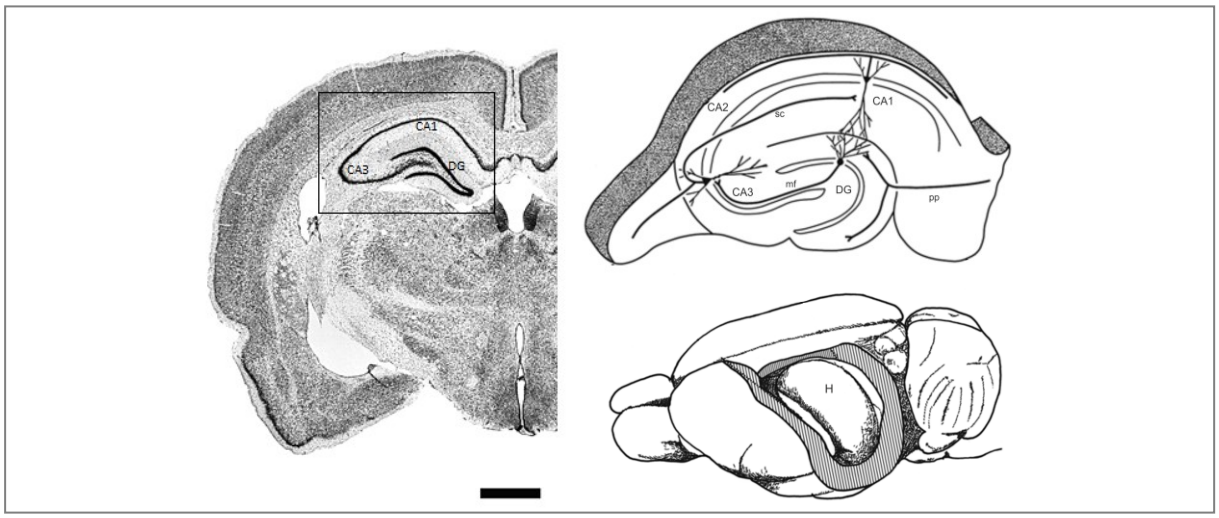

Figure 7. Hippocampal structure in rats. **Left**: Coronal section of rat brain at the rostral level containing the right dorsal hippocampus. (Bar = 1 mm, relevant only for the left picture) **Right top**: Drawing of rat hippocampus. **Right bottom**: Rat brain with removed part of the left hemisphere to expose the hippocampus (H). In rodents, the hippocampus is oriented along the dorsoventral axis. (adapted from Andersen et al., 2009)

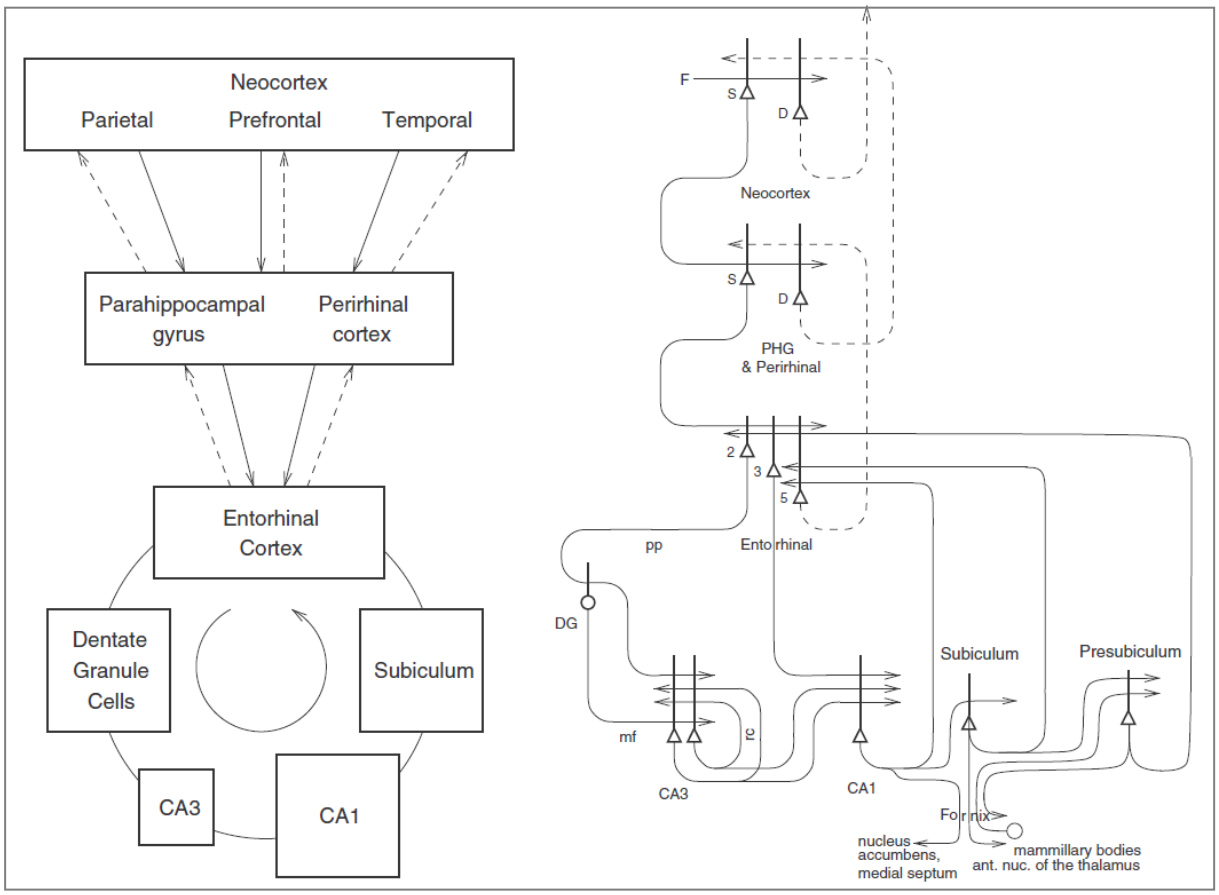

Figure 8. Hippocampal circuitry: inputs, outputs and connections. Solid lines: forward connections from neocortex to the hippocampus. **Dashed lines**: backprojections from the hippocampus to the neocortex. **Left**: flow-chart diagram. **Right**: more detailed diagram of some of the principal excitatory neurons in the pathways. **D**: Deep pyramidal cells. **DG**: Dentate Granule cells. **F**: Forward inputs to areas of the association cortex from preceding cortical areas in the hierarchy. **mf**: mossy fibres. **PHG**: parahippocampal gyrus and perirhinal cortex. **pp**: perforant path. **rc**: recurrent collateral of the CA3 hippocampal pyramidal cells. **S**: Superficial pyramidal cells. **2**: pyramidal cells in layer 2 of the entorhinal cortex. **3**: pyramidal cells in layer 3 of the entorhinal cortex. The thick lines above the cell bodies represent the dendrites. (from Rolls, 2016)

Such autoassociative networks are capable of 'pattern completion', i.e. reproducing previously encountered pattern from partial or degraded input, which may also provide mechanism for generalization. These patterns of connectivity are crucial factors in determining behavior of neural networks in DG and CA3 (Rolls, 2016), which allow groups (ensembles) of hippocampal pyramidal neurons to dynamically represent specific specific place in specific environments, to respond distinctly to similar stimuli (pattern separation) and to maintain consistent representations despite perturbations in the environment (pattern completion). Hippocampal projections beyond the subiculum and EC include parahippocampal structures (perirhinal, postrhinal cortices) and prefrontal cortex (including orbitofrontal cortex, which also provides hippocampal input).

4.2 Place cells

Pyramidal neurons in CA3 and CA1 in freely moving rodents display location-specific activity, which earned them the name **Place cells** (Bird and Burgess, 2008; Muller, 1996; Nakazawa et al., 2004; O'Keefe and Dostrovsky, 1971). These neurons are vigorously active if the animal occupies a certain location in the environment and virtually silent when the animal is outside of it. This specific location is called the cell's firing field (FF). Basically, different place cells will be active at different parts of the environment eventually covering the entire area. Individual locations are not encoded by single neurons, but rather by a population of simultaneously active cells (Nakazawa et al., 2004). In open spaces, firing fields are independent of the rat's orientation or head direction, therefore place cell code for location and do not simply reflect immediate sensory input (Bird and Burgess, 2008). Moreover, place cells maintain their FFs in complete darkness, despite strong control that visual cues exert over FF location in light. There is no topographic mapping of the environment by the place cells, so firing fields of two neighbouring place cells can have arbitrary locations.

Firing fields form rapidly in novel environments (ususally within five minutes) (Nakazawa et al., 2004). The same place cell can be active in many different environments showing that it can take part in many different representations similarly to one light in a jumbotron, which can also be part of many different messages (Fenton, 2015). FFs may be stable for weeks hence multiple different representations (cell ensembles) must remain stable in parallel. Unlike dentate GCs, which commonly display multiple FFs in regular environments, place cells in CA1 and CA3 typically have a single FF. However, they also display multiple firing fields in sufficiently large environments (Fenton et al., 2008; Park et al., 2011). About 40% of CA1 pyramidal cells display place-specific activity in a given environment and NMDA receptors are essential for pattern separation, pattern completion, and formation of highly tuned CA1 place cells (Nakazawa et al., 2004).

4.3 Ensemble coding and dynamic grouping

A group of place cells active in a certain location can be considered a neuronal ensemble. Sequence of such ensembles reflects trajectory of the animal during exploration and forms a 'superensemble' (ensemble of ensembles), which constitutes hippocampal neuronal representation of that environment. These ensembles arise and are maintained by means of neuronal coordination. The fact that the same place cell can take part in different representations and the fact that it can have multiple firing fields in sufficiently large environments demonstrate (Fenton, 2008, 2015) that place cells use distributed **ensemble coding** mechanism rather than dedicated coding to convey information about animal's location within an environment as well as to convey information about the environment identity. Dedicated coding is based on the concept of cardinal units or cardinal cells where each cell is dedicated to a particular higher-order stimuli (such as face of a specific person or a specific location in space) so that information about this stimulus is represented in the activity of a single cell. In ensemble coding paradigm based on Hebb's concept of cell assembly (Hebb, 1949), information is represented by a simultaneous activity of a functionally related group of cells, which together constitute a cell assembly (neuronal ensemble) (Fenton, 2008).

Because two ensembles can share place cells (nodes), there is a need to avoid simultaneous activation of two representations that have cells in common. Otherwise, the two representations will merge into one, a situation called **superposition catastrophe** (Von Der Malsburg, 1994) (Figure 9), interference will occur and information will be lost with all cognitive and behavioural consequences. To avoid this interference, it is necessary to activate different representations at distinct times in a process of neural coordination referred to as **dynamic grouping** (Kelemen and Fenton, 2010), which has been demonstrated in the hippocampus by Kelemen and Fenton (2016). Fenton defines **neural coordination** as "the processes that coordinate the timing of responses by neurons participating in representing multiple distinct items, without changing the responses of such neurons to individual items"(Fenton, 2008, p.391). For example, place cells of one ensemble represent a distinct 'item', namely a particular place. In this instant, Fenton describes neural coordination as the 'coordinating processes', which act as to enable (cause) a pattern of neural activity/representation to arise at a particular time and not other, without changing the representation itself. The term neural coordination can, in our understanding, also be used to refer to an occurrence of 'coordinated neural activity', i.e. some meaningful pattern per se is itself an instant of neural coordination. According to our read of the literature, both meanings are used.

Overall, if an appropriate representation should be formed or activated, it is important for the animal to distinguish, if it is in a new environment or one it has already encountered. In the latter case, it is necessary to activate the correct neuronal representation of previously encountered environments, which corresponds to the current situation.

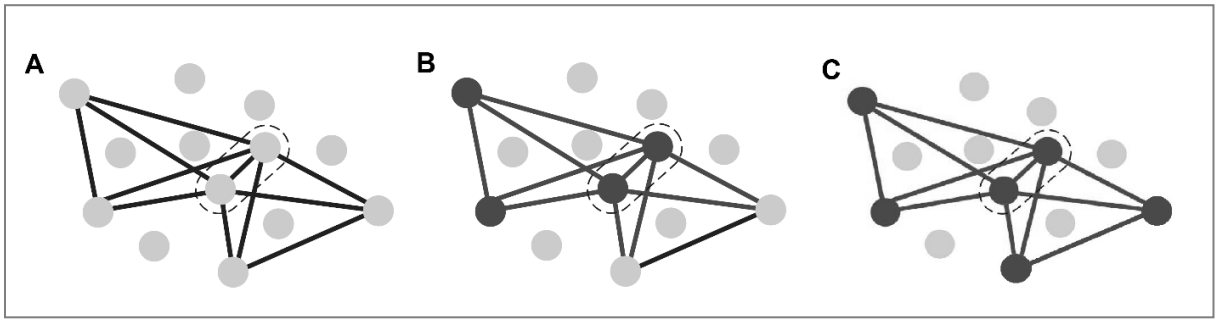

Figure 9. Interference between two overlapping associative networks.
Only pyramidal neurons are shown, inhibitory interneurons are not depicted. Active neurons dark grey, inactive neurons light grey. **A** – Two overlapping inactive cell assemblies. Some neurons are involved in both cell assemblies (dashed line). This increases the number of assemblies that can be stored in the network. However, activation of one cell assembly could potentially spread to the other assembly and corrupt the information. **B** – Only one cell assembly is active, information is preserved. If the treshold for neural activation is sufficiently high, neurons belonging to the second cell assembly remain below treshold and activation of one assembly will not spread to the other. **C** – Two overlapping cell assemblies active simultaneously, superposition catastrophe, information is lost. If the treshold for neural activation is low, activating one assembly will also cause activation of the other assembly (as shown). This might be the case in schizophrenia if pyramidal neurons are disinhibited. (adapted from Kandel et al., 2012)

Discrimination between similar environments is enhanced by **pattern separation** in the DG, which prevents superposition catastrophe and increase memory capacity by decorrelating representations in CA3 (Rolls, 2016). Decorrelation (orthogonalization) of input patterns to CA3 result from divergent projection from the EC to DG sparse but strong projections from the DG to CA3 and extremely sparse activity of dentate GCs (Jung and McNaughton, 1993). In vivo experimental evidence for pattern separation was provided by Leutgeb (2007) and McHugh (2007). Conversely, **pattern completion** enables reactivation of an entire representation in a situation when only partial or noisy cues are available (Guzman et al., 2016) (for example viewing the same environment from a different angle or with minor modifications). Pattern completion arise from the recurrent connectivity in CA3 (Rolls, 2016), where a given pyramidal cell is directly connected to approximately 2% of other CA3 pyramidal cells (Nakazawa et al., 2004). When only a subset of nodes of ensemble are stimulated, recurrent connections between nodes of a representation will lead to mutual excitation between its neurons, activating the entire ensemble and enabling recognition of a familiar environment. Because of this property, CA3 is often referred to as **'autoassociation network**'. CA3 also displays **attractor dynamics**. Generally, "an attractor refers to a set of states of a system that have the property that they 'attract' neighbouring states to move toward the stable states … like a ball rolls down the slope of a hill toward a valley" (Knierim and Neunuebel, 2016). Once the basin of attraction is reached it is resistant to change. Attractor dynamics would be characterised by abrubt switching between two ensembles (each constituting a basin of attraction), unlike gradual change of standard autoassociation network. Whether CA3 operates according to attractor dynamics or not is an active area of research. For example, experimental evidence provided by Colgin et al. (2010) doubts that CA3 acts as a standard autoassociation network (where CA3 neurons are supposed to associate the co-occurrence of input features), but rather act as an attractor-map in which attractor states are preconfigured during

development and transition between attractor states is primarily governed by self-motion information (path integration). According to Knierim and Neunuebel (2016), variation in similarity of input and output patterns of the CA3 region (from EC and DG to CA1) indicates that attractor dynamics of CA3 may oscillate between pattern completion and pattern separation. In vitro experimental evidence for pattern completion in CA3 was provided by Jackson (2013). **Dynamical systems hypothesis** have also been applied to schizophrenia and propose that cognitive, positive, and negative symptoms of schizophrenia result from shallower basins of attraction in cortical networks, which make switching between attractor states abnormally easy (Loh et al., 2007). **CA1** region lacks recurrent collaterals and shows more linear dynamics between input and output patterns, making pattern completion in this region unlikely (Knierim and Neunuebel, 2016). CA1 is involved in recognition of familiar and novel contexts or objects (Nakazawa et al., 2004).

Coordinated activation of individual representations in the hippocampus depends on inhibition by GABAergic interneurons. Inhibition allows disambiguation of similar (overlapping) representations. This is important because individual place cells participate in multiple representations. Recurrent connections in CA3 are structured in a way that incoming excitation activates only a subset of neurons with strong mutual excitatory connections, which, in turn, activate interneurons and cause widespread inhibition of the other neurons. Appropriate **excitation-inhibition balance** creates a competitive winner-take-all configuration of the network (Fenton, 2015). This way, only the appropriate representation is activated at a given time, while the irrelevant ones are suppressed. Depending on changing behavioral needs, activation/suppression of different representations can occur very quickly, on a timescale from 25 ms to several seconds, as was shown in rats trained to simultaneously avoid both room- and arena- defined sectors (double avoidance) on the Carousel (Kelemen and Fenton, 2016).

4.4 Hippocampal pathology in schizophrenia

Abnormalities in several brain regions are commonly found in patients with schizophrenia. These include enlarged ventricles, abnormalities in the temporal, frontal and parietal lobes and subcortical structures such as basal ganglia, corpus callosum and thalamus (Shenton et al., 2001). In addition, abnormalities in white matter are also present, including reduced connectivity between frontal and medial temporal lobe (Ellison-Wright and Bullmore, 2009). Medial temporal lobe (MTL), which is critical for declarative memory, has been implicated in most MRI and post-mortem studies of patients with schizophrenia and includes the hippocampus, amygdala and parahippocampal gyrus (Shenton et al., 2001). Hippocampus is often reduced bilaterally in size not only in chronic patients, but also in first-episode patients, their non-psychotic siblings, and individuals at high-risk of schizophrenia (Tamminga et al., 2010). Crucially, hippocampal reduction is not caused by neuropathological processes of neurodegeneration or gliosis (Harrison, 2004). The lack of degenerative changes

implicates that hippocampal alterations observed in schizophrenia are of neurodevelopmental origin and are rather quantitative than qualitative in nature (in comparison to healthy population) (ibid.). Prolonged stress, especially during the critical period of brain maturation in adolescence, can negatively affect the hippocampus and cause dendritic shrinkage, and loss of neurons including PV+ interneurons (Grace, 2016). Unmedicated patients with schizophrenia show decreased basal perfusion of the MTL, which is, to some extent, normalized after antipsychotic treatment (Tamminga et al., 2010). The anterior hippocampus (corresponding to ventral hippocampus in rodents) is hyperactive in patients. This overactivity correlates both with psychosis and loss of hippocampal PV+ inhibitory interneurons (Grace, 2016). Studies in humans (Stone et al., 2010) and animals (Grace, 2012; Lipska et al., 1993) provide evidence that hyperactivity of the hippocampus can increase dopamine release in the striatal region, which may lead to aberrant salience and psychosis (Howes and Kapur, 2009).

In addition, task-related activation of the MTL is decreased in schizophrenia patients (Tamminga et al., 2012). Activity-dependent glutamatergic signaling from the DG to CA3 region is reduced (ibid.). This might sensitize the CA3 (via homeostatic plasticity) to incoming signals, decrease the threshold for LTP generation and cause higher level of CA3 activation (ibid.). Dentate gyrus and its projections to the CA3 are critical for pattern separation, while recurrent collaterals in the CA3 are responsible for pattern completion. Disruption of balance between pattern separation and pattern completion (Guzowski et al., 2004) could increase synaptic strength in the CA3 and inapproriately increase pattern completion (Tamminga et al., 2012). Overactive pattern completion can enhance cross-linking of unrelated hippocampal representations (Kubík et al., 2014), leading to excessive associations, distorted perception or hallucinations (Behrendt, 2010; Tamminga et al., 2012). This model is illustrated in Figure 10.

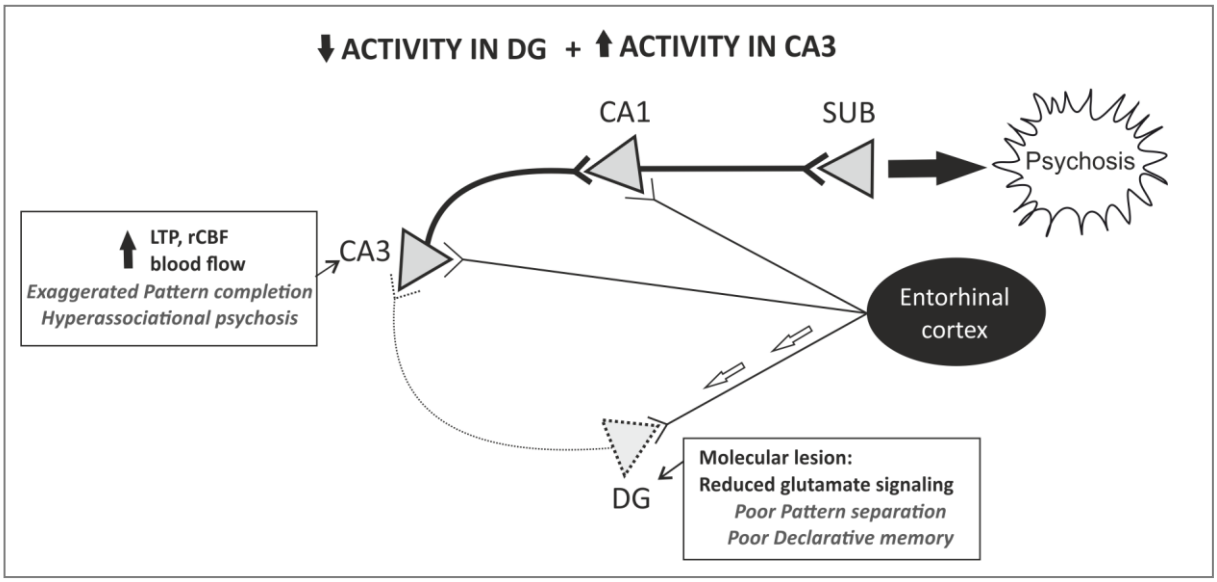

Figure 10. Hippocampal plasticity model of psychosis.
Reduced pattern separation and increased pattern completion leads to excessive associations, distorted perception and hallucinations. **DG** – dentate gyrus, **SUB** – subiculum, **rCBF** – regional cerebral blood flow, **LTP** – long-term potentiation. (adapted from Tamminga et al., 2012)

5 IMMEDIATE-EARLY GENES (IEGs)

Visualizing neural activity is possible using various methods (from real-time calcium imaging to positron emission tomography), which differ in spatial (μm to cm) and temporal resolution (real-time to hour) and each have their own specific limitations (accessibility of brain structures for imaging, inflicted collateral tissue damage, spatio-temporal resolution trade-off and other). To visualize neuronal ensembles, cellular resolution combined with a high number of simultaneously recorded cells is necessary. Methods utilizing activity-dependent expression of immediate early genes (IEGs) have such properties and allow imaging of active neuronal ensembles.

5.1 IEG expression: Between neuronal activity and synaptic plasticity

Immediate early genes are a group of genes that do not require *de novo* proteosynthesis for expression of their RNA. Operationally, IEGs are defined as genes that can be expressed as RNA transcripts in the presence of protein synthesis inhibitors (Guzowski et al., 2005). Expression of IEGs in neurons of resting animals is extremely low, but rises rapidly to high levels in response to growth factors and mitogens(Loebrich and Nedivi, 2009), application of patterned, LTP-inducing neural stimulation (Cole et al., 1989) and, most importantly, also after behavioural experience (Guzowski et al., 1999, 2005). Most IEGs (such as *c-fos*, *c-jun*, *zif268*) are regulatory transcription factors (RTFs), but some IEGs also possess effector function (*Arc, Homer1a*). RTFs control expression of other genes (Loebrich and Nedivi, 2009). Effector IEGs can be further divided into four functional subgroups: effector IEGs related to cellular growth (*BDNF*, *Narp*), intracellular signaling (*RheB*, *RGS-2*, *Homer1a*), synaptic modification or other structural changes (*Arc*, *Homer1a*, *Narp*, *BDNF*) and metabolism (*COX-2*) (Kubik et al., 2007). The most parsimonious account of behaviorally induced IEG expression is that it is induced by neural activity associated with learning and its products take part in stabilizing changes in synaptic efficacy which are necessary for long-term memory (Guzowski, 2002). Their expression does not simply reflect recent neuronal activity but instead reflects the induction of activity-related plasticity (Holtmaat and Caroni, 2016). Because of their above mentioned properties, various IEGs are used as markers of neural activity, allowing visualization of active neuronal ensembles.

Arc (activity-regulated cytoskeleton-associated gene/protein, also known as *Arg3.1*) is an effector IEG which is essential for stabilization of activity-dependent synaptic plasticity, synaptic consolidation and long-term memory (Kubik et al., 2007). It is implicated many different forms of synaptic plasticity and the temporal profile of its expression and intracellular distribution is the most precisely regulated of known IEGs (Korb and Finkbeiner, 2011). Signaling pathways involved in *Arc* expression include PKA, ERK, and PKC-mediated cascades (Korb and Finkbeiner, 2011). *Arc* Transcripts appear in the nucleus very rapidly, within 5 minutes after stimulation (Guzowski et al., 1999) and half-life of *Arc* mRNA is short, approximately 45 minutes (Korb and Finkbeiner, 2011).

Disruption of *Arc* by either genetic knock-out (Plath et al., 2006) or administration of *Arc* antisense RNA-oligonucleotides, which selectively block synthesis of Arc protein) (Guzowski, 2002), impairs consolidation of long-term memory but spares short-term learning and memory. Localization of Arc protein is strictly regulated. It can be found in the nucleus, dendrites and postsynaptic densities, but is not present in axons or axon terminals. After six hours, Arc protein is ubiquitinilated, degraded in the protesome and its levels return to baseline (ibid). Arc is essential both for LTP and LTD (types of Hebbian plasticity). Its role in LTP is most probably mediated by regulating cytoskeleton dynamics, spine morphology or its role in other signaling cascades (ibid). On the other hand, it facilitates endocytosis of synaptic AMPA receptors, leading to decrease in synaptic efficacy, which may directly underlie LTD (Chowdhury et al., 2006). in the same mechanism can also support cell-wide plasticity called homeostatic scaling (Shepherd et al., 2006), which is a non-Hebbian „form of synaptic plasticity that adjusts the strength of all of a neuron's excitatory synapses up or down to stabilize firing" (Turrigiano, 2008). Given these disparate effects, it has been suggested that specific effect of Arc may depend on the amount of its expression, its location in the cell, postranslational modifications, and binding partners (Bramham et al., 2010; Korb and Finkbeiner, 2011).

Homer1a (H1a) is an effector IEGs that is expressed in response to learning-related behaviour and patterned stimulation of neurons. Homer 1 a belongs to the Homer family of adaptor proteins which are located mainly in PSD of neurons but is also present in non-neuronal tissues (Shiraishi-Yamaguchi and Furuichi, 2007). Sala et al. (2003) found that expression of Homer 1a reduces the size of PSD-95 clusters, density and size of hippocampal dendritic spines, the number of NMDAR clusters, the level of surface AMPARs, and reduces postsynaptic AMPA and NMDA receptor synaptic currents. Authors suggest that H1a is involved in a negative feed-back loop regulating synaptic structure and function in a use-dependent manner. Later, Hu et al. (2010) showed Homer 1a evokes agonist-independent activation of group I mGluRs, which decreases AMPAR expression, thereby scaling down the level of membrane AMPARs in a cell-wide manner along with inducing homeostatic scaling. In addition, Homer 1a is also implicated in sleep loss and consolidation of contextual memory by synaptic remodeling during sleep (Diering et al., 2017; Maret et al., 2007). Upregulation of Homer 1a was found after several antidepressant treatments (including non-pharmacological) (Serchov et al., 2016) and treatment with antipsychotics (Iasevoli et al., 2011).

5.2 IEG imaging of neuronal ensemble activity

In 'conventional' IEG imaging of neural circuit activity related to cognition (Guzowski et al., 2005), one group of animals recieves experimental treatment and the other serves as a control group. Depending on whether IEG mRNA or protein is to be detected, animals are sacrificed either 30 minutes or 1-2 hours afterwards, respectively. After brain sectioning, *in situ* hybridization or immunohistochemical methods are performed. Detection of IEG mRNA is thought to provide a more

direct information about neuronal activity than levels of IEG proteins, which are, on the other hand, closer related to resulting synaptic plasticity (Kubik et al., 2007). To ensure neuronal activation changes detected in the experimental group are due to the specific cognitive component of the behavioral task, it is necessary to control other intervening factors (Guzowski et al., 2005). These include stress and arousal, attention, motor activity and sensory input experienced by animal engaged in the experimental task. Ideally, all components experienced by the experimental animal, except the cognitive process of interest, should be recreated in the control group. This can be very challenging. In addition, interindividual differences in the baseline of neuronal activation might exist, making between-subject comparisons even more difficult. Furthermore, experimental task may not necessarily activate more cells in the region of interest, because neural coding may depend more on which cells are active rather than how many of them (Guzowski et al., 2005). Some of these problems could be circumvented if within-animal comparison was available. However, conventional IEG imaging does not allow measurement at two distinct times and cannot detect changes where different populations of neurons are active, but the overall numbers of active cells remain the same. To address these shortcomings, Guzowski et al. have developed an imaging method using the IEG *Arc*, which allows visualization of two neuronal populations active during two distinct epochs called catFISH (cellular compartment analysis of temporal activity by fluorescent in-situ hybridization, Figure 11) (Guzowski et al., 1999, 2001, 2005; Vazdarjanova and Guzowski, 2004).

5.3 Two time-point IEG imaging: catFISH

***Arc* catFISH** (Guzowski et al., 1999) utilizes the strict temporal dynamics of *Arc* expression and mRNA localization. *Arc* pre-mRNA appears in the cell nucleus as bright intranuclear foci (INF) within 2 minutes following behavioural stimulus (exploratory behaviour). After 15 minutes, *Arc* INF signal disappears and after 25-30 min *Arc* mRNA can be found in the cytoplasm. Behavioural procedure used to trigger *Arc* expression involves two exploratory sessions lasting 5 min (for induction of IEG expression), separated by 20 minutes of rest period in the home cage (when *Arc* expression is very low). Immediately after the second behavioural epoch, the rats are sacrificed, their brains quickly removed and flash-frozen (to prevent RNA degradation or diffusion). Following *in situ* hybridization, the INF signal marks neurons active during the second ecpoch and the cytoplasmic *Arc* signal marks neurons active during the first epoch. Neurons with both INF and cytoplasmic signal were active during both sessions. This allows within-animal comparison of two neuronal ensembles active during two distinct episodes.

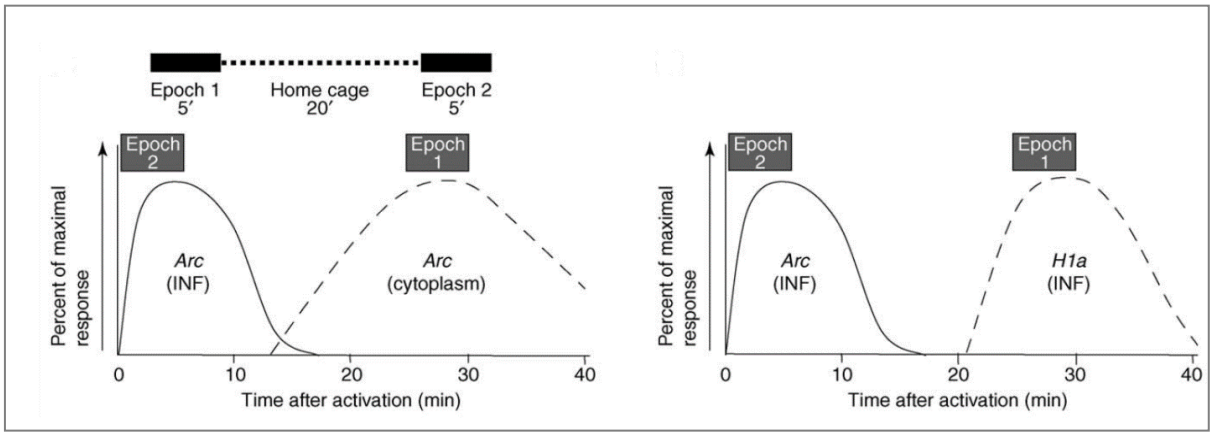

Figure 11. *Arc* catFISH compared to *Arc/H1a* catFISH. For the induction of IEG expression, rat expolores an open field (objects might be present) during Epoch 1 (five minutes) and Epoch 2 (five minutes). Environments during first and second expoloratory session might or might not be the same. Epoch 1 and 2 are separated by 20 minutes of rest in a home cage, which does not stimulate IEG expression beyond the level found in control animals. The animal is sacrificied immediately after the second expolratory session (Epoch 2) and brain is flash-frozen. Brain sections are labeled by *in situ* hybridization using *Arc* riboprobes (Left: *Arc* catFISH) or double-labeled by *Arc* and *H1a* riboprobes (Right: *Arc/H1a* catFISH). **Top** – time course of behavioural protocol; **INF** = intranuclear foci. (adapted from Guzowski et al., 2005)

***Arc/H1a* catFISH** is an advanced version that combines labeling *Arc* and *Homer1a* RNA transcripts. Note that *Arc* and *Homer 1a* are expressed in the same neurons of rat hippocampus and neocortex and in both exploratory sessions after exploration of a novel environment (Vazdarjanova et al., 2002). However, *Arc* mRNA is produced from short primary transcript (~3.5 kb), while *Homer 1a* mRNA is derived from a much longer primary transcript (~55 kb) (Guzowski et al., 2005). Due to the length of the *Homer 1a* primary transcript, 3′UTR (untranslated region) of the *Homer 1a* pre-mRNA does not appear in the nucleus until ~25-30 after the behavioural stimulus. Therefore, *Homer 1a* 3′UTR-specific riboprobe marks neurons active during the first, but not the second, exploratory session. On the other hand, *Arc* INF appear in 2 minutes after the exploratory behaviour, thus *Arc* riboprobes mark neurons active during the second epoch. Double-label *Arc/H1a* catFISH provides two INF signals, making subsequent image analysis much easier to quantify than in *Arc*-only catFISH, where attributing *Arc* cytoplasmic signal to a specific neuron can be difficult.

5.4 Ensemble similarity and contextual specificity

CA1 neurons expressing *Arc* and *Homer 1a* after exploration are most likely place cells, which express their firing fields in that environment. First, percentage of *Arc* and *Homer 1a*-expressing neurons in CA1 after exploration of an environment is similar to the percentage of neurons displaying place fields in electrophysiological recordings (Guzowski et al., 1999). Second, both IEG expression and place cell activity are environmental context-specific (Kubík et al., 2007). Guzowski et al. (1999) used *Arc* catFISH to show that in animals exposed twice to the same environment (A/A), majority of the CA1 cells activated in the first session were reactivated in the second session and the ensemble similarity was high. In contrast, if the environments were different (A/B), then most neurons were only active in

one of the sessions and the ensemble similarity was low. *Arc/H1a* catFISH was used to show that CA3 ensembles were even more dissimilar than CA1 ensembles in rats exploring different environments (A/B), but in slightly modified environments (A/A'), active ensembles in CA3 remained more similar than in CA1 and matched the same environment (A/A; Vazdarjanova and Guzowski, 2004). Together with unit recording studies (Lee et al., 2004; Leutgeb et al., 2004) these findings suggested that CA3 maintains dynamic balance between pattern separation and pattern completion, which de-emphasizes minor deviations, but accentuates substantial (supra-threshold) changes (Guzowski et al., 2004). CA1 ensembles did not display this non-linearity and this departure likely emerges from different properties of their respective inputs.

Kelemen and Fenton (2010) demonstrated that cognitive control in double avoidance on the Carousel (shock defined simultaneously in both frames on a rotating arena) requires dynamic grouping (neural coordination) of hippocampal ensemble activity, i.e. selective activation of the behaviourally more relevant spatial representation at each moment. Switching between room- and arena-frame representations (measured electrophysiologically in cell discharge) occurred on a time-scale of milliseconds to seconds. Authors conclude that „cognitive control is mediated by a dynamic functional grouping“ (Kelemen and Fenton, 2010, p.2). They also hypothesize that the hippocampus might be a key structure (together with frontal cortex) for cognitive control in rodents, because unilateral inactivation of dorsal hippocampus by tetrodotoxin impairs avoidance with high demand for cognitive control, but not spatial memory and navigation (Cimadevilla et al., 2001; Kubik, 2005; Wesierska et al., 2005).

5.5 Deficits in cognitive control and ensemble disambiguation

Acute administration of MK-801 (0.15 mg/kg) increases similarity between CA1 ensembles representing different environments (A/B) and obliterates the difference from the same environment (A/A). In addition, the same dose of MK-801 also impaired cognitive control on the Carousel (Kubík et al., 2014). The increased similarity is reminiscent of superposition catastrophe, where increased overlap of neuronal representations leads to loss of information and disrupted cognitive control.

Another line of evidence in support of the hypothesis that disrupted cognitive control in schizophrenia might be caused by abnormal neural coordination comes from studies using an established neurodevelopmental model of schizophrenia by neonatal lesion of the ventral hippocampus (NVHL), a model originally developed by Lipska and Weinberger in 1990’s (Lipska et al., 1993; Tseng et al., 2009). Rats with neonatal ventral lesion of the hippocampus showed both abnormal inter-hippocampal synchrony and diminished cognitive control on the Carousel (Lee et al., 2014). Targeting neural synchrony by an anticonvulsant ethosuximide was sufficient to normalize task-associated inter-hippocampal synchrony in the theta and beta range, increase synchrony between the hippocampus and medial prefrontal cortex, and improve cognitive control on the Carousel.

Since schizophrenia is conceptualized as a neurodevelopmental disorder, Lee et al. (2012) reasoned that the limited success of cognitive remediation in schizophrenia might be due to suboptimal timing of the therapy, which is initiated after the first psychotic episode (i.e. in adulthood). Early prophylactic cognitive remediation therapy during adolescence (characterized by major neuroplastic changes) might have greater impact on cognition. Rats with NVHL, which received cognitive training on the Carousel during adolescence, later demonstrated spared cognitive control and improved inter-hippocampal synchrony in adulthood, despite persisting brain lesion (Lee et al., 2012). In addition, the procognitive effect of adolescent training generalized beyond the specific training task to an unrelated T-maze alternation task.

EXPERIMENTAL PART

6 AIMS OF WORK

Previous work at the Department of Neurophysiology of Memory, Czech Academy of Sciences (Kubík et al., 2014) showed that 0.15 mg/kg MK-801 (i.p.) disrupts both cognitive control on the Carousel and neuronal coordination in that it increased ensemble similarity in rats that explored two different environments (A/B) and eliminated ensemble specificity to spatial context in CA1. Acute MK-801 administration is an established animal model of schizophrenia (Adell et al., 2012) and reduced cognitive control represents a core cognitive deficit observed in people with schizophrenia (Barch and Ceaser, 2012). Other authors (Lee et al., 2012) used neonatal ventral hippocampal lesion (NVHL) model of schizophrenia to demonstrate that cognitive training on the Carousel during adolescence has protective effect later in adulthood both on cognitive control performance (Carousel, T-maze alternation) and on neural coordination measured as inter-hippocampal synchrony of neural oscillation in the beta and gamma range.

Our major goal is to test the hypothesis that adolescent cognitive training on the Carousel in high cognitive control conditions (Room+Arena- avoidance) will mitigate the effect of acute MK-801 (0.15 mg/kg, i.p.) on neuronal coordination in rats. For our purpose, we operationally define neuronal coordination as a situation when the similarity (overlap) between two CA1 ensembles representing exploration during two distinct sessions (and marked by the presence of *Homer 1a* or *Arc*, respectively) in rats exploring the same environment twice (A/A) is greater compared to animals exploring two different environments (A/B).

To address this question, we trained rats on the Carousel during adolescence and examined their ensemble activity patterns using *Arc/H1a* catFISH procedure later in adulthood. We compared the effect of adolescent training on MK-801-induced deficit in neural coordination to handled controls. Specifically, we tested if cognitive training in adolescence prevents MK-801-induced increase in ensemble similarity in distinct environments (A/B) in adulthood.

A minor goal of this study is to find if the effect of adolescent cognitive training can transcend to other domains than representation of spatial context. Specifically, we tested if it also reduced increased similarity in home cage controls after MK-801.

7 MATERIALS AND METHODS

7.1 Subjects

Forty-eight male (n=48) Long-Evans acquired from the Institute of Physiology breeding colony at postnatal (PD) 30 were used. Rats were housed in transparent plexiglass cages (first by four then by two, depending on age), kept at 12/12 h light and dark cycle (lights on at 7 am), with water and food available *ad libitum*. Rats were acclimatized to the laboratory vivarium for 10 days (PD30-39), handled for 4 days (PD 40-43), and habituated to the arena (1 day, PD 44). A half of the animals was trained in the Room+Arena- (AAPA) task on the Carousel for 5 days (PD 47-51) while the other half was only handled. At PD 92-93 all rats were handled and at PD 94 they received either physiological saline or MK-801 injection (i.p., see Drug treatment). 30 minutes after the injection, animals received two exploration sessions (5 min) separated by 20 min interval in their home cages to induce IEG expression for *Arc/H1a* catFISH analysis of hippocampal ensemble activity patterns. Immediately thereafter, they were deeply anesthetized with isoflurane, decapitated, and their brains were flash-frozen and stored at -80°C for later analysis. All animal treatment complied with the Czech Animal Protection Act and EU directive 2010/63/EC.

7.2 Experimental design and groups

Twelve different experimental treatments were used for 48 animals. In general, some animals received pro-cognitive training in the AAPA task during adolescence, while others were only handled during this perion. In adulthood, either MK-801 was administered to induce schizophrenia-like behaviour or a physiological saline. Shortly after drug administration, a behavioural procedure to induce immediate-early gene (IEG) expression was used, after which rats were sacrificed, their brains quickly removed and stored at –80ºC for later processing. After slide preparation, mRNA of IEGs was labeled by fluorescent *in situ* hybridization. Experimental treatments are in more detail depicted in Figure 12. Overall design of the experiment is illustrated in Figure 13. For practical reasons, animals were divided into six cohorts (I-VI) of eight animals. Assignment of the experimental treatment for each animal within the group was done semi-randomly to increase within- and between-group comparisons (supplement 1).

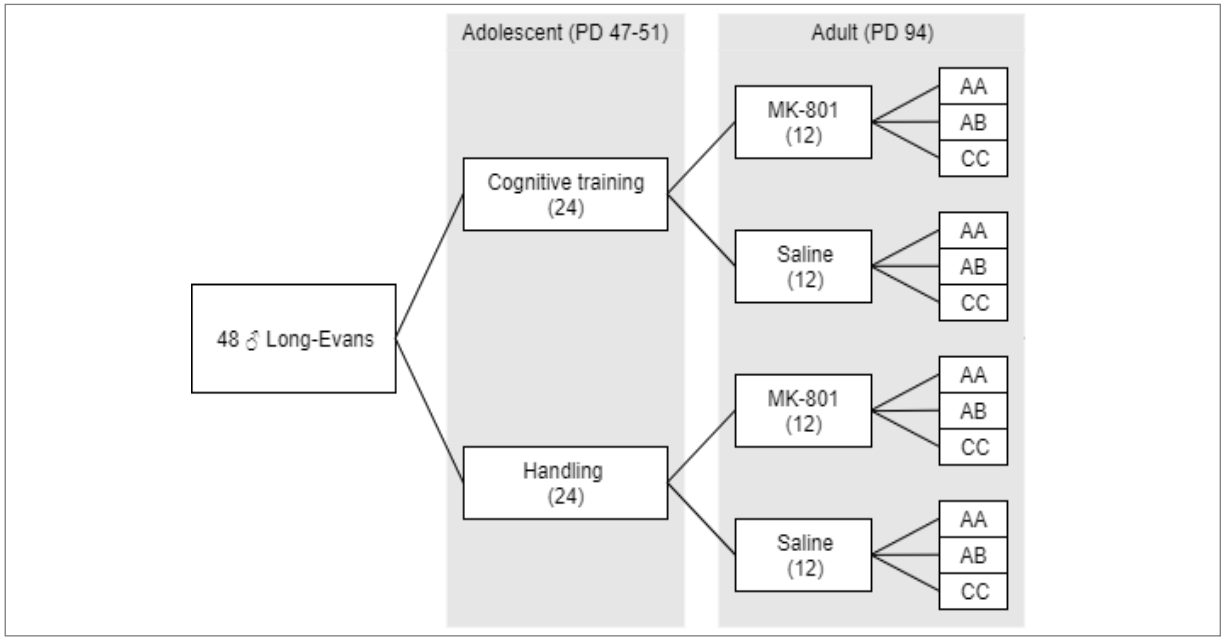

Figure 12. Experimental treatment. First, 48 male Long-Evans rats were divided into two groups during adolescence, one was subjected to the pro-cognitive training using AAPA task, other half served as a control group and was only handled during this period. Later, in adulthood, each group was further divided into two, intraperitonaly receiving either a solution of MK-801 0.15 mg/kg or an equivalent volume of physiological saline (1 ml/kg). Next, each group (MK-801 or Saline) was further divided into 3 groups: AA, AB, CC (each contained 4 animals). For example, one treatment group was Cognitive training/MK-801/AA.
AA – animal explored twice the same environment, **AB –** animal explored two different environments, **CC –** cage control animal, no exploratory session. **Numbers in brackets** refer to the number of animals receiving the treatment. **PD –** Postnatal day.

7.3 Adolescent pro-cognitive training

During adolescence, rats received cognitive training, which consisted of 5 days of training on the Carousel with high demand for cognitive control (Room+Arena- alias AAPA). Control animals were only handled during this period. Behavioural data were analysed by Track Analysis software (Biosignal Group Inc., USA).

7.3.1 Carousel apparatus

The apparatus consists of a smooth, elevated, metallic, circular arena, 82 cm in diameter, with 5 cm high lip at the outer edge and a transparent plexiglass wall on the perimeter to prevent rats from accidental or deliberate fall off the arena, and a motor for arena rotation (1 rpm). Arena was placed in a small room and indirectly lit by 40 W light bulb. One infrared (IR) LED was attached to the outer edge of the arena to compute a virtual “arena frame” view of the arena. Other IR LEDs were fixed to a harness carried by the rats on their backs. A tracking system (iTrack, Biosignal Group, Inc. New York, USA) was used to record position of the rat via an overhead IR camera, deliver shocks to reinforce place avoidance behaviour and store data for offline analysis. The harness was attached to a cable carrying power for the LEDs and the shock current (50 Hz, 0.5 s, 0.3-0.7 mA). AC shock current was delivered from a constant current source via an alligator clip connected to a subcutaneous electrode

located between rat's shoulders. The electrode was made by piercing the skin with a hypodermic needle and twisting the sharp end to secure the needle from slipping out.

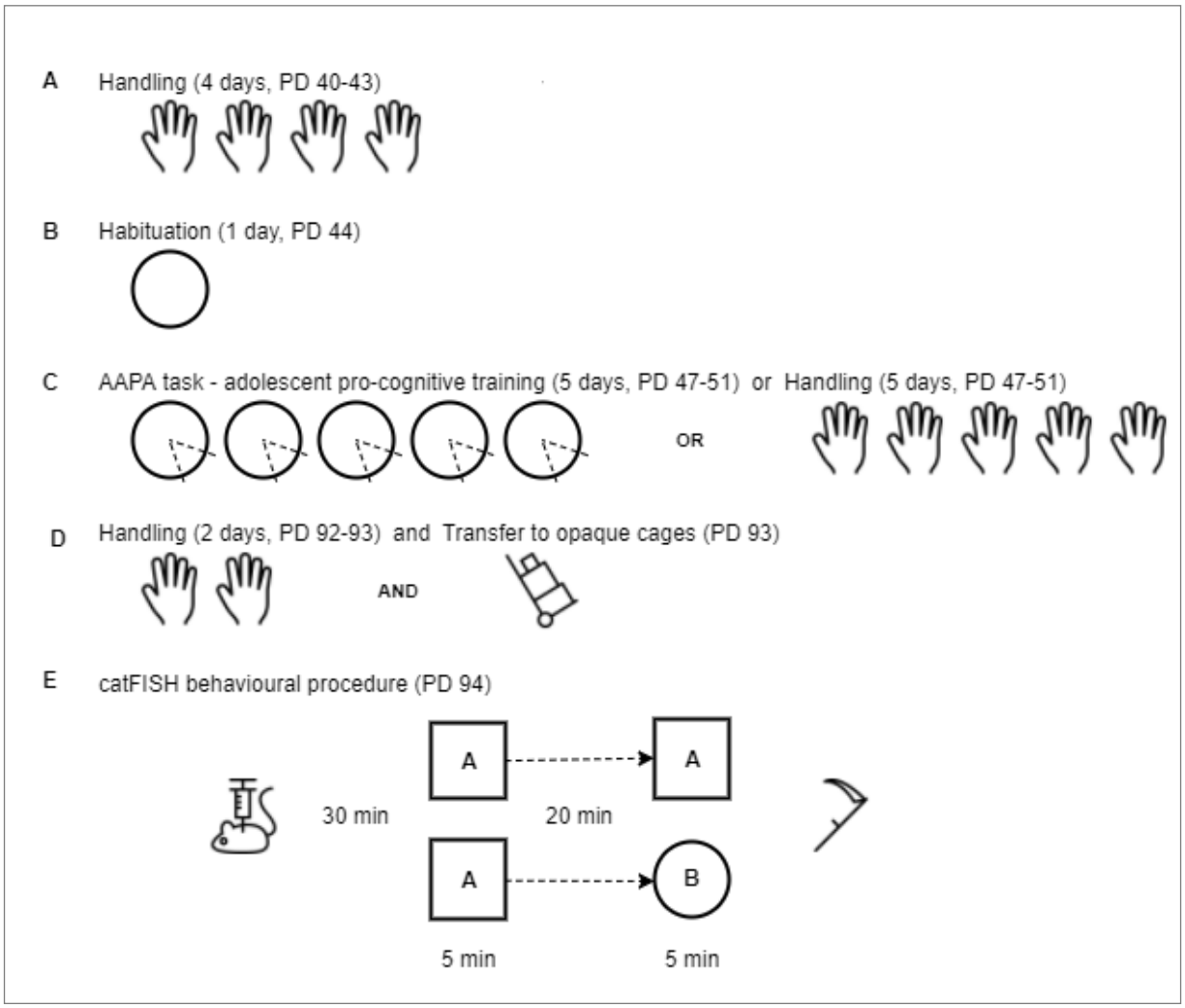

Figure 13. Experimental design. Rats (n=48) were recieved at postnatal day (PD) 30 and acclimatized to the laboratory vivarium for 10 days (PD30-39).
A – All rats were handled 5 min/day for four days (PD 40-43).
B – All animals were habituated to the Carousel apparatus, 1 session for 10 min, with light and rotation turned on, harness attached to rat's back but shock was turned off.
C – Adolescent rats (PD 47-51) were subjected either to pro-cognitive training (AAPA task, Room+Arena– condition, n=24), recieving one 20 min session/day for 5 days, or were handled 5 min/day for 5 days (control group, n=24).
D – All adult rats (PD 92-93) were handled 5 min/day for two days. After PD 93 handling, rats were moved from the basement laboratory vivarium to the ground floor vivarium and housed individually in opaque plexiglass cages to avoid high IEG expression background resulting from social interaction.
E – At PD 94, rats were subjected to the catFISH behavioural protocol (for details see below). During light phase of the day (morning till noon) all rats recieved a single MK-801 (0.15 mg/kg) or saline injection (see Drug treatment). After 30 minutes, rats experienced two 5 min exploratory sessions in open field for induction of IEG expression (twice the same environment AA or different environments AB condition). Exploratory sessions were separated by 20 min in homecage. Rats were sacrificed immediately after the second exploration. One third of the animals was kept as a cage control group (CC, not depicted) and did not recieve any exploration session (this group of animals was sacrificed at the corresponding time).

7.3.2 Room+Arena- (AAPA) task

In line with the literature (Lee et al., 2012), we chose the Room+Arena- task (AAPA) as the pro-cognitive training in adolescence. Computer-based tracking system (iTrack, Biosignal Group, USA) counted an entrance and delivered a mild but aversive shock whenever a rat entered a 60º shock sector for at least 500 ms and additional shocks were delivered every 1500 ms until the rat left the shock zone. New entrance was counted when rat left the shock zone for at least 1500 ms. Because size, weight and shock sensitivity varied between subjects, intensity of the shock was set individually for each rat (and was usually increased in later days of the training to keep animals motivated). Intensity was set to the lowest value sufficient to elicit an escape response, but not freezing, which could interfere with the task.

7.4. *Arc/Homer1a* catFISH mapping of neuronal ensemble activity patterns

To map active neuronal ensembles in the CA1 region of right dorsal hippocampus, we used double-label *Arc/Homer1a* catFISH technique (Guzowski et al., 2005; Kubík et al., 2007). The overall process consisted of the following: drug treatment, catFISH behavioural protocol, cryosectioning and slide preparation, riboprobe synthesis, fluorescent *in situ* hybridization, confocal microscopy, image acquisition, and image analysis. CA1 neurons were then classified as negative, *Arc*+, *H1a*+ and double (*Arc&H1a*)+.

7.4.1 Drug treatment

Adult rats (PD 94) received single injection of either sterile saline solution or MK-801 [dizocilpine; (+)-5-methyl-10,11-dihydro-5*H*-dibenzocycloheptene-5,10-imine maleate; Sigma] diluted in saline at a dose of 0.15 mg/kg. Injections were administered 30 minutes prior to the catFISH behavioural protocol (1ml/kg, i.p.).

7.4.2 catFISH behavioural protocol

Rats, housed individually in opaque plexiglass cages to reduce IEG expression induced by social interaction, received a single injection of either saline (n=24) or MK-801 solution (n=24). Half of these rats received cognitive training during adolescence and the other half was handled. After 30 minutes, some rats received two exploratory sessions lasting 5 min and separated by 20 min in the home cage. Two different environments were used for the exploratory sessions: environments A and B. Sixteen rats explored the same environment twice (A/A) and 16 explored different environments (A/B). Sixteen rats remained in their home cages as cage controls (CC). The behavioural procedure is illustrated in fig. XY1 (image E).

Environment A was a white square open field with 72 cm long sides, non-transparent walls, softly lit with dispersed light, containing three identical objects (wooden blocks with size 6.5 cm × 5.5 cm × 4.5 cm) and treated with ethanol. Environment B (located in another room) was a circular arena 82 cm in diameter with white plastic floor and transparent plexiglass wall, softly lit with dispersed light, containing three identical dark plastic cylinders (6 cm in height and 5.5 cm in diameter), and treated with acetic acid solution. Neither of the two rooms (with A or B environments) was used during cognitive training in adolescence. Immediately after the second exploratory session (or at a corresponding time point for the CC group), rats were deeply anesthetized with isoflurane and decapitated. To prevent IEG pre-mRNA diffusion and degradation, which would compromise subsequent image analysis, brains were quickly removed and flash-frozen in isopenthane bath cooled by dry ice and stored in −80ºC for later processing.

7.4.3 Tissue and slide preparation

Frozen brains were cut in coronal plane to obtain four millimeter thick segments of the right hemisphere with the right dorsal hippocampus. Eight such segments (one for each animal from the same I-VI group) were embedded in optimal cutting temperature medium (OCT; Sakura) to create one block. Arrangement of brain segments in each block was done in a semi-random fashion to increase within- and between-group comparisons and to counterbalance potential effects of position within a block. In total, six such blocks were prepared as illustrated by supplement 2. The blocks were cryosectioned at 20 µm in a cryostat (Leica CM 1850, Germany), thaw-mounted on gelatine-coated slides (Fisher) and stored at –80ºC for later processing.

7.4.4 Riboprobe synthesis

Digoxigenin- and fluorescein-labeled RNA probes (riboprobes) for fluorescent *in situ* hybridization were synthesized according to literature (Guzowski et al., 2005; Kubik et al., 2012) using commercial transcription kits (MaxiScript; Ambion, Thermo Fisher Scientific) and RNA labeling mixtures (Roche/Sigma). *Arc* antisense riboprobes were labeled with digoxigenin during synthesis. To reduce background and nuclear staining, shorter fluorescein-labeled riboprobes were generated for *H1a* detection (full pre-mRNA of *H1a* is ~55kb long). This shorter (1002 bp) *H1a* antisense riboprobe targeted 5' end of the 4.4 kb long 3'UTR of the *Homer 1a* pre-mRNA.

Plasmid linearization and purification. Circular plasmids containing template sequences for riboprobe *in vitro* synthesis were linearized with restriction enzymes (Hind III for *Arc*, Eco RI for *H1a*) using commercial kit from New England Biolabs. After restriction digest, linearized plasmids were purified. First, proteins were degraded by protease K (150 µg/ml, 30 min at 37ºC).

DNA was isolated using phenol-chlorophorm-isoamlyl alcohol (PCIA) extraction. Organic phase of PCIA mixture was added to the sample, mixed, let 1 min to rest and centrifuged for 3 min. Top layer containg DNA was removed and the PCIA extraction process repeated. After second extraction and centrifugation 10% of extracted volume of 3M sodium acetate and 250 % of extracted volume of 100% ethanol was added. Solution was incubated for 30 min at -20ºC and then centrifuged for 30 min at 4ºC. Ethanol (75%) was added to the pellet and centrifuged for 15 min at 4ºC. Supernatant was removed and the remaining pellet was incubated at 56ºC in heatblock until all ethanol evaporated. Rnase-free water (1 µl/µg of purified DNA) was added and solution incubated for 5 min at 56ºC to dissolve the pellets.

The presence of linearized DNA in the solution was verified by DNA agarose-gel electrophoresis. Approximate concentration of the DNA in the sample was calculated by comparing integrated density signal of linearized DNA bend to the signal of the ladder band on the agarose gel (using ImageJ).

Riboprobe synthesis and purification. Linearized and purified plasmids, digoxigenin- or fluorescein-labeled mixture of RNA nucleotides, T3 RNA polymerase, transcription buffer and RNase-free water were mixed and incubated at 37°C. After 1 hour, DNAse I was added to the sample to degrade plasmid DNA. After 15 min of incubation, 0.5M EDTA and RNase-free water were added.

To purify riboprobes from DNA and protein residues, Mini Quick Spin Columns (Roche) were used. Spin columns were centrifuged for 1 min. RNase-free water was added to the spin columns and centrifuged again (this process was repeated once more). Then, two samples containing riboprobes were added to their respective spin columns and centrifuged for 4 min. The presence of riboprobes was determined by RNA agarose-gel electrophoresis.

7.4.5 Double-label RNA IEG FISH

Three highest-quality slides from each block were selected (in total 18 slides) and fluorescent *in situ* hybridization was performed according to literature (Kubik et al., 2012; Vazdarjanova and Guzowski, 2004).

Fixation and dehydration. Samples (slide-mounted brain sections) were fixed in 4% solution of cooled and buffered paraformaldehyde, washed in 2× SSC, incubated in 0.5% acetic anhydride/ 1.5% triethanolamine, washed in RNase-free water, dehydrated by acetone/methanol at –20ºC, rehydrated and equilibrated in 2× SSC.

Pre-hybridization. PAP-lines were drawn at the shorter sides of each slide to prevent applied solutions from leaking off the slide. Samples were treated with pre/hybridization-buffer (without

riboprobe), coverslips were added and samples incubated for 30 min at room temperature. Incubation tray contained solution of 2× SSC/ 50% formamide of the same osmolarity as the pre-hybridization buffer.

Riboprobe denaturation. Riboprobes were diluted in hybridization buffer for final concentration 1 ng of riboprobe per 1 µl of solution, heat denatured (90ºC/5 min) and cooled at –20ºC.

Hybridization. Coverslips were washed off in 2× SSC solution, 120 µl of hybridization buffer with riboprobes was applied and coverslips added. The digoxigenin-labeled *Arc* antisense riboprobes and fluorescein-labeled *Homer1a* 3′ UTR antisense riboprobes were added together. Samples in incubation tray were incubated overnight at 56ºC.

Post-hybridization wash. Coverslips were removed in 2× SSC, samples were washed twice in 2× SSC at room temperature, incubated for 15 min (37ºC) in RNase A (10 µg/ml of 2× SSC), washed twice in 2× SSC, then 0.5× SSC (all at room temperature), incubated for half an hour in 0.5× SSC at 56ºC and finally washed in 0.5× SSC at room temperature.

Peroxidase activity quenching and blocking. Samples were incubated in 3% solution of H_2O_2 (2× SSC), washed in 2× SSC and incubated in blocking buffer for 10 min.

Detection of fluorescein-labeled *H1a* 3′UTR riboprobes. Anti-fluorescein horseradish peroxidase (HRP) -antibody conjugate (Jackson labs) was centrifuged for 20 min at 4ºC. Detection solution was prepared by diluting the antibody conjugate in blocking buffer (1:1000), applied to samples, coverslipped and incubated for 2 h at room temperature. Coverslips were washed off in TBS, slides were washed three times in TBS-T.

Tyramide-fluorescein signal amplification for *H1a* 3′UTR riboprobes. Tyramide-fluorescein solution (TSA kit, PerkinElmer) was applied to each sample for signal amplification of *H1a* 3′UTR riboprobes, coverslipped and incubated for 30 min at room temperature. Coverslips were washed off in TBS, samples washed twice in TBS-T.

Detection of digoxigenin-labeled *Arc* riboprobes. Residual peroxidase activity was quenched immediately after the second TBS-T wash by incubating the samples in 3% solution of H_2O_2 (2× SSC) for 15 min. Samples were then washed three times in TBS-T. Anti-digoxigenin HRP-antibody conjugate (Roche) was centrifuged for 20 min at 4ºC. Detection solution (anti-digoxigenin conjugate diluted in blocking buffer, 1:300) was applied to samples, coverslipped and incubated for 2 h at room

temperature. Coverslips were washed off in TBS, slides were washed three times in TBS-T.

Tyramide-CY3 signal amplification for *Arc* riboprobes. Tyramide-CY3 solution (TSA kit, PerkinElmer) was applied to each sample for signal amplification of *Arc* riboprobes, coverslipped and incubated for 30 min at room temperature. Coverslips were washed off in TBS, samples washed twice in TBS-T and once more in TBS.

Dapi staining and sealing. Cell nuclei were stained by DAPI (Invitrogen), 1:10000 (DAPI/TBS), samples were washed twice in TBS. Slides were coverslipped with antifade medium (Vectashield, Vector labs) and sealed with nail polish.

7.4.6 Confocal microscopy, image acquisition and analysis

Eighteen slides labeled by *in situ* hybridization were used for image acquisition (three slides from each I-VI block). Each slide contained right hemispheres from 8 animals (same I-VI group). Three microscope fields (regions) from each of the CA1 region present on the slide were selected and captured in a Z-stack of 21 images (sampling frequency 1 μm). In total 432 Z-stacks (of equal number of regions) were acquired (18 slides ×8 hemispheres per slide ×3 areas per CA1).

Confocal stacks from CA1 were acquired on an inverted Leica TCS SP8 AOBS WLL confocal laser scanning microscope with 20× oil immersion objective. Blue signal (DAPI) was imaged with 405 nm excitation and a 415-490 bandpass, green signal (TSA-Fluorescein) with 488 nm excitation and 510-550 bandpass, and orange/red signal (TSA-Cy3) with 561 nm excitation and 610-680 bandpass. Laser power, gain and offset were always set at the same value for the whole slide and settings were optimized to obtain bright intranuclear foci (INF) signal of the IEGs pre-mRNA. Image acquisition from each slide was completed in a single session.

Image analysis was performed semi-automatically using ImageJ and a custom macro. Only **neuronal nuclei** in the median 20% of the remaining Z-stack were (manually) selected to avoid inclusion of partial cell nuclei and possible false negative result (due to cut-off INF signal). Putative glial cell nuclei, identified by their smaller size and bright, unstructured nuclear counterstaining, were not included in the analysis.

After cell nuclei selection, all optical planes in a stack were projected into a single plane (each channel (blue, green, red) was z-projected into a separate image). **Background** in green and red channel was subtracted by selecting four sample areas (previously selected cell nuclei) in each channel with high background noise and subtracting their average integrated density from all pixels in the image. Selection of four sample areas for background removal was done manually for each region and channel to maximize signal to noise ratio (researcher was blinded to the experimental treatment of animals). Z-projected images from all three channels were then combined into a single **composite**

image (432 composite images were obtained). Number of selected cells and signal parameters for each probe (integrated, average, minimum and maximum intensities) were measured automatically.

Next, **thresholds** for determination of *Arc* and *H1a* INF positive cells in composite images were set. Cells were automatically **classified** as *Arc*+ or *Arc*– and *H1a*+ or *H1a*– (or double-positive/-negative) depending on the input threshold values. These threshold values were set separately for each region, each probe (green and red channel) and each slide by manually selecting four cells (in each region and probe channel) with the lowest INF signal. Previous experiments at our department showed that MK-801 lowers IEG expression (both number of positive cells and signal strength). Also, IEG expression in cage controls was expected to be low. To avoid false positive signal by setting thresholds too low (by including cage control animals and MK-801 treated group), only animals that explored novel environment (A/A or AB) and received saline were selected for threshold setting. Then, average threshold values of integrated and maximum intensities from one slide were applied to all images from a given slide. Images from CC animals were not used for setting the threshold values to avoid false positive results (therefore, at this point in the image analysis, researcher was blinded only to the non-CC animal samples).

To infer the degree of overlap between ensembles activated by each behavioural epoch, we calculated the similarity score. The **similarity score** (as described by Vazdarjanova and Guzowski, 2004) reduces four types of cell-staining values (negative, *Arc*+, *H1a*+ and *Arc*/*H1a*+) from 2 behavioural epochs into a single value and allows comparison of neuronal activity patterns across multiple brain regions. Similarity score with a value of 1 indicates that the same population of neurons was activated in both epochs. A score of 0 signify that two statistically independent neuronal populations were active during the two epochs.

Similarity scores were calculated as follows (Vazdarjanova and Guzowski, 2004):

(1) **Epoch 1 active cells** = ($H1a^+$ plus $Arc/H1a^+$)/total cells
This is the fraction of total *H1a* positive cells

(2) **Epoch 2 active cells** = (Arc^+ plus $Arc/H1a^+$)/total cells
This is the fraction of total *Arc* positive cells

(3) **p(E1E2)** = epoch 1 active cell fraction × epoch 2 active cell fraction.
This is the probability of cells being active in both epochs ($Arc/H1a^+$ cells), assuming the two epochs activated statistically independent neuronal ensembles (i.e. p(E1E2) is random overlap)

(4) **diff(E1E2)** = ($Arc/H1a^+$) – p(E1E2).
This is a measure of deviation from the independence hypothesis.

(5) **Least epoch** = the smaller of the ensembles activated by epoch 1 or epoch 2.

(6) **Similarity score** = diff(E1E2)/(least epoch - p(E1E2).
This normalizes the diff(E1E2) fraction to a perfect A/A condition.

A perfect A/A is 1, and a perfect A/B is 0.

8 RESULTS

8.1 Cognitive training on the Carousel (Room+Arena–; AAPA)

Level of significance was set at alpha = 0.05, two-tailed. We analyzed performance on the Carousel in rats that received cognitive training on the Carousel in adolescence (other animals were only handled). Repeated measures-ANOVA (or its non-parametric equivalent related-samples Friedman's ANOVA) was used to assess the number of errors (entrances to the to-be-avoided sector), maximum time of avoidance and distance walked by animals during each session on the Carousel. Sessions were used as a repeated within-subject measure. Results are illustrated in Figure 14.

The number of **errors** (E) was not normally distributed and no transformation was able to correct the issue. Thus, related-samples Friedman's ANOVA was used with Bonferroni correction applied to the level of test significance (for 5 learning days it makes new level of significance $p = 0.01$). The ANOVA showed that the number of errors decreased across sessions [$\chi^2(4) = 56.836$, $p < 0.001$]. Related samples Wilcoxon sums ranks post-hoc tests revealed significant improvements between sessions 1 and 2 [$W+ = 45$; $z = -2.833$, $p = 0.05$], 2 and 3 [$W+ = 33$; $z = -3.181$, $p = 0.001$], and 4 and 5 [$W+ = 48$; $z = -2.349$, $p = 0.05$]. No significant improvement was found between sessions 3 and 4 [$W+ = 87$; $z = -1.285$, $p = 0.199$].

Values of the **Maximum avoidance time** (maxT) had to be logarithmically transformed to meet parametric assumptions. The rm-ANOVA found a significant increase in maximum time of avoidance throughout sessions [$F(4,88) = 15.86$, $p < 0.001$]. Repeated planned contrasts showed a significant increase of maxT between sessions 1 and 2 [$p = 0.007$], 2 and 3 [$p = 0.041$], sessions 3 and 4 [$p = 0.024$] but not sessions 4 and 5 [$p = 0.130$].

Distance (D) walked by animals on each day was analyzed using rm-ANOVA since data met all parametric assumptions. The ANOVA showed that overall locomotion did not change during the course of the training [$F(4,88) = 1.805$, $p = 0.135$].

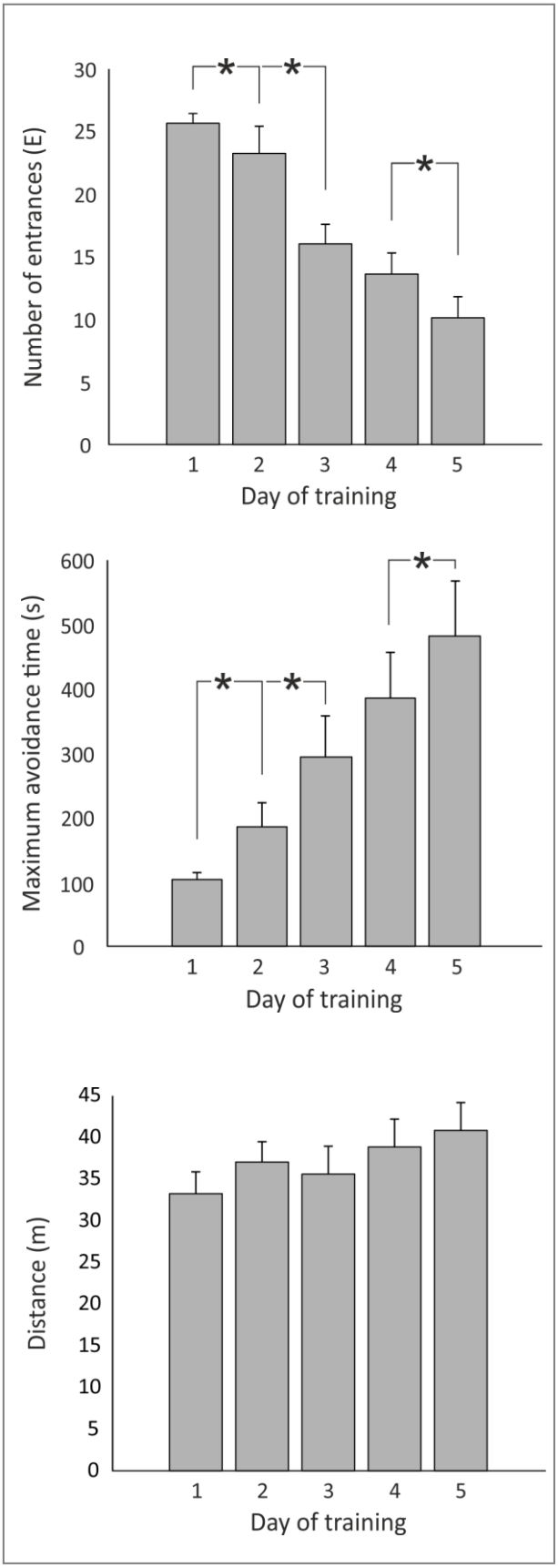

Figure 14. Place avoidance training on a rotating arena during adolescence.
For the purpose of pro-cognitive training, 24 adolescent rats were received one 20 min session/day for 5 days of place avoidance task on a rotating arena (Room+Arena– variant; AAPA).

Average number of entrances to the shock sector per each day of training. Subjects entered the shock zone significantly less after each day of training. The only exception was day 4, which did not significantly differ from the previous training day. Average number of entrances on day 1 was 25.6 ± 0.8 (SEM) and decreased to an average of 10.1 ± 1.8 on day 5.

Average Maximum avoidance time (s) per each day of training. Subjects avoided the shock sector for significantly longer after each training day. The only exception was day 4, which did not significantly differ from the previous day. Average maximum time of avoidance on day 1 was 102.9 ± 10.3 s and decreased to an average of 481.3 ± 85.5 on the last training day.

Average distance (m) walked by rats on the Carousel each training day was not significantly different between sessions. On day 1, average distance was 33.3 ± 2.5 m and increased to 40.7 ± 3.3 m on day 5.

8.2 IEG expression and ensemble similarity

To determine if exploration and MK-801 affected IEG expression, and to evaluate similarity between neuronal ensembles under different conditions, we used double-label *in situ* hybridization for *Arc*/*H1a* mRNA (Figure 15). After confocal microscopy, we selected neuronal nuclei for later image analysis. Three slides per animal, and three different positions in CA1 region were analysed from each slide. Each position was captured as a Z-stack of 21 images. Next, by selecting only the nuclei present in the middle 20 % of Z-stack, 51 neurons were chosen for further analysis. The number of CA1 neurons selected for each rat (all 9 positions summed) ranged from 335 to 581 (mode 440). For 48 animals, we analysed a total of 21 801 neurons for the presence of *Arc*, *Homer1a* or both intranuclear foci signals (INF). At the last step of image analysis, CA1 neurons were determined to be *Arc*+, *H1a*+, double-positive (*Arc*+/ *H1a*+) or negative (no INF signal present).

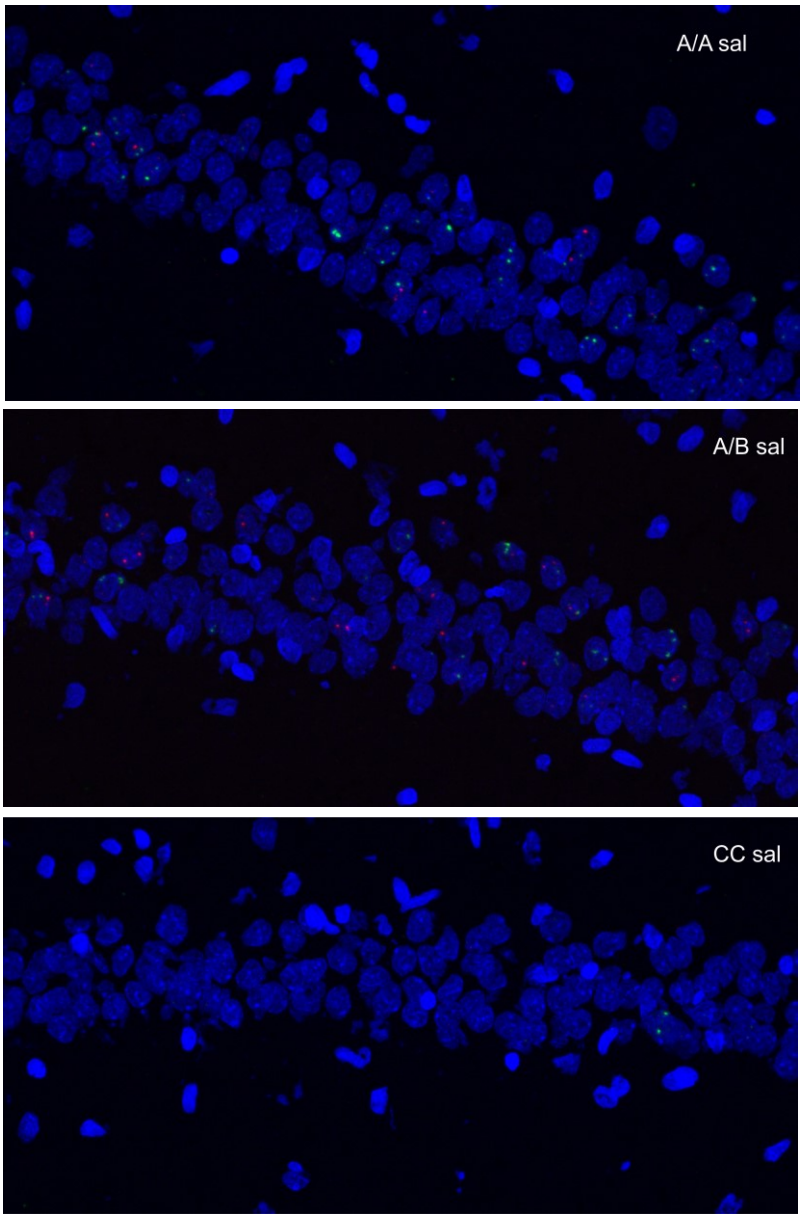

Figure 15. Immediate-early gene (IEG) expression in hippocampal CA1 neurons.
Samples of Z-projected composite images with labeled *Arc* (**red**) and *Homer1a* (**green**) mRNA from saline-treated rats (**sal**). Expression of IEGs was induced by exploration of the same environment twice (**A/A**) or two different environments (**A/B**). Some animals served as cage controls (**CC**) and did not explore novel environment.
Samples from animals that received MK-801 (0.15 mg/kg) i.p. injection are not depicted. In general, MK-801 decreased the intensity and number of intranuclear foci (bright intranuclear signal of labeled *Arc* and *Homer1a* mRNA).

8.2.1 Exploration increased and MK-801 decreased expression of IEGs

Previous studies show that exploration of a novel environment causes massive induction of IEGs *Arc* and *Homer 1a* expression in hippocampal pyramidal neurons (Guzowski et al., 1999; Vazdarjanova et al., 2002). Acute administration of MK-801 decreased this exploration-induced expression (Kubik et al., 2014). We first examined if our data meet these assumptions using a three-way ANOVA with repeated measures on proportions of *Arc*+ and *Homer 1a*+ cells and main factors of Treatment (MK-801 or saline) and Behaviour (cage controls vs. exploration, A/A+A/B environments pooled). The ANOVA confirmed that exploration increased IEG expression compared to cage controls [$F(1,44) = 15.111$, $p < 0.001$] and that MK-801 decreased IEG expression relative to saline [$F(1,44) = 10.103$, $p = 0.003$]. Results are illustrated in Figure 16.

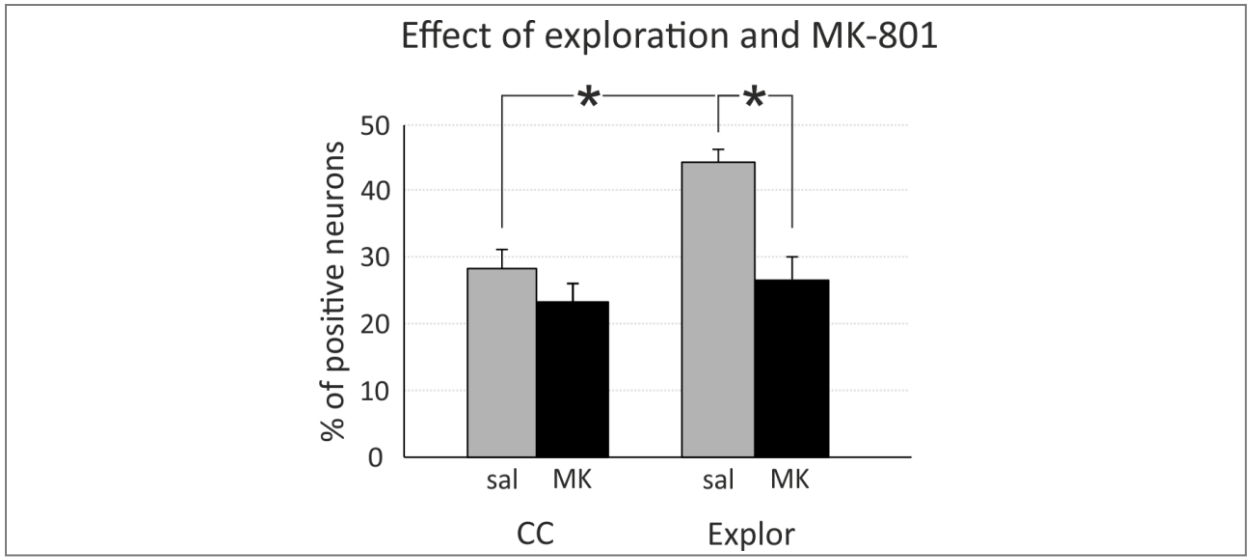

Figure 16. Effects of exploration and MK-801 on IEG expression (*Arc, Homer 1a)*.
Effect of exploration on IEG expression. In saline-treated animals, exploration (A/A+A/B) significantly increased the proportion of IEG+ neurons (from 28.6 % to 44.3 %). In MK-801-treated animals, no significant increase was observed in the exploratory group.
Effect of MK-801 on the percentage of IEG+ neurons. In cage controls (CC), no significant difference was observed between saline and MK-801-treated animals. In group that explored novel environment (A/A+A/B), the proportion of IEG+ neurons was significantly smaller in MK-801 group than in saline-treated animals (26.5 % to 44.3 %).
In addition, a significant **interaction** between environment and pharmacological treatment (saline/MK-801) was revealed by statistical analysis suggesting that MK-801 eliminates the increase of IEG+ cells caused by exploration.
CC – cage control animal (n = 16); **Exploration** (**Explor**) – both animal groups that explored the same environment twice (A/A) or two different environments (A/B) are added together (n = 32); **sal** – physiological saline; **MK**- MK-801

8.2.2 MK-801 abolished the increase of IEG expression induced by exploration

A significant interaction between treatment and exploration [F(1,44) = 4.506, p = 0.039] suggested that MK-801 affected IEG expression after exploration differently than in CC. Post-hoc tests with significance level adjusted by Bonferroni correction to alpha = 0.025 revealed a significant exploration-induced increase in numbers of *Arc*+ and *Homer 1a*+ cells after saline [t(22)= -4.881, p <0.001], but not after MK-801 [t(22) = -0.629, p = 0.536].

8.2.3 CA1 ensembles showed contextual specificity after saline, but not after MK-801

An important assumption of this study was that ensembles activated in the same environment (A/A) are more similar than in different environments (A/B) in the absence of MK-801 (Guzowski et al., 1999, Kubík et al., 2014). To test this assumption, we compared saline-treated animals exploring the same or different environments using two-way ANOVA with Environment (A/A, A/B, CC) and Adolescent training (training or handling only) as between-subject factors. Cage controls were included in the analysis to compare ensemble similarity after explicit exploration with that induced by spontaneous behaviour during unsignaled epochs in the home cage (exploratory sessions were signaled by experimenter handling the animals). The ANOVA found no effect of training [F(1,18) = 0.535, p = 0.474], but a significant effect of environment [F(2,18) = 24.394, p < 0.001].Post hoc tests showed that all groups were different from each other. Importantly, similarity was highest in the A/A group, lower in A/B, and lowest in CC. No such effect were observed after MK-801, in agreement with previous observation that MK-801 eliminates contextual specificity (difference in ensemble similarity between A/A and A/B exploration) in CA1. The analysis confirmed previously published results in line with our assumptions. Results are illustrated in Figure 17.

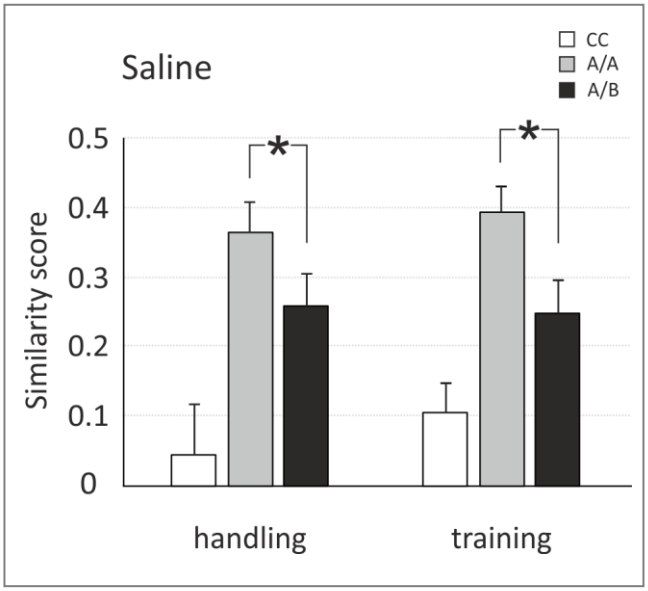

Figure 17. Ensemble similarity in saline-treated animals.
Saline-treated animals. A significant difference in similarity scores between A/A and A/B was present both in handling and training group. Similarity scores of cage controls (CC) were significantly different from both A/A and A/B (not marked in the figure).

8.2.4 Effect of adolescent pro-cognitive training on ensemble similarity

We tested if cognitive training during adolescence protects neuronal coordination (expressed as ensemble similarity A/A > A/B) from the detrimental effect of MK-801 (expressed as ensemble similarity A/A = A/B). If training was protective, ensemble similarity in A/A and A/B should remain significantly different even after MK-801. We analyzed MK-801-treated animals separately, because no effect of pre-training was predicted in saline controls. Despite a relatively large decrease in ensemble similarity observed in the A/B and CC groups after training compared to handling (see Figure 18), a two-way ANOVA on similarity scores with main factors of Environment (A/A, A/B, CC) and Training (training, handling) failed to find an effect of environment [$F(2,18) = 0.797$, $p = 0.466$], training [$F(1,18) = 0.624$, $p= 0.440$], nor interaction [$F(2,18) = 0.973$, $p = 0.397$].

We also conducted hypothesis-driven independent samples t-tests to directly test the hypotheses that cognitive pre-training during adolescence reduces ensemble similarity increased by MK-801 in rats subjected to a) A/B exploration or b) home cage. Nonetheless, neither of these tests proved significant [$t(6) =1.3$, $p = 0.241$. Results are illustrated in Figure 18.

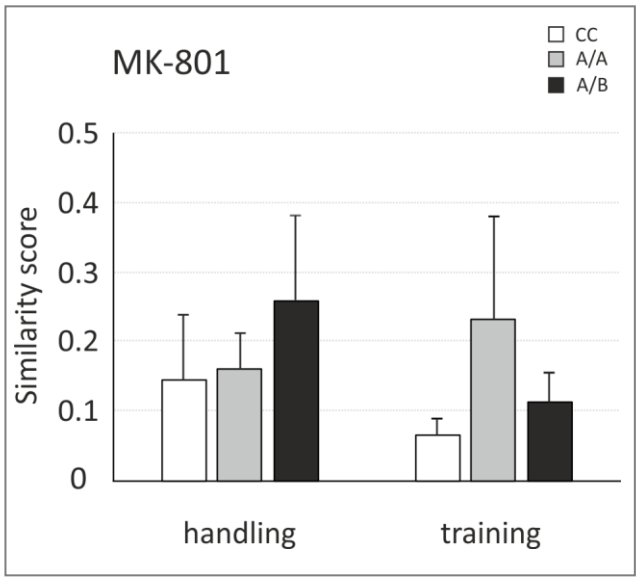

Figure 18. Effect of adolescent training in MK-801-treated animals.
No significant difference in similarity scores was revealed by two-way ANOVA between handled and trained animals (all MK-801-treated). Comparison of A/B similarity score between handled and trained groups (t-test) did not show any significant difference, despite A/B similarity score being twice smaller in trained animals (0.11 ± 0.05 SEM) than in handled animals (0.25 ± 0.12).

9 DISCUSSION

The aim of this thesis was to evalute the protective effect of adolescent cognitive training (Carousel task) on NMDAR antagonist MK-801-induced acute impairment of neuronal coordination in adulthood. The protective effect would manifest as preserved contextual specificity of neuronal ensembles activated during exploration of two distinct environments (A/B), which has been demonstrated to be deficient in MK-801-treated rats (Kubík et al., 2014). Acute MK-801 administration is an established animal model of schizophrenia (Bubeníková-Valešová et al., 2008) and disrupted neural coordination is supposed to underlie cognitive deficits observed in patients with schizophrenia (Fenton, 2008).

9.1 Adolescent cognitive training on Carousel

We first examined if animals showed learning during adolescent cognitive training on the Carousel. Half of the animals received the training to stimulate processes of cognitive control and potentially prevent neural coordination deficit in adulthood. Cognitive control allows focusing attention on goal-relevant information and ignoring irrelevant distractions (Amer et al., 2016), which is impaired in schizophrenia. We consider the number of entrances and maximum time of avoidance to be sufficient behavioural indicators of the engagement of cognitive control in the Carousel task. We demonstrated a clear learning curve both using the **Number of entrances (E)** and the **Maximum avoidance time (MaxT)**, yet animals in this study did not reach the same asymptotic level of performance established in previous studies (Kubik et al., 2014). This deficiency may be age-related, as other studies from our laboratory revealed suboptimal place avoidance in rats under six weeks of age (unpublished observations). Another factor that could have contributed to better performance in other studies is increased motivation in rats trained to simultaneously collect randomly distributed food on the arena (**foraging**; Kubik et al., 2014), which was not used in this study. In contrast to place avoidance, overall locomotion (**Total distance traversed - D**) did not significantly change during the course of training, although average distance gradually increased from 33.3 m to 40.8 m. In addition, we also recorded the number of **shocks**. The average number of shocks delivered significantly decreased with training from 119 on day 1 to 46 on day 5. This is still relatively high compared to adult rats trained with foraging (Kubík et al., 2014). After the first shock resulting from an entrance, additional shocks were delivered every 1.5 s until rat left the shock sector. A small number of rats displayed a strategy of directly running through the shock sector, which limited the number of shocks per entrance to 1, instead of running against the direction of rotation and away from the sector, which limited the number of entrances compared to a passive strategy (sitting) that would result in 20 entrances per session and 4-5 shocks per entrance. Two types of the run-through strategy occurred. Some rats ran through the sector before receiving the shock (‘anticipators’), but others escaped only after receiving the first

shock (‘responders’). Although both strategies are suboptimal, the anticipators were able to predict the approaching shock sector, which required cognitive control, whereas the responders learned to simply escape in response to the shock, without the need for cognitive control. These strategies result in similar numbers of entrances and shocks, but they differ in engagement of cognitive control. Therefore, we prefer the Number of entrances which is not affected by these strategies.

9.2 IEG expression, MK-801 and exploration

In agreement with previous reports (Kubík et al., 2014; Buchtová et al., 2016, Vazdarjanova and Guzowski, 2004) novel environment **exploration** significantly increased the proportion of *Arc*+ and *Homer 1a*+ neurons compared to cage controls. Aproximately 44% of neurons were IEG+ in saline-treated rats exploring novel environment, which is similar to those reported by Vazdarjanova et al. (2002), but slightly higher than reported by Kubík et al. (2014)

However, the fraction of **IEG+** neurons in **cage control** animals was very high, 29.6% and 23.4% for saline and MK-801-treated animals, respectively. In studies by other authors, only 5% of neurons (on average) were IEG+ in cage control animals (Kubík et al. 2014; Vazdarjanova et al. 2002; Guzowski et al. 1999; Nomura et al. 2012). We suspect that **suboptimal conditions** at the vivarium, where rats were housed prior to and between behavioural testing for IEG induction and accidental noise are responsible for the high background IEG expression in CC animals and their lack of decrease after MK-801. Each animal cohort was moved to the local vivarium the night before behavioural testing to ensure calm conditions and prevent overt IEG induction prior to testing. Probably the most severe of the disturbances were illegitimate stress experiments using social defeat paradigm carried out in the vivarium during the course of our study. **This fact was a direct breach of the directions for animal housing and it was not known to us at the onset of our study**. More than half of cohorts were present at the same vivarium, a fact that we discovered only later. During aggressive encounters and stressful situations rats vocalize both in sonic and ultrasonic range (Castelhano-Carlos and Baumans, 2009). Ultrasound emmission is an important communication medium in rats and it can activate the limbic system in rats even at low intensities (ibid.). Limbic system includes basolateral amygdala (BLA) that projects to the hypothalamic paraventricular nucleus (PVN), which in turn regulates the hypothalamo-pituitary-adrenocortical (HPA) axis responsible for stress response (Jankord and Herman, 2008). BLA activation has been shown to induce *Arc* expression in the hippocampus (Huff, 2006). These facts strongly suggest that IEG expression and ensemble similarities were influenced by uncontrolled variable of stressful conditions and accidental noise

Next, NMDAR antagonist **MK-801** (0.15 mg/kg, i.p.) significantly decreased proportions of IEG expressing neurons in exploring animals (**A/A+A/B**). This finding is consistent with previous experiments by Kubík et al. (2014), who showed that 0.10 and 0.15 mg/kg of MK-801 substantially reduced both *Arc* and *Homer 1a* expression in the CA1 hippocampal region. This reduction is likely

due to direct effect of MK-801 on pyramidal neurons, because IEG expression depends on NMDAR activity (Kubík et al., 2014; Link et al., 1995). NMDARs are crucial for many types of synaptic plasticity (Vyklicky et al., 2014) and both *Arc* and *Homer 1a* are implicated in various types of neuroplastic changes (Bramham et al., 2010; Sala et al., 2003). Inhibition of NMDARs on pyramidal cells by the psychotomimetic MK-801 is not necessarily in conflict with the proposition that psychotic disorganization (Fenton, 2008) and disrupted cognitive control is caused by disinhibition of pyramidal neurons. Reduced IEG expression can result from direct NMDAR inhibition on pyramidal neurons and reflect general impairment of plasticity resulting in learning deficits. In contrast, increased ensemble similarity may result from disinhibition due to inhibition of NMDAR on GABAergic interneurons and reflect impaired neural coordination and cognitive control.

MK-801 did not significantly decrease IEG expression in **cage controls** (**CC**). This result contrasts with observations of Kubík et al. (2014). Only 5 % of neurons were IEG+ in MK-801 CCs in the study by Kubík et al. compared to 24 % in ours. We suspect that this lack of decrease in IEG expression after MK-801 in cage controls also results from stressful vivarium conditions and noise. Besides decreasing IEG expression via NMDAR blockade (as mentioned above), MK-801 also decreases sensory gating (Bast et al., 2000). Impaired sensorimotor gating increases sensitivity to environmental and self-generated stimuli, which can subsequently increase IEG expression – if IEG-inducing stimuli such as stress or noise are present.

9.3 Ensemble similarity

In both trained and non-trained saline-treatead animals, esembles acitvated in two eploratory sessions were more similar if the same environment was explored twice (**A/A**) than in two different encirenments (**A/B**). Our observations are consistent with previous research (Guzowski et al., 1999; Kubík et al., 2014, Vazdarjanova and Guzowski, 2004). The difference in A/A to A/B similarity we observed was significant, although not as stark as reported by Guzowski et al. (1999) or Kubík et al. (2014). We suppose that stressful vivarium conditions, which likely increased IEG expression in CC (see above), also contributed to IEG expression in exploring animals in environment-independent fashion, and thus reduced the difference between ensembles activated in different environments (A/B) and weakened the contextual specificity of CA1 ensembles. . Interestingly, a study using *Arc* catFISH in mice has shown that contextual fear conditioning (CFC) increased ensemble similarity in different environments and thus eliminated the contextual specificity of IEG-expressing ensembles in CA1 (Nomura et al., 2012). Importantly, CFC uses inescapable shocks to induce fearful behaviour (freezing) accompanied by substantial stress, which could alter the salience of environmental stimuli and redirect the animals' attention to other variables than spatial context. In line with this notion, CFC substantially altered location-specific activity of hippocampal pyramidal neurons in electrophysiological recordings (Moita et al., 2004). However, this place cell remapping was observed

only in the (unchanged) training context, but not in a neutral context.

Our main hypothesis was that adolescent cognitive training would protect neuronal coordination from the detrimental effects of MK-801 (0.15 mg/kg). Specifically, we expected that pre-trained rats will show lower ensemble similarity after MK-801 administration than handled controls. To our dismay, these comparisons failed significance in two-way ANOVA as well as planned t-tests, despite the promisingly-looking trend in the CC and A/B exploration groups (see Figure 18). We do not think that the pattern of the data is necessarily in conflict with our main hypothesis. Instead, we believe that the lack of significance was due to the small number of experimental subjects (n=4) combined with the suboptimal housing conditions described above, which increased variance in the data. We chose the n=4 because it was sufficient in previous studies (Vazdarjanova and Guzowski, 2004, Lee et al. 2012), because we wanted to minimize the numbers of animals used and keep the laborious data collection and image analysis manageable (total n = 48), and because we could not have foreseen the technical difficulties in the vivarium. We consider two approaches to the current data set. The first and quick will be to refine the catFISH analysis. If the lack of significance is due to increased variance rather than a lack of effect, then higher quality image data may reduce the variance and help to reveal an effect. Alternatively, if the increased variance is due to higher inter-individual differences it may be necessary to increase the n=5, which was sufficient in Kubik et al. (2014).

10 CONCLUSIONS

Neural coordination is considered to be necessary for cognitive control – the ability to use goal-relevant information and to ignore misleading stimuli. Cognitive control is diminished in patients with schizophrenia and in animal models of the disease. Since schizophrenia is a neurodevelopmental disorder, timing of the intervention might be critical. We investigated if adolescent cognitive training on Carousel prevents the detrimental effects of acute MK-801 administration on neuronal coordination in adult rats. This protective effect would manifest in MK-801-treated rats as smaller overlap between hippocampal CA1 ensembles activated by exploration of two different environments (A/B) in trained rats compared to those without training. To map active neuronal ensembles, we used *Arc/H1a* catFISH, which utilizes the expression of immediate-early genes (IEGs) *Arc* and *Homer 1a*.

According to our findings, we conclude:

1) In agreement with previous studies, expression of IEGs *Arc* and *Homer 1a* is increased by exploration of a novel environment and decreased by acute i.p. administration of MK-801 at a dose of 0.15 mg/kg. The effects of exploration and MK-801 are antagonistic to each other in regard to IEG expression.
2) In saline-treated control animals, exploration of the same environment twice (A/A) activates ensembles that are more similar to each other than ensembles activated by exploration of two different environments (A/B). The difference we observed was significant, but not as stark as reported by other authors.
3) In adult MK-801 treated rats, similarity of neuronal ensembles representing two different contextual experiences (A/B) was reduced by more than half in trained animals compared to rats that did not receive the adolescent pro-cognitive training. The difference, however, did not reach statistical significance.

Taken together, our results suggest that adolescent training in cognitive control might be protective against disruption of neuronal coordination in adult pharmacological model of schizophrenia. In addition, we also replicated several previously published findings. However, our results were probably negatively affected by the suboptimal conditions during IEG-induction phase of our experiment. For this reason, we intend to extend the number of subjects by adding new cohort of animals and repeat *in situ* hybridization in the near future. A significant effect of adolescent training would lend further support to the neurodevelopmental and discoordination theories of schizophrenia.

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12 SUPPLEMENTS

Supplement 1. Animal cohorts I-VI.

For practical reasons, animals (n=48) were divided into six cohorts (I-VI) by eight. Treatment assigned to specific animal was semi-random to increase within- and between group comparison.

COHORT	ANIMAL No.	COGNITIVE TRAINING, HANDLING	DRUG	ENVIRONMENT
Cohort I	1	Handling	MK-801	AA
	2	Handling	saline	AA
	3	Cognitive training	saline	CC
	4	Handling	saline	AB
	5	Cognitive training	saline	AB
	6	Handling	MK-801	CC
	7	Cognitive training	MK-801	AB
	8	Cognitive training	MK-801	CC
Cohort II	9	Cognitive training	MK-801	CC
	10	Handling	MK-801	AA
	11	Cognitive training	saline	AA
	12	Handling	saline	CC
	13	Handling	saline	AB
	14	Handling	MK-801	CC
	15	Cognitive training	saline	AB
	16	Handling	MK-801	AB
Cohort III	17	Cognitive training	MK-801	CC
	18	Handling	saline	AA
	19	Handling	saline	CC
	20	Handling	MK-801	AB
	21	Cognitive training	saline	AA
	22	Cognitive training	saline	AB
	23	Cognitive training	MK-801	AA
	24	Cognitive training	saline	CC
Cohort IV	25	Cognitive training	saline	AA
	26	Handling	MK-801	AB
	27	Cognitive training	MK-801	AA
	28	Cognitive training	MK-801	AB
	29	Handling	MK-801	CC
	30	Handling	saline	AA
	31	Handling	saline	AB
	32	Cognitive training	saline	CC
Cohort V	33	Handling	MK-801	AB
	34	Cognitive training	saline	AA
	35	Handling	saline	AB
	36	Cognitive training	MK-801	AB
	37	Cognitive training	MK-801	AA
	38	Handling	MK-801	AA
	39	Handling	saline	CC
	40	Cognitive training	MK-801	CC
Cohort VI	41	Handling	MK-801	CC
	42	Handling	saline	CC
	43	Handling	MK-801	AA
	44	Cognitive training	saline	AB
	45	Cognitive training	MK-801	AB
	46	Handling	saline	AA
	47	Cognitive training	saline	CC
	48	Cognitive training	MK-801	AA

Supplement 2. Arrangement of samples in blocks I-VI.

Four millimeter thick segments of the right hemisphere containing right dorsal hippocampus (RDH) were used to create blocks (from which slides for IEG imaging were later prepared). Each of the six blocks (corresponding to the I-VI animal cohorts) consisted of eight RDH segments (one for each animal from the group). RDH samples were semi-randomly arranged in each block to maximize within- and between-block comparison.
Numbers **01-48** refer to the specific animal. Treatment recieved by each animal is coded as follows: **AA**, **AB**, **CC** – environments explored during catFISH behavioural exposition; **tr** – adolescent pro-cognitive training; **h** – adolescent handling (no training); **MK** – MK-801 injection before exploratory session; **sal** – physiological saline injection befory exploratory session.

Block I	05 AB tr sal	06 CC h sal	03 CC tr sal	01 AA h MK
	07 AA tr MK	02 AA h sal	08 CC tr MK	04 AB h sal
Block II	09 CC tr MK	11 AA tr sal	16 AB h MK	14 CC h MK
	12 CC h sal	15 AB tr sal	10 AA h MK	13 AB h sal
Block III	18 AA h sal	22 AB tr sal	17 CC tr MK	21 AA tr sal
	20 AB h MK	19 CC h sal	23 AA tr MK	24 CC tr sal
Block IV	32 CC tr sal	27 AA tr MK	25 AA tr sal	28 AB tr MK
	26 AB h MK	29 CC h MK	31 AB h sal	30 AA h sal
Block V	33 AB h MK	456-34 AA tr sal	456-35 AB h sal	456-37 AA tr MK
	40 CC tr MK	456-38 AA h MK	456-39 CC h sal	456-36 AB tr MK
Block VI	43 AA h MK	47 CC tr sal	41 CC h MK	44 AB tr sal
	46 AA h sal	45 AB tr MK	48 AA tr MK	42 CC h sal