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Presented by
Anja Aloysia Goepfrich (M.Sc.)
born in Tettnang, Germany
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Impact of Endocannabinoid Signaling on Cognitive Processing throughout Adolescence and Adulthood

Referees: PD Dr. Miriam Schneider

Prof. Dr. Rainer Spanagel

To my beloved husband Tobias

This thesis was written in the Central Institute of Mental Health in Mannheim at the Institute of Psychopharmacology in the period of 11th of April 2011 to 31th of August 2014 under supervision of PD Dr. Miriam Schneider.

I herewith declare that I wrote this thesis independently under supervision and used no other sources and aids than those indicated.

Mannheim, September 2014 Anja Goepfrich

Content

Zusammenfassung

Die vorliegende Doktorarbeit untersuchte grundlegende kognitive Fähigkeiten, den Einfluss des endocannabinoiden Systems (ECS) und Entwicklungsaspekte während der Adoleszenz männlicher Wistarratten. Zunächst wurden mögliche Verhaltensunterschiede zwischen drei verschiedenen Wistar Han Rattenlinien analysiert und die W[rcc] Linie für weitere Experimente auf Grund ihrer besten Leistung im Wiedererkennungstest für Objekte und ihrer guten Leistung der Präpulsinhibition (PPI) der akustischen Schreckreaktion (ASR) ausgewählt.

Im zweiten Projekt wurde die basale Ontogenese verschiedener kognitiver Fähigkeiten untersucht und die Ergebnisse zeigten unterschiedliche Entwicklungsmuster während der Adoleszenz und des jungen Erwachsenenalters. Die Erinnerungsleistung für das Wiedererkennen von Objekten zeigte eine nicht-lineare Entwicklung mit einem Leistungsabfall am postnatalen Tag (pd) 40, am ungefähren Beginn der Pubertät. Dieser Abfall konnte durch die Verabreichung des Cannabinoidrezeptor 1 (CB1R) Antagonisten/inversen Agonisten SR141716 verbessert werden, was auf eine Beteiligung des sich entwickelnden ECS zu diesem Zeitpunkt hindeutet. Das Unterscheidungsvermögen für kürzlich und weiter in der Vergangenheit zurückliegend erkundete Objekte entwickelte sich später als für das Wiedererkennen von Objekten, zeigte aber keine Unterschiede während der Adoleszenz. Im Gegensatz hierzu entwickelte sich die PPI der ASR sukzessive und zeigte einen stetigen Anstieg der Startleamplitude und sensomotorischer Filterfähigkeiten bis hinein ins frühe Erwachsenenalter (pd 100). Molekulare Analysen zeigten einen erhöhten CB1R Gehalt im Hippocampus (Hip) in der frühen Adoleszenz, was auf ein erhöhtes ECS um dieses Alter hinweist. Die Myelinisierung, welche mit der Verbesserung kognitiver Fähigkeiten in Zusammenhang steht, scheint sukzessive im Hip und Caudauten Putamen (CPu) anzusteigen. Zusammengefasst zeigen verschiedene kognitive Fähigkeiten unterschiedliche Entwicklungszeitverläufe und Änderungen des sich entwickelnden ECS scheinen einigen dieser Entwicklungsverläufe zu Grunde zu liegen.

Langzeiteffekte einer chronischen pubertären WIN 55,212-2 Behandlung auf kognitive Fähigkeiten im Erwachsenenalter beinhalteten eine verringerte Wiedererkennungsleistung von Objekten in der W[rcc] Rattenlinie, was dieses Tiermodell der Schizophrenie für diese Rattenlinie bestätigte. Darüber hinaus wurden komplexe kognitive Leistungen in einem sog. „Aufmerksamkeits Set Shift Test“ (ASST) untersucht, in welchem eine Beeinträchtigung der Fähigkeit beobachtet wurde, eine zuvor gelernte Regel umzudrehen, während andere Befähigungen Regeln zu lernen sich nicht von Vehikel behandelten Tieren unterschieden. Dies deutet eine spezielle Beeinträchtigung der Fähigkeit Regeln umzudrehen nach der chronischen Beeinflussung des sich entwickelnden ECS an. Außerdem zeigten molekulare Analysen einen

erhöhten CB1R Gehalt im medialen präfrontalen Cortex (mPFC), was auf einen möglichen kompensatorischen Entwicklungsmechanismus in dieser Hirnregion nach der Störung in diesem speziellen Zeitfenster hindeutet.

Im vierten Projekt wurden keine Langzeiteffekte einer chronischen pubertären Methylphenidat (MPH) Behandlung auf verschiedene Verhaltenstests, inklusive der lokomotorischen Aktivität, dem Angst-ähnlichem Verhalten, der Aufnahme verschieden schmackhafter Flüssigkeiten oder kognitiver Fähigkeiten im Erwachsenenalter festgestellt. Daher schien das hier benutzte MPH Behandlungsschema keine Langzeiteffekte zu verursachen, jedoch könnten weitere Untersuchungen andere für eine MPH Behandlung anfällige Entwicklungszeitfenster aufzeigen.

Zusammenfassend zeigte die vorliegende Doktorarbeit unterschiedliche Entwicklungsmuster kognitiver Fähigkeiten während der Adoleszenz männlicher Wistarratten, die scheinbar mit der Entwicklung des ECS gekoppelt sind und die Beeinflussung dieses Systems während dieses anfälligen Zeitraums kann die kognitiven Fähigkeiten im Erwachsenenalter beeinträchtigen.

Summary

The present thesis investigated the basal cognitive abilities, the influence of the endocannabinoid system (ECS), and developmental aspects throughout the adolescence in male Wistar rats. First, possible behavioral differences in three different lines of Wistar Han rats were analyzed and the W[rcc] line was selected for the subsequent experiments based on its best performance in the object recognition test and good performance in the prepulse inhibition (PPI) of the acoustic startle reflex (ASR).

In the second project the basic ontogeny of various cognitive abilities was analyzed and the results revealed differential patterns of development across adolescence and early adulthood. Object recognition memory showed a non-linear development with a decrease in performance on postnatal (pd) 40, at the approx. onset of puberty. This decrease was ameliorated by the administration of the cannabinoid receptor 1 (CB1R) antagonist/inverse agonist SR141716 indicating an involvement of the developing ECS at this time point. Recency discrimination developed later than recognition memory but did not display variations across adolescence. In contrast, PPI of the ASR developed gradually revealing a continuing increase of startle amplitude and sensorimotor gating abilities until early adulthood (pd 100). Molecular analysis showed increased CB1R levels in the hippocampus (Hip) in early adolescence indicating an increased ECS around that age. Myelination, which is linked to improved cognitive skills, appeared to increase gradually in the Hip and in the caudate putamen (CPu). Altogether, different cognitive abilities displayed differential time-courses of development and alterations of the developing ECS are implicated underlying some of these behavioral patterns.

Long-term effects of a chronic pubertal WIN 55,212-2 treatment on cognitive abilities in adulthood included a decreased object recognition memory in the W[rcc] line, which confirmed this animal model of schizophrenia for this rat line. Moreover, complex cognitive skills were investigated in an attentional set shift test (ASST) and an impaired reversal learning ability was found while other learning abilities did not differ from vehicle treated animals. This indicates specific deficits in reversal learning upon chronic interference with the developing ECS. Furthermore, molecular analysis showed increased CB1R levels in the medial prefrontal cortex (mPFC) indicating a possible developmental compensatory mechanism in this brain region after disturbances in this particular developmental time-window.

In the fourth project no long-term effects of a chronic pubertal methylphenidate (MPH) treatment on various behavioral tests were observed in adulthood for locomotor activity, anxiety-related behavior, intake of liquids of variable palatability or cognitive processing. Thus, the presently employed MPH administration paradigm did not appear to cause long-term effects but further

investigations may reveal other time-windows of vulnerability to MPH administration during development.

Altogether, the present thesis revealed differential developmental patterns of cognitive abilities during adolescence of male Wistar rats which appear to be linked to the developing ECS and interference with this system at this vulnerable time period may impair cognitive skills in adulthood.

1. Introduction

1.1 Cognition

Cognition is an umbrella term for various mental processes. Shettleworth (2009) refers to cognition as “mechanisms by which animals acquire, process, store and act on information from their environment”. So at first an organism perceives sensory stimuli from its surroundings and then associations about the relevant information are made. These associations are then encoded and stored in the brain (D'Mello and Steckler, 1996). The successful integration of information is called binding (Treisman, 1996) and it is closely related to the formation of mnemonic representations (Sander et al., 2012). Cognitive processes therefore include perception, learning, memory, attention, planning, and decision making but also pre-attentional sensory gating (e.g. prepulse inhibition of the acoustic startle reflex; PPI) and meta-cognition (thinking and knowledge about cognition; (reviewed by Millan et al., 2012; see Figure 1)). Cognitive abilities can be conscious or unconscious processes and depend on motivation, attention, and prior experience (D'Mello and Steckler, 1996).

Learning and memory processes can be either perceived as psychological processes or as changes in synaptic neural connectivity (Vanderwolf and Cain, 1994). Learning can be described as an adaptation to the environment which is based on information transfer and subsequent changes in interneural communication (Wotjak, 2005). Memory can be referred to as the relative persistence of these changes. Learning and memory cannot be measured *per se* but can only be inferred from behavior (Cahill et al., 2001).

Usually three stages are considered when learning and the creation of memory occurs: *Encoding* happens during the presentation of learning material. As a result information is stored during a *consolidation* stage and the recall of learned information can be accessed during *retrieval* (Eyseneck, 2000, Straube, 2012). Memory can be divided according to type (*declarative (or explicit) memory* like facts and episodic events or *procedural (or implicit) memory* like motor skills and habits), or according to temporal categories (like *short-term memory* (STM) and *long-term memory* (LTM) (Purves, 2004)). From the concept of STM the term *working memory* (WM) evolved (Baddeley, 2012). STM is often referred to as the storage of information for seconds to minutes whereas WM implies the possible mental manipulation of this information. Miller (1960) employed the term WM for memory used to plan and carry out behavior. Olton (1979) later adopted this term for the behavior of rats in a radial arm maze but his use of WM is now often seen as LTM.

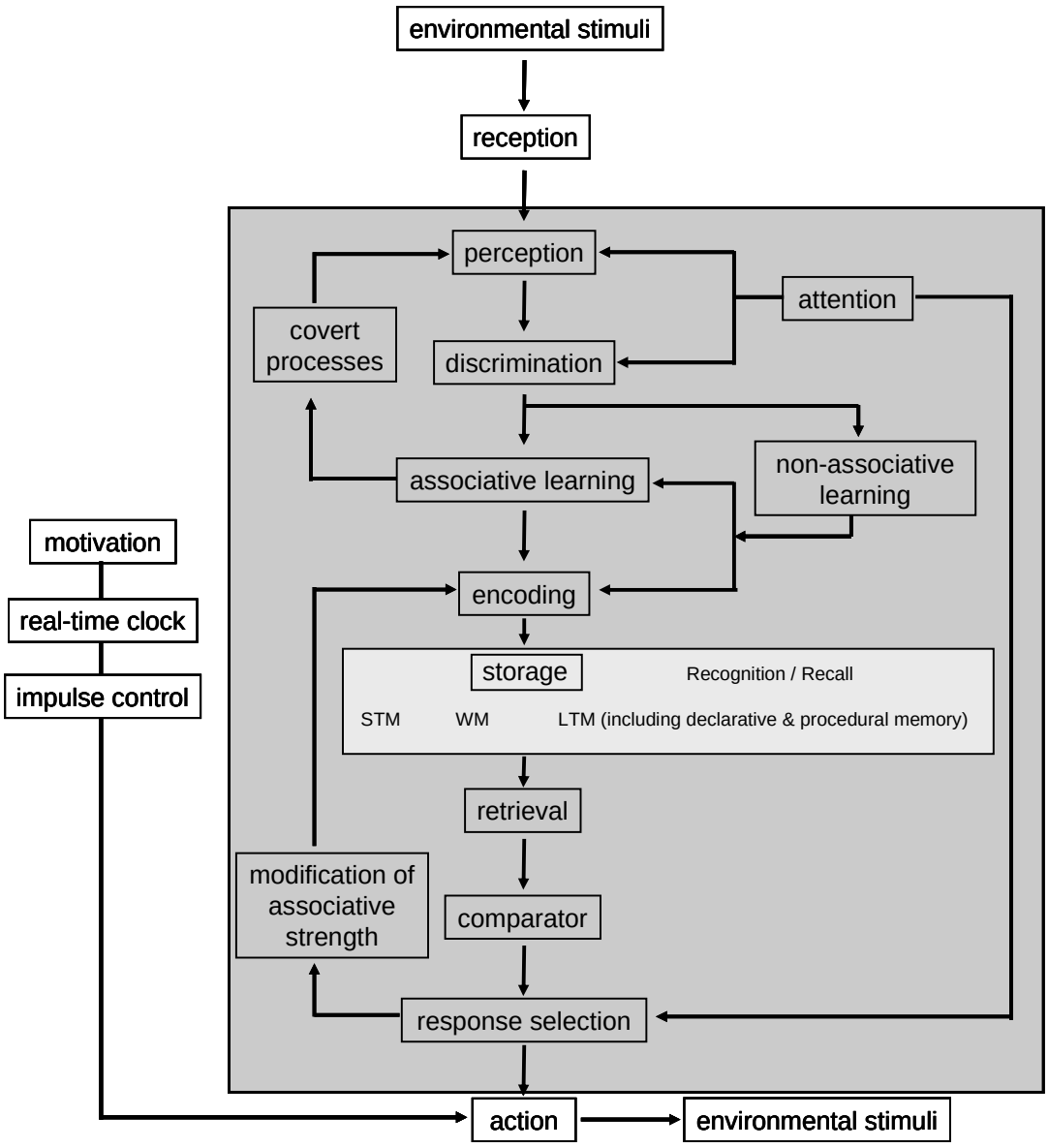

Figure 1: Schematic diagram of processes implicated in learning and memory (Adapted from D'Mello and Steckler, 1996). Arrows represent possible interactions and direction of information flow. Abbreviations: STM: short-term memory; LTM: long-term memory; WM: working memory.

Various models of memory were established and need to be constantly revised. Atkinson and Shiffrin's multi-store model (1986) comprised three types of memory: sensory stores, which hold information briefly and are modality-specific, a short-term store, and a long-term store (Eyseneck, 2000). Information from the environment is received via the sensory stores and transferred into the short-term store. This process depends on attention. Then some of this information is transferred into the long-term store often requiring rehearsal. However, the multi-store model was gradually regarded as oversimplified and needed to be improved.

In Baddeley and Hitch's multicomponent model (Baddeley, 2010) an attentional control system (the central executive) is supported by two STM storage systems (the visuo-spatial sketch-pad and

the phonological loop for verbal and acoustic material; see Figure 2A). The episodic buffer, as a further component, was added later on and is supposed to combine information of various sensory inputs as a temporary store. The other components are able to interact through this buffer which also contains links to LTM (Baddeley, 2010). It is supposed to have a limited capacity (of approximately four so called "chunks") and to be consciously accessible (Baddeley, 2010). However, because of the importance of language in this model, it is only partially applicable when studying memory in animals.

In Cowan's embedded processes model (2008), STM is a part of a temporarily activated LTM (see Figure 2B). A further subset of this is the focus of attention, which has a limited chunk or item capacity. WM is defined as cognitive processes that keep information in a very accessible state and comprises therefore both the focus of attention and the central executive (attention control) processes (Cowan, 2008).

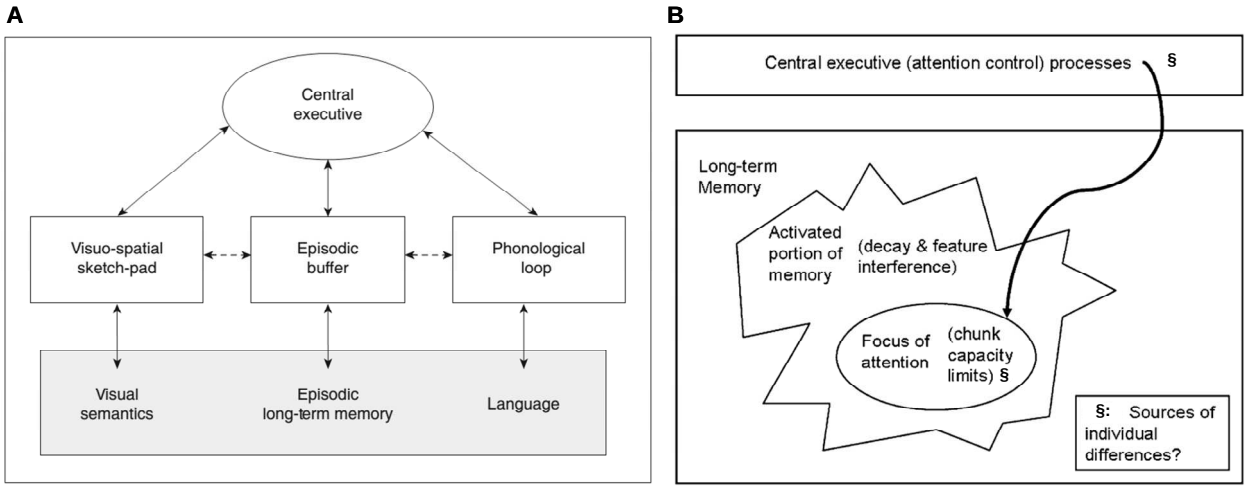

Figure 2: Baddeley and Hitch's multicomponent model (A; Figure from Baddeley, 2010) and Cowan's embedded process model (B; Figure from Cowan, 2008, Baddeley, 2010). A: The multicomponent model includes links to LTM. The visuo-spatial sketch pad, the episodic buffer and the phonological loop as three STM components interact with the central executive (attentional control system). B: In the embedded process model STM is a part of the activated LTM.

1.1.1 Neuronal Structures

Various brain regions are involved in different cognitive processes among which are the striatum, the prefrontal cortex (PFC), and the hippocampus (Hip). The striatum is involved in motivation and habit formation (White, 1997). The PFC is particularly involved in higher order executive functions like WM, planning, attentional set shifting, inhibitory response control, temporal integration of voluntary behavior, and goal directed behavior (Dalley et al., 2004, Pattij et al., 2008). The Hip is involved in spatial learning, episodic memory, consolidation, and in forming multi-modal representations of environmental cues (Sweatt, 2004, Fanselow and Dong, 2010). In addition, the PFC-Hip pathway appears to be very important in cognition related to executive

functions and emotional regulation (Vertes, 2006, Euston et al., 2012, Godsil et al., 2013). Furthermore, structural anomalies as well as irregular functional coupling have been observed in psychiatric disorders like schizophrenia (Godsil et al., 2013).

Striatum

The striatum belongs to a group of subcortical nuclei called the basal ganglia. It consists of a dorsal part (comprising the nucleus caudatus and the putamen; CPu) and a ventral part (consisting of the nucleus accumbens (NAc); reviewed by Grahn et al., 2009). The NAc is implicated in reward processing and motivational behavior (Salamone and Correa, 2002, O'Doherty, 2004) and the dorsal striatum is involved in stimulus-response (habit) and egocentric learning (Packard and McGaugh, 1996, White, 1997). The striatum is also considerably connected to the PFC, receiving afferents from various cortical areas and projecting back to the PFC via the substantia nigra or globus pallidus and the thalamus (Wise et al., 1996, White, 1997). Therefore, the striatum can integrate sensory information (received from the PFC) with previously learned responses and appropriate behavior in a particular context or situation. In turn, output from the striatum to the frontal cortex may influence the latter to execute rules in the presence of a particular context (White, 1997).

Prefrontal Cortex

The PFC is the association cortex of the frontal lobe and one of the latest cortices to develop phylogenetically (Fuster, 2001). It also undergoes late development during ontogeny (Huttenlocher, 1990) and imaging studies suggest that full maturity is not reached until end of adolescence (Paus et al., 1999, Sowell et al., 1999). Early definitions of the PFC were based on the cytoarchitectonic criterion of having a granular layer IV and a location rostral to the agranular motor areas (Uylings et al., 2003). But when comparing different species these criteria could no longer be used exclusively. Rose and Woolsey defined it as the cortex with reciprocal connections of the mediodorsal nucleus of the thalamus (Rose and Woolsey, 1948). Today other criteria are additionally used for defining the PFC, e.g. functional properties, presence and distribution of neurotransmitters and receptors, embryological development, and for closely related species, cytoarchitectonic characteristics (Uylings et al., 2003).

The primate PFC can be divided into a dorsolateral, a medial, and an orbital part (Uylings et al., 2003). The rodent PFC is divided into a medial, a ventral, and a lateral part with further subdivisions as follows (see Figure 3): the medial part consists of a dorsal region including the precentral and the anterior cingulate cortices as well as a ventral region comprising the prelimbic (PL), the infralimbic (IL) and the medial orbital coritces. The ventral region comprises the ventral

orbital and the ventral lateral orbital cortices. The lateral part includes the dorsal and ventral agranular insulas and the lateral orbital cortices (Dalley et al., 2004).

Connections of the PFC to other subcortical structures include the brainstem, the thalamus, the basal ganglia, and the limbic system (Fuster, 2001). Additionally, there are many internal connections within the PFC which are often reciprocally and topologically well organized (Fuster, 2001). Hence, the PFC is provided with information about the internal environment (e.g. arousal, drives, and motives) by afferent connections from the brainstem, the diencephalon, and the limbic system, while information about the motivational relevance of sensory stimuli is conveyed from the ventral tegmental area (VTA), the amygdala and the hypothalamus.

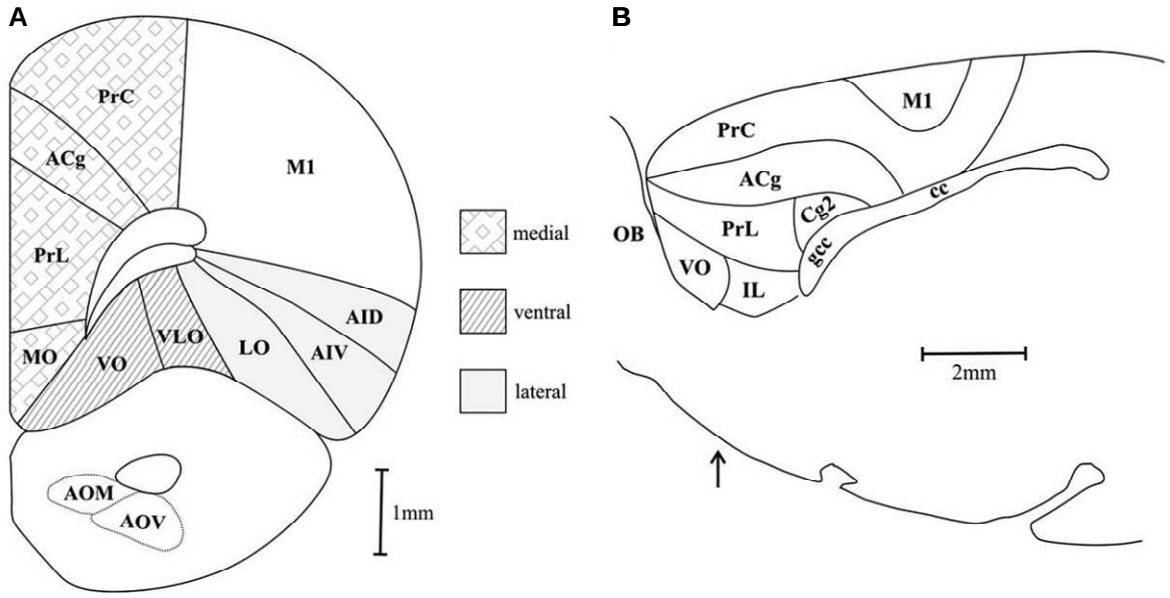

Figure 3: Coronal (A) and sagital (B) sections of the rat PFC (Figures from Dalley et al., 2004). Abbreviations: PrC: precentral cortex, ACg: anterior cingulate cortex, PrL: prelimbic cortex, IL: infralimbic cortex, MO: medial orbital cortex, VO: ventral orbital cortex, VLO: ventrolateral orbital cortex, LO: lateral orbital cortex, AIV: ventral agranular insular cortex, AID: dorsal agranular insular cotex, M1: primary motor area, OB: olfactory bulb, cc: corpus callosum, Cg2: cinglate cortex area 2, gcc: genu of cc.

Based on anatomy and function there appears to be a dorsal-ventral gradient of the medial part of the PFC (mPFC). The dorsal regions appear to be specialized in the control of actions while the ventral regions seem to be specialized in autonomic and emotional control (Heidbreder and Groenewegen, 2003).

When environmental demands shift, the PFC is essential in planning, controlling, and directing behavior accordingly and in helping to select and process information (Miller and Cohen, 2001, Holmes and Wellman, 2009). Thus, the PFC takes the inputs of current contexts and events and predicts the most adaptive responses based on previous experiences (Euston et al., 2012). Furthermore, the rapidly acquired input-output mappings in the PFC are most likely initially supported by the Hip but later become independent of it (Euston et al., 2012).

Due to its manyfold connections with other structures and within itself it is argued that the functions of the PFC cannot be taken out of this broad connectionist context (Fuster, 2001). A particular function could not be localized within a discrete portion of the PFC. However, lesion studies are showing that the mPFC is important in attentional set shifting (Birrell and Brown, 2000) whereas the orbifrontal cortex (OFC) is important in reversal learning (see also 1.4.5; McAlonan and Brown, 2003).

Hippocampus

The Hip is part of the limbic system and situated in the temporal lobe. It comprises the subiculum, the Hip proper (also termed *cornu ammonis* (CA1-4)), and the dentate gyrus (DG). The Hip receives information from the entorhinal and perirhinal cortex via the perforant path to the DG (see Figure 4; Sweatt, 2004, Dokter and von Bohlen und Halbach, 2012). From the DG mossy fibers relay information to the CA3 field and from here to CA1 via the Schaffer collaterals. The major output neurons in the CA1 field are glutamatergic pyramidal cells. These neurons project mainly to the ipsi- and contralateral entorhinal cortices but also to the contralateral Hip via the fornix (Sweatt, 2004). There are also direct projections from the CA1 and the subiculum to the mPFC (IL/PL) but there are no direct connections back from the mPFC to the Hip (Warburton and Brown, 2010). Connections from the mPFC reach the Hip indirectly via the entorhinal cortex and via the nucleus reuniens of the thalamus (Vertes, 2006).

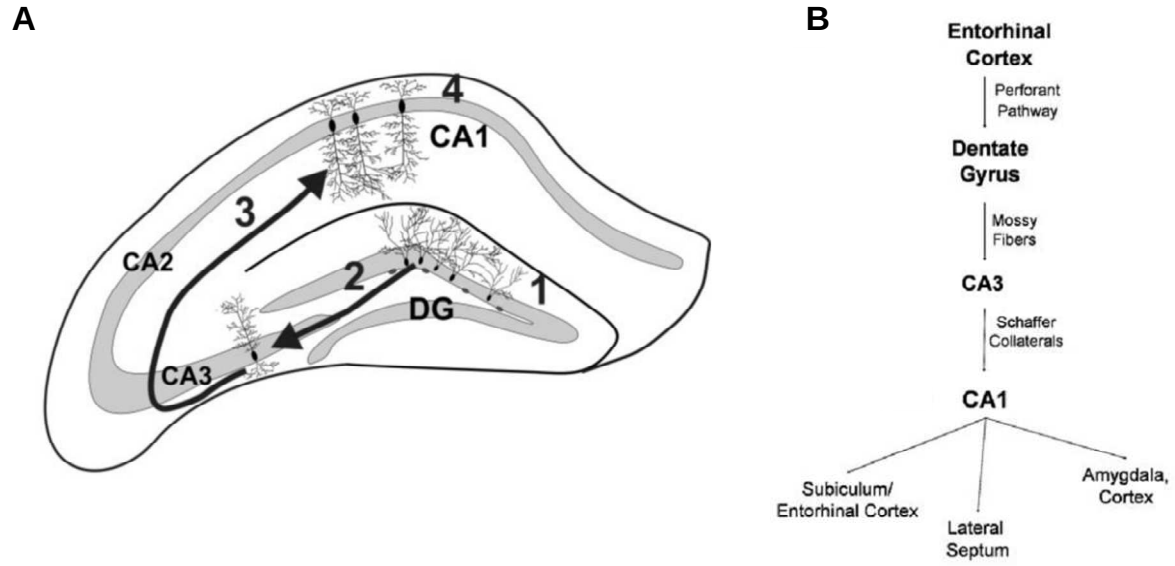

Figure 4: Schematic pathway of sensory signals through the Hip. A: (1) Fibers from the entorhinal cortex project to the DG via the perforant path. (2) Mossy fibers relay from DG to CA3. (3) CA 3 and CA 1 are connected via the Schaffer collaterals. (4) CA 1 projects back to the entorhinal cortex. B: Schematic overview over Hip pathways. Abbreviations: CA 1 – 3: *cornu ammonis*; DG: dentate gyrus. Figure A from Doktor and von Bohlen und Halbach (2012); Figure B adapted from Sweatt (2004).

The importance of the Hip in memory became evident by the well-known case of the hippocampectomy in the patient H.M. (Scoville and Milner, 1957). In order to help treating his severe epilepsy he underwent experimental surgery and had both hippocampi removed. Consequently he suffered from anterograde amnesia and had selective deficits in certain types of memory (i.e. declarative) whereas other types (i.e. procedural memory) were spared (Milner et al., 1998). The formation of declarative memory is critically dependent on the Hip and the medial temporal lobe (Sweatt, 2004). But there is also evidence that the Hip contributes to WM and planning together with the PFC (Godsil et al., 2013). The connection between PFC and Hip is mainly ipsilateral (Godsil et al., 2013). Investigated in asymmetric pathway disconnection analysis ("crossed lesions"), the PFC is compromised in one hemisphere and the Hip in the other hemisphere. One can study the influence of these lesions in comparison to unilateral control lesions in a variety of behavioral tests. Floresco (1997) revealed that this connection is needed in a delayed condition in a win-shift radial arm maze task in rats. Involvement in cognitive flexibility of goal-directed behavior was also shown in a reward-discounting choice task (Gruber et al., 2010). In this test animals with neonatal lesions of the ventral Hip displayed neuronal hyperactivity in the PFC in adulthood and deficits in cognitive flexibility when reward contingencies were changed. The Hip-PFC pathway is thought to be critically involved in information transfer when acquiring new rules in goal-oriented reward learning (Godsil et al., 2013). Asymmetric disconnection analysis also indicated an involvement of the Hip-PFC pathway in object recognition (OBJR) memory, particularly in tests for contextual and temporal recognition of objects (see also 1.4.3; Barker and Warburton, 2011).

Altogether, the integrated activity of various brain regions appears to be involved in distinct types of memory processes. Inactivation or lesion of a particular brain region might interfere with one particular aspect of memory. However, compensation by other intact regions might help to maintain mnemonic functioning to a certain degree and, hence, cannot be excluded in behavioral performance.

1.1.2 Neurotransmitter Systems

The involvement of distinct neurotransmitter systems in cognition appears to be rather complex. Similar as to the hypothesis that multiple brain regions act in a coordinated manner in various cognitive processes, various neurotransmitters seem to be differently involved in them. A meta-analysis for the involvement of several neurotransmitters in four established behavioral paradigms for cognitive abilities (Morris water maze, radial maze, passive avoidance, and spontaneous alternation) revealed that glutamate, γ-aminobutyric acid (GABA), dopamine (DA), and acetylcholine (ACh) seemed to have a powerful impact on cognitive processes but no

relationships between a specific transmitter system and a particular task was found (Myhrer, 2003). Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) whereas GABA is considered the major inhibitory neurotransmitter (McEntee and Crook, 1993, Krnjevic, 2004). Glutamate and GABA are important in long-term potentiation (LTP) which is considered the neurophysiological model of learning and memory (Brown et al., 1988, McEntee and Crook, 1993, Myhrer, 2003). In LTP repetitive high frequency stimulation (e.g. in the Hip) induces the potentiation of synaptic transmission (Bliss and Lomo, 1973, McEntee and Crook, 1993). Two major glutamate receptors, NMDA (N-Methyl-D-Aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), are required for induction and expression of LTP, respectively (see Myhrer, 2003). Furthermore, GABA antagonists facilitate the induction of LTP and additionally, changes in the DAergic, cholinergic, noradrenergic, and serotonergic systems influence LTP.

In addition to amino acids like glutamate and GABA, monamines are also important neurotransmitters in learning and memory. DA is considered to be involved in motivational behavior but also in response selection and habit formation (Romanides et al., 1999, Myhrer, 2003). Serotonin is connected to emotional behavior but its influence in learning and memory seems to be less strong (Hashimoto et al., 1999). Although ACh has often been considered as essential in learning and memory processes, it is now rather considered to have an important role in attention processes (Blokland, 1995). Furthermore, interactions between neurotransmitter systems are widely observed in various cognitive tests, e.g between ACh and glutamate (Levin et al., 1998), between ACh and DA (McGurk et al., 1988), and between ACh and serotonin (Steckler and Sahgal, 1995).

A very important modulatory neurotransmitter system involved in cognition is the endocannabinoid system (ECS, see 1.3.4). The ECS is also known to interact with various neurotransmitter systems, e.g. with DA (reviewed by El Khoury et al., 2012), ACh (Bura et al., 2007), glutamate and GABA (reviewed by Lopez-Moreno et al., 2008). Thus, as Myhrer (2003) mentioned: “the multiple memory systems in the rat brain can hardly be related to specific transmitter systems because of the great extent of interactions between the systems”. Optimal performance in a behavioral task most likely depends on plural memory systems and monaminergic projections exert modulatory functions like attention, emotion, and motivation (Myhrer, 2003). In addition, interactions of various neurotransmitters with the ECS have an important influence in cognition.

1.1.3 Neuropsychiatric Disorders

Cognitive dysfunctions are very common in a range of neurpsychiatric disorders including schizophrenia, attention deficit hyperactivity disorder (ADHD), Alzheimers's disease, bipolar disorder, and many more (reviewed by Millan et al., 2012). Traditionally, emotional symptoms like anxiety, hallucinations, and depression have been primarily investigated in these disorders but the cognitive deficits are now also considered a core deficit and a major component decreasing the quality of life in affected individuals (Millan et al., 2012). In schizophrenia a broad pattern of cognitive deficits can be observed, e.g. deficits in WM, executive functions, PPI, and attention. Impairments in bipolar disorder are similar but less severe. Interestingly, bipolar disorder shares certain genetic risk factors with schizophrenia (Millan et al., 2012). In addition, schizophrenia patients often display co-morbidity for anther disorder like obsessive-compulsive or anxiety disorders (see Braga et al., 2013, Lepage et al., 2014). Observations like these led to a novel approach of integrating the research on pathophysiology of mental disorders into a new classification scheme to provide a better match between research findings and clinical decision making (Insel et al., 2010). This RDoC (Research Domain Criteria) project is designed to complement the current DSM 5 (Diagnostic and Statistical Manual of Mental Disorders) for characterizing mental disorders. The goal is to eventually identify new targets for treatment developments and detect subgroups for treatment selection by incorporating data obtained from genetics, imaging, and cognitive science. Regarding schizophrenia, a recent study found an association between a higher genetic risk for the disorder and treatment outcome (Frank et al., 2014). In this study the patients with a higher genetic load also displayed a higher risk for treatment resistance. The authors suggest that early treatment with clozapine (which is usually prescribed only if positive response to two other antipsychotics has failed due to unwanted side effects) might be useful to be considered in this subgroup.

Similarly, the endophenotype-based approach seeks to establish biological underpinnings for diagnosis and classification of psychiatric disorders in order to improve understanding of their neurobiology and genetics (Gottesman and Gould, 2003). An endophenotype is considered a heritable vulnerability trait mediating between genes and phenotype, thus forming a causal connection between genes and observable symptoms (Rommelse et al., 2011). Due to the enormous complexities of psychiatric disorders, this approach tries to identify simpler clues to genetic underpinnings than the disease syndrome itself (Gottesman and Gould, 2003). Endophenotypes are characterized by several criteria (e.g. association with the disorder, heritability, state-independency and higher rate of occurrence in non-affected family members than in the general population). Using this approach, it has, for example, been proposed that autism spectrum disorder and ADHD partly share heritability traits and might be considered as

different manifestations of the same disorder (Rommelse et al., 2010, Rommelse et al., 2011). The authors therefore suggest that treatment for ADHD might also be beneficial in autism spectrum patients and collaborating networks of experts are needed for cross-fertilization of future research (Rommelse et al., 2011).

Cognitive deficits in neuropsychiatric disorders are impacting everyday life of affected individuals and these deficits appear to be more responsible than other clinical symptoms for impaired functional outcome of, for example, schizophrenia (Lepage et al., 2014). Functional outcome describes abilities like living independently, planning and altering basic activities, and being employed (Warner, 2009, Lepage et al., 2014). Studies showed associations between cognition and social adjustments in schizophrenia patients (reviewed by Green, 1996, Lepage et al., 2014). Particularly, impairments in information processing and executive functioning were linked with social skills, problem solving, and community adjustment (Green, 1996). Other studies found that affected individuals with specific cognitive deficits were most likely to experience difficulties with elementary social behavior and first-episode psychosis patients with poor clinical outcomes showed difficulties in overall social cognition (Smith et al., 1999, Montreuil et al., 2010). Additionally, cognitive abilities appear to influence the abilities of patients for independent living and this influence exists in a reciprocal fashion (Lepage et al., 2014). For example, one study found improved sustained attention and verbal memory in formerly homeless mentally ill people after moving to a residence. Interestingly, moving to supported housing revealed greater executive function improvements than moving to independent apartments (Caplan et al., 2006).

Altogether, elucidating the underlying pathophysiologies of mental disorders, specifically cognitive deficits, is a crucial step to help improve clinical and functional outcomes of affected patients.

1.1.4 Neuroenhancement

When investigating cognitive abilities the idea to improve these skills is often discussed. The pharmacological improvement of cognitive skills without therapeutic intent is called neuroenhancement or brain doping, and some of the agents used are called “smart drugs” or “smart pills”. Some of the cognitive enhancing substances like caffeine and nicotine are freely available and their use is wide-spread throughout society. For example, caffeine helps staying awake, preventing fatigue, and reducing decline in cognitive performance by blocking adenosine A_1 and A_{2A} receptors, hence blocking increasing adenosine levels (Cauli and Morelli, 2005, Muller and Schumann, 2011). Nicotine is an agonist at the nicotinic ACh receptor that appears to improve attention and cognitive performance (Markou, 2008, Muller and Schumann, 2011). In

addition to increasing ACh, nicotine also increases norepinephrine and modulates the mesolimbic DA system (Mitchell, 1993, Wonnacott, 1997, Markou, 2008). Other employed substances can be obtained by medical prescription, e.g. Methylphenidate (MPH; see below) or modafinil. Both of these substances bind and inhibit the DA and norepinephrine transporter, however, modafinil also influences GABA, serotonin, and glutamate levels (Rasetti et al., 2010). Modafinil is usually prescribed to treat narcolepsy, sleep apnoea and shift-work sleep disorder (Repantis et al., 2010). Furthermore, illegal drugs like cocaine and amphetamines are also used to improve cognitive performance. These psychostimulants interact with the norepinephrine transporter, thereby blocking the uptake of norepinephrine in the cell (Ritz and Kuhar, 1989, Ritz et al., 1990).

In a recent study the twelve-month prevalence of cognitive enhancing drug use (including freely available ones, medically prescribed, and illegal drugs; all only for the purpose to improve cognitive performance) among German university students was estimated to range around 20% (Dietz et al., 2013). Similar numbers were found in a poll among the readers of the magazine "nature" (Maher, 2008). Although the beneficial effects on cognitive performance are controversial and the long-term effects for healthy individuals have not been fully investigated, an increasing number of people appear to take substances like MPH (Repantis et al., 2010, Finger et al., 2013).

Through blocking the DA and norepinephrine transporter and thereby preventing the reuptake of DA into the cell, MPH increases the DA concentration in the extracellular space (Volkow et al., 1998, Challman and Lipsky, 2000, Madras et al., 2005). This effect was observed in the PFC and in its cortical and subcortical projection regions as well as in the NAc and the Hip (Kuczenski and Segal, 2001, Pliszka, 2005, Wilens, 2006). Because of its action on the catecholaminergic system MPHs effects during development appear to be especially pronounced on cognition, motivation and emotional behavior (Rosso et al., 2004, Britton, 2011).

It is often prescribed for the treatment of ADHD and narcolepsy. It was shown to improve attentional focus, WM, and flexible control responses and is therefore also abused as a smart drug taken by healthy students to improve their academic performance (Greely et al., 2008).

The increasing awareness for ADHD, which is characterized by inattention, hyperactivity and impulsivity (Faraone et al., 2003), in recent years led to a rise in diagnosis and prescription of pharmacological treatment agents such as Ritalin or Concerta which are trade names for MPH (Madras et al., 2005). This also causes the inadvertent treatment of healthy children misdiagnosed with ADHD (Carlezon and Konradi, 2004). Only little is known about the effects of MPH on healthy individuals, so far results imply improved vigilance, effects on spatial working memory and planning and improved predicted visually-guided saccades (Camp-Bruno and Herting, 1994, Elliott et al., 1997, Allman et al., 2012). However, these studies only investigated acute effects on

young adult individuals, not long-term effects on adolescents. Due to this and because of the rising number of adolescents and young adults using neuroenhancers, it is important to elucidate any possible behavioral changes caused by a chronic treatment with MPH.

1.2 Adolescence

1.2.1 Terminology and Characteristics

The transitional time period from childhood to adulthood is termed adolescence. During this phase an individual gradually acquires the skills and abilities it needs for an independent life. It is also a time when the individual achieves sexual maturity. In addition to hormonal changes, also neuroanatomical, -functional and behavioral changes occur. Puberty (lat. *pubertas* = maturity) as a part of adolescence describes the achievement of sexual maturation. Here, measurable, biological signs allow the determination of its beginning and endpoint. In contrast, adolescence (lat. *adolescere* = to grow up) describes the broader transitional period from a juvenile to an adult individual and includes achievements of adult social and cognitive behaviors (Spear, 2000, Sisk and Foster, 2004, Schneider, 2013).

Elevation of the Gonadotropin releasing hormone initiates the onset of puberty which leads to a rise of the sex hormones luteinizing hormone and follicle-stimulating hormone (Sisk and Foster, 2004, Schneider, 2013). In rodents puberty can be estimated by external physical signs (Korenbrot et al., 1977, Schneider, 2008). In male rats the balano-preputial separation (BPS), which describes the complete separation of the prepuce from the glans penis, indicates the onset of puberty around pd 40 (usually between pd 38 and pd 45; Korenbrot et al., 1977, Schneider, 2008). Around pd 60 fertility is usually reached and indicated by the presence of mature spermatozoa in the vas deference and completion of spermatogenesis (Swerdloff et al., 1971, Schneider, 2008). An overview about the timing of puberty and adolescence is given in Figure 5.

Furthermore, individual differences for the beginning of puberty exist between members of the same species and sex. Here, multiple permissive signals appear to be involved in the timing of puberty onset including internal signals (e.g. metabolic cues – has the individual attained sufficient energy stores for successful reproduction?) and external ones (e.g. environmental cues – is it the right seasonal time for mating?) (Sisk and Foster, 2004).

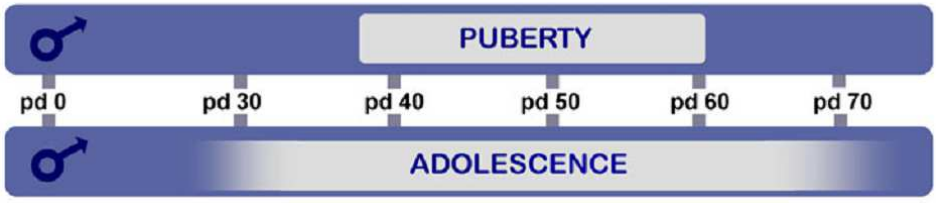

Figure 5: Timing of puberty and approximated timing of adolescence in female and male rats (pd: postnatal day; Figure from Schneider, 2013).

1.2.2 Brain Changes during Adolescence

During adolescence both progressive and regressive changes in the brain can be observed: synaptic overproduction and subsequent pruning, the maturation of neurotransmitter systems, as well as myelination (see Figure 6 and reviewed in Schneider, 2013). Synaptic overproduction and pruning, serving as refinement processes in development, have been analyzed for example in post mortem studies (Huttenlocher, 1979, Lewis, 1997) but also in MRI studies where regional differences in gray matter changes have been investigated (Giedd et al., 1999, Gogtay et al., 2004, Sowell et al., 2004). Overall, gray matter changes display an inverted U-shape maturation over adolescence whereas white matter maturation continues in a more linear fashion (Paus et al., 2008, Brenhouse and Andersen, 2011). Pruning in the cortex occurs in a back to front direction with sensorimotor cortices maturing first, association cortices next and the frontal poles at the last stage. Subcortical regions like the NAc, CPu and cerebellum display gray matter changes that peak during adolescence (Durston et al., 2001). Within a structure, differential patterns of development can also be observed, e.g. in the Hip, posterior subregions show an increase in volume over time whereas anterior regions a reduction (Gogtay et al., 2006).

Furthermore, alterations of receptor systems during adolescence have been observed and during that time receptor expression levels appear to rise, peak, and decline towards adult levels (see e.g. Brenhouse and Andersen, 2011, Schneider, 2013). These mechanisms are also regionally-dependent and include receptors of a variety of systems like DA, endocannabinoid, glutamate, and GABA (Lidow et al., 1991, Andersen et al., 2000, Eggan et al., 2010). However, detailed information about receptor expression during the ontogeny of adolescence is scarce so far. Regarding DA receptor 1 and 2 (D1 and D2) density, a peak was observed at pd 40 in the striatum and at pd 50 in the PFC of male rats compared to lower levels before (pd 21) and after adolescence (pd 100 and pd 120) (Teicher et al., 1995, Andersen et al., 1997, Andersen et al., 2000).

Changes in receptor densities have also been observed in the ECS. Increased cannabinoid receptor 1 (CB1R) densities have been observed between pd 30 and pd 40 in the striatum, limbic forebrain, mPFC and mesencephanlon of male rats (see also 1.3.5; Rodriguez de Fonseca et al., 1993, Klugmann et al., 2011b). Thus, both the DA system and the ECS appear to be increased around puberty (reviewed in Schneider, 2013). Close interactions between the ECS and the DA system and their role in reward have been shown previously (e.g. van der Stelt and Di Marzo, 2003, Gardner, 2005) and the changes in these systems during adolescence probably underlie some of the observed behavioral alterations.

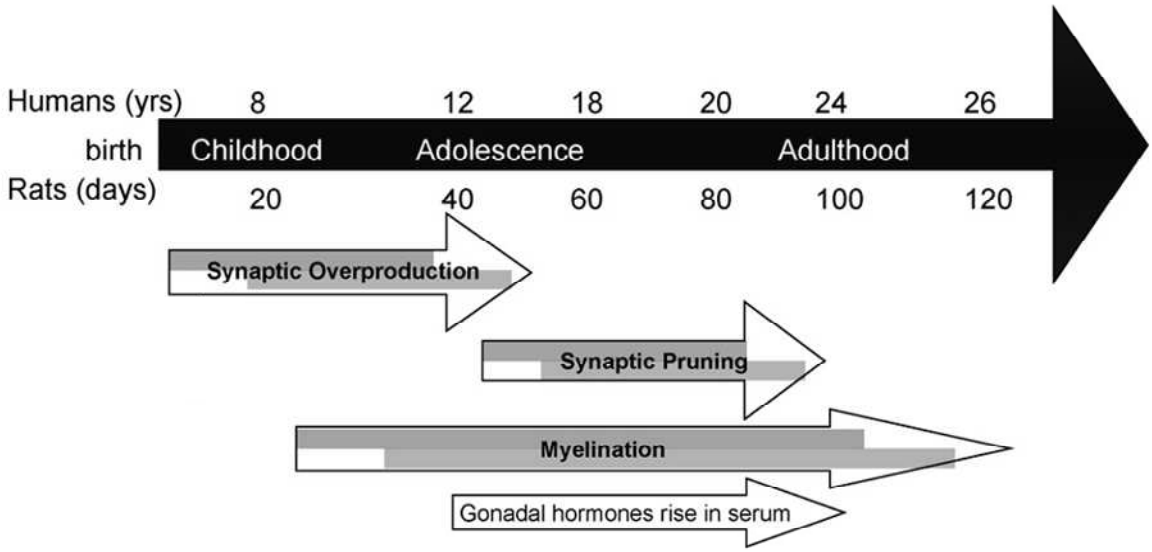

Figure 6: Timeline of developmental processes in humans and rodents. In females puberty and adolescence usually start earlier than in males, represented here in dark gray and light gray, respectively. Figure adapted from Brenhouse and Andersen (2011).

The formation of a myelin sheath (i.e. white matter) around neuronal axons by oligodendrocytes increases the speed of information exchange and also modulates timing and synchrony of neuronal firing patterns creating functional networks in the brain (Fields and Stevens-Graham, 2002, Giedd and Rapoport, 2010, Brenhouse and Andersen, 2011). Myelin acts as an electrical insulator on the axon and because of this, action potentials are generated only at the nodes of Ranvier along the axon, leading to a fast signal propagation in a so-called saltatory fashion (Purves, 2004). Improvements in WM are associated with increased white matter maturation (Bava et al., 2010). In humans the majority of myelination occurs within the first two years of life in most subcortical regions and primary motor and sensory regions but is ongoing in telencephalic areas, including frontal cortex and hippocampus, during adolescence and young adulthood. This region-specific protracted maturation has been confirmed by post-mortem and imaging data (Sowell et al., 1999, Lenroot and Giedd, 2006). In the largest myelin tract, the corpus callosum, a rostral to caudal pattern of myelination can be observed (Giedd et al., 1996).

However, detailed information about the ontogeny of myelination in adolescent rodents is still lacking. Most studies in rodents omit the adolescent period or use animals that are still developing (i.e. adolescent animals) as adult reference points (Norton and Poduslo, 1973, Meier et al., 2004). One recent study used a longitudinal design combining MRI and histology to investigate the development of myelination and volume changes in total brain, cortex, and striatum of male rats (Mengler et al., 2014). They found a considerable increase in myelination until the third postnatal

month and this continued until the sixth postnatal month in the cortex. However, in this study, again, the adolescent period was not studied in detail: tested ages were 3 weeks (approximately pd 21), 1, 2, 3 and 6 months (approximately pd 30, 60, 90, and 180).

1.2.3 Behavioral Changes and Cognitive Development during Adolescence

In many animals an age-related migration can be observed and this seems to be an adaptive process to avoid inbreeding (Spear, 2000). Thus, the migrating individuals must acquire skills to survive outside the territory they have been raised in. They need to interact with unfamiliar individuals of their species, avoid predators while searching for food in an unknown territory and find a mate. Adolescence is the time period where individuals learn to attain skills needed for independency away from their family. Adolescents display a variety in behavioral differences compared to juveniles or adults. These behaviors include increased social interaction with peers, risk taking, novelty-seeking, reward sensitivity and initiation of drug use and can be seen in various species (Spear, 2000, Casey et al., 2008, Schneider, 2013).

In humans, basic cognitive functions like selective attention are established in early childhood. Yet other more complex functions like planning, cognitive control, problem solving, and reasoning continue to develop well into adolescence and involve increasing WM abilities (Catts et al., 2013). During achievement of increased WM capacity, the PFC and the parietal cortex show an increased activation and white matter connectivity (Klingberg et al., 2002, Nagy et al., 2004). There is supposedly a shift from using ventral to more dorsolateral regions of the PFC in WM tasks as children mature into adolescence (Catts et al., 2013). Also the protracted maturation of several association cortices has been linked to the development of mature cognition (Giedd et al., 1999, Casey et al., 2000, Gogtay et al., 2004). Thus, an important part of adolescent cognitive development is to increase the efficiency of information transfer across widely distributed neural networks (Catts et al., 2013). This appears to be reflected by progressive myelination and faster and more synchronized axonal firing across long distances.

Behaviorally, although some aspects of cognition appear to develop in a linear fashion, others do not. For example, in a sample of children between 7 and 13 years of age a linear increase in abilities of executive function and memory was observed, however, the employed strategy for task completion revealed a regression in the subgroup of children aged 12 and 13 (Anderson et al., 2001). Accordingly, children aged 11 exhibited a better organizational ability compared to 12 and 13 year-olds. In another study, performance in a face recognition test in children increased between 6 and 10 years of age but then remained at a fixed level or declined for several years before increasing further by the age of 16 (Carey et al., 1980). In a comprehensive study of the normal brain development of healthy children (an NIH MRI study; Waber et al., 2007) structural

and metabolic brain development as well as behavior was followed longitudinally. A steep improvement from age 6 to 10 was observed for several raw score measures but also a deceleration during adolescence for others.

For rodents very little is known about the development of cognition during adolescence. Particularly longitudinal studies are scarce (e.g. Mengler et al., 2014, Molenhuis et al., 2014). Instead, most studies use separate groups of animals usually for a limited number of varying time points (e.g. Heyser and Ferris (2013): pd 21, 35, 42, and 90; Reger et al. (2009): pd 20-23, 29-40, and 50+ (as adults); Cyrenne and Brown (2011): pd 28, 40, and 80). However, some of the obtained results were rather contradicting. For example, while one study observed sex differences at mid adolescence in rats (Cyrenne and Brown, 2011), another study found similar recognition memory abilities across the investigated time points (Heyser and Ferris, 2013). These studies investigated the behavior of Lister Hooded and Spargue-Dawley rats respectively, whereas, apparently, so far no studies have investigated the ontogeny of recognition memory in Wistar rats. Furthermore, while a different study found that pd 18 old mice were not able to detect object novelty, suggesting immaturity in information processing at early ages, (Ricceri et al., 2000), Heyser and Ferris (2013) observed reliable object recognition memory skills in rats as early as pd 21. More comprehensive studies are needed to understand the development of cognitive abilities in rodents, particularly to understand any possible subtle alterations throughout adolescence.

1.3 The Endocannabinoid System

The endocannabinoid system (ECS) is a lipid signaling system involved in the modulation of neurotransmission. It consists of cannabinoid receptors (e.g. CB1R and CB2R), corresponding ligands (endogenous cannabinoids (eCBs)), and enzymes for their synthesis and degradation. Because both the CB1R and the CB2R are widely distributed in the body and the CNS, the ECS is involved in mediating a range of central and peripheral functions including neuronal development, hormone release and action, inflammation, cardiovascular, respiratory and reproductive functions, bone formation and energy metabolism, as well as cellular functions, such as cell architecture, proliferation, adhesion, motility, and apoptosis (Di Marzo, 2009). Additionally, it is involved in cognitive, motivational and affective processes (Di Marzo, 2009).

There are also a number of exogenous ligands that can activate the receptors like the plant-derived cannabinoids (e.g. Δ^9-THC (Δ^9-Tetrahydrocannabinol)) or synthetic ligands like WIN 55, 212-2 (WIN). These have been important in elucidating the molecular mechanisms of the system.

Plant extracts of *Cannabis sativa*, and cannabis preparations like marijuana, have been used for recreational and therapeutic purposes for thousands of years (for a review see Kano et al., 2009). However, it was not before in 1964 that the most psychoactive component, Δ^9-THC was identified (Gaoni and Mechoulam, 1964). In addition to the psychoactive components of *Canabis sativa*, there are also non-psychoactive components like cannabinol and cannabidiol. Δ^9-THC is highly lipophilic, allowing the passage across the blood brain barrier (see Ameri, 1999). Thus, prior to the identification of specialized cannabinoid receptors, effects of cannabinoids were thought to be due to cellular membrane disruptions or inhibition of membrane-associated enzymes (Hillard et al., 1985, Martin, 1986).

1.3.1 Receptors

CB1R was characterized by binding of the synthetic cannabinoid agonist CP55,940 (Devane et al., 1988) and was subsequently cloned from rat brain in 1990 (Matsuda et al., 1990). Three years later CB2R was identified by sequence homology (Munro et al., 1993). Both receptors are $G_{i/o}$ protein-coupled receptors (GPRs) with seven transmembrane domains (see Ameri, 1999, Castillo et al., 2012). Their N-terminal extracellular domain is glycosylated and the intracellular C-terminal domain couples to the G protein complex (Svizenska et al., 2008). Human CB1R and CB2R share only 44% amino acid sequence identity (Munro et al., 1993), but the human and murine CB1R share 97-99% of sequence homology (Kano et al., 2009). CB1R is suggested to exist as homodimers and to also form heterodimers with other classes of GPRs (e.g. D2 receptor

or orexin 1 receptor; see Kano et al., 2009) probably leading to a more direct cross-talk between the ECS and other neurotransmitter systems. CB1R is mainly found in the CNS implying its involvement for the main psychoactive effects of cannabinoids. In contrast, the CB2R is mainly found in the periphery and on immune cells (Kano et al., 2009).

CB1R is one of the most abundantly expressed GPRs in the brain (Ameri, 1999). High densities are found in the olfactory bulb, the Hip, the lateral parts and the target nuclei of the striatum as well as the cerebellar molecular layer (Herkenham et al., 1990). Additionally, moderate levels are found in the forebrain regions, the amygdale, and the hypothalamus. High receptor expression in the frontal cortex and the cerebellum explains the effects of cannabinoids on memory and motor functions. Very sparse receptor expression in the lower brain stem areas (controlling cardiovascular and respiratory functions) are in accordance with the observation that high levels of Δ^9-THC are not lethal (Herkenham et al., 1990, Kano et al., 2009). Cellular and subcellular distribution of CB1R was revealed at the perisynaptic sites of presynaptic cells (Kano et al., 2009). Both excitatory and inhibitory synapses contain CB1Rs.

Other ECS associated receptors include the TRPV1 (transient receptor potential vanilloid 1) and the orphan GPR55 receptors. The first is a Ca^{2+}-permeable, non-selective cation channel, expressed on primary sensory neurons and involved in thermal hyperalgesia and pain sensation (Caterina et al., 2000). In the brain, however, TRPV1 is activated by endovanilloids (including anandamide (AEA); see 1.3.2 and Starowicz et al., 2007), implying a mechanism of interaction between the ECS and the endovanilloid system. The orphan GPR55 can be activated by endogenous (e.g. AEA) as well as exogenous cannabinoid ligands (e.g. Δ^9-THC, CP55,940) eliciting a Ca^{2+} response (Lauckner et al., 2008). However, with some antagonists acting as agonists at this receptor while other ligands seem not to bind at all, it has a distinct, controversially discussed, pharmacological profile. Additionally, GPR55 mRNA was detected in the brain but functional receptor activation is still not proven (Ryberg et al., 2007).

1.3.2 Endogenous Ligands

The discovery of the cannabinoid receptors encouraged the research for eCB ligands (see Figure 7). The first isolated lipid was called AEA, a mixture of the Sanskrit word for bliss (*ananda*) and “amid”, reflecting its chemical structure (Ameri, 1999). AEA belongs to one of the best characterized eCB families, N-acylethanolamines, or fatty acid ethanolamides (Fonseca et al., 2013, Piomelli, 2014). Endogenous cannabinoids are synthesized on demand and, because of their lipophilic nature, are not stored in vesicles. Both AEA and the subsequently identified eCB 2-Arachidonoylglycerol (2-AG; belonging to the family of monoacylglycerols), are derivates of

arachidonic acid and bind to CB1R and CB2R with different affinities and efficacies (Pagotto et al., 2006).

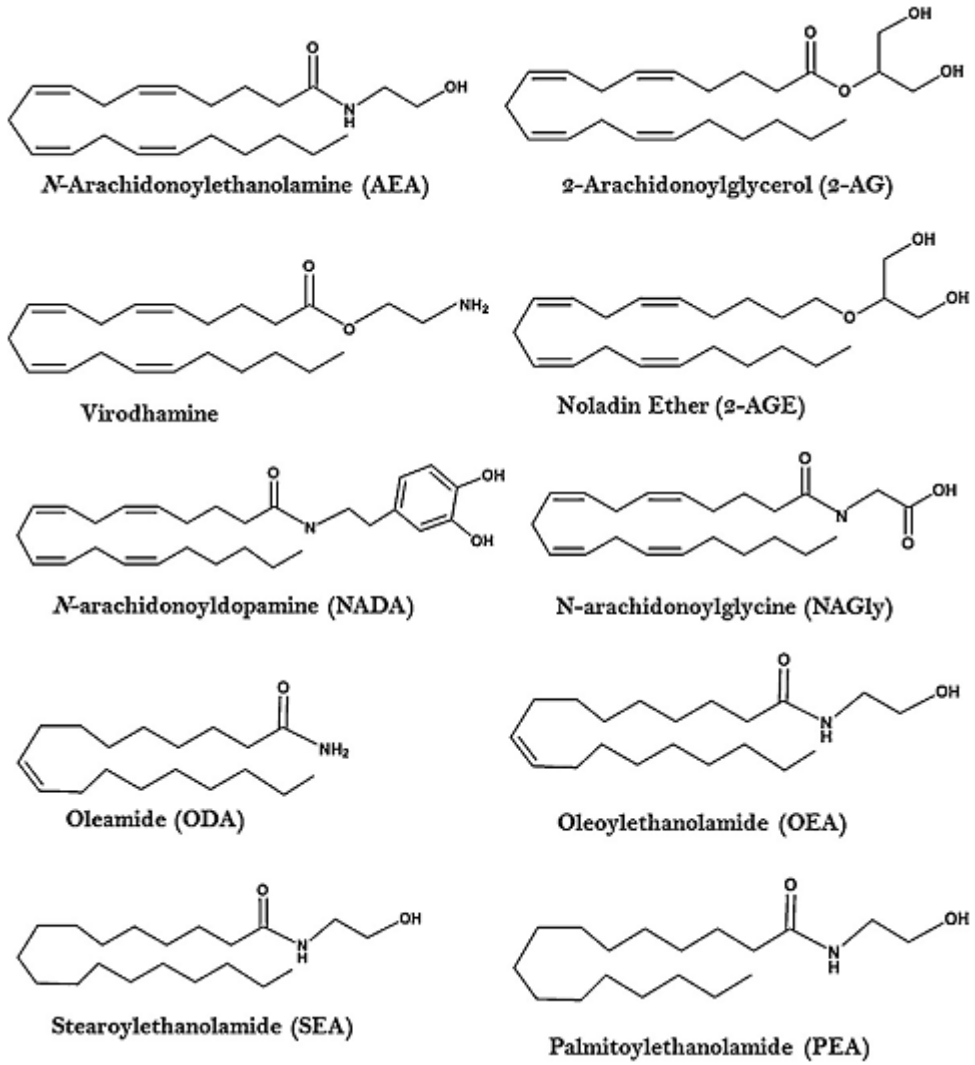

Figure 7: Chemical structure of the main endogenous cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG). Additionally, various endogenous cannabimimetic molecules have been identified so far (virodhamine, noladin ether, N-arachidonoyldopamine, N-arachidonoylglycine and oleamide). Furthermore, some endocannabinoid-like compounds are shown (oleoylethanolamide, palmitylethanolamide and stearoylethanolamide). Figure from Fonseca et al. (2013).

A two step, enzymatic, Ca^{2+} -requiring process must occur to form AEA. First, arachidonic acid must be transferred from the *sn*-1 position of phospholipids to the primary amino group of phosphatidylethanolamine (PE) yielding N-arachidonoyl - PE (NAPE). This step is catalysed by N-acyltransferase (NAT). The next reaction is the hydrolysis of NAPE to AEA and phosphatic acid catalysed by NAPE- phopholipid D (NAPE-PLD). However, NAPE-PLD independent pathways of generating AEA have also been proposed (for review see Piomelli, 2014). AEA is a lipid-derived messenger among which alternative routes of their synthesis are common.

The activity of AEA is rapidly terminated by its re-uptake into cells and enzymatic degradation by fatty acid amino hydrolase (FAAH). This membrane-bound hydrolase breaks down AEA yielding

arachidonic acid and ethanolamine (Deutsch and Chin, 1993). Although other lipid hydrolases can also degrade AEA, FAAH seems to be the most important mechanism for this process since interrupting FAAH activity, genetically or pharmacologically, cause a profound enhancement of AEA mediated CB1R signaling (Cravatt et al., 2001, Kathuria et al., 2003).

The monoacylglycerol 2-AG was first isolated in 1995 (Mechoulam et al., 1995, Sugiura et al., 1995) and its levels in the CNS are higher than those of AEA (Sugiura et al., 1995). It binds both CB1Rs and CB2Rs and acts as a full agonist at the first one (Childers and Breivogel, 1998). 2-AG is also an important precursor and degradation product of phosphoglycerides (De Petrocellis et al., 2004) and there are also several synthesis pathways known. One is the formation of diacylglycerol (DAG) from phosphatidylinositol (PI) by phospholipase C (PLC). Then DAG lipase hydrolyses DAG to 2-AG (Kano et al., 2009). Inactivation of 2-AG is catalyzed by degradation via monoacylglycerol lipase (MAGL) yielding glycerol and arachidonic acid, which can then be recycled into membrane phospholipids (Piomelli, 2014). Both AEA and 2-AG can also be subject to oxidation by COX-2 (cyclooxygenase 2) and various LOXs (lipoxygenases) (Kano et al., 2009, Fonseca et al., 2013, Piomelli, 2014).

Other endogenous cannabimimetic molecules are virodhamine, noladin ether, N-arachidonoyldopamine, N-arachidonoylglycine, and oleamide. Additionally, some endocannabinoid-like compounds have also been identified, for example oleoylethanolamide, palmitylethanolamide, and stearoylethanolamide (see Figure 7 and Fonseca et al., 2013 for a review).

1.3.3 Signaling

Synthesized either upon postsynaptic activation or constitutively, eCBs are released from the post synaptic membrane and travel across the synaptic cleft to the presynaptic CB1Rs in a retrograde fashion (Ohno-Shosaku et al., 2001, Mukhopadhyay et al., 2002). Activation of CB1Rs inhibits neurotransmitter release including glutamate, GABA, serotonin, glycin, ACh, and can indirectly modify DA transmission (reviewed by van der Stelt and Di Marzo, 2003, Kano et al., 2009, Castillo et al., 2012). Thus, neurotransmitter release can be modulated both at excitatory as well as inhibitory synapses by eCBs. These mechanisms are termed depolarization-induced suppression of inhibition or excitation (DSI or DSE respectively; Kreitzer and Regehr, 2001, Ohno-Shosaku et al., 2001, Wilson and Nicoll, 2001) and have been observed in various brain regions (Kano et al., 2009). Both short-term (eCB-STD) and long-term (eCB-LTD) neurotransmitter release suppression are modulated by eCBs.

Upon ligand binding to the cannabinoid receptors various intracellular signaling cascades are activated (see Figure 8; Fonseca et al., 2013). Activation of the CB1R coupled $G_{i/o}$ proteins

inhibits adenylyl cyclase (AC). Therefore the conversion of ATP in cyclic AMP (cAMP) is reduced. However, cAMP is needed for the regulation of protein kinase A (PKA) which in turn inhibits A-type potassium channels (K^+_A) via phosphorylation. The overall effect of cannabinoid receptor activation therefore results in an activation of K^+_A channels. Other effects of activated $G_{i/o}$ proteins (e.g. CB1R) are inhibition of N- or P/Q type Ca^{2+} channels and activation of inwardly rectifying potassium channels (K_{ir}) (De Petrocellis et al., 2004). This is regulated by protein kinase C (PKC) which can, after activation, phosphorylate CB1R and uncouple the receptor from the ion channels. Additionally, through the adapter protein FAN (factor associated with neutral sphingomyelinase activation), sphingomyelin (SM) is hydrolyzed via the sphingomyelinase (SMase) and consequently ceramide (an ubiquitous lipid second messenger) accumulates in the cell (Velasco et al., 2005).

Intracellular kinases like focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPK (p38) can also be stimulated by CB1R activation and can therefore mediate CB1R-induced expression of immediate early genes (IEG) like c-fos. Less is known about signaling associated with CB2R activation. However, ion channels do not seem to be modulated upon ligand binding (Felder et al., 1995).

Altogether, the ECS can exert various effects via the complexity of possible metabolic pathways and signaling cascades it is involved in and it thus modulates a range of physiological functions.

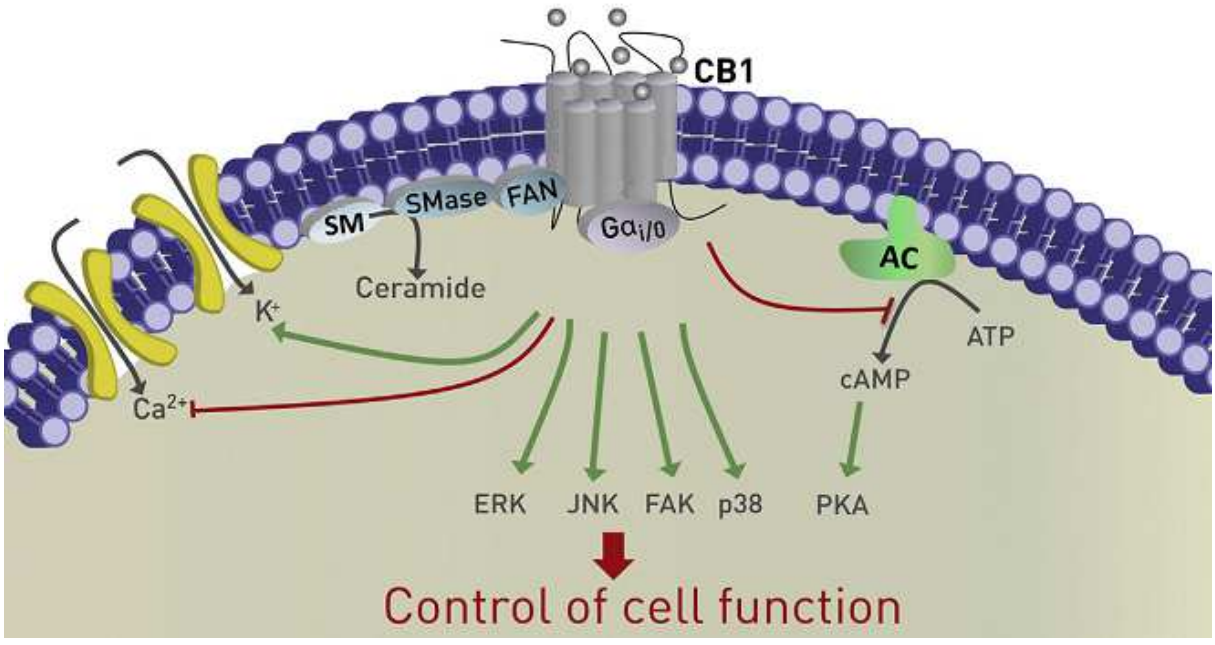

Figure 8: Main intracellular signaling pathways of CB1R activation. Stimulation of $G_{i/o}$ protein-coupled receptor signaling inhibits adenylyl cylcase (AC), which mediates the conversion of adenosine triphosphate (ATP) into cyclic AMP (cAMP). cAMP binds to PKA. Modulated ion channels include activation of inwardly rectifying potassium channels (K_{ir}) and inhibition of N- or P/Q type Ca^{2+} channels. Furthermore, ceramide accumulation is mediated via sphingomyelin (SM) hydrolysis by sphingomyelinase (SMase; activated by the adapter protein factor associated with neutral SMase activation: FAN). Additionally, intracellular kinases are stimulated including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), focal adhesion kinase (FAK), and p38 MAPK (p38). Figure from Fonseca et al. (2013).

1.3.4 Behavioral and Cognitive Effects of Cannabinoids

In rodents a “tetrad” of symptoms is often observed after cannabinoid intoxication consisting of hypothermia, rigid immobility (catalepsy), analgesia, and decreased motor activity (hypolocomotion). In addition, euphoria, enhancement of sensory perception, difficulties in concentration, and impairment of memory have been observed in humans (Ameri, 1999). The initial period of euphoria can be followed by depression. Furthermore, anxiety, panic, and psychotic reactions can occur (Moreira and Lutz, 2008). Thus, behavioral effects of cannabinoids appear to vary depending on individual vulnerability to certain effects as well as concentration and route of administration. Low doses often produce stimulatory effects followed by sedation whereas high doses mainly produce sedative effects (Ameri, 1999). For example, in rodents the application of high doses of Δ^9-THC increased anxiety-like behavior whereas low doses showed anxiolytic-like effects (Moreira and Lutz, 2008).

Overall, the ECS exerts a modulatory role on diverse emotional, cognitive, and physiological regulatory circuits.

Cognitive disturbances of acute effects of the CB1/CB2R agonist Δ^9-THC in humans include STM and WM impairments (Ranganathan and D'Souza, 2006). On the other hand, cannabidiol, a CB1/CB2R antagonist, prevents these effects (Morgan et al., 2010). Therefore, the ratio of Δ^9-THC/cannabidiol might influence the effects of cannabis in humans. Additionally, differing methodologies (different tasks employed), small sample sizes and a lack of appropriate control groups make the comparison of human studies difficult. Further confounding factors in human studies are possible polydrug use, pre-existing cognitive differences between cannabis users, as well as the amount and potency of cannabis used (see Jager and Ramsey, 2008, Mechoulam and Parker, 2013). Therefore, animal studies provide essential and often better comparable insights into the impact of cannabinoids on mechanisms of learning and memory. For example, in OBJR tests in rats STM was impaired after acute administration of synthetic cannabinoid receptor agonists like WIN and CP55,940 (Schneider and Koch, 2003, O'Shea et al., 2004). Also, WM was impaired in an 8-arm radial maze task and in the Morris water maze after Δ^9-THC administration (Lichtman and Martin, 1996, Varvel et al., 2001). Cannabinoid receptor antagonists (e.g. SR141716A; SR) appear to enhance STM possibly due to a prolonged retention of memory (e.g. Terranova et al., 1996, Lichtman, 2000). CB1R knockout mice displayed longer retention in an OBJR test (Reibaud et al., 1999).

Manipulations of the ECS also influence the performance on more complex cognitive tasks in rodents. For example, acute application of Δ^9-THC or overexpression of the CB1R (by viral mediated gene-transfer) impaired reversals in an attentional set shifting task (ASST; see 1.4.5 and

Egerton et al., 2005a, Klugmann et al., 2011a). Increased preservative errors were observed in a strategy set shifting task after application of the CB1R agonist HU-210 (Hill et al., 2006).
In addition to acute effects of cannabinoids, long-term effects on cognition have also been observed. Particularly chronic treatment during developmentally vulnerable time periods like adolescence (see 1.3.5) impair recognition for objects and social partners, PPI, and progressive ratio performance (Schneider and Koch, 2003, 2007, Schneider et al., 2008, Leweke and Schneider, 2011). Interestingly, chronic treatment in adulthood did not reveal these effects on cognition. Earlier treatment periods also revealed impairments in learning and memory that persisted into adulthood (Mereu et al., 2003, Antonelli et al., 2005). Thus, critical developmental periods like the juvenile period and adolescence appear to be especially vulnerable to cannabinoid treatment (Jager and Ramsey, 2008, Schneider, 2008, 2009).

1.3.5 The Endocannabinoid System during Development

CB1Rs are already present during early development as demonstrated in binding studies of rat and human studies (Berrendero et al., 1999, Mato et al., 2003). Additionally, eCBs and mRNA expression of the CB1R have been demonstrated in gestational and early postnatal stages in the rat (Berrendero et al., 1999). However, the distribution pattern has been shown to be transient and atypical in distinct brain regions compared to adulthood. For example, CB1R has been demonstrated in white matter areas during early postnatal ages but not in adulthood implicating an important role in developmental processes (Berrendero et al., 1999, Mato et al., 2003).
During embryonic development, eCBs appear to be involved in migration, cell proliferation, specification, differentiation, and survival of neuronal progenitors as well as in axonal growth and guidance and establishing synaptic communication (reviewed by Harkany et al., 2007, Saito et al., 2013). Due to the important role of the ECS in these processes disturbances of the system by exogenous application of cannabinoids interfere with normal development (for a review see Trezza et al., 2008). This renders the developing organism particularly vulnerable to perturbations, for example by maternal cannabis abuse.
During adolescence only few studies investigated the expression and distribution of ECS components. An increase in CB1R expression in the striatum, limbic forebrain, mPFC, and mesencephanlon has been demonstrated in rat brain between pd 30 and pd 40 (Rodriguez de Fonseca et al., 1993, Klugmann et al., 2011b). Additionally, levels of AEA, 2-AG, and CB1R are dynamically altered in the NAc, CPu and PFC as observed at pd 29, pd 38 and pd 50 after injection of vehicle or THC (Ellgren et al., 2008). In the PFC, AEA was found to gradually increase throughout adolescence, whereas 2-AG levels were lower in later stages compared to earlier ones both in the PFC and the NAc. Additionally, increased AEA levels have been observed

in the hypothalamus immediately before the onset of puberty in female rats (Wenger et al., 2002). Importantly, these alterations in the ECS take place while concomitantly other neurodevelopmental processes occur, including synaptic pruning, myelination, and maturation of other neurotransmitter systems with which the ECS interacts (Viveros et al., 2011; see also 1.2.2). Therefore, interference with this system during puberty and adolescence can lead to long lasting behavioral and neurobiological alterations as observed in a number of human and animal studies (see 1.3.4 and Grant et al., 2003, Jager and Ramsey, 2008).

1.4 Behavioral Paradigms

1.4.1 Rat Strain and Line Differences in Behavioral Testing

The Wistar rat is an outbred strain very commonly used in behavioral analyses. A strain can be defined as "a group of animals of known ancestry maintained by a deliberate mating system" (Sabourdy, 1965). For example, outbred strains are bred by deliberately avoiding mating of closely related individuals (in contrast to inbreed strains). A line is considered as "part of a family of animals separated from other parts by one or more generations of independent ancestry." (Sabourdy, 1965). Differences between different animal strains (like Wistar, Sprague-Dawley or Fischer rats) have been extensively investigated both behaviorally as well as neurochemically (e.g. Rex et al., 1999, Yilmazer-Hanke, 2008, Brand et al., 2012). However, even within the same line of a rat strain variations in behavior have been observed (Hirate et al., 1989, Honndorf et al., 2011, Langer et al., 2011, Palm et al., 2011b, Goepfrich et al., 2013).

As most laboratories receive all or parts of their animals from commercial breeders they are at least partially dependent on their conditions and breeding schedules. Many experimenters gain a lot of expertise about the behavior of a particular line for the testing paradigms they specialize in because they keep using the same line of animals. However, researchers can be forced to switch lines if the supplier abandons the breeding of that particular line. Consequently, the expertise gained in many years of previous research must be transferred to a new animal line. For the selections of the appropriate animal line for one's experimental hypothesis it is important to be aware that there are differences between rat strains and lines. Additionally, important other factors like age, gender, holding, and handling conditions can impact the results of behavioral testing. Therefore, the more valuable information about these details is given (as well as line-specific behavioral information) the better the comparability between studies will be.

1.4.2 Open Field Test

The open field test, introduced by Hall (1934), was originally employed to measure emotionality in rats. In this test, an animal is put into an unknown arena from which it cannot escape for a certain time (reviewed by Prut and Belzung, 2003). Open spaces, particularly brightly lit ones, are usually avoided by rodents. They tend to stay in the corners or near the walls (also termed thigmotaxis; see e.g. Simon et al., 1994) and spend less time in the center area. Therefore, the open field is often used as an anxiety-related paradigm (although this has frequently been debated; see e.g. Ennaceur, 2014). However, rodents also have a tendency to explore novel environments due to their natural foraging behavior. Therefore, the distance traveled in the arena can also be measured and is commonly used as an indicator of locomotor activity. In the context of this thesis the open field arena was not brightly illuminated and in addition, to obtain a locomotor index, the open field session often served as habituation for further testing in the same environment (e.g OBJR, object recency, or social recognition). Upon repeated exposure the locomotor activity decreases as the arena becomes familiar (see e.g. Thiel et al., 1999). This in turn renders any newly introduced objects or social partners in the arena as novel and directs novelty exploration towards these stimuli.

1.4.3 Recognition Memory

The one-trial OBJR test (Ennaceur and Delacour, 1988) is also called spontaneous OBJR test (Winters et al., 2008), or novel OBJR test (Anderson et al., 2004, Reger et al., 2009). It is used to test declarative memory, STM, as well as the preference for novelty, and is widely employed in rodent studies (Steckler et al., 1998, Dere et al., 2007, Winters et al., 2008, Ennaceur, 2010). The cognitive processes required in this type of memory integrate multimodal sensory information. To recognize a previously encountered item, two processes seem to be important: *recollection* and *familiarity*. In recollection, specific details about the context in which an item was encountered are remembered whereas familiarity entails the knowledge of having encountered an item before without more additional information (Squire et al., 2007). Two theories are trying to explain the involvement of these two processes in OBJR memory: According to the *dual process theory*, recollection depends on the Hip and familiarity depends on the adjacent perirhinal cortex (Brown and Aggleton, 2001, Eichenbaum et al., 2007). However, according to the *unitary process theory*, (an alternative view; see e.g. Squire et al., 2007) the structures of the medial temporal lobe and other brain areas operate rather cooperatively and the neuroanatomical distinction of recollection and familiarity might be too simple. It is argued that, instead, strong and weak memories are targeted by methods investigating separate processes of recognition memory.

1. Introduction

In rodents, the OBJR test utilizes their natural tendency to explore novel stimuli (i.e. objects or social partners). Typically, it consists of a sample and a choice phase (Ennaceur and Delacour, 1988, Dere et al., 2007). In the sample phase (usually ranging from 2 - 10 min; Dere et al., 2007) the animal encounters two identical objects in an open field arena. After a retention delay of various time intervals (from 1 min (Ennaceur and Delacour, 1988) to 24 h (Dere et al., 2007)) the animal encounters one familiar object and one that has not been seen before. The animal usually spends more time exploring the novel object in contrast to the time spend exploring the familiar object. The longer the retention delay the poorer the performance of the animal, because the familiar object tends to be forgotten and could be regarded as novel again (Dere et al., 2007). This depends on the time span of the sample phase and the rodent strain used but can also be influenced by pharmacological agents.

This test does not require any pretraining or learning of reward-associations or other rules and is less stressful than, for example, hidden-platform tests in the morris water maze (Dere et al., 2007). It only depends on a natural behavior of the animal. It is therefore comparable to test situations for memory tests in humans (e.g. visual paired comparison; Ennaceur and Delacour, 1988, Ennaceur, 2010).

A number of variations for the OBJR test exist. For example in the object location test (see Ennaceur and Meliani, 1992b, Mumby et al., 2002) the animal encounters two identical objects one of which has a changed position in the choice phase. Usually more time is spent exploring the object in the new location. In the object recency (OBJRecency) variation of the test (Mitchell and Laiacona, 1998, Hannesson et al., 2004) the animal at first encounters two identical objects (sample phase 1), then, after a delay, another set of two novel, identical objects (sample phase 2) and in the choice phase it encounters one of the first encountered (less recent) and one of the recently encountered (more familiar) objects. Usually more exploration is directed towards the less recently encountered objects compared to the more recently (i.e. more familiar) encountered objects. In the social recognition test (SOCR; Everts and Koolhaas, 1997, Bielsky and Young, 2004, Schneider et al., 2008), instead of objects, the test animal encounters novel conspecifics that can be explored for a certain time and, after a delay period, exploration of a novel versus a familiar social partner can be determined.

A number of brain regions seem to be involved in the OBJR test and its variations (reviewed by Dere et al., 2007, Warburton and Brown, 2010). The Hip has been considered essential for OBJR memory for a long time; however, several studies using various techniques (e.g. lesions, pharmacological interference, immediate early gene expression) revealed controversial results (reviewed by Mumby, 2001, Dere et al., 2007, Warburton and Brown, 2010). One explanation might be that after damage of this structure, extra-hippocampal structures can possibly act in a

compensatory way for lost recognition abilities (Dere et al., 2007). More recently, the Hip has been suggested to be important particularly in OBJR tests when spatial and contextual cues are relevant during the encoding stage of the test (Winters et al., 2004, Forwood et al., 2005). The perirhinal cortex is also implicated in encoding and retrieval of OBJR memory (most likely involving AMPA receptors) as well as consolidation (possibly involving NMDA receptors; Dere et al., 2007). Memory of temporal order or relative recency is believed to rely on the mPFC in humans, non-human primates and rodents (Kesner and Holbrook, 1987, Mitchell and Laiacona, 1998, Fuster, 2001, Hannesson et al., 2004) but additionally, a role for the perirhinal cortex has also been suggested in rats (Barker et al., 2007). Furthermore, a functional connection between the mediodorsal thalamus and the mPFC was implicated in OBJRecency but not in OBJR memory *per se* (Cross et al., 2012).

In contrast to OBJR, in which visual and haptic perception of the objects seem to be critical, the SOCR test relies on olfactory cues (including pheromones), the action of the neuropeptides oxytocin, and vasopressin and possibly involves differential brain regions (see Bielsky and Young, 2004). One hypothetical brain circuit involves the vomeronasal organ and olfactory epithelium which project to the accessory and main olfactory bulbs, respectively. These structures both project to the medial amygdala and from here projections to the lateral septum and the Hip appear to be critical for SOCR memory. Oxytocin is critical for mediating SOCR in the medial amygdala and implicated in the initial processing or encoding of social cues (Ferguson et al., 2001), while vasopressin is critical for SOCR memory in the lateral septum (Engelmann and Landgraf, 1994, Everts and Koolhaas, 1997). Moreover, these neuropeptides are suggested to affect SOCR memory by activating the norepinephrin neurotransmitter system (Dluzen et al., 1998).

Different neurotransmitter systems are implicated in OBJR memory. For example, the NMDA receptors of the glutamatergic system are involved in various brain regions and variations of the OBJR test (reviewed by Warburton et al., 2013). The involvement of the DAergic system has also been investigated by several pharmacological studies (Hotte et al., 2005, Hotte et al., 2006, de Lima et al., 2011). D1 receptor activation appears to enhance long-term OBJR memory (> 4h) but impairs OBJR memory after short intervals (15 min; Hotte et al., 2005, Hotte et al., 2006, de Lima et al., 2011). However, under certain conditions, DA receptors do not appear to be critical for memory formation as neither D1 receptor blockade nor D2 receptor activation impaired memory in another study (de Lima et al., 2011). Acute pharmacological inhibition of the cholinergic system (by administration of muscarinic ACh receptor antagonist scopolamine) impairs OBJR memory, possibly by decreasing attentional processes (Ennaceur and Meliani, 1992a, Dere et al., 2007). On the other hand, nicotine improved OBJR memory (Puma et al., 1999). However, as

mentioned in 1.1.2 this is possibly due to interactions with other neurotransmitter systems and these observations are in accordance with observations in other behavioral tests (see Blokland, 1995). Furthermore, the ECS is important in OBJR performance. As mentioned in 1.3.4, acute application of exogenous cannabinoids can impair OBJR performance (Schneider and Koch, 2003, O'Shea et al., 2004) while chronic application during critical developmental periods can exert long-term impairing effects (Schneider et al., 2008). CB1R knock out mice displayed enhanced OBJR memory (Reibaud et al., 1999).

Altogether, it appears that an interacting network of the Hip, the perirhinal cortex, and the mPFC are involved in successful OBJR memory. The perirhinal cortex is believed to be particularly of interest for the judgement of the occurrence of an item whereas the recollection of an item in context or of multiple items involves interaction of perirhinal cortex, Hip, and mPFC (Warburton and Brown, 2010). In addition, multiple neurotransmitter systems appear to be involved in OBJR memory and its variations, partially influencing or modulating each other. However, there is also a debate about how animals solve the task and what they perceive while exploring the objects (Ennaceur, 2010) which makes it difficult to judge if the animals perceive an item without the context of its presentation (e.g. the arena, the experimental room, experimenter, room etc.).

1.4.4 Acoustic Startle Response and Prepulse Inhibition

In mammals, the response to an abrupt, intense auditory stimulus is a fast contraction of the skeletal and facial muscles called the acoustic startle reflex (ASR) (Geyer, 1999, Koch, 1999, Leumann et al., 2001). During this reaction the eye-lids are closed, the muscles contract, the body length is shortened, ongoing behaviors are interrupted and the heart rate accelerates (Koch, 1999). This behavior is believed to serve as a protective function to unexpected, aversive events and/or as preparation of a fight/flight response. The ASR is triggered by auditory stimuli > 80 dB and has a non-zero baseline which means it can be modulated by various stimuli which enhance or attenuate the magnitude of the ASR (e.g. PPI see below). A reduction of ASR magnitude after repeated presentation of the stimulus can also occur which is termed habituation. Sensitization is the enhancement of a response following a strong stimulus e.g. electric footshock (Koch, 1999). The ASR is very fast with a measured onset latency of 5 - 10 msec in rats and 14 - 151 msec in humans (Leumann et al., 2001). Therefore, only few synaptic relays participate in the neuronal circuit of this reflex, supposedly involving the auditory nerve, the ventral cochlear nucleus, the nuclei of the lateral lemniscus, the nucleus reticularis pontis caudalis (PnC), a spinal interneuron, and a neuromuscular junction (Davis et al., 1982). To date several models have been proposed in which the PnC is thought to have a central role. It receives inputs from the auditory pathway

(Davis et al., 1982) and projects to facial, cranial, and spinal motor neurons (Lingenhohl and Friauf, 1992). As a result the PnC is regarded as a sensorimotor interface of the ASR.

If a weak, non-startling sensory event precedes the startle-eliciting signal by 30 - 500 msec, the magnitude of the ASR is reduced (Hoffman and Ison, 1980). This has been termed PPI and is used as an operational measure of sensorimotor gating mechanisms. Sensorimotor gating describes the ability of weak sensory events to inhibit or "gate" a motor response otherwise triggered by intense stimuli (Swerdlow et al., 2001).

Several midbrain nuclei appear to be involved in a hypothetical circuit mediating the PPI of the ASR (see Figure 9; Koch, 1999, Fendt et al., 2001). An auditory perpulse activates the inferior colliculus and from here information is relayed to the superior colliculus. This in turn receives input from auditory, somatosensory and visual structures and projects to the pedunculopontine tegmental nucleus. This nucleus sends cholinergic projections to the PnC which mediates the PPI of the ASR. Lesions of the pedunculopontine tegmental nucleus do not completely block PPI, therefore an alternative pathway is also likely to exist (Koch, 1999). Additionally, other brain regions appear to be involved in modulating PPI including the NAc, the ventral Hip and the mPFC as well as the basolateral amygdala and the mediodorsal thalamus (Koch, 1999, Swerdlow et al., 2001). Various neurotransmitter systems influence the PPI of the ASR and manipulations in these systems disrupts PPI (Geyer et al., 2001). For example, DA agonists disrupt PPI which can be reversed by the antipsychotic haloperidol (Swerdlow et al., 1994). Serotonin agonists and NMDA antagonists also cause PPI deficits (Mansbach and Geyer, 1989, Sipes and Geyer, 1994).

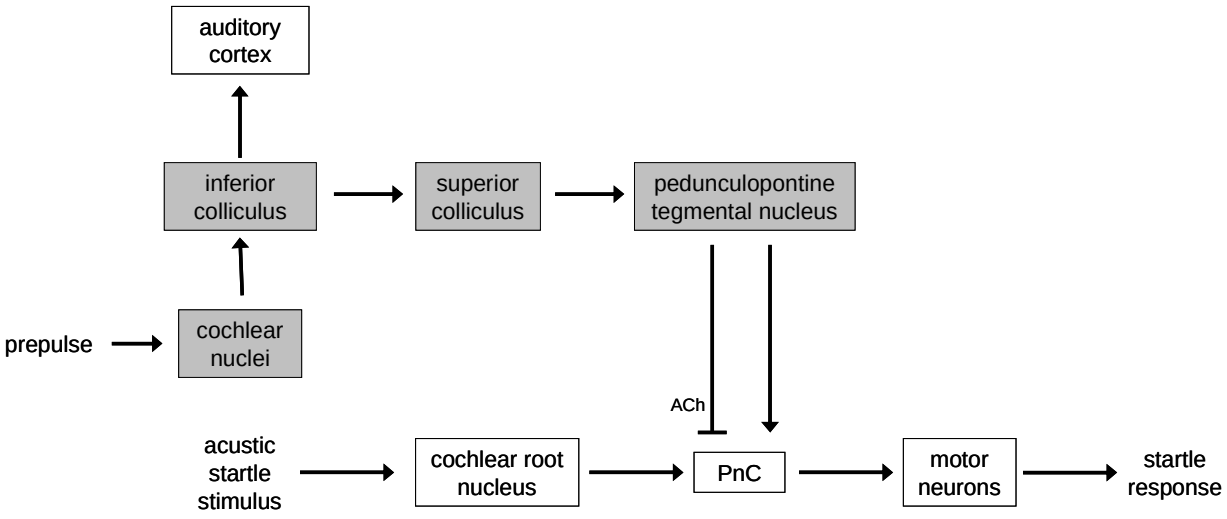

Figure 9: A simplified hypothetical circuit mediating the PPI of the ASR. Gray shaded boxex represent brain nuclei mediating PPI while the lower white boxes are involved in the ASR. PnC: nucleus reticularis pontis caudalis. Figure adapted from (Koch, 1999).

PPI occurs after the first exposure to a prepulse suggesting that it does not involve learning mechanisms. Increased prepulse intensity leads to an increase in PPI. Other parameters like the

background noise level can influence startle and PPI and hence a background noise between 50 - 70 dB and prepulses 1 - 20 dB above that are often used (Leumann et al., 2001). Prepulse stimuli of different intensities are given in random order prior to startle stimuli during the test phase. The inhibition of the prepulse is calculated in percentage to the amplitude of the startle without prepulse. In neuropsychiatric disorders like schizophrenia and Huntington's disease PPI and ASR are impaired (Koch, 1999).

1.4.5 Attentional Set Shift Test

The ASST is a complex cognitive test measuring behavioral flexibility. According to Tait et al (2013) an attentional set is a hypothetical "store" that maintains "the reward-predicting aspects of a stimulus, and the contents of an attentional set must be updated when new dimensions become relevant" (Tait et al., 2013). Successful performance in this test therefore requires a range of aspects of cognitive abilities including rule learning, updating of rules when changes occur, suppressing inappropriate responses, focused attention, and resisting distractibility. It was adapted for rodents by Birrell and Brown (2000) as an analog for the Wisconsin Card Sorting Test (WCST). The performance in the latter is often impaired in patients suffering from schizophrenia, ADHD, Parkinson's, or Alzheimer's disease (reviewed by Tait et al., 2013). In the WCST the test subject must sort a deck of cards according to shape, color, or number of the displayed symbols. The rule is not explicitly told but a feedback after each sorting is given. After a certain amount of cards the rule changes, thus the test subject has to find the new rule and adapt the sorting strategy. For rats often a digging task is employed (Birrell and Brown, 2000). The animal has to retrieve a reward from a pot containing scented digging material by either focusing on the odor or the type of digging medium. Newly introduced rules can, for example, reverse reward contingencies (reversal stage of the test). A new rule can also require the animal to employ the learned rule to a new situation (e.g. facing new odors and digging materials but relying on the rule that the type of digging media always predicts the reward) which is called an intradimensional shift (IDS). An extradimensinal shift (EDS) would then require the animal to shift the focus of attention from one stimulus dimension (e.g. type of digging material) to another (e.g. always to focus on the odor for finding the buried reward).

Lesion studies in rodents revealed an important role of the mPFC for the EDS of the test (Birrell and Brown, 2000). The OFC seems to be particularly involved in the reversal stage (McAlonan and Brown, 2003) and the cingulate cortex is implied in set formation (Ng et al., 2007). In addition, pharmacological studies implied various neurotransmitter systems in performance in the ASST (Tait et al., 2013), like the DAergic, noradrenergic, and ECS (Egerton et al., 2005a, Egerton et al., 2005b, Lapiz and Morilak, 2006). Considering the tremendous interactions between

these neurotransmitter systems and their modulating influence on PFC functions a disruption by lesion or pharmacological intervention would reasonably affect normal PFC function (see Tait et al., 2013).

1.4.6 Anxiety-related Paradigms

Rodents have a natural tendency to explore a new environment. However, open spaces or heights are usually avoided, like the center of a brightly illuminated open field. This spontaneous exploratory behavior versus the retreat and staying in dark or enclosed compartments can be observed in a variety of anxiety-related tests, e.g. the light/dark emergence test (EMT) or the elevated plus maze (EPM; for a review see Ennaceur, 2014).

In the EMT, the most commonly used apparatus consists of a dark compartment (1/3 of apparatus size) and a bright compartment (2/3 of apparatus size) (Crawley and Goodwin, 1980). The test animal can freely move between both compartments for a defined time. Emergence latency, number of movements between the two different compartments, as well as rearing can be measured to evaluate anxiety and exploratory behavior. Enhanced activity in the illuminated compartment as well as increased transitions between the compartments (without increased general motor activity) is considered as low anxiety-related behavior (Bourin and Hascoet, 2003). Modulation of EMT behavior can be observed after administration of various drugs (e.g. benzodiazepines act anxiolytic while amphetamines elicit anxiogenic behavioral effects). Interestingly, basal behavioral differences in EMT for different rat strains have been reported (e.g. van der Staay et al., 2009).

The precursor of the EPM is a Y-maze with alternating open and closed arms invented by Montgomery (1955). In the EPM the test subject is placed in an elevated (50 -70 cm above ground level), plus-shaped maze which consists of two opposing open and closed arms (Handley and Mithani, 1984, Dawson and Tricklebank, 1995). Usually, animals spend more time in the closed arm of the maze. The EPM was validated physiologically and pharmacologically (Pellow et al., 1985) suggesting that anxiolytic drugs increase the number of entries as well as the time spent in the open arms. In contrast, anxiogenic drugs reduce these behaviors. The test duration is commonly limited to 5 min because the avoidance effect is mainly observed during this time (Montgomery, 1955). The EPM is widely used, although the influence of various test parameters, for example the illumination level or height above ground, are still discussed (Garcia et al., 2005). However, the system has some clear advantages such as not requiring any pretraining, food or water deprivation, or the use of noxious stimuli and is conducted easily and quickly (Pellow et al., 1985).

1.4.7 Intake Paradigms

Intake of various foods or liquids can be measured in limited free access paradigms (Files et al., 1994, Wong et al., 2009, Friemel et al., 2010, Goepfrich et al., 2013). The individual animal usually has *ad libitum* access to edible substances or liquids of variable palatability for a limited time span and the intake is measured and corrected for the animal's bodyweight. Often the animals are habituated to the new food or liquid beforehand and the test is conducted in single cages after a short habituation period. Highly palatable substances like sucrose, sweetened condensed milk (SCM), and casein pellets (CPs) are known to be eagerly consumed by rats and therefore often used as rewards in positively reinforced test (e.g. ASST or radial arm maze). It is, however, important to verify that test and control groups of animals devote the same valence to the used reward. If one group of animals consumes less of the reward at basal levels, it is difficult to assign differences in performance of a learning task to cognitive instead of possible motivational or hedonic differences. Possible differences in the amount of intake could arise from different caloric requirements or differences in taste perception but could also be influenced by stress or satiety (Strouthes et al., 1974, Gronli et al., 2005). In contrast to palatable foods and liquids, ethanol (EtOH) is often used to model drug intake. Since it has a bitter taste (depending on the concentration) and less impact on the caloric demands than the above mentioned rewards, it is often used to analyze the hedonic values of a drug (reviewed by Green and Grahame, 2008). In free choice paradigms animals are presented EtOH solutions of varying concentrations along with normal water. Differences in EtOH intake have been observed between different rat strains and lines (Wilson et al., 1997, Palm et al., 2011b, Goepfrich et al., 2013).

1.5 Study Aims

In the present thesis, four associated projects were conducted, which investigated cognitive abilities and their development in the Wistar laboratory rat.

In the first project possible differences between three different Wistar Han rat lines were investigated. Two rat lines (i.e. W[hsd] and W[rcc]) were obtained from the same breeder (i.e. Harlan Laboratories), and were compared with each other and to a line from another breeder (i.e. W[Jan] from Janvier). As Harlan plans to replace all W[hsd] colonies with W[rcc] (Harlan Laboratories, 2011), possible behavioral differences in these two lines were investigated in a variety of behavioral paradigms as well as in the Wistar line W[Jan]. Their performance in several behavioral paradigms including an open field test, an OBJR test, as well as the PPI of the ASR was analyzed and based on the results, one rat line was selected for the best performance in the cognitive tasks employed for the subsequent projects.

The second project addressed the ontogeny of cognitive abilities from before the onset of puberty throughout the period of adolescence until adulthood of the rat. Only few rodent studies have investigated the basic cognitive development during adolescence so far and particularly longitudinal studies are scarce. Therefore, animals were tested repeatedly for abilities in recognition memory and sensorimotor gating function by employing an OBJR and an OBJRecency tests, as well as ASR and PPI throughout adolescence and young adulthood. Based on these results, the influence of a pharmacological intervention by the CB1R antagonist/inverse agonist SR on OBJR and PPI performance on pd 40 was investigated. Due to the vast neuroanatomical changes in the brain during adolescence, including the proposed changes in the ECS and the alterations in myelination, the abundance of the CB1R and myelin was investigated histologically and the protein content of the CB1R was analyzed at corresponding time points to the behavioral tests.

The third project investigated the long-term effects of a chronic pubertal WIN treatment in adulthood, particularly in cognitive tasks in the Wistar rat. The chronic pubertal WIN treatment is considered as an animal model of schizophrenia (Leweke and Schneider, 2011), yet little information is available on the influence of this treatment on complex cognitive abilities. Therefore, the performance of adult rats was investigated in the ASST. Additionally, it was intended to examine if the treatment yields the same effects in the W[rcc] line as in the W[hsd] line in recognition memory and sensorimotor gating, tested in an OBJR test and PPI, respectively.

In the fourth project the possible long-term effects of a chronic pubertal MPH treatment was investigated in a broader range of behavioral tests. As most studies focused on the juvenile or the early adolescent period for MPH treatment, little is known about ongoing treatment throughout the period of adolescence. Here, the effects of a chronic pubertal MPH treatment of Wistar rats were investigated by behavioral characterization in adulthood (> pd 80) to elucidate long-term effects in a longitudinal design.

2. Material and Methods

2.1 Animals

296 male albino Wistar™ Han (Wistar) rats with known birth dates (date of birth considered as pd 0) were purchased from Harlan Laboratories GmbH (Horst, Netherlands) or Janvier (Le Genest St Isle, France). They were delivered shortly after weaning and then housed in groups of four to six in Makrolon™ cages (Eurostandardtype IV) under a 12/12 hr light-dark cycle with the light phase starting at 7 am. During the light phase, a radio provided background noise. Animals had *ad libitum* access to food ('V1536-000 ssniff R/M-H, ssniff Spezialdiäten GmbH, Soest, Germany) and tap water. All experiments were conducted in accordance with the ethical guidelines for the care and use of laboratory animals and were approved by the local animal care committee (Regierungspräsidium Karlsruhe, Germany).

Behavioral testing was conducted between 10 am and 5 pm. For the ASST animals were maintained on a mild food restriction (12g / animal / day) from the first day of habituation and body weight was measured every day before experiments started. For behavioral tests the test subjects were transported from the holding room to an experimental room. The animals were conveyed to the room individually in Makrolon™ cages (Eurostandard type III) or in their home cage before being put in the test apparatus. In most experiments a radio provided constant background noise except during PPI of the ASR where white noise was used. After removing urine and excrements, the test apparatus was cleaned with water and antifect N liquid (Schülke & Mayr GmbH, Norderstedt, Germany) thoroughly between each subject to eliminate the smell of the former test subject.

2.2 Experimental Design

2.2.1 Project I: Differences of Cognitive Abilities in Three Wistar Han Rat Lines

To assess the differences in cognitive abilities in the three Wistar Han rat lines, adult animals were obtained from commercial breeders and group housed in our vivarium for at least one week prior to testing. Three groups (n = 12 for each) were obtained from two different suppliers: In addition to Wistar Han animals obtained from Janvier (RjHan:WIST termed W[Jan]; Le Genest St Isle, France), HsdHan:WIST (termed W[hsd]) and RccHan:WIST (termed W[rcc]) animals were both obtained from Harlan Laboratories GmbH (Horst, Netherlands). After recovering from transport and habituating to their new environment the animals were tested in the open field test for locomotor activity, in an OBJR test for short-term memory and in a test of PPI of the ASR for

sensorimotor gating (see Figure 10). Based on the results Wistar Han rcc animals were employed for all further tests.

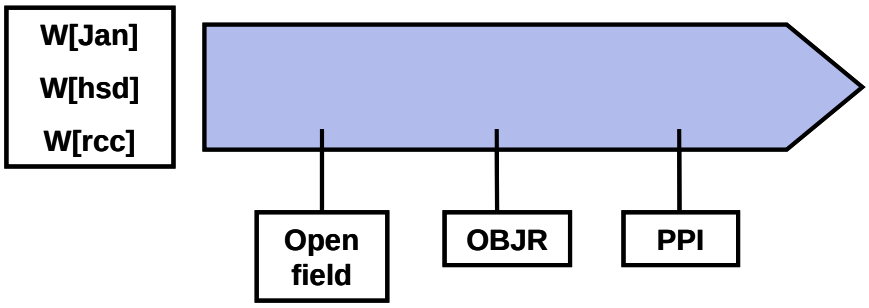

Figure 10: Test sequence for the comparison of cognitive abilities in three different Wistar Han rat lines: RjHan:WI (W[Jan]) obtained from Janiver, RccHan:WIST (W[rcc])] and HsdHan:WIST (W[hsd]) both obtained from Harlan Laboratories GmbH. All animals were tested for in the open field, OBJR and PPI.

2.2.2 Project II: Ontogeny of Cognitive Abilities

To check for the possible influence of the ITI length during the OBJR test, two groups of animals were tested with different ITIs in a preliminary study. Therefore four animals aged pd 45 (n = 4) and six adult animals (> pd 100; n = 6) were tested in the OBJR test with ITIs of either 10 or 20 min.

To investigate the cognitive development during adolescence, one group (n = 16) of animals was tested in the open field test, in the OBJR test, in a PPI paradigm, and in an OBJRecency test every ten days from pd 30 to pd 80 as well as in adulthood (pd 100 and pd 130; see Figure 11). The animals were tested in an open field for 15 min on one experimental day (starting on pd 29) which also served as a habituation session for the OBJR test. The OBJR test was conducted starting 3 h after lights on the following day (starting on pd 30). After a break of approx. 4 h, the animals were tested for PPI. The next day the animals were tested for OBJRecency (starting on pd 31). This testing sequence took place throughout the period of adolescence until pd 80. After a break the animals were then tested in the same test sequence at two adult time points: pd 100 and pd 130.

Another group of adult animals (n = 16; > pd 100) underwent the same behavioral test battery as a control group for a possible impact of repeated testing. This group was tested in an open field, OBJR, PPI and OBJRecency test three times every ten days at the same intervals as the adolescent animal group (see Figure 11).

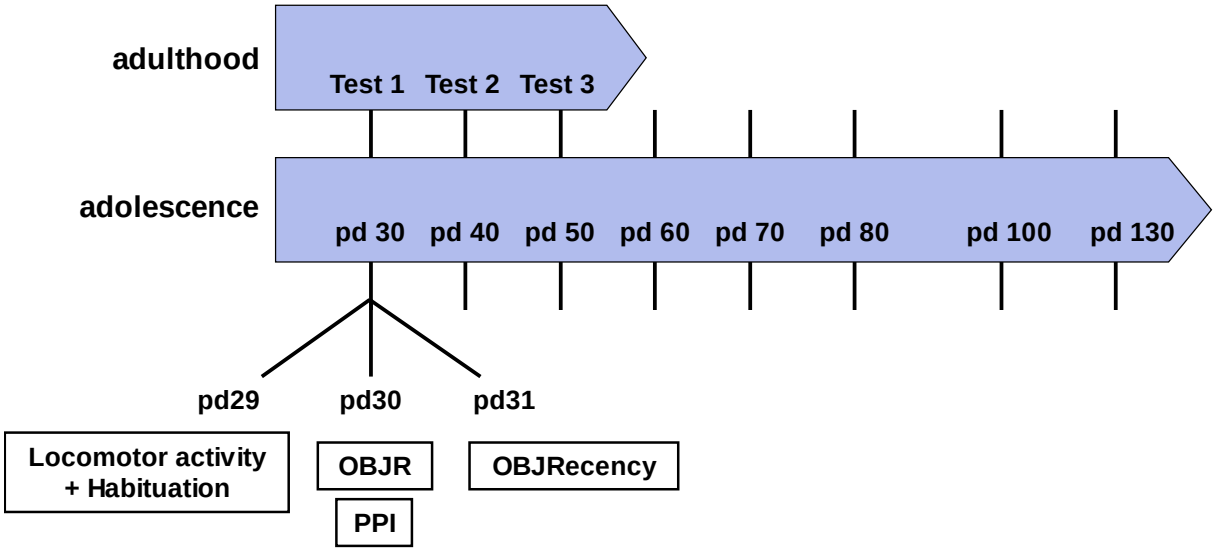

Figure 11: Test sequence for the development of cognitive abilities during adolescence. Animals were tested in the open field, OBJR, PPI, and OBJRecency on three consecutive days every ten days from pd 30 to pd 80. After a break they were tested again at two adult time points (i.e. pd 100 and pd 130). In parallel, one control group of adult animals was tested in the same sequence three times for possible effects of repeated testing.

Based on the results, three other groups of animals (pd 30, pd 40, and pd 130) were tested for OBJR and PPI under pharmacological treatment with the synthetic CB1R antagonist / inverse agonist SR (0.3 mg/kg SR or vehicle; see Figure 12 and Table 1). Thus, SR was administered intraperitoneally (i.p.) 30 min prior to testing in the OBJR. PPI was tested immediately after OBJR.

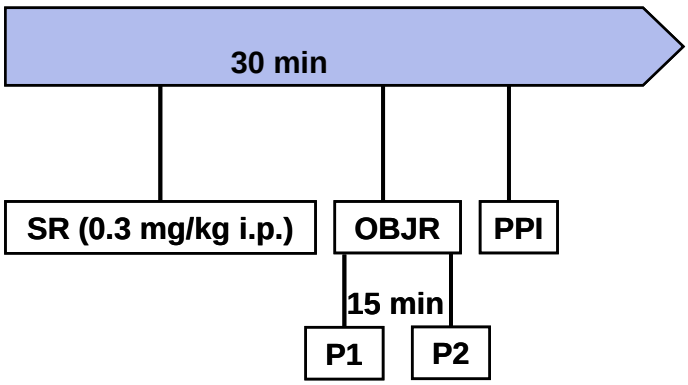

Figure 12: Experimental design of tests for pharmacological treatment of SR (0.3 mg/kg) at pd 30, pd 40, or pd 130. Animals were injected vehicle or SR i.p. 30 min prior to testing. Afterwards they underwent OBJR and PPI.

Table 1: Numbers of animals for SR (0.3 mg/kg) or vehicle treatment in the OBJR and PPI tests.

Number of animals per group	Vehicle	SR
pd 30	12	12
pd 40	16	17
Pd 130	18	14

In addition, the brain tissue of behaviorally naïve animals were collected for molecular analysis at the same time points during development (pd 30, 40, 50, 60 and 130; n = 5 for each time point). One group of animals was perfused and cryo-sectioned for staining of myelin and CB1R. Another group of animals (n = 6 for the time points pd 30, 40, 50 and 130) was CO_2-anesthetized and brain regions (mPFC, CPu, NAc, Hip) were dissected for western blot analysis of CB1R.

2.2.3 Project III: Long-term Effects of Chronic Pubertal WIN 55, 212-2 Treatment on Cognition

To assess the long term-effects of chronic pubertal WIN treatment on cognition, 24 animals were tested in adulthood for OBJR, PPI, CP intake, and in an ASST (see Figure 13). Before testing, animals received either the synthetic cannabinoid WIN (1.2 mg/kg; n = 12) or vehicle (n = 12) for 25 days from pd 40 to pd 65 (Schneider et al., 2008). During this period the rats irregularly received 20 injections (i.p.) (Schneider and Koch, 2003, Leweke and Schneider, 2011) with either 1, 2 or 0 injections every day (in total 10 times one injection, 5 times two injections and 10 times no injection). This chronic intermittent treatment schedule mimics the irregular consumption of cannabinoids in human users. Long-term effects of treatment were assessed with behavioral tests starting on pd 80.

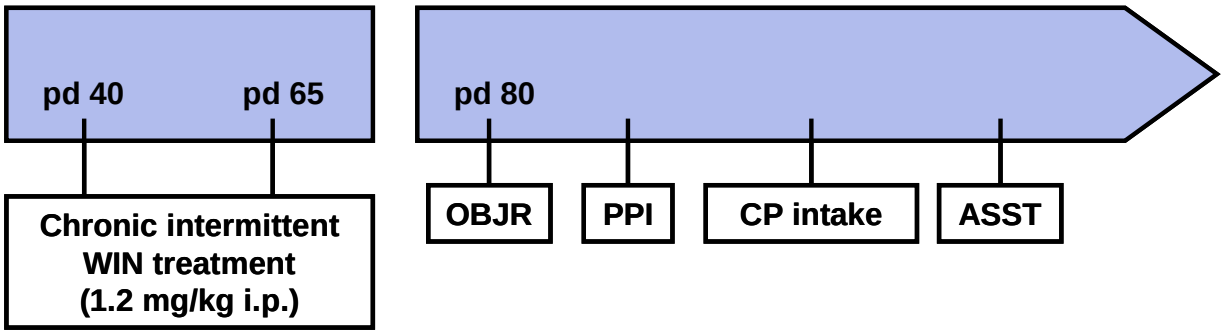

Figure 13: Experimental design of chronic, intermittent pubertal WIN treatment from pd 40 to pd 65 followed by tests of OBJR, PPI, CP intake, and ASST after pd 80.

After behavioral testing animals were CO_2-anesthetized in adulthood (> pd 100) and brain regions (mPFC, Cpu, Hip) were dissected for western blots analysis of CB1R, FAAH and MAGL.

2.2.4 Project IV: Long-term Effects of Chronic Pubertal Methylphenidate Treatment on Cognition

To study the long-term effects of chronic pubertal MPH treatment, 24 rats underwent chronic treatment of either MPH (2 mg/kg; n=12) or saline (i.p.; n=12) from pd 40 to pd 55 (see Figure 14). Injections were conducted at 9 am every day for 16 days. All animals remained undisturbed after treatment cessation except for changing of cages and weighing twice a week. The following

behavioral tests were conducted after all animals had reached pd 80: Open field, EPM, EMT, SCM and EtOH intake, PPI, OBJR and SOCR.

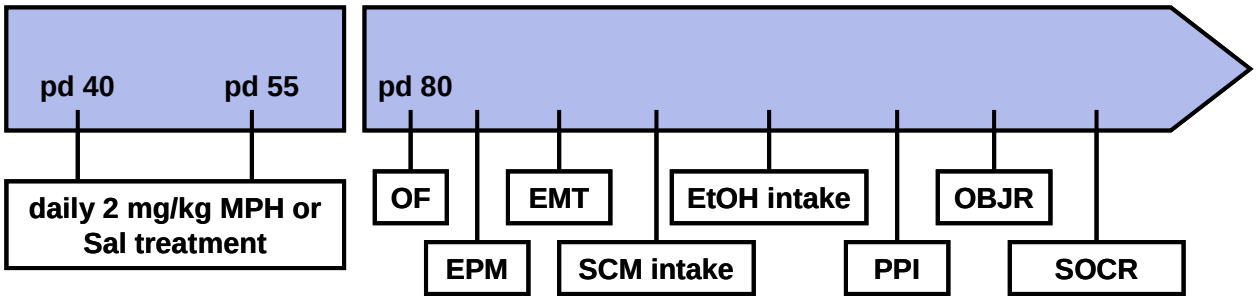

Figure 14: Experimental design of chronic pubertal MPH (2mg/kg) or saline (Sal) treatment was conducted from pd 40 to pd 55. After pd 80 the following tests were conducted: Open field (OF), EPM, EMT, SCM intake, EtOH intake, PPI, OBJR, SOCR.

2.3 Drugs

2.3.1 SR141716

The cannabinoid antagonist SR141716 (SR; RTI International, North Carolina, USA) was dissolved in EtOH, Tween 80 (0.1%) and saline (0.9% NaCl) in ratios of 1:5:54. First it was dissolved in EtOH and Tween 80 in a glass vial on a magnetic stirrer. Saline was added later to a final concentration of 0.3 or 0.6 mg/kg and administered i.p. Animals were weighed before treatment and the injection volume (1 ml/kg) was adjusted according to their current body weight.

2.3.2 WIN 55, 212-2

Cannabinoid agonist WIN 55,212-2 (WIN) (Sigma-Aldrich, Germany) was dissolved in 0.1% Tween 80 and diluted in NaCl (0.9 %). The drug was administered i.p. in a dose of 1.2 mg/kg. Animals were weighed daily before treatment and the injection volumes (1 ml/kg) were adjusted according to their current body weight.

2.3.3 Methylphenidate

The DA transporter / norepinephrin transporter blocker methylphenidate (MPH) (Sigma-Aldrich, Germany) was dissolved in saline (0.9 % NaCl). The drug was administered i.p. in a dose of 2 mg/kg. Animals were weighed daily before treatment and the injection volumes (1 ml/kg) were adjusted according to their current body weight.

2.3.4 Anesthetics

The NMDA receptor antagonist ketamin (10% hydrochloride injection solution, CP-Pharma GmbH, Burgdorf, Germany) was administered in combination with the alpha-2 receptor agonist xylazin (Rompun 2%: xylazinhydrochloride injection solution, Bayer Vital GmbH, Leverkusen, Germany) as an anesthetic 100 mg : 5 mg i.p. before perfusion. Animals were weighed before treatment and the injection volume (1 ml/kg) was adjusted according to their current body weight.

2.4 Behavioral Testing

2.4.1 Open Field Test

Locomotor activity was measured in a dark gray PVC open field apparatus consisting of four equally sized arenas (51 cm x 51 cm x 50 cm) for parallel analysis of four animals. The light in the center of the arena was adjusted to 50 lx. The animal was placed in the center of the arena at the beginning of the test. The behavior was analyzed by using the observation program Viewer^2 (Biobserve GmbH, Bonn, Germany). In project I and IV, the distance traveled [cm] was recorded digitally for a period of 30 min while in project II, the distance traveled [cm] was recorded for a period of 15 min.

2.4.2 Object Recognition Test

The OBJR test was performed in one of the open field arenas in which the open field test had been conducted on the previous day (see 2.4.1). The open field test also served as a habituation session in which the animal could familiarize itself with the testing environment in order to avoid a lack of exploring the object in the subsequent test sessions. The light was adjusted to 50 lx. The objects were placed in the arena 10 cm away from the wall in order to allow the animal to walk around it. The OBJR test consisted of two exploration trials: a sample (P1) and a test trial (P2) separated by an ITI. First the animal was placed into the arena containing two identical, clean, unfamiliar objects which it was allowed to explore for 3 min (sample trial). Then the animal was put back into its home cage for an ITI of 15 min (except for a varying ITI in the preliminary study of 10 or 20 min). After that the animal was again placed into the same open field arena which contained one identical copy of the object from the sample trial and a second, unfamiliar object and was allowed to freely explore for 3 min (test trial). The animals were always placed into the arena with its back turned to the objects. The arena and the objects were thoroughly cleaned with antifect N liquid (50% diluted with water), then with water and subsequently dried between animals and trials. Both trials were recorded by a video camera and later analyzed by an experienced

experimenter. Exploration behavior was defined as sniffing, touching with whiskers or nose, and licking of the object but not by standing or sitting on it. The time spent with each object as well as the total exploration time were determined. The percentage of the time spent with the new object versus the familiar one was calculated as

$$\% = \frac{100}{(\text{time exploring familiar object} + \text{time exploring new object}) * \text{time exploring new object}}$$

and a discrimination index was calculated as the difference of time spent exploring the new object versus the familiar one.

2.4.3 Object Recency Test

The OBJRecency test was performed in an open field arena and conducted similarly to the OBJR test (see 2.4.2). Different objects from the OBJR test were used to avoid any possible confounding effects of familiarity. It consisted of two sample trials (P1 and P2) and a test trial (P3). Each trial lasted 3 min and was separated by an ITI of 45 min in which the animal was transferred back into its home cage. In P1 the animal was allowed to explore two identical, clean, and new objects. In P2 it was again allowed to explore two identical, clean objects which were different from the objects of P1. In P3 a clean copy of an object from P1 (an old familiar object) and one from P2 (a recently familiar object; (Dere et al., 2007) were placed in the arena. The time spent with the old familiar vs. the recently familiar object was calculated as in the OBJR test (as percentage discrimination and discrimination index).

2.4.4 Social Recognition Test

The SOCR test was conducted in the open field arena and the design and time course were similar to the OBJR test (see 2.4.2). The time intervals of P1 and P2 were both 3 min and the ITI was 15 min. All animals were habituated to the environment separately on the previous day. On the test day, the social partner animal was placed into the arena shortly before the test animal so the social partner could adapt to the environment. After P1 both the test animal and the social partner animal were put back into their individual home cages. Following an ITI of 15 min, the familiar social partner and a new social partner animal were placed into the open field arena. The test animal was then also placed into the open field arena for P2.

Again the behavior was recorded by a camera and social investigation (anogenital exploration, non-anogenital exploration and approach/following) of the test animal was later analyzed (see also Schneider et al., 2008). The time spent with the familiar vs. the novel social partner was calculated as percentage social discrimination as in the OBJR test.

2.4.5 Acoustic Startle Reflex and Prepulse Inhibition

The PPI of the ASR was measured in a startle chamber (SR-LAB; San Diego Instruments, San Diego, USA). A white noise pulse with an intensity of 115 dB sound pressure level (SPL) and a duration of 40 msec (0 msec rise/fall times) was used as a startle stimulus. Four pulses with different intensities (72, 76 80, 84 dB SPL, duration 20 msec) were used as prepulses and were presented 100 msec before the startle stimulus. White noise (65 dB SPL) served as background noise. The PPI program consisted of an acclimatization period, an initial startle exposure and the test period. In the acclimatization period, the animals were only exposed to the background noise for 5 min. Five initial startle stimuli followed. The test program comprised six different trial types in a pseudorandomized order: startle pulse alone, startle pulse preceded by prepulses of different intensities (see above) or background noise alone. The ITI varied between 10 and 20 sec. All combinations were presented 10 times thus resulting in a series of 60 trials.
The initial five startle pulses were excluded from further analysis. PPI was calculated as the percentage decrease of the ASR magnitude in trials when the startle stimulus was preceded by a prepulse:

$$\%\ PPI = 100 - \frac{100 * \text{mean ASR amplitude on prepulse-pulse trials}}{\text{mean ASR amplitude on pulse alone trials}}$$

2.4.6 Casein Pellet Intake

CPs were purchased from Bio Serve Dustless Precision Pellets®, Bilaney, Kent, UK, and also used as a reward in the ASST (see below). All rats were habituated once in their home cage to the pellets and the experimenter observed that each rat consumed at least one pellet during habituation. The test was conducted in single cages (Makrolon™, Eurostandard type III). For testing, the body weight was assessed and animals were placed in the single test cages. After an initial cage habituation for 5 min, the rats had free access to the CPs for 5 min. The amount of consumed CPs was measured by weighing CPs before and after animals had access to them. Testing was always conducted at the same time in the middle of the light phase. CP intake was then calculated as [g] intake per kg body weight (g/kg BW).

2.4.7 Attentional Set Shift Test

The ASST was adapted from Birrell and Brown (2000) and Colacicco et al. (2002); (see Figure 15). Extensive habituation to the test apparatus, the food reward, and some materials is required to ensure good response of the animals during the test. Therefore, the animals were acquainted with

the reward (CPs; Dustless Precision Pellets®, Bio Serve, see 2.4.6), the ceramic pots, and different digging materials. For a few nights the pots were filled with home cage bedding and with rewards placed on top of them, as well as buried in them. The experimenter assured that each animal consumed at least one pellet during the habituation. The pots were rebaited several times and left in the home cage overnight (not more than three pots per cage). The following nights some of the digging materials were introduced in the same manner (up until the intradimensional shift (IDS); see below). On the second day of habituation, two animals were placed in the test apparatus to explore it with a familiar animal. The test apparatus was made of dark gray PVC (see Figure 16). First, the animals were placed into the small start compartment for 30 sec. Then the sliding door was lifted to give them access to the test chamber. As soon as they were both in the test chamber the sliding door was lowered again. They were left in the apparatus for 15 min before the sliding door was lifted again and the animals were gently pushed back into the start compartment. This was done to familiarize them with the course of events during the testing procedure. They were then returned to the home cage. The next day each rat was placed in the apparatus individually and had the possibility to explore the box again for 15 min.

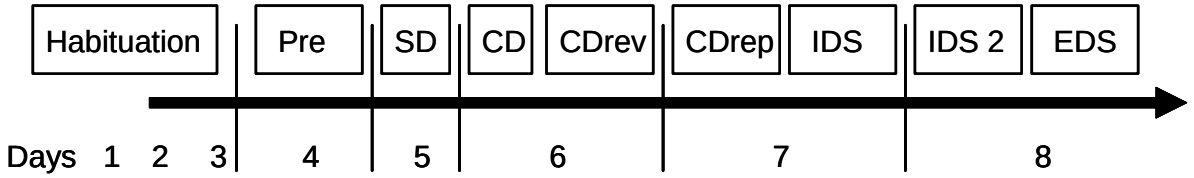

Figure 15: Timeline of the ASST. After a habituation phase, 1-2 rules per day were tested over a period of 8 days in total. Pre: pretraining; SD: simple discrimination, CD: compound discrimination, rev: reversal, rep: repetition, IDS: intradimensional shift, EDS: extradimensional shift.

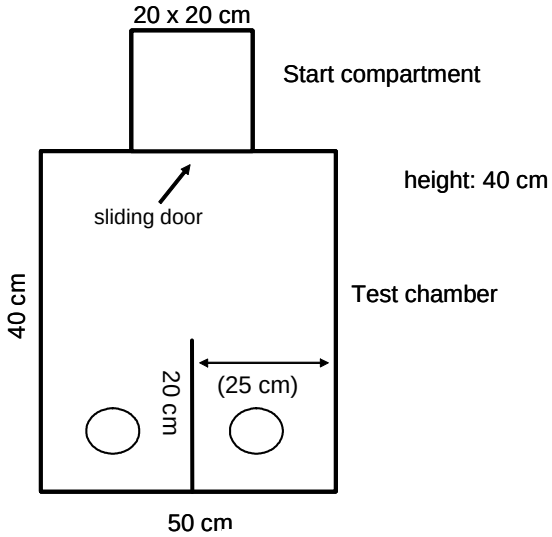

Figure 16: Dimensions of the ASST box. The small compartment on top is considered the start compartment. The test chamber below contains two fixed rings in which the ceramic pots with the scented digging material and reward were placed into.

The next day the rats completed the pretraining (Pre). Therefore they were placed into the apparatus individually. After entering the test chamber they were now facing two otherwise empty pots with one CP in every one. The experimenter recorded the time from lifting the door until the animal retrieved the reward. It was then gently pushed back into the start compartment to eat its reward. During a 30 sec ITI, the pots were replaced by pots filled with familiar, unscented digging material. Each had a reward on top of the digging material. The animal had to retrieve the reward again while the time was recorded. Then the reward was increasingly buried deeper in the pot. The animal had to retrieve it four times in less than one minute after it had been completely buried. The rat was then returned to its home cage.

The next day each rat was presented two bowls containing two different odors (but the same digging material). The rats had to learn that only in the bowl with a certain odor a reward could be obtained (simple discrimination, SD). This perceptual stimulus would be important in the next trial. In the following trial, the rat was presented two bowls each containing an odor and a diverging digging material (complex stimuli). Only by attending to the previously important dimension, it would find the food reward (compound discrimination, CD). This was ensured by changing material odor combinations. In the next trial, the previously learned rule was reversed (CDReversal, CDRev) meaning that the cue that had previously been unimportant was now leading to the food reward (for example the previously unimportant smell of basil now led to the reward whereas the previously important smell of capsicum did not regardless of the type of digging material presented with it).

Then two new complex stimuli were introduced (intradimensional shift, IDS) and the rat had to learn that, again, one of the cues with the same perceptual dimension led to the food reward. Two new odors and materials were then introduced applying the same rule (IDS 2). In the next trial, again, two new complex stimuli were presented but this time a cue of the other perceptual dimension was important to find the food reward (extradimensional shift, EDS). For example: if the rat had previously learned that the odor had always led to the reward, it now had to focus on the type of digging material to obtain the reward. An example of employed combinations of digging materials and odors is shown in Table 2.

Table 2: Employed combinations of digging materials and odors for different trials. The rewarded material or odor is shown in italics. The colored silica sand had a grain size of 3-4mm whereas the black silica sand had a grain size of 1-2mm. Pre: pretraining; SD: simple discrimination, CD: compound discrimination, rev: reversal, rep: repetition, IDS: intradimensional shift, EDS: extradimensional shift.

Trial	Digging material 1	Digging material 2	Odor 1	Odor 2
Pre	*Seramis ®*			
SD	*colored silica sand*	hamster bedding		cumin
CD	*colored silica sand*	hamster bedding	capsicum	cumin
CD rev	colored silica sand	*hamster bedding*	capsicum	cumin
CD rep	colored silica sand	*hamster bedding*	capsicum	cumin
IDS	*beech chipping*	rough stones	nutmeg	basil
IDS 2	*straw pellets*	pine bark	thyme	dill
EDS	cork granules	black silica sand	*rosemary*	curcuma

A test subject completed one rule stage when it obtained the reward within one minute in six consecutive trials (trails to criterion). The position of the rewarded pot was randomized and the experimenter ensured that the animal did not follow a false rule (e.g. always retrieving the reward from the left pot).

2.4.8 Light/Dark Emergence Test

The EMT was conducted in a light/dark box made of pale gray PVC. The apparatus consisted of two compartments, separated by a dividing wall with a 10 cm x 15 cm wide opening which enabled the test subject to move freely between the compartments. The smaller, dark compartment with black walls (25 cm x 25 cm x 40 cm) could be closed by a lid and was used as start box. The larger, bright compartment had gray walls (25 cm x 50 cm x 40 cm) and was brightly illuminated (80 lx). Rats were initially placed in the dark, closed compartment which was opened after 1 min and their behavior was videotaped for 5 min. Subsequent video analysis manually scored the latency of the animals to emerge from the dark compartment into the lit compartment [sec] (an entry was defined when the animal entered the compartment with all four limbs), the emergence frequency, the duration of time spent in the lit compartment [sec], the number of rearings, and risk assessment behavior (only head or forepaws are placed in the open compartment without concomitant movement of the hind limbs, even if the rat subsequently entered the area). The apparatus was thoroughly cleaned with antifect N liquid between the sessions.

2.4.9 Elevated Plus Maze

The EPM is a plus-shaped apparatus consisting of dark gray PVC elevated 50 cm above the floor with two opposing open arms (12 cm x 50 cm x 50 cm) which were illuminated by 80 lx and two enclosed arms (12 cm x 50 cm x 50 cm) extending from a central square (10 cm x 10 cm). At the beginning of each trial, each rat was placed on the central platform facing a closed arm. Each rat was videotaped for 5 min and the following behaviors were analyzed: number of entries into open or closed arms (an entry was defined if all four paws were placed on that arm), time spent in open and closed arms [sec], head dips (the whole head is lowered beneath the edge of an open arm), risk assessment (only head or forepaws are placed in an open arm without concomitant movement of the hind limbs, even if the rat subsequently entered the arm), self grooming frequency, and self grooming time [sec]. Percentage of open arm entries (open arm entries / (open + closed arm entries) x 100) and percentage of time spent in open arms (open arm time / (open + closed arm time) x 100) were calculated as well. The apparatus was thoroughly cleaned with antifect N liquid between the sessions.

2.4.10 Sweetened Condensed Milk Intake

The SCM (Nestle AG, Frankfurt, Germany) was freshly diluted 1:3 with water on the day of use. All rats were habituated once in their home cage to the SCM overnight, at least 48 h before testing. The test was conducted in single cages (Makrolon^{TM}, Eurostandard type III). On the test day, the body weight was assessed and the animals were placed in the single test cages. After an initial cage habituation for 5 min, the rats had free access to the SCM bottle for 15 minutes. The weight of the bottles was assessed before and after the trial and SCM intake was then calculated as intake in [ml] per kg body weight (ml/kg BW).

2.4.11 Ethanol Intake

The initial consumption of an EtOH solution (6%) was measured in a 24 h experiment, followed by a 24 h experiment for the consumption of a higher concentrated EtOH solution (10%). The rats were separated in single cages (Makrolon^{TM}, Eurostandard type III) in the experimental room. During the test session the animals were given *ad libitum* access to water or EtOH and laboratory food. After 24 h the animals were returned to their home cage. The weight of the water and EtOH bottles was recorded before and after the test session and the drinking volume as well as the EtOH intake in [g] per kg bodyweight was calculated for each rat. Additionally, preference scores (EtOH volume / total drinking volume) were calculated.

2.5 Molecular Analysis

Immunohistochemistry

2.5.1 Tissue Preparation and Fixation

Behaviorally naïve animals were used for histology at the same age (pd 30, 40, 50, 60, and 130) as the behaviorally analyzed animals. Animals were anaesthetized with Ketamine : Xylazin (100 mg : 5 mg i.p.) (Meinhardt et al., 2013) and then transcardially perfused with 80 ml ice cold phosphate buffered saline (PBS; including 10.000 I.E. Heparin-Natrium (Ratiopharm GmbH, Ulm, Germany) / 1000ml PBS) followed by 100 ml 4% paraform aldehyde (PFA). Brains were removed and stored at 4°C in 4% PFA over night for post-fixation. Brains were then put into a 10% sucrose solution for 2 days for dehydration before they were snap-frozen in isopentane and stored at -80°C before sectioning. Sectioning was conducted at a cryostat into 30 µm slices, collected in a cryoprotect solution (30% glycerol, 30% ethylenglycol, 10% 2xPO4), and stored at -80°C.

2.5.2 Goldchloride Staining

Goldchloride is used for staining of myelin (Schmued, 1990, Wahlsten et al., 2003, Schneider and Koch, 2005). Sections were washed in PBS, mounted on Superfrost Plus slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) and airdried over night. Subsequently, sections were placed in a 0.2% goldchloride solution (1 g Tetrachlorogold(III)säure-Trihydrat, Carl Roth, Karlsruhe, Germany, dissolved in 0.02M PBS with 4.5 g NaCl, pH 6.8-7) for 2 h. Sections were washed in *aqua dest*. and then fixed in freshly mixed 2.5% sodium thiosulfate solution for 5 min. Sections were then washed with running tap water for 30 min and briefly rinsed in *aqua dest*. Then the sections were dehydrated in rising EtOH steps (70, 80, 90, 100, 100%; 10 min each), and xylol (10 min) and embedded in Eukitt (O. Kindler GmbH, Freiburg, Germany).

2.5.3 Cannabinoid Receptor 1 Staining

The staining was conducted with free floating sections adapted from (Egertova and Elphick, 2000). Sections were washed with PBS (2 x 50 min and 2 x 20 min) on a stirrer at room temperature (RT). Then sections were blocked with normal goat serum (NGS; 5%) and H_2O_2 (0.3%) in PBS containing 0.1% Tween (PBS-T) for 4 h at RT. After 2 x quick washes and 3 x 15 min washes in PBS-T, sections were incubated over night at 4°C with antiserum against the CB1R (a gift from Prof. Elphick at Queen Mary University, London) 1:1000 in PBS-T and 5% NGS. On the next day the antibody was recycled and the sections washed 4 x 20 min in PBS-T. A

secondary antibody (goat-anti-rabbit IgG HRP, Vectastain Elite ABC Kit (Rabbit IgG) Vector Laboratories, Inc. Burlingame, CA, USA) was applied 1:500 in PBS-T and 2,5% NGS for 3 h at RT. Sections were washed 2 x 20 min in PBS-T followed by 2 x 20 min in PBS. ABC solution was prepared 30 min prior to use by mixing solution A and solution B 1:400 in PBS (Vectastain Elite ABC Kit, Vector Laboratories, Inc. Burlingame, CA, USA). Sections were then incubated in ABC solution for 1 h at RT. After that sections were washed 3 x quickly in PBS while the 2,4 diaminobutyric acid (DAB, Sigma-Aldrich, Taufkirchen, Germany) solution was being prepared. Sections were incubated in DAB for approximately 5 min until staining could be observed. Care was taken to expose all sections for the same time to the DAB solution. The DAB reaction was stopped with PBS. Sections were then mounted on Superfrost Plus slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) and airdried over night. Following dehydration in rising EtOH steps and xylol (see above), sections were embedded in Eukitt.

2.5.4 Histological Analysis

Histological images were captured using an Axioskop 2 microscope (Zeiss, Jena, Germany) with a 2.5x air objective and an Olympus ColorView 3 camera. Semiquantitative analysis of the myelin and CB1R staining was performed using ImageJ software (Schneider et al., 2012).

The region of interest (ROI: mPFC, CPu, Hip) was outlined manually for each image based on the rat brain atlas by (Paxinos G., 1998). At least three sections (right and left hemisphere) of each ROI were analyzed per brain.

The captured images were uniformly processed for the analysis of staining intensity. For the myelin staining each image was converted into a binary image after the contrast was enhanced by 40%. The outside of the ROI was cleared and the myelin staining was visible as black staining. If the colors were switched the image was inverted. Subsequently, the white [0] and black [255] values were extracted from the histogram. Percentage of myelination per ROI was calculated as

$$\% = \frac{\text{value [255]}}{(\text{value [255]} + \text{value [0]}) * 100}$$

For the CB1R staining, images were first converted to 8-bit grayscale images. Subsequently, the ROI was outlined and its mean gray value (optical density; OD) was measured. A rectangle of constant size in corpus callosum was used as a background control and its mean gray value was subtracted from the mean value of the ROI. The mean OD was then divided by the mean area size of the ROI.

$$\text{CB1R staining} = \frac{\text{OD (ROI)} - \text{OD (background)}}{\text{mean area size (ROI)}}$$

2.5.5 Western Blots

Western blot analysis was conducted as described before (Schneider et al., 2014). Rats were briefly anesthetized with CO_2 and decapitated. Brains were removed and mPFC, CPu, NAc, and Hip were quickly dissected and homogenized in 500 µl lysis buffer (10 nM Tris-HCl, 2 mM EDTA, pH 8.0) containing protease inhibitors (cOmplete Tablets Mini, EDTA-free EASYpack, Roche Diagnostics, Penzberg, Germany) and stored at -80°C. Protein content was measured by Bradford Protein Assay (BioRad Laboratories GmbH, Munich, Germany) using bovine serum albumin as a standard (Sigma-Aldrich, Munich, Germany). Protein samples (25 µg for mPFC, CPu, and NAc, 50 µg for Hip) were mixed with 2 x mercapto/SDS sample buffer (Sigma-Aldrich, Munich, Germany) and heated at 95°C for 5 min. Electrophoresis was subsequently carried out at 200 V in NuPage® Novex Bis-Tris Mini Gel 4-12% gels (Invitrogen, Darmstadt, Germany). Separated proteins were then blotted (400 mA for 90 min) onto PVDF membranes (BioRad Laboratories, Munich, Germany) using Towbins buffer.

Membranes were then blocked with Odyssey™ blocking buffer (LiCor Biosciences GmbH, Bad Homburg, Germany; 1:1 in tris-buffered saline (TBS) solution) on a shaker at RT for 30 min (FAAH) or 1 h (MAGL). For CB1R staining membranes were blocked in 2.5% nonfat dry milk / TBS solution at RT for 1 h.

Membranes were subsequently incubated with either rabbit polyclonal anti-FAAH antibody (1:1000, item number 101600, Cayman Chemical Company, Ann Arbor, Michigan, USA) or with rabbit polyclonal anti-MAGL antibody (1:500, item number 100035, Cayman Chemical Company, Ann Arbor, Michigan, USA) at 4°C over night in Odyssey™ blocking buffer / TBS solution. For CB1R analysis a rabbit polyclonal anti-CB1R antibody (1:1000, IMG-pAb001, ImmunoGenes, Zug, Switzerland) was incubated in 2.5% nonfat dry milk / TBS solution for 24 h at 4°C. Every blot was simultaneously co-incubated with goat polyclonal ß-Actin antibody (1:2000, sc-1615, Santa Cruz Biotechnology Inc., Heidelberg, Germany).

After 3 washing steps with TBS containing 0.1% Tween (TBS-T), the secondary antibody was incubated in Odyssey™ blocking buffer / TBS solution for 1h at RT. To allow simultaneous detection of MAGL/FAAH/CB1R and ß-Actin, secondary antibodies with two different fluorescent wavelengths were used: donkey-anti-rabbit 800 (1:10000 for FAAH and MAGL, 1:15000 for CB1R; Cat. Nr. 926-32213 LiCor Biosciences GmbH, Bad Homburg, Germany) and donkey-anti-goat 680 (1:10000 for 25 µg protein and 1:15000 for 50 µg protein; Cat. Nr. 926-68024, LiCor Biosciences GmbH, Bad Homburg, Germany). Membranes were washed with TBS-T, rinsed with ultrapure water, and subsequently scanned with the Odyssey Imaging System (LiCor Biosciences GmbH, Bad Homburg, Germany). The band density was quantified using the LiCor Imaging system. Background-corrected values of FAAH, MAGL and CB1R were corrected

for the corresponding ß-Actin contents and expressed as arbitrary units. To allow group comparisons between different blots without the interference of gel variations, percentage changes were calculated separately for each blot (with mean of control group = 100%).

2.6 Statistical Analysis

Project I:

Differences between W[Jan], W[hsd] and W[rcc] animals were analyzed by one-way analysis of variance (ANOVA) for OF and OBJR. Differences in PPI were analyzed by repeated measurement (RM) ANOVA. For post-hoc testing Tukey's test was used.

Project II:

In the preliminary study of assessing the influence of varying ITIs on OBJR, RM ANOVA was employed. Differences in the development of adolescent rats and repeated testing in adult animals were analyzed by RM ANOVA and by subsequent time point comparison for OF, OBJR, OBJRecency, PPI and ASR (except for adult controls in OF and ASR; here, paired Student's t-test was used). The pharamcological influence of SR was analyzed seperately for each age group with Student's t-tests. For PPI, RM ANOVA with subsequent post-hoc testing was used. The development of myelination and western blots for CB1R were analyzed by one-way ANOVA with subsequent Fisher LSD post-hoc tests.

Project III:

Student's t-test was used to analyze OBJR, PPI, CP intake and western blots. MANOVA followed by multiple ANOVAs was applied for ASST.

Project IV:

Student's t-test was employed for the analysis of EMT, EPM, SCM, and EtOH intake, PPI, OBJR, and SOCR.

All data are presented as means with standard error (means + standard error of the mean (S.E.M.)). Significant differences ($p < 0.05$) between groups are marked with *, statistical trends ($p < 0.1$) are marked with #. The level of significance was set at $p \leq 0.05$. All calculations were performed in SPSS software 21.0 (IBM, Somers, USA).

3. Results

3.1 Project I: Differences of Cognitive Abilities in Three Wistar Han Rat Lines

3.1.1 Open Field

Locomotor activity measured in the open field differed significantly between the rat lines regarding the distance traveled (Figure 17; $F_{2,33} = 5.154$; $p = 0.011$). Post-hoc Tukey test revealed that W[Jan] were more active in the open field than W[rcc] rats ($p = 0.008$), while the other lines did not differ (W[Jan] vs. W[hsd]: $p = 0.265$; W[hsd] vs W[rcc]: $p = 0.251$).

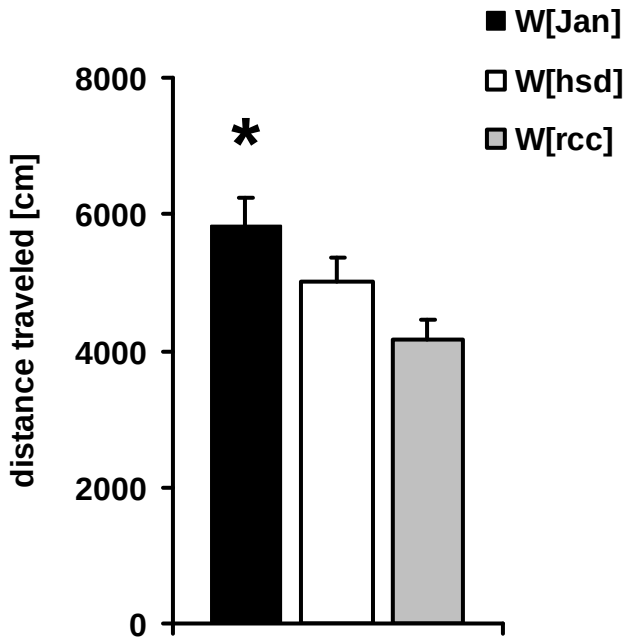

Figure 17: Distance traveled in the open field test of W[Jan], W[hsd] and W[rcc] animals. W[Jan] animals showed a significantly higher locomotor activity than W[rcc] rats. Data are expressed as mean + S.E.M. (* $p \leq 0.05$, $n = 12$)

3.1.2 Object Recognition

There was a significant difference between the rat lines regarding the percentage discrimination observed in the OBJR test (Figure 18; $F_{2,33} = 4.565$; $p = 0.018$). Post-hoc Tukey's test revealed a higher object discrimination for W[rcc] compared to W[Jan] animals ($p = 0.013$;), while no difference was found between the other rat lines (W[rcc] vs W[hsd]: $p = 0.334$; W[hsd] vs. W[Jan]: $p = 0.267$).

Initial exploration time (during P1) differed significantly between the animals ($F_{2,33} = 15.064$; $p < 0.001$). Post-hoc Tukey's test revealed that W[hsd] displayed a higher exploration time

compared to W[rcc] and W[Jan] ($p \leq 0.001$), while no difference was found between the other lines (W[rcc] vs. W[Jan]: $p = 0.979$). Total exploration values in P1 ± S.E.M: W[Jan] = 21.9 sec ± 2.9; W[rcc] = 22.8 sec ± 2.1; W[hsd] = 42.5 sec ± 3.8).

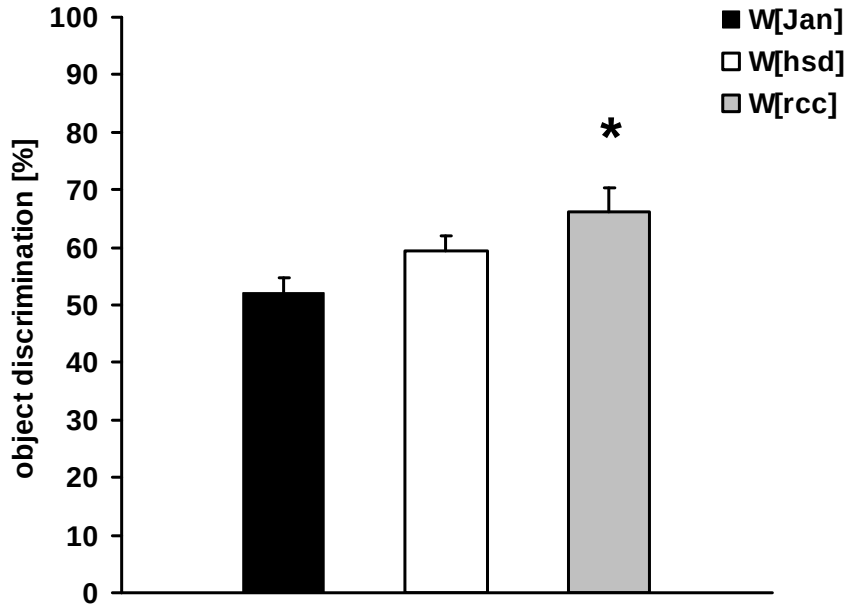

Figure 18: Percentage object discrimination in the OBJR test of W[Jan], W[hsd] and W[rcc] animals. W[rcc] animals displayed a higher object discrimination than W[Jan] animals. Data are expressed as mean + S.E.M. (* $p \leq 0.05$, n = 12).

3.1.3 Prepulse Inhibition of the Acoustic Startle Reflex

Measurement of the PPI revealed a significant difference between groups (Figure 19; $F_{2,33} = 3.435$; $p = 0.044$). Post-hoc Tukey's test showed that W[Jan] displayed a higher PPI compared to W[hsd] ($p = 0.05$) while the other rat lines did not differ (W[Jan] vs W[rcc]: $p = 0.114$; W[hsd] vs W[rcc]: $p = 0.925$). Additionally, the rat lines also differed in their initial ASR amplitude ($F_{2,33} = 22.822$; $p < 0.001$) with W[rcc] displaying a significantly higher ASR than W[hsd] and W[Jan] ($p \leq 0.001$) while no difference was observed between W[hsd] and W[Jan] rats ($p = 0.6$). Total ASR values [arbitrary units] ± S.E.M: W[Jan] = 792.2 ± 77.2; W[rcc] = 4511.5 ± 649.4; W[hsd] = 1368.1 ± 314.8).

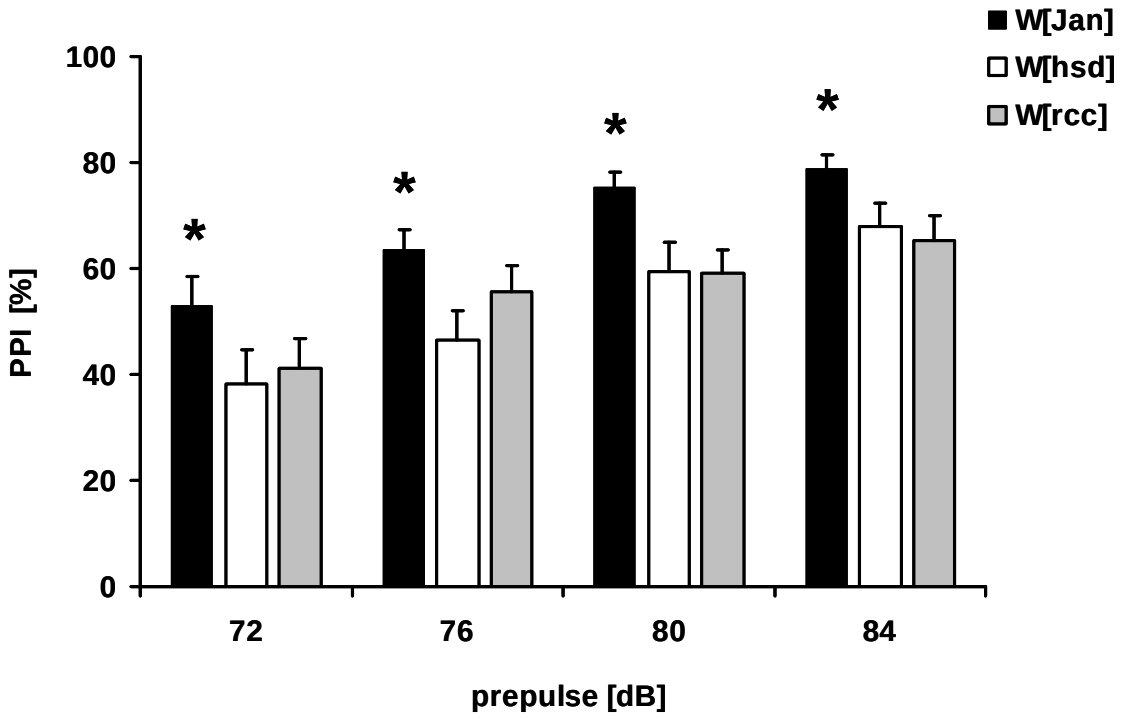

Figure 19: PPI of W[Jan], W[hsd] and W[rcc] animals. W[Jan] showed a higher PPI compared to W[hsd] animals. Data are expressed as means + S.E.M. (* $p \leq 0.05$, n = 12).

3.2 Project II: Ontogeny of Cognitive Abilities

3.2.1 Influence of Intertrial Interval Length on Object Recognition Performance

The influence of varying ITIs in an OBJR test was investigated in a preliminary study. Adult animals showed a similar object discrimination performance and discrimination index for both 10 min and 20 min ITIs (Figure 20). However, pd 45 animals showed a higher object discrimination performance and a higher discrimination index at an ITI of 10 min compared to 20 min (Figure 20). Although the results were not statistically significant (possibly caused by the limited sample size of n = 4 for pd 45 and n = 6 for adult) based on these observations an ITI of 15 min was chosen for the following study design.

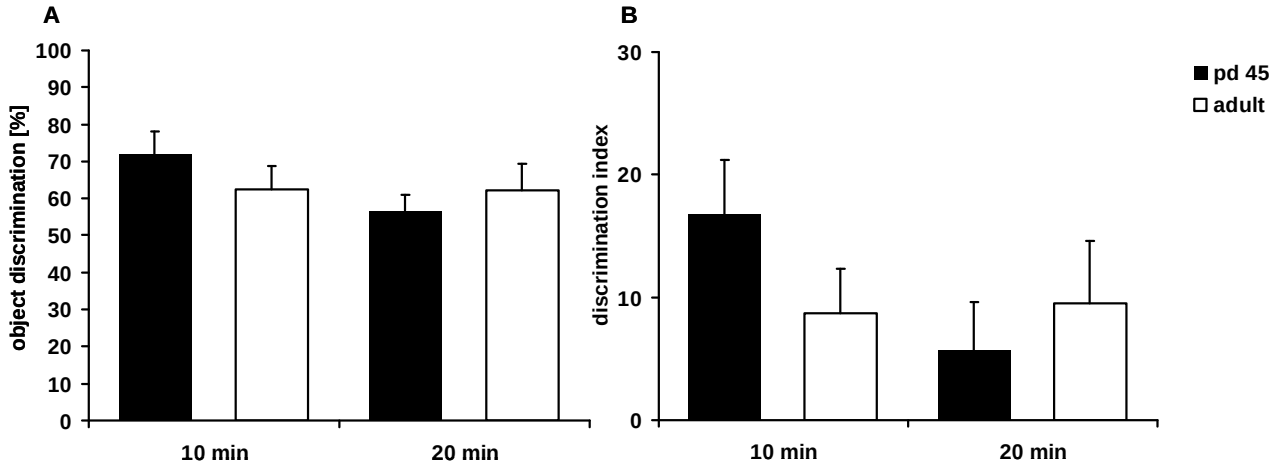

Figure 20: Percentage object discrimination (A) and discrimination index (B) in the OBJR for pd 45 and adult animals with varying ITIs of 10 and 20 min. Although not statistically significantly different, object discrimination was lower at an ITI of 20 min in pd 45 animals compared to an ITI of 10 min (A). In addition, the discrimination index showed a reduction after 20 min ITI compared to 10 min ITI in pd 45 animals (B). In adult animals both percentage object discrimination and discrimination index did not differ with varying ITIs. Data are expressed as mean + S.E.M. (pd 45: n = 4, adult: n = 6).

3.2.2 Locomotor Behavior during Development

A significant variation in the distance traveled in the open field was observed over the course of development during repeated testing (Figure 21; $F_{4.7,70.0} = 4.384$; $p = 0.002$). Time point comparisons showed a significantly higher locomotion of the animals on pd 59, pd 69, pd 79, and pd 99 compared to pd 129 ($p \leq 0.05$; Figure 21A), while no difference was observed in the same animals on pd 29, pd 39, and pd 49 compared to pd 129 ($p \geq 0.113$). Locomotor behavior was also repeatedly tested in a separate group of adult control animals. Due to technical difficulties one test point had to be excluded from the analysis. However, for two repeated test points paired Student's t-test did not show any differences in the distance traveled in the open field ($t_{15} = -0.553$; $p = 0.588$; Figure 21B).

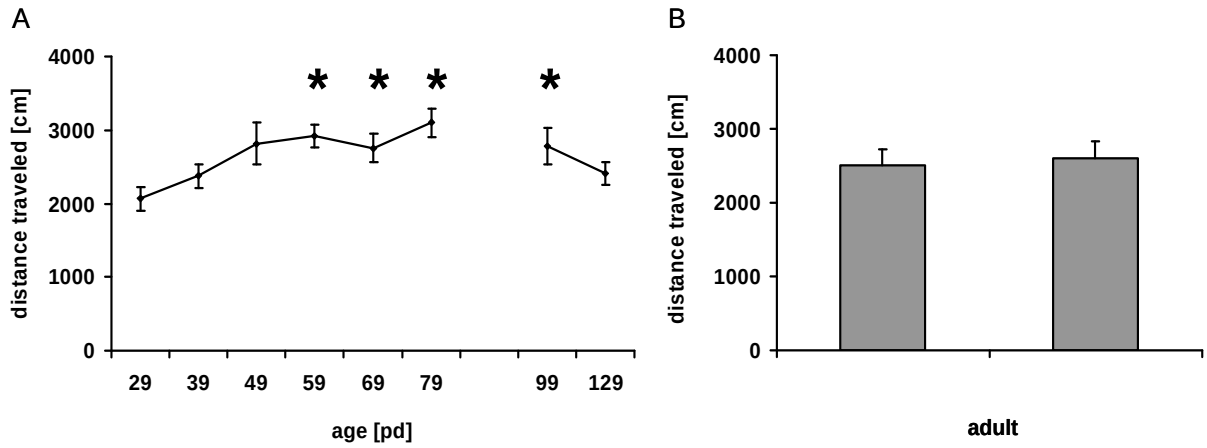

Figure 21: Mean distance traveled in the open field between age pd 29 and pd 129 (A) and in adult rats (B). Locomotor activity was significantly increased at pd 59 - 99 compared to pd 129 (A). Adult animals displayed no difference in total distance traveled upon repeated testing (B). Data are expressed as mean + S.E.M. (* $p \leq 0.05$, $n = 16$).

3.2.3 Basal Development of Object Recognition and Object Recency

In the OBJR test, RM ANOVA revealed no significant effect for the development of the animals' abilities to discriminate novel versus familiar object (Figure 22A; $F_{5.7,85.9} = 1.667$; $p = 0.142$). However, time point comparison revealed a significantly lower percentage object discrimination on pd 40 ($p = 0.041$) as well as a trend for a lower percentage object discrimination on pd 50 ($p = 0.085$) compared to percentage performance on pd 130. No difference was observed in the same animals on any other time point compared to pd 130 ($p \geq 0.478$).

No significant effect was shown for the discrimination index (Figure 22B; $F_{5.4,80.8} = 1.438$; $p = 0.216$) however, time point comparison found a trend for a lower discrimination index on pd 40 ($p = 0.059$) compared to pd 130. No difference in discrimination index was found in the same animals on any other time point compared to pd 130 ($p \geq 0.129$).

In addition, object exploration times during P1 differed significantly over the course of development (Table 3; $F_{5.6,83.5} = 12.296$; $p < 0.001$). Animals tested between pd 30 and pd 80 displayed significantly higher exploration times ($p \leq 0.01$) compared to when the same animals were tested on pd 130. In contrast, no difference was observed in the same animals between pd 100 and pd 130 ($p = 0.373$).

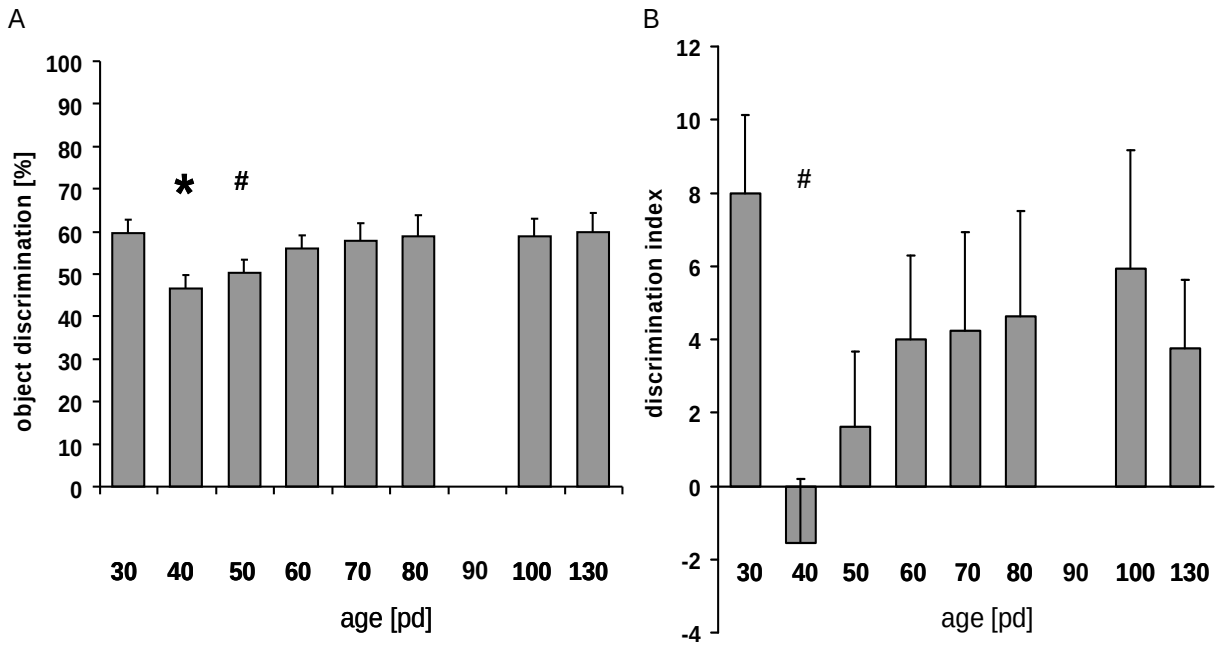

Figure 22: Percentage object discrimination (A) and discrimination index (B) in the OBJR test during the development from pd 30 - 130. Object discrimination was significantly lower at pd 40 compared to pd 130 and at pd 50 a trend for a lower object discrimination compared to pd 130 was detected (A). A trend for a reduced discrimination index was found at pd 40 compared to pd 130 (B). Data are expressed as mean + S.E.M. (* $p \leq 0.05$; # $p \leq 0.1$, n = 16).

Table 3: Exploration time during P1 for the age of pd 30 – 130 in the OBJR test. Exploration time was significantly higher during pd 30 - 80 compared to pd 130. Data are expressed as mean [sec] and S.E.M. (* $p \leq 0.05$, n = 6).

Time P1	**pd 30**	**pd 40**	**pd 50**	**pd 60**	**pd 70**	**pd 80**	**pd 100**	**pd 130**
mean	36.81 *	36.56 *	31.44 *	29.56 *	27.31 *	24.00 *	19.06	16.69
S.E.M.	2.54	2.88	2.43	2.16	2.02	1.26	1.75	2.07

Repeated testing in adult control animals revealed neither significant changes in percentage object discrimination nor in discrimination index (Figure 23; $F_{2,30} = 0.143$; $p = 0.867$ and $F_{2.0,29.5} = 0.704$; $p = 0.501$ respectively). Also exploration time was similar over three repeated test sessions (Table 4; $F_{2,30} = 1.929$; $p = 0.163$).

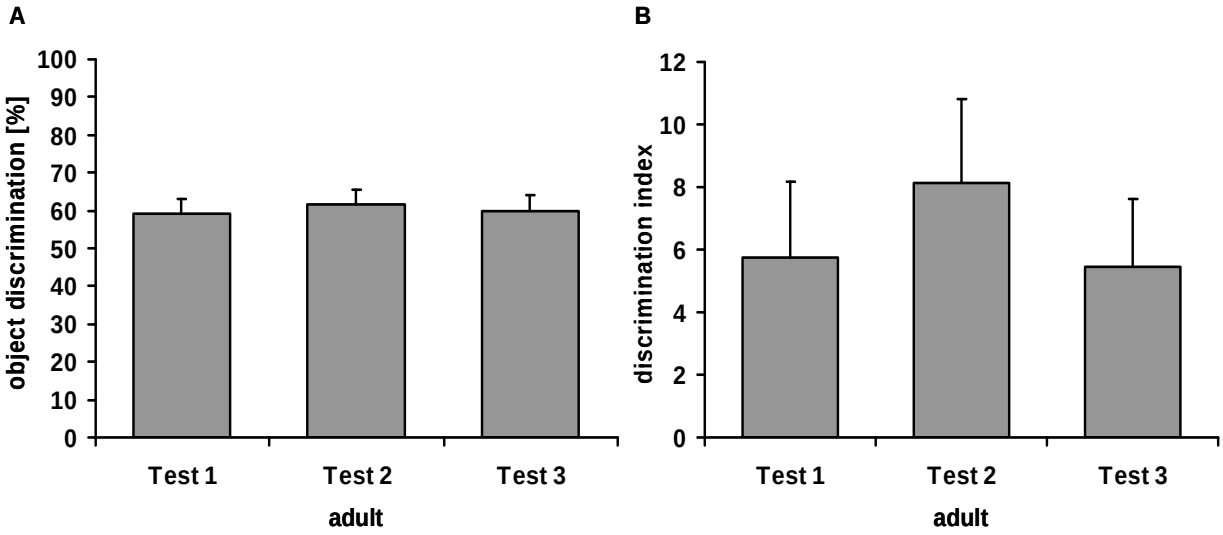

Figure 23: Percentage object discrimination (A) and discrimination index (B) of adult rats upon repeated testing in the OBJR test. Neither for the percentage object discrimination nor for discrimination index could a significant difference be observed. Data are expressed as mean + S.E.M. (n = 16).

Table 4: Exploration time during P1 of adult animals in the OBJR test. No difference in exploration time was observed upon three repeated test sessions. Data are expressed as mean [sec] and S.E.M. (n = 16).

Time P1	Test 1	Test 2	Test 3
adult animals			
mean	30.31	25.75	27.44
S.E.M.	2.17	1.91	2.46

The OBJRecency performance revealed no global effect over the course of development (Figure 24A; $F_{7,98} = 1.924$; $p = 0.074$). However, time point comparison revealed that the percentage object recency discrimination performance on pd 31 compared to pd 131 was significantly lower than in the same animals on any other test point ($p = 0.026$).

Comparison of the discrimination index revealed no significant variation in performance over the course of development (Figure 24B; $F_{6.8,95.1} = 1.453$; $p = 0.196$).

The exploration time during P1 varied significantly over the course of development (Table 5; $F_{6.4,89.7} = 7.442$; $p < 0.001$). Time point comparison showed that adolescent and early adult animals (pd 41, pd 51, pd 61 and pd 71) displayed a higher exploration time compared to pd 131 ($p \leq 0.004$) but the same animals tested on pd 31, pd 81, and pd 101 showed no significantly higher exploration time compared to pd 131 ($p \geq 0.1$).

One animal had to be excluded from the analysis due to low performance in one trial (exploration time < 1sec).

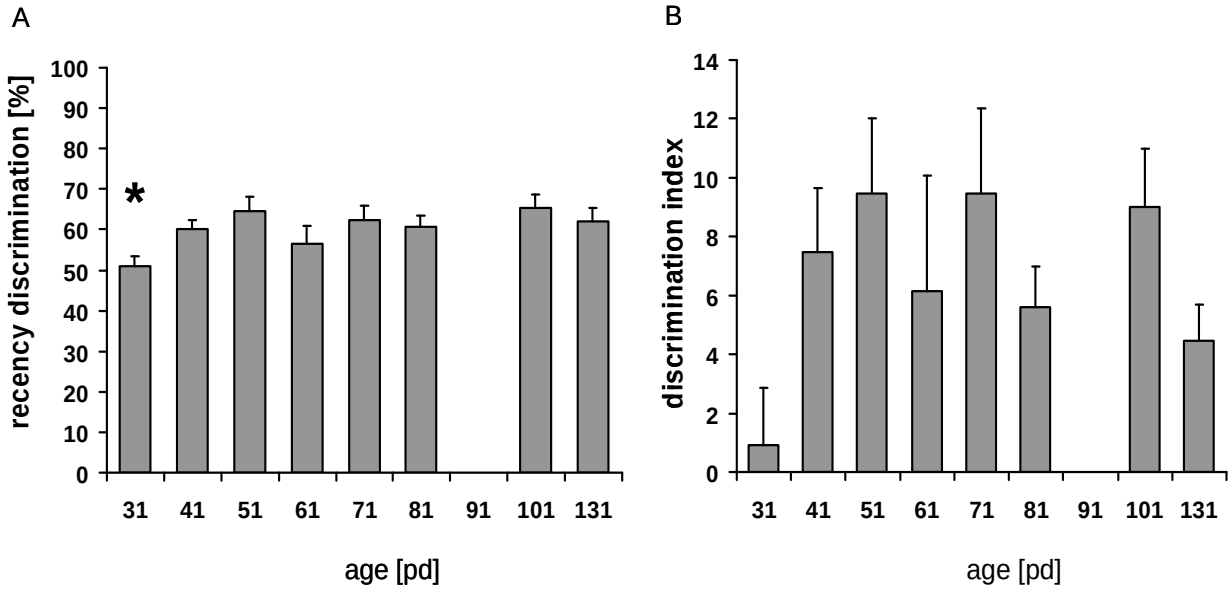

Figure 24: Percentage object recency discrimination (A) and discrimination index (B) of the OBJRecency test during the development from pd 31 – 131. A significantly lower percentage object recency discrimination at pd 31 compared to pd 131 was observed (A). Discrimination index did not vary over the course of development (B). Data are expressed as mean + S.E.M. (* $p \leq 0.05$, n = 15).

Table 5: Exploration time during P1 for the age of pd 31-131 in the OBJRecency test. Exploration time was significantly higher during pd 41-71 compared to pd 131. Data are expressed as mean [sec] and S.E.M. (* $p \leq 0.05$, n = 15).

Time P1	pd 31	pd 41	pd 51	pd 61	pd 71	pd 81	pd 101	pd 131
mean	40.40	55.53 *	53.13 *	44.40 *	45.67 *	33.33	33.53	31.47
S.E.M.	5.28	4.51	2.83	2.95	2.63	2.42	3.21	3.50

Repeated testing in adult control animals revealed neither significant changes in percentage object recency discrimination nor in discrimination index (Figure 25; $F_{2,30} = 0.795$; $p = 0.461$ and $F_{1.7,25.3} = 0.062$; $p = 0.914$ respectively). Also exploration time was similar over three repeated test sessions (Table 6; $F_{1.5,22.2} = 0.084$; $p = 0.866$).

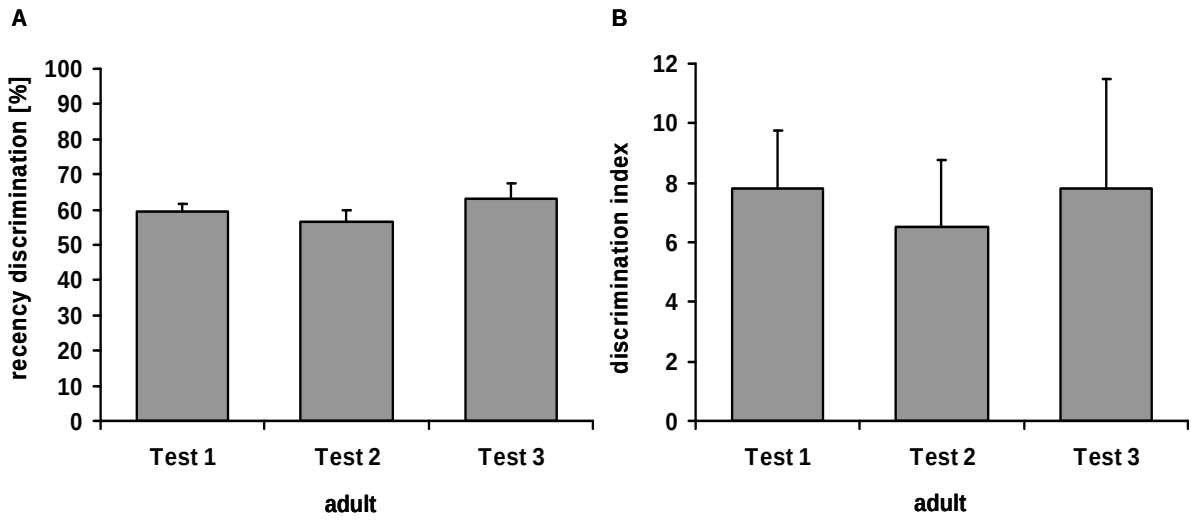

Figure 25: Percentage object recency discrimination (A) and discrimination index (B) of adult rats upon repeated testing in the OBJRecency test. Neither for the percentage object recency discrimination nor for discrimination index could a significant difference be observed. Data are expressed as mean + S.E.M. (n = 16).

Table 6: Exploration time during P1 of adult animals in the OBJRecency test. No difference in exploration time was observed upon three repeated test sessions. Data are expressed as mean [sec] and S.E.M. (n = 16).

Time P1	Test 1	Test 2	Test 3
adult animals			
mean	35.94	37.63	37.44
S.E.M.	3.16	3.39	3.60

3.2.4 Basal Development of Prepulse Inhibition of the Acoustic Startle Reflex

The PPI performance varied significantly over the course of development (Figure 26A; $F_{6.2,93.1} = 32.726$; $p < 0.001$). Compared to when tested in adulthood (pd 130), the same animals tested in adolescence and as early adults (pd 30 - pd 80) displayed significantly lower mean PPI values ($p \leq 0.001$), whereas no difference was observed in the same animals when tested between pd 100 and pd 130 ($p = 0.197$) or pd 120 and pd 130 ($p = 0.803$). Adult control animals displayed no significant alterations of mean PPI values over three repeated test sessions (Figure 26B; $F_{2,30} = 0.369$; $p = 0.695$).

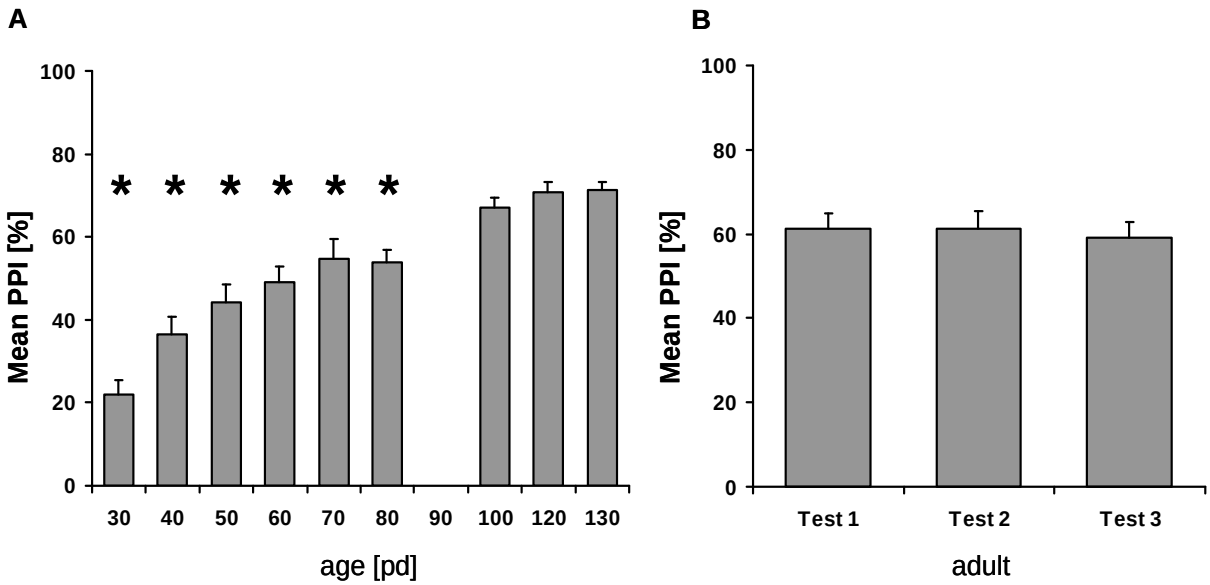

Figure 26: Mean PPI values over the age of pd 30 – 130 (A) or of adult rats (B). PPI of pd 30 – pd 80 was significantly reduced compared to pd 130 (A). In adult rats no difference in PPI was observed upon three repeated tests (B). Data are expressed as mean + S.E.M. (* $p \leq 0.05$, n = 16).

Comparison of ASR values also revealed a significant change over the course of development (Table 7; $F_{1.8,26.8} = 20.109$; $p < 0.001$). Time point comparison showed that when tested in adolescence and as early adult rats (pd 30 - pd 80), animals displayed significantly lower ASR value ($p \leq 0.005$) compared to when the same animals were tested in adulthood (pd 130). No differences were observed between pd 100 and pd 130 or pd 120 and pd 130 ($p = 0.344$ and $p = 0.803$ respectively).

Due to technical difficulties, test 1 of the ASR in adult controls could not be analyzed however, paired Student's t-test did not show any differences between tests 2 and 3 (Table 8; t_{15}=-0.303; $p = 0.766$).

Table 7: Mean ASR values [arbitrary units] over the age of pd 30 – 130. ASR of pd 30 – pd 80 was significantly reduced compared to pd 130. Data are expressed as mean [sec] and S.E.M. (* $p \leq 0.05$, n = 16).

ASR	pd 30	pd 40	pd 50	pd 60	pd 70	pd 80	pd 100	pd 130
mean	481.98 *	903.87 *	1208.17 *	1466.57 *	1361.61 *	2848.51 *	4342.87	4814.94
S.E.M.	33.56	57.50	93.90	187.63	172.98	502.79	723.43	895.38

Table 8: Mean ASR value [arbitrary units] of adult control animals. No difference was observed between two test sessions. Data are expressed as mean [sec] and S.E.M. (n = 16).

ASR	Test 2	Test 3
adult animals		
mean	4823.60	4920.89
S.E.M.	651.45	607.83

3.2.5 Pharmacological Influence of SR141716 on Behavior during Development

Student's t-test revealed a significantly improved object discrimination at pd 40 in SR (0.3 mg/kg) compared to vehicle treated animals (Figure 27B; t_{31} = -2.594; p = 0.014). No difference was observed between groups on pd 30 or 130 (Figure 27A and C; t_{21} = 1.012; p = 0.323 and t_{30} = 0.77; p = 0.447 respectively). Exploration time during P1 was significantly reduced in SR compared to vehicle treated animals on pd 30 (Table 9; t_{21} = 5.736; p < 0.001). No differences in exploration time during P1 were observed for any other time point (t_{31} = 0.609; p = 0.547 on pd 40 and t_{30} = 0.771; p = 0.446 on pd 130). One animal had to be excluded from OBJR analysis due to low object exploration time (< 1 sec) of one of the objects in P1.

RM ANOVA revealed no difference between treatment groups for PPI (Figure 27D-F; pd 30: $F_{1,22}$ = 2.083; p = 0.163; pd 40: $F_{1,31}$ = 0.003; p = 0.954 and pd 130: $F_{1,30}$ = 2.045; p = 0.163). However, in line with the previous results (see 3.2.4), a gradual development of the PPI from pd 30 through pd 40 to pd 130 was observed. No difference in ASR amplitude between treatment groups was found at any time point (Table 10; Student's t-test: p ≥ 0.2).

Table 9: Exploration time of P1 [sec] for pd 30, 40 and 130 old animals after vehicle or SR (0.3 mg/kg) treatment. Exploration time on pd 30 was significantly reduced in SR treated animals compared to vehicle. No differences were observed for any other time point. Data are expressed as means +S.E.M. (***** p ≤ 0.05, pd 30: vehicle n = 12; SR n = 11; pd 40: vehicle n = 16; SR n = 17; pd 130: vehicle n = 18; SR n = 14).

Time P1	pd 30		pd 40		pd 130	
	Vehicle	SR	Vehicle	SR	Vehicle	SR
mean	37.92	21.55 *	35.69	33.41	32.33	28.79
S.E.M.	1.67	2.36	2.71	2.58	3.52	2.58

Table 10: ASR amplitude [arbitrary units] of pd 30, 40 and 130 old animals after vehicle or SR (0.3 mg/kg) treatment. No differences were observed between vehicle or SR treated animals for any time point. Data are expressed as mean + S.E.M. (pd 30: vehicle n = 12; SR n = 12; pd 40: vehicle n = 16; SR n = 17; pd 130: vehicle n = 18; SR n = 14).

ASR	pd 30		pd 40		pd 130	
	Vehicle	SR	Vehicle	SR	Vehicle	SR
mean	618.22	532.59	776.55	785.11	5572.78	4697.46
S.E.M.	60.34	32.14	97.78	77.99	677.09	553.67

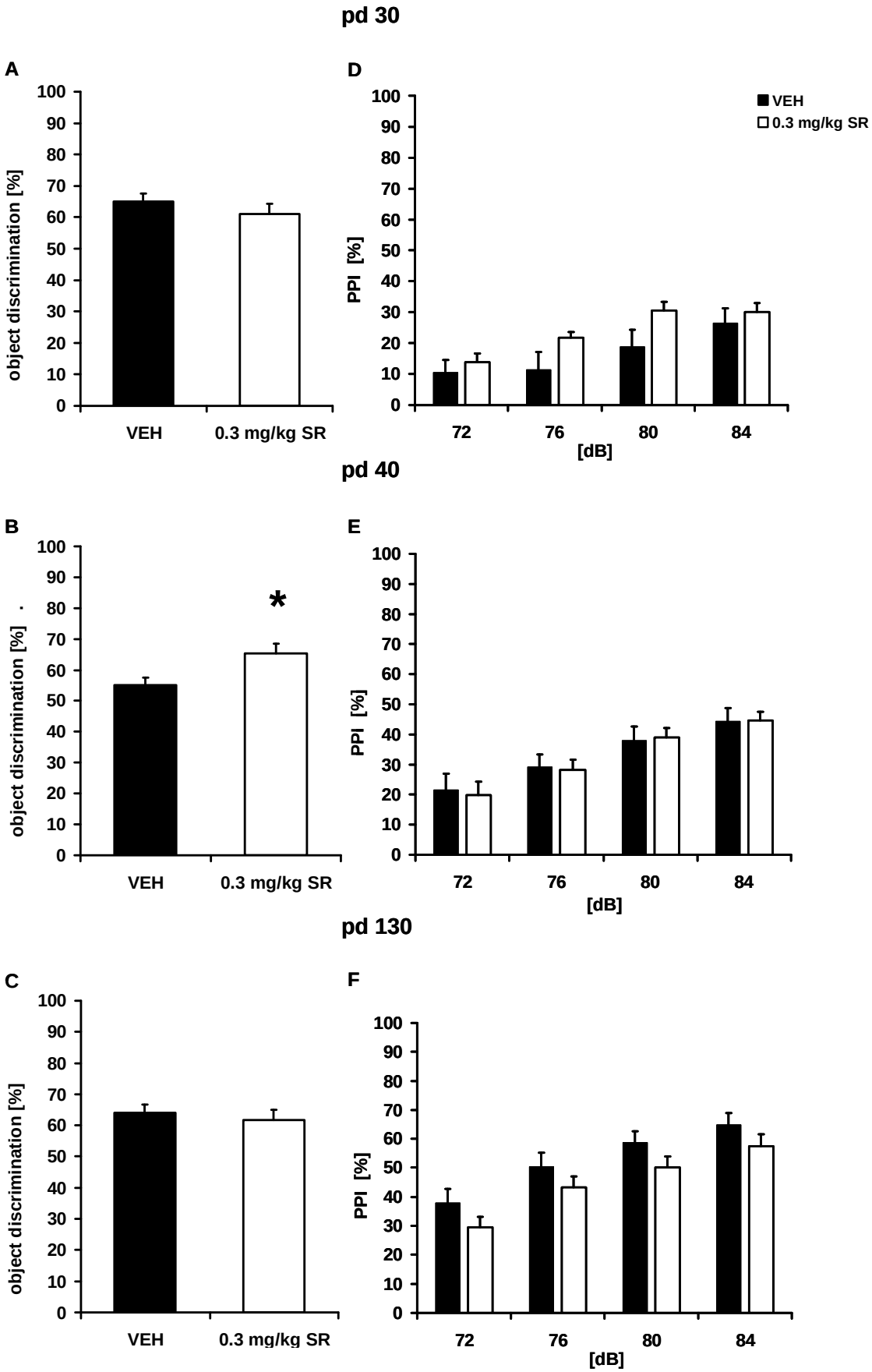

Figure 27: Percentage object discrimination of the OBJR test (A-C) and PPI (D-F) of pd 30 (A+D), pd 40 (B+E), and pd 130 (C+F) old animals after vehicle (VEH) or SR (0.3 mg/kg) treatment. In pd 40 animals a significantly increased object discrimination was observed after SR treatment (B). No significant differences were found for various prepulses in the PPI (D-F). Data are expressed as mean + S.E.M. (* $p \leq 0.05$, pd 30: n (VEH) = 12; n (SR) = 11; pd 40: n (VEH) = 16; n (SR) = 17; pd 130: n (VEH) = 18; n (SR) = 14).

3.2.6 Molecular Analysis

Myelination

Brain sections of different age points were stained with goldchloride to assess myelination across the development (for representative brain sections see Figure 28). Analysis of the CPu revealed significant differences of myelination at different ages (Figure 29A; $F_{4,20} = 5.549$ $p = 0.004$). Post-hoc Fisher LSD test showed that on pd 30, pd 50, and pd 60 animals displayed significantly less myelination than on pd 130 ($p \leq 0.004$).

In the NAc, no differences of myelination at the different ages were found (Figure 29B; $F_{4,20} = 1.784$ $p = 0.172$).

Analysis of the Hip revealed a trend for changes of myelination across different age points (Figure 29C; $F_{4,20} = 2{,}507$ $p = 0.075$). Post-Hoc Fisher LSD test showed that, at all tested time points, animals displayed significantly less myelination than at pd 130 ($p \leq 0.031$).

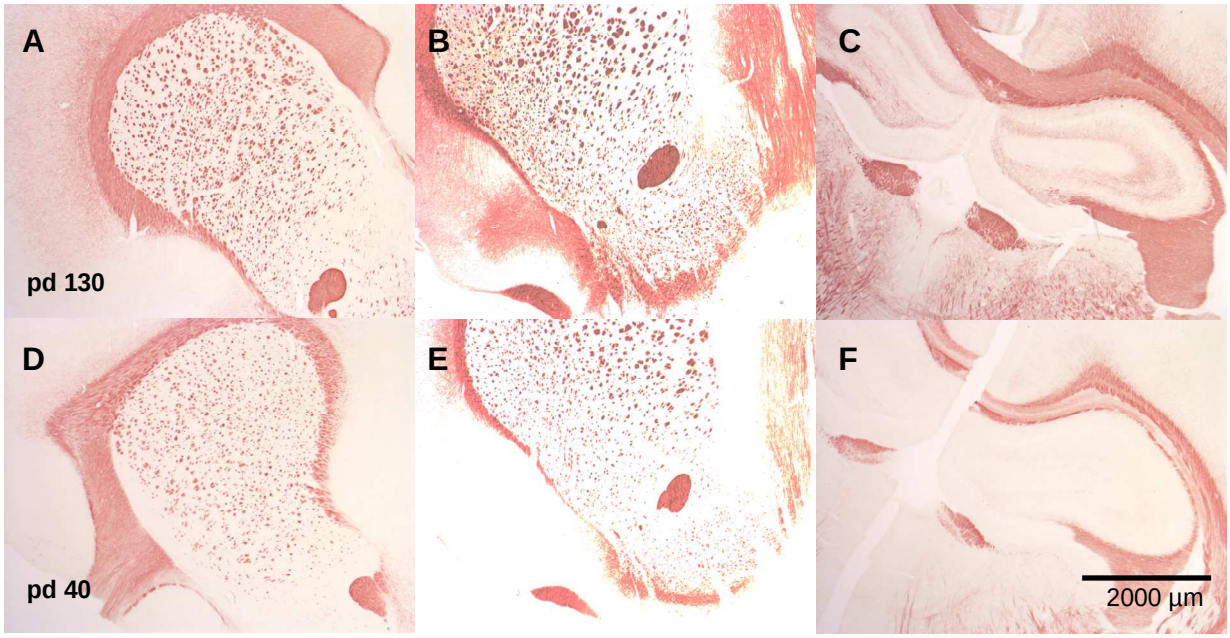

Figure 28: Representative brain sections of pd 130 (A-C) and pd 40 (D-F) stained for myelin. A,D: CPu, B,E: NAc, C,F: Hip. Pictures were taken with a 2.5x magnification.

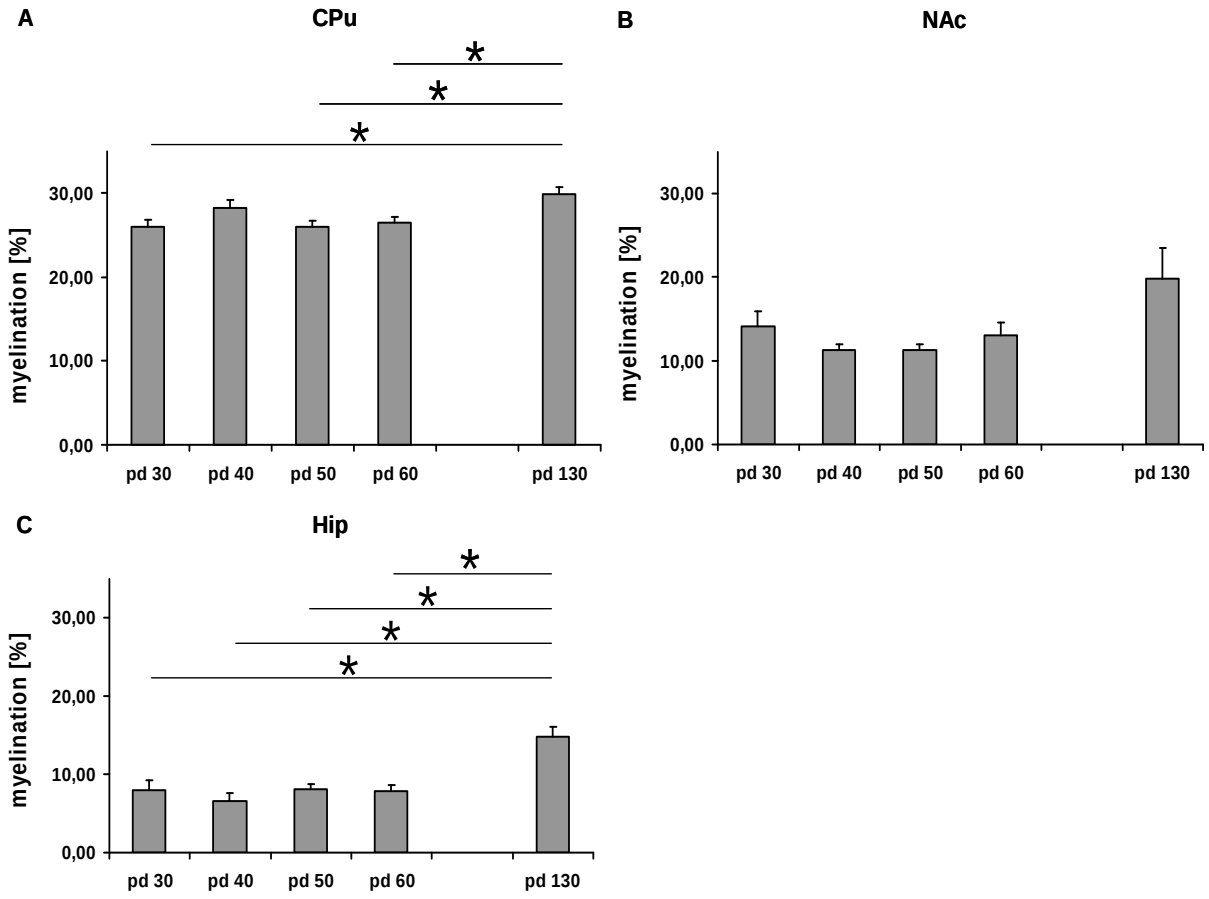

Figure 29: Percentage myelination in CPu (A), NAc (B) and Hip (C) over the development from pd 30 – pd 130. In the CPu, myelination was significantly lower at pd 30, 50, and 60 compared to pd 130 (A). No difference in myelination was observed in the NAc (B).In the Hip, myelination was significantly lower at all time points compared to pd 130 (C). Data are expressed as mean + S.E.M. ($p \leq 0.05$, $n = 5$ for each time point).

Cannabinoid Receptor 1 Staining

Staining for the CB1R were done for pd 40 and pd 130 and brain sections are representatively shown in Figure 30. Student's t-test showed no difference in CB1R density in the mPFC (t_6 = 0.088; p = 0.932), CPu (t_6 = -0.449; p = 0.669), and Hip (t_6 = 1.27; p = 0.251) between pd 40 and pd 130 old animals (Figure 31). For the analysis of CB1R staining two animals had to be excluded due to technical difficulties.

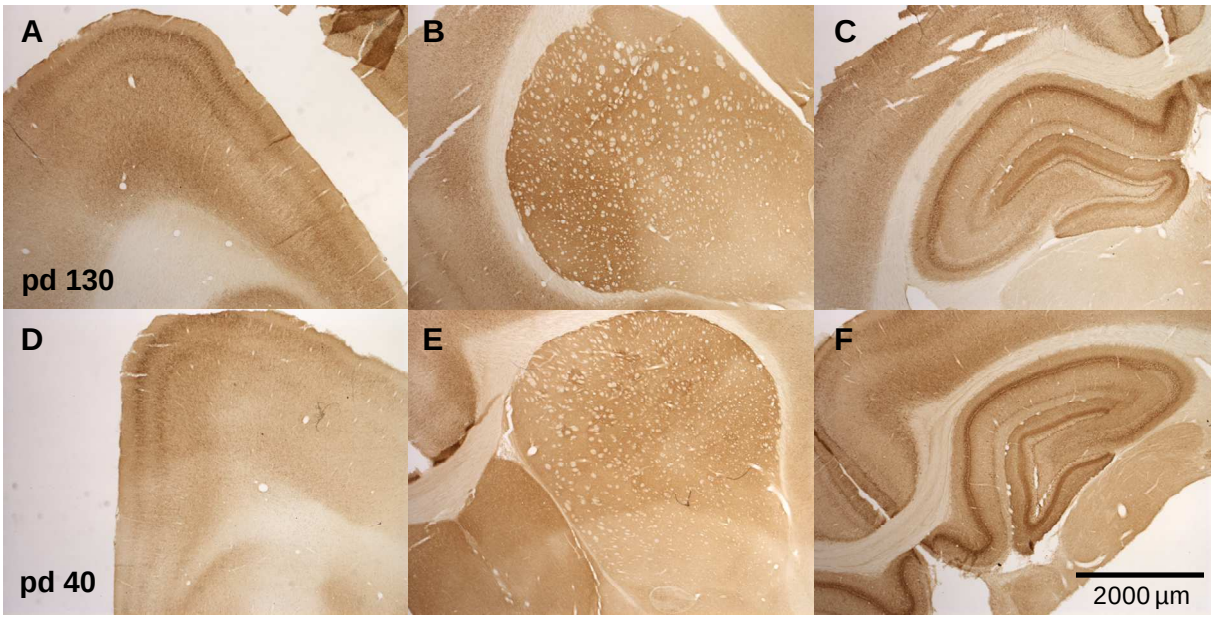

Figure 30: Representative brain sections of pd 130 (A-C) and pd 40 (D-F) stained for CB1R. A,D: mPFC, B,E: CPu, C,F: Hip. Pictures were taken with a 2.5x magnification.

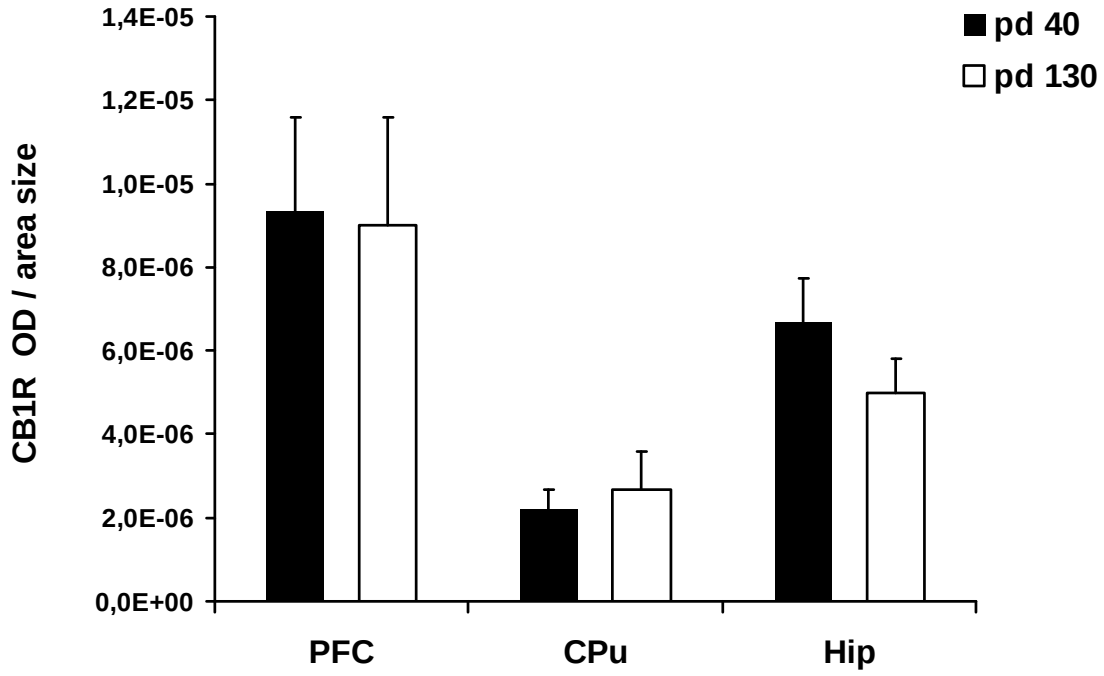

Figure 31: Mean CB1R optical density (OD) per area size in pd 40 (n = 4) and pd 130 (n = 4) old animals. Data are expressed as mean + S.E.M.

Western Blot of the Cannabinoid Receptor 1

Western blot analysis revealed a differential protein expression of CB1R in the analyzed brain regions (see Figure 32 for representative western bands of mPFC). In the mPFC CB1R expression was significantly different at distinct age points (Figure 33A; $F_{3,22} = 3.386$; $p = 0.036$) and post-hoc analysis showed a significantly lower expression of CB1R of adolescent animals (on pd 30, pd 40, and pd 50) compared to adult rats at pd 130 ($p \leq 0.033$.) In the CPu no significantly different expression was detected (Figure 33B; $F_{3,22} = 2.171$; $p = 0.120$) although, in the NAc a trend for a differential expression was observed (Figure 33C; $F_{3,22} = 2.642$; $p = 0.075$). Post-hoc testing revealed an increasingly higher expression of CB1R with further development which was significant at pd 40 ($p = 0.026$) and pd 130 ($p = 0.019$) compared to pd 30 (pd 50 vs pd 30: $p = 0.163$). Analysis of the Hip also showed a trend for a changing CB1R expression (Figure 33D; $F_{3,22} = 2.407$; $p = 0.095$) and post-hoc showed a higher CB1R expression in adolescent animals, significant at pd 30 ($p = 0.05$) and pd 40 ($p = 0.047$) compared to pd 130 (pd 50 vs pd 130: $p = 0.681$).

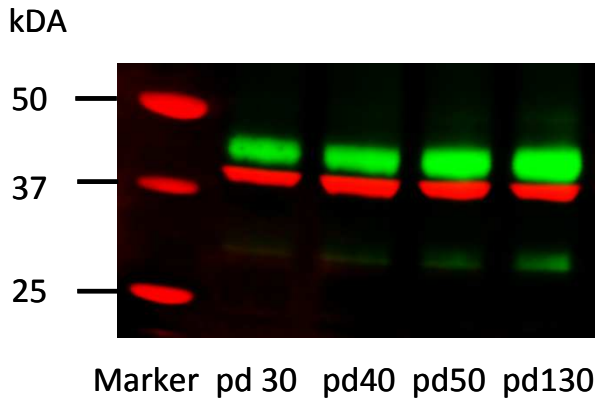

Figure 32: Representative western blot of mPFC samples of animals aged pd 30, 40, 50, and 130. First lane contains Odyssey Molecular Protein marker followed by mPFC samples of indicated ages. CB1R is visible in green and the internal standard ß-Actin in red.

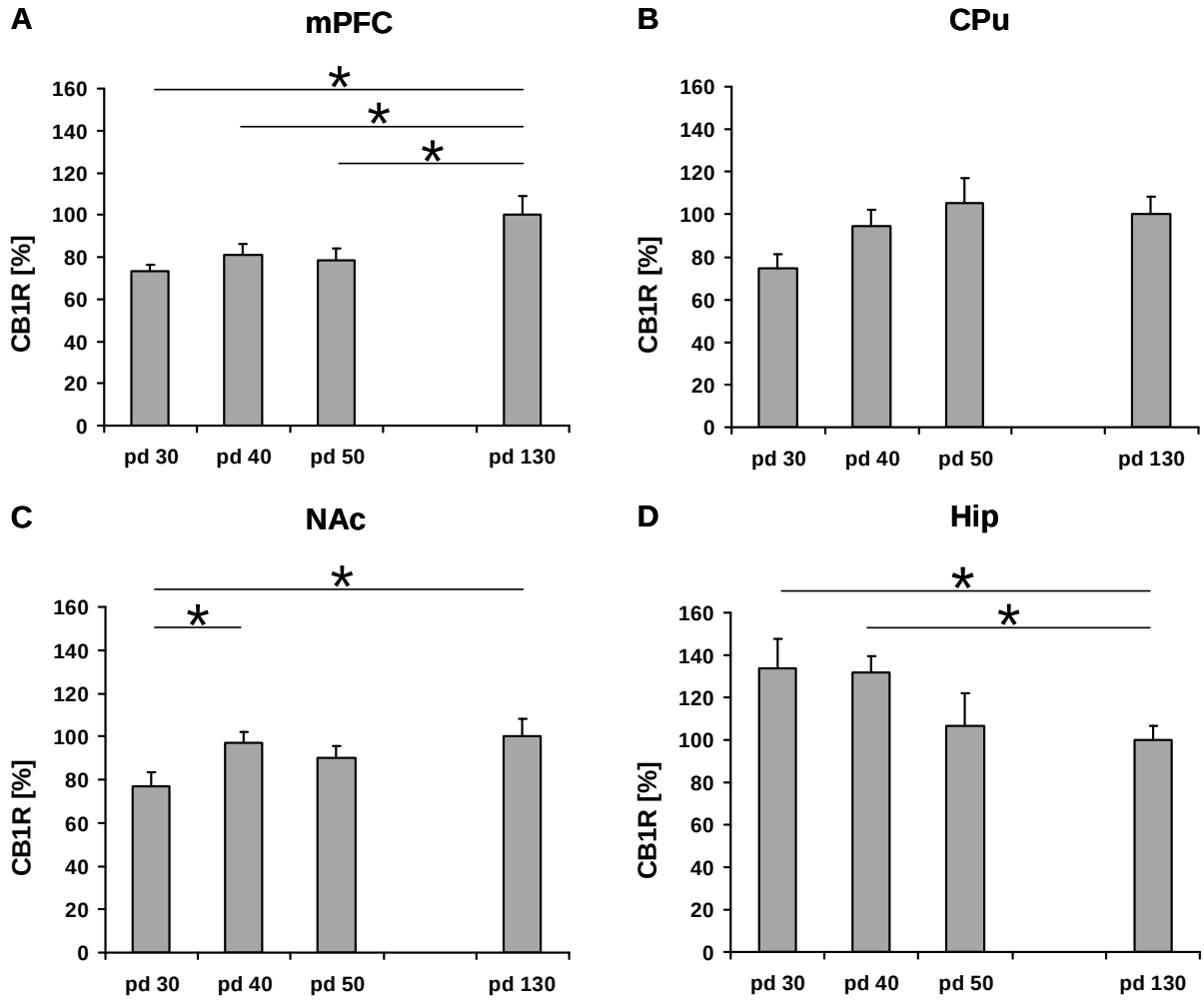

Figure 33: Western blot analysis of CB1R (percentage of pd 130) in mPFC (A), CPu (B), NAc (C), and Hip (D) over the development from pd 30 until pd 130. CB1R in the mPFC was significantly lower at pd 30, pd 40, and pd 50 compared to pd 130 (A). In the CPu no differential expression of CB1R was found at any age point (B). In the NAc, CB1R was significantly lower at pd 30 compared to pd 130 and compared to pd 40 (C). In the Hip, CB1R was significantly higher at pd 30 and pd 40 compared to pd 130 (D). Data are expressed as mean + S.E.M. (* $p < 0.05$, $n = 6$ for each time point).

3.3 Project III: Long-Term Effects of Chronic Pubertal WIN 55, 212-2 Treatment on Cognition

3.3.1 Object Recognition

A significant difference between vehicle and WIN (1.2 mg/kg) treated animals was found in the OBJR test. Student's t-test revealed a significantly impaired object discrimination for WIN treated animals after 15 min (Figure 34A; $t_{22} = 2.843$ $p = 0.0095$). Additionally, the discrimination index was significantly lower in WIN treated animals (Figure 34B; $t_{22} = 2.658$ $p = 0.014$) although, the initial exploration did not differ between groups ($t_{22} = 0.444$ $p = 0.661$; P1 ± S.E.M: WIN = 44.83 sec ± 3.03; vehicle = 46.5 sec ± 2.22).

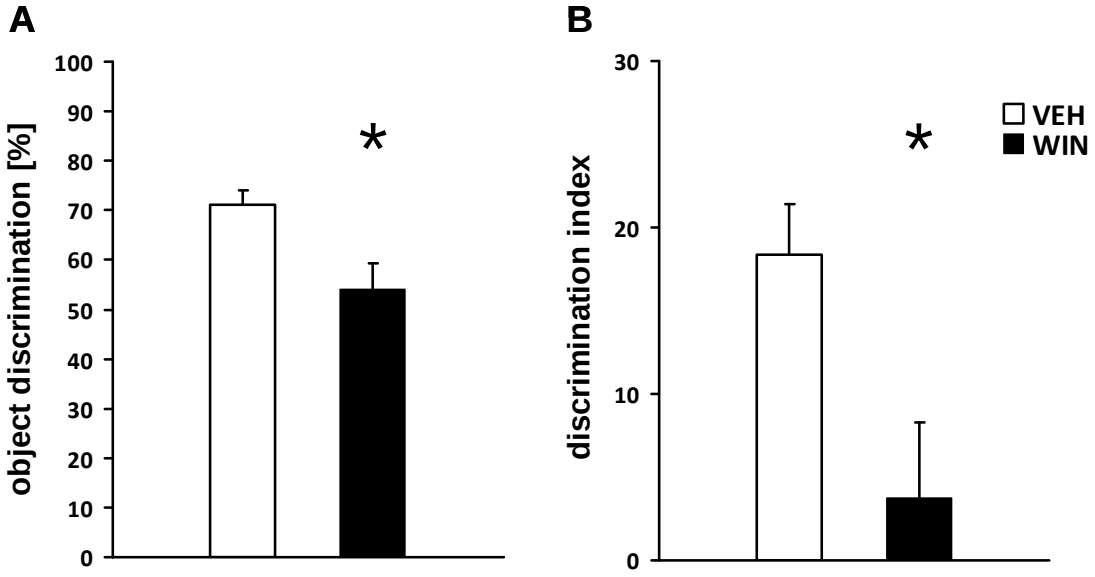

Figure 34: Percentage object discrimination (A) and discrimination index (B) in the OBJR test of chronically WIN and VEH treated animals. Both object discrimination and discrimination index were significantly reduced in WIN treated animals compared to VEH treated ones. Data are expressed as mean + S.E.M. (* $p < 0.05$, $n = 12$).

3.3.2 Prepulse Inhibition and Acoustic Startle Reflex

No difference was observed for mean PPI values between vehicle and WIN treated animals (Figure 35A; $t_{22} = -0.196$ $p = 0.846$). Neither did ASR differ between the two groups (Figure 35B; $t_{22} = -0.481$ $p = 0.635$).

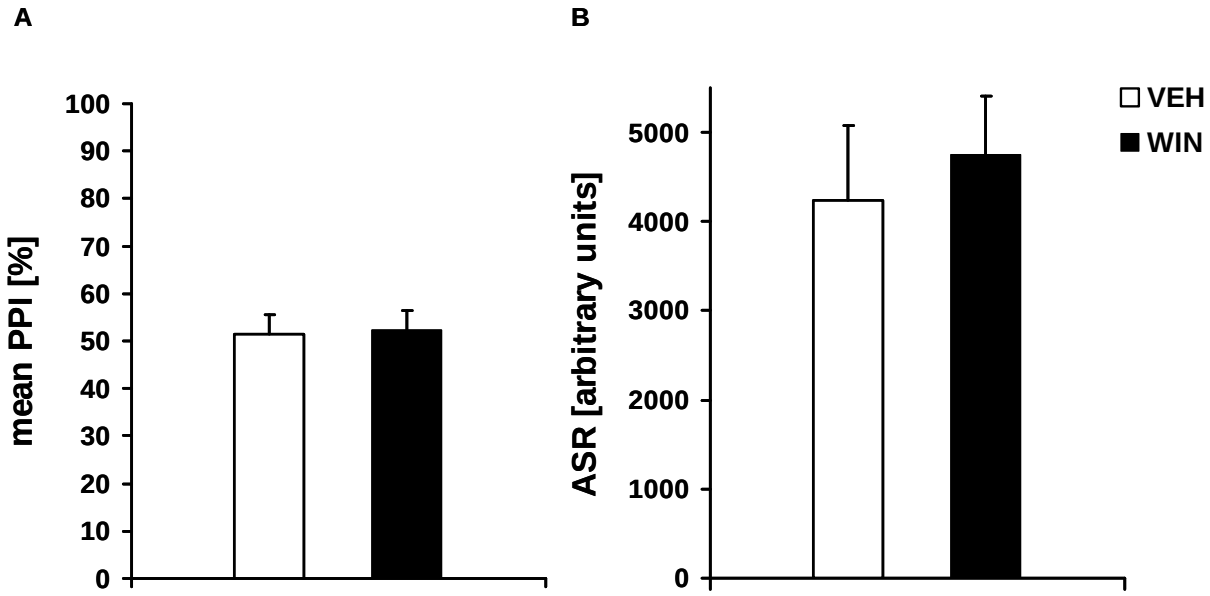

Figure 35: Mean PPI (A) and ASR (B) of chronically WIN and VEH treated animals. No difference was observed between treatment groups. Data are expressed as mean + S.E.M. (n = 12).

3.3.3 Casein Pellet Intake

CP intake did not differ significantly between vehicle and WIN treated animals (Figure 36; $t_{22} = -0{,}454$ $p = 0.655$).

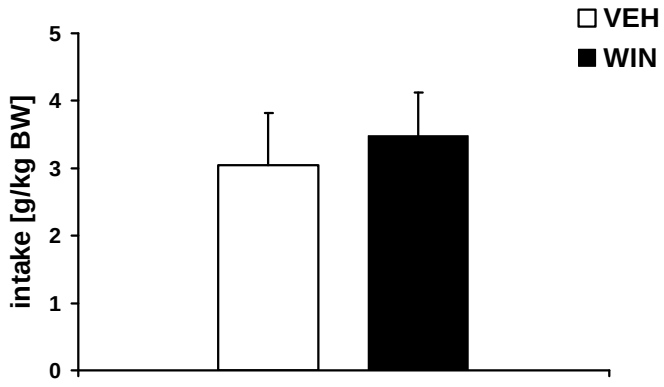

Figure 36: CP intake of chronically WIN and VEH treated animals. No difference was observed between treatment groups. Data are expressed as mean + S.E.M. (n = 12).

3.3.4 Attentional Set Shifting

All animals learned to perform a series of six consecutively correct trials at each stage of the set shifting paradigm (Figure 37). Over the course of the whole training, animals needed gradually less trials to reach the learning criterion for Pre, SD and CD as well as for IDS. To successfully complete the EDS, all rats needed more trials to criterion. MANOVA analysis revealed a significant treatment difference between vehicle and WIN treated animals over the whole course of set shifting training (Wilk's $\lambda = 0.008$ $F_{8,15} = 238.073$ $p = 0.000$). Further analysis by multiple ANOVAs indicated a significant difference between vehicle and WIN treated animals for CDrev ($F_{1,23} = 6.993$ $p = 0.015$) with WIN animals needing more trials to criterion (14.33 ± 1.3) than vehicle animals (10.42 ± 0.6) to complete the CDrev. No statistical differences between vehicle and WIN rats were found in the learning performance at the other stages ($p \geq 0.155$).

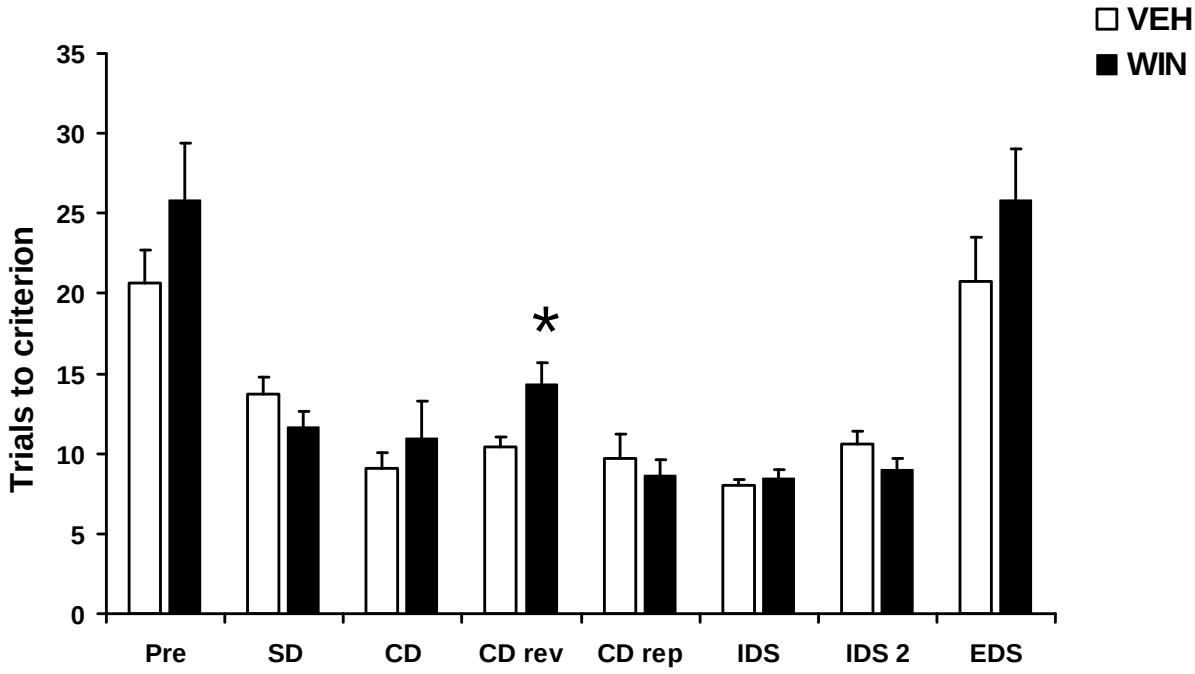

Figure 37: Behavioral performance in the ASST of chronically WIN and VEH treated animals. WIN treated animals needed significantly more trials to reach criterion in the CDrev stage of the test compared to VEH treated ones. Pre: pretraining; SD: simple discrimination, CD: compound discrimination, rev: reversal, rep: repetition, IDS: intradimensional shift, EDS: extradimensional shift. Data are expressed as mean + S.E.M. (* $p < 0.05$, $n = 12$).

3.3.5 Western Blot of Cannabinoid Receptor 1, Fatty Acid Amino Hydrolase, and Monoacylglycerol Lipase

CB1R protein content was significantly elevated in the mPFC of WIN treated animals compared to vehicle treated controls (Figure 38; $t_{13} = -3.343$; $p = 0.005$). No differences were observed in the CPu or Hip ($t_{22} = 0.424$; $p = 0.676$ and $t_{20} = -0.389$; $p = 0.702$ respectively) for the percentage protein content.

Neither were there any differences between WIN and vehicle treated rats for the percentage protein content of FAAH or MAGL in the mPFC, CPu and Hip (see Table 11). For the western blot analysis, several samples had to be excluded due to too low protein contents (see Figure 38 and Table 11).

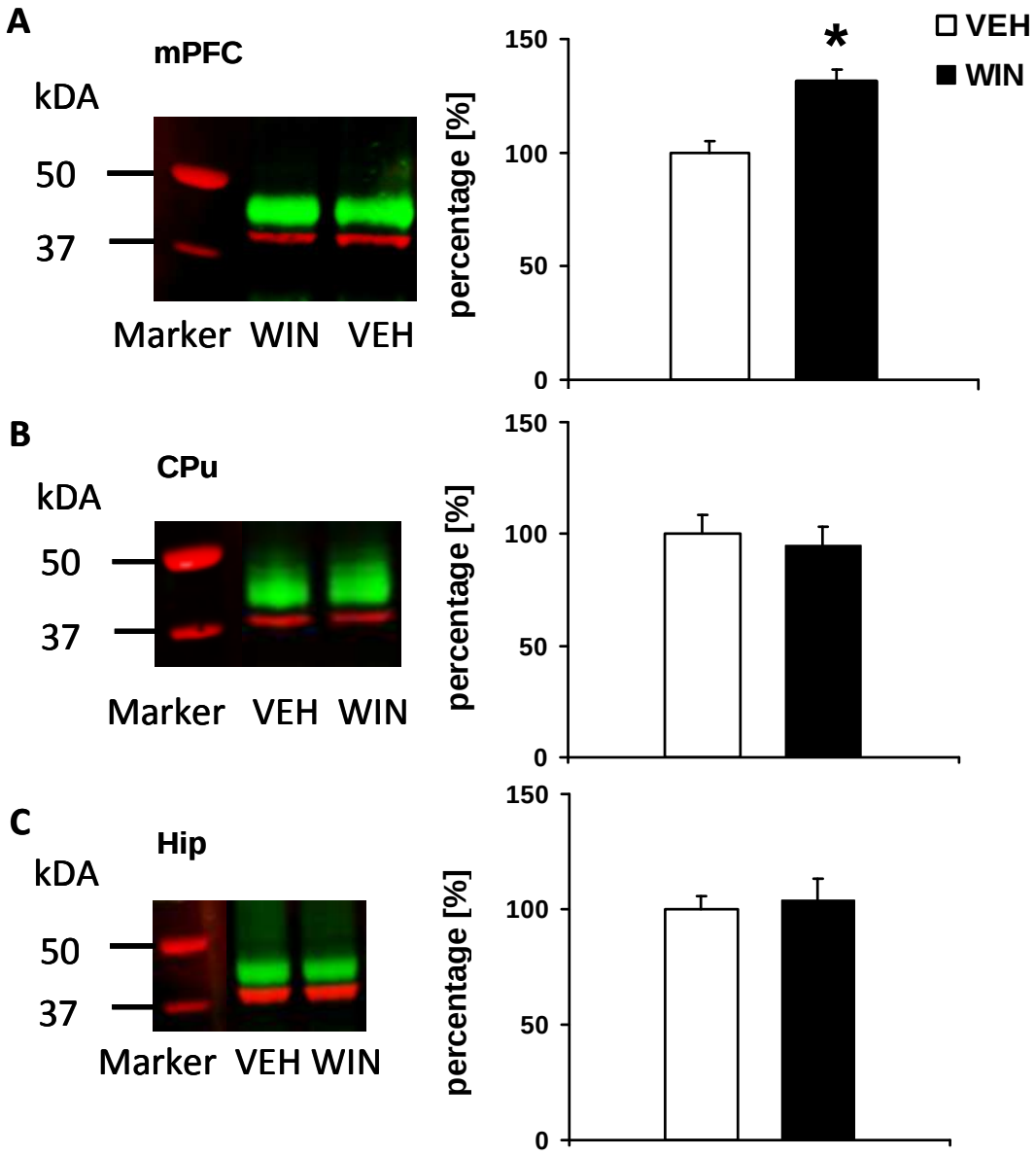

Figure 38: CB1R protein expression (percentage of VEH treatment group) in mPFC (A), CPu (B), and Hip (C) for WIN and VEH treated animals. Left column shows representative western bands for the respective indicated brain regions. CB1R is visible in green and the internal standard ß-Actin in red. A significantly higher percentage CB1R protein content was found in the mPFC of WIN treated animals compared to VEH treated ones (A). No differences were observed in the CPu (B) or Hip (C). Data are expressed as mean + S.E.M. (* $p < 0.05$, mPFC: n (VEH) = 8, n (WIN) = 7, CPu: n (VEH) = 12 n (WIN) = 12, Hip: n (VEH) = 11, n (WIN) = 11).

Table 11: Western blot analysis of mPFC, CPu, and Hip samples for FAAH, and MAGL of vehicle or WIN treated animals. No significant differences in protein expression of FAAH and MAGL were observed in mPFC, CPu and Hip. Data are expressed as mean ± S.E.M. FAAH: mPFC: n (VEH) = 12, n (WIN) = 11, CPu: n (VEH) = 12, n (WIN) = 12, Hip: n (VEH) = 11, n (WIN) = 10. MAGL: mPFC: n (VEH)=12, n (WIN) = 11, CPu: n (VEH) = 12, n (WIN) = 12, Hip: n (VEH) = 11, n (WIN) = 11.

	mPFC		CPu		Hip	
	Vehicle	**WIN**	**Vehicle**	**WIN**	**Vehicle**	**WIN**
FAAH						
%	100 ± 12.02	116 ± 18.58	100 ± 13.32	127 ± 10.45	100 ± 5.96	108.95 ± 8.02
MAGL						
%	100 ± 16.25	123 ± 39.29	100 ± 12.26	124 ± 11.65	100 ± 6.17	102 ± 9.47

3.4 Project IV: Long-Term Effects of Chronic Pubertal Methylphenidate Treatment on Cognition

3.4.1 Open Field

The open field test did not show any differences in locomotion between the groups ($t_{18} = -0.185$; $p = 0.856$). Mean values of total distance traveled: saline treated animals: 4340.27 cm ± 556.64; MPH (2.0 mg/kg) treated animals: 4480.79 cm ± 426.75. Due to technical difficulties 4 animals were excluded from the analysis (n = 3 of the saline group and n = 1 of the MPH treated group).

3.4.2 Anxiety-Related Behavior

In the EMT no differences between saline injected control and MPH treated animals were found for latency, rearing, time spent in the lit compartment, emergence frequency, or risk assessment (Table 12; $p \geq 0.4$). Similarly, no differences were observed in the EPM for percentage open arm time, head dips, risk assessments, self grooming, self grooming time, and closed arm entries (Table 13; $p \geq 0.1$). Only a trend for a higher number of open arm entries by MPH treated animals was observed (total open arm entries: $t_{22} = -1.787$; $p = 0.088$; percentage open arm entries: $t_{22} = -1.85$; $p = 0.078$).

Table 12: Performance of saline and MPH treated animals in the EMT. Latency [sec], number of rearings, time spent in the lit compartment [sec], emergence frequency, and number of risk assessments did not differ between groups. Data are expressed as mean ± S.E.M. (Saline n = 12, MPH n = 12).

EMT	**Saline**	**MPH**
Latency	223.25 ± 22.61	208.08 ± 32.09
Rearing	2.5 ± 1.12	1.83 ±0.97
Time spent in lit compartment	21.92 ± 8.03	18.75 ± 9.62
Emergence frequency	1.75 ± 0.52	1.25 ± 0.48
Risk assessment	4.08 ± 0.86	4.5 ± 0.69

Table 13: Performance of saline and MPH treated animals in the EPM. Percentage open arm time, number of head dips, risk assessments, self grooming, self grooming time [sec], and open arm entries did not differ between groups. A trend for a higher number of open arm entries and a higher percentage of open arm entries was observed for MPH treated animals indicated by #. Data are expressed as mean ± S.E.M. (# $p \leq 0.1$, saline n = 12, MPH n = 12).

EPM	**Saline**	**MPH**
percentage open arm time	5.14 ± 2.00	8.96 ± 3.13
head dips	3.33 ± 0.70	5.25 ± 1.26
risk assessments	7.83 ±0.77	8.92 ± 0.69
self grooming	0.75 ± 0.22	0.92 ± 0.19
self grooming time	6.33 ± 2.1	8.41 ± 1.9
closed arm entries	6.67 ± 0.82	7.33 ± 0.7
open arm entries	0.92 ± 0.31	2.17 ± 0.63 #
percentage open arm entries	10.85 ± 3.56	21.17 ± 4.29 #

3.4.3 Intake of Liquids of Variable Palatability

No difference in SCM intake between the saline and the MPH treated group was found (Figure 39A; t_{22}= -0.407; p = 0.688). Similarly, the preference for both 6% and 10% EtOH intake did not differ in a 24 h free choice intake paradigm (Figure 39B and C; t_{22} = 0.272; p = 0.788 and t_{22} = 0.288; p = 0.776 respectively).

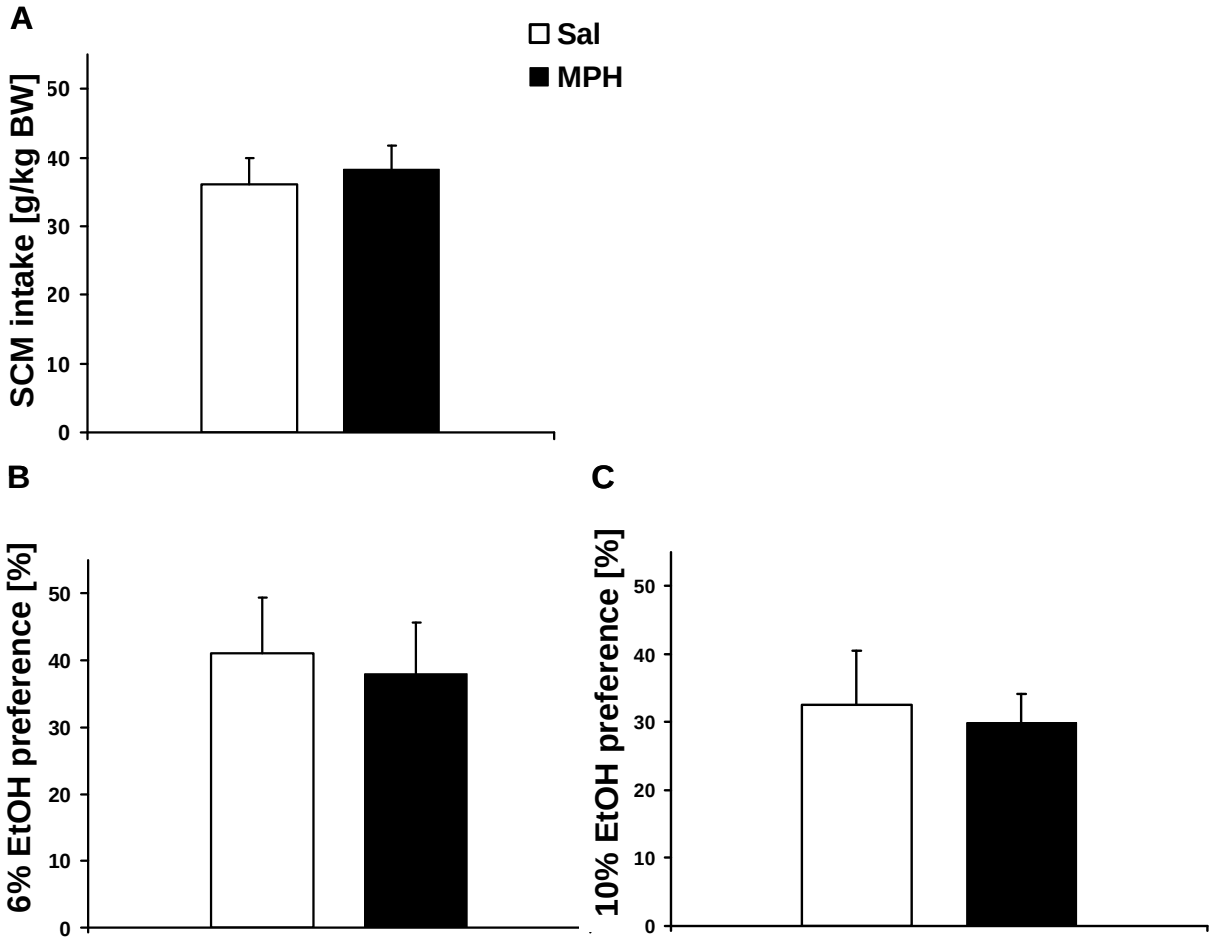

Figure 39: SCM intake (A) in a 15 min intake paradigm, preference for a 6% (B) and 10% (C) EtOH solution of Saline (Sal) and MPH treated animals in a 24 h free choice intake paradigm. No difference was observed between groups. Data are expressed as mean + S.E.M. (Sal n = 12, MPH n = 12).

3.4.4 Cognitive Tests

There was no difference between the saline and the MPH treated group in mean PPI (Figure 40A; $t_{22} = -0.653$; $p = 0.52$) and ASR (Figure 40B; $t_{22} = 1.55$; $p = 0.878$).

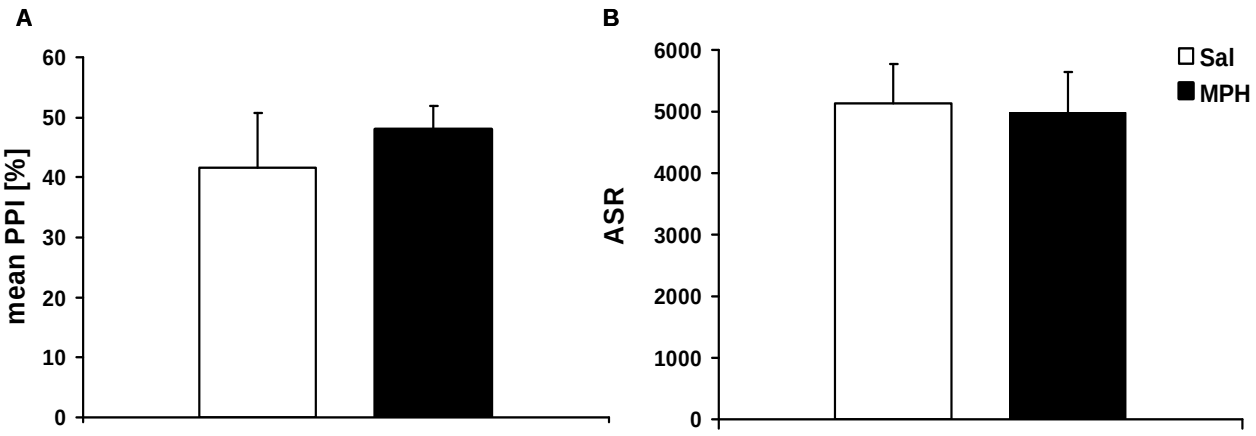

Figure 40: Mean PPI (A) and ASR amplitude [arbitrary units] (B) performance of saline (Sal) and MPH treated animals. No difference was observed between groups. Data are expressed as mean + S.E.M. (Sal n = 12, MPH n = 12).

The percentage of OBJR and discrimination index did also not differ between groups (Figure 41; $t_{22} = 0.578$; $p = 0.569$ and $t_{22} = 0.482$; $p = 0.635$ respectively). In addition, total exploration times during P1 in the OBJR test did not differ between groups ($t_{22} = -0.135$; $p = 0.894$).

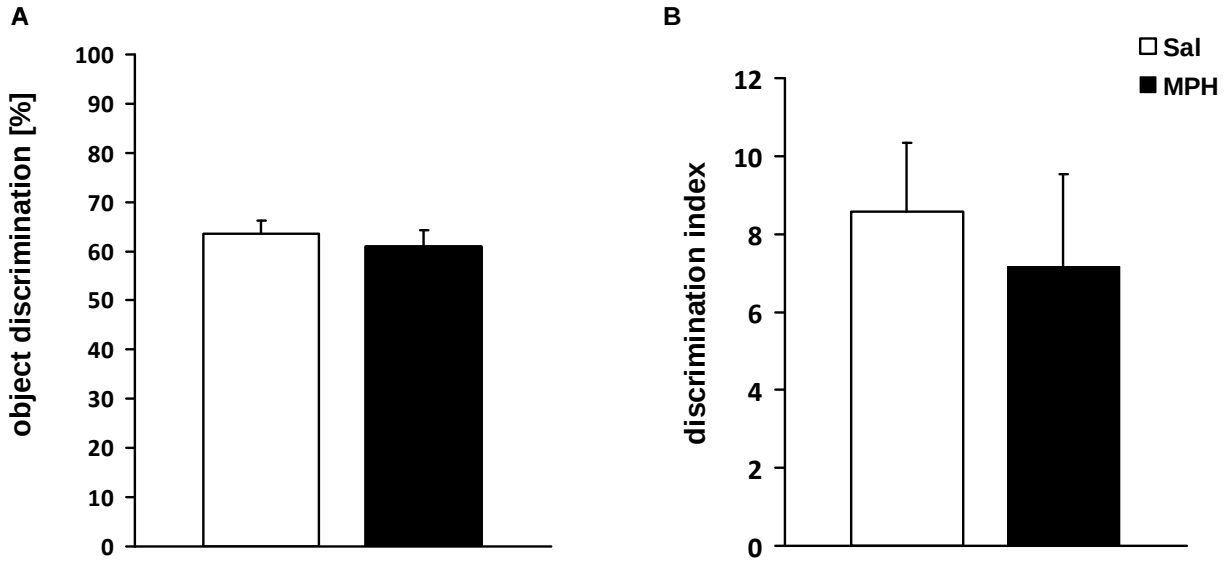

Figure 41: Percentage discrimination (A) and discrimination index (B) of saline (Sal) and MPH treated animals in the OBJR test. No difference was observed between groups. Data are expressed as mean + S.E.M. (Sal n = 12, MPH n = 12).

Likewise, the percentage of SOCR did not differ between groups (Figure 42; $t_{22} = 0.215$; $p = 0.832$). And additionally, total exploration time during P1 in the SOCR test did not differ between groups ($t_{22} = 0.489$; $p = 0.629$).

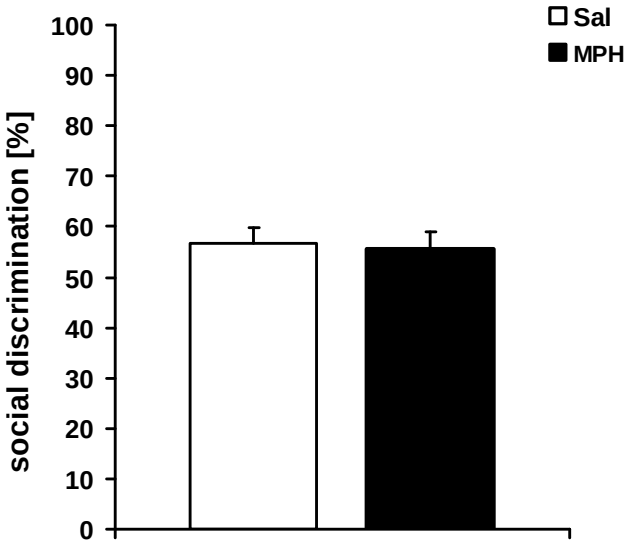

Figure 42: Percentage discrimination of saline (Sal) and MPH treated animals in the SOCR test. No difference was observed between groups. Data are expressed as mean + S.E.M. (Sal n = 12, MPH n = 12).

4. Discussion

4.1 Project I: Differences of Cognitive Abilities in Three Wistar Han Rat Lines

This project revealed behavioral differences between the three Wistar rat lines W[hsd], W[rcc] and W[Jan]. W[Jan] animals were obtained from a different supplier (Janvier) than W[hsd] and W[rcc] ones (both obtained from Harlan Laboratories). Behavioral variations were observed in locomotor activity during an open field test, in cognitive processing during an OBJR test, and during PPI of the ASR (the two latter paradigms representing tests for STM and sensorimotor gating abilities, respectively).

Altogether, the results of the present project add to the increasing picture of line and supplier variations observed in the field of behavioral research in rodents. The main goal of this project was to evaluate possible behavioral differences in three Wistar rat lines, particularly in cognitive tests. The Wistar rat strain is commonly employed in scientific, particularly in behavioral research (Clause, 1993, Palm et al., 2011b). For many researchers who have been using the W[hsd] line, the intended substitution of W[hsd] line by W[rcc] animals by Harlan Laboratories poses an unknown variable to their experimental practice.

In the open field test W[Jan] rats displayed a higher locomotor activity compared to W[rcc] rats. In addition to measuring locomotor activity, the open field arena is also used to measure anxiety-like behavior under aversive conditions e.g. when brightly lit. For example, one study found differences in female Wistar rats of different suppliers in an open field arena and in an EPM test (Honndorf et al., 2011). They observed higher locomotor acitivity of Wistar rats from Janvier compared to those from Charles River which appear to be in line with the presently observed higher locomotor activity of the W[Jan] rats.

The object recognition test is often employed to analyze cognition and short-term memory because it can be easily performed and is based on the natural tendency of rodents to prefer novel objects over familiar ones (Ennaceur and Delacour, 1988). W[rcc] rats showed the best performance in the OBJR test, significantly better than W[Jan] animals. This result indicates a better STM processing in the W[rcc] rat line. W[hsd] animals displayed an intermediate object discrimination performance but the highest exploration time during P1, significantly higher than W[rcc] and W[Jan], who displayed similar exploration times. Therefore, exploration times do not appear to be linked to object discrimination performance.

Strain differences in object recognition memory have been investigated before (for review see Andrews, 1996). However, a comparison of Long-Evans, Wistar, and Sprague-Dawley rats from

Harlan, Netherlands, revealed a sufficient discrimination of the new vs. familiar object in an object discrimination task only in Long-Evans rats (Andrews et al., 1995). In contrast, several studies have found that Wistar rats can be tested successfully in this paradigm (Schneider and Koch, 2002, Kosiorek et al., 2003, Ennaceur et al., 2005, Schneider et al., 2008, de Bruin et al., 2011). Furthermore, one study compared both male and female, Wistar and Hooded Lister rats, that were all obtained from Charles River, Kent, UK (Ennaceur et al., 2005). In this study, all animals displayed a sufficient discrimination of novel vs. familiar objects except for female Wistar rats. One possible explanation could be the confounding effect of a higher neophobia towards novel objects in female rats (Ennaceur et al., 2005). In the present project, W[rcc] rats were able to discriminate a novel object from a familiar one presented 15 min earlier. However, the performance of W[Jan] animals reached only chance level and these animals therefore, appear to have difficulties performing this task.

The reduction of the ASR by a preceding prepulse, termed PPI, is generally considered an operational measure of sensorimotor gating (Hoffman and Ison, 1980, Koch, 1999). Several studies have investigated strain and supplier differences in ASR and PPI before (Rigdon, 1990, Varty and Higgins, 1994, Swerdlow et al., 2000). For example, in one study differences between Wistar rats and Sprague Dawley derived CD rats under the influence of the dopamine agonist apomorphine were detected (Rigdon, 1990). Apomorphine injection led to a blockade of PPI without affecting the startle amplitude in Wistar rats. In contrast, apomorphine caused an increase in the startle amplitude without affecting PPI in CD rats. Another study examined differences between Wistar and Spargue Dawley rats of the two different suppliers: Harlan laboratories, USA and Bantin-Kingman (UK) (Swerdlow et al., 2000). Supplier differences in the sensitivity to the disruptive effects of dopamine agonists were observed in this study. Specifically Harlan derived Sprague Dawley rats displayed greater sensitivity towards PPI disruptive effects of apomorphin compared to Bantin-Kingman derived Sprague-Dawley rats. One more study analyzed the effect of different prepulse parameters on Wistar, Lister Hooded, and Sprague-Dawley rats (Varty and Higgins, 1994). A lower sensitivity of Wistar rats compared to Lister Hooded and Sprague-Dawleys for various prepulse durations and intensities was observed. In the present project, differences in startle amplitude and PPI between the three Wistar lines tested were detected. W[hsd] and W[rcc] animals were indeed found to differ in their behavioral performance for startle-reactivity. Although the Wistar line obtained from a different supplier (Janvier, W[Jan]) was found to show similarities in startle reactivity with the W[hsd] line, a distinct difference was observed for sensorimotor gating, indicating that this Wistar line constitutes no adequate alternative, either.

4. Discussion

In general, behavioral differences in Wistar rats, all originating from the original colony at the Wistar Institute at the beginning of the last century, were analyzed in the context of this project. Since this time, many generations of rats have been bred as offspring from the founder colony. Considering how quickly behaviors can change when selectively bred for, the observations detected here, along with the observations of many other researchers, are not unexpected. When selecting an appropriate rat line for a particular hypothesis and testing paradigm, researchers should be aware of possible differences in the animal's behavior and further evaluations of line and supplier differences will help to make studies more comparable. The present results therefore emphasize the high behavioral diversity even among Wistar rat lines. Due to the best performance of W[rcc] animals in the OBJR test and their intermediate performance in PPI, this line was selected for all following projects.

4.2 Project II: Ontogeny of Cognitive Abilities

In this project, the longitudinal development of cognitive abilities was measured behaviorally in Wistar rats from adolescence to adulthood and additionally, molecular analysis investigated the development of myelination and CB1R levels and distribution throughout this age period.
The main finding was that developmental trajectories for the behavioral tests applied differed greatly throughout adolescence. For OBJR a non-linear course in performance was observed that appeared to vary with onset of puberty whereas the PPI of the ASR displayed a gradual increase until early adulthood. Performance in the OBJRecency test showed a later onset than in the OBJR test but displayed less variation throughout development. Findings from a pharmacological treatment with a low dose of the CB1R antagonist/inverse agonist SR implicated the involvement of the ECS in OBJR performance specifically on pd 40. CB1R protein level measurements displayed a brain region-dependent variation including increases in development (in the mPFC and the NAc) and decreases (in the Hip). The development of myelination also displayed a gradual increase with increasing age and variations within these regions.

Influence of Intertrial Interval Length on Object Recognition Performance

In a preliminary study the possible influence of varying ITI lengths on OBJR performance revealed that adolescent animals displayed better recognition memory at shorter ITIs. Animals tested on pd 45 showed a higher percentage object discrimination and a higher discrimination index after an ITI of 10 min compared to an ITI of 20 min. Adult animals (> pd 100) did not show differences for both measurements after either ITI length.
It has been shown before that the length of the ITI during an OBJR test can influence performance of the test animal (e.g. Baker and Kim, 2002, Winters and Bussey, 2005, Bertaina-Anglade et al., 2006). For example, one study in rats and mice showed that the ability to discriminate the novel from the familiar object decreases when the ITI is extended (Bertaina-Anglade et al., 2006). They observed successful novel object discrimination for short ITIs (10 min, 1, 2, and 3h) but not after long ITIs (4 and 24h after the first exposure to two objects) in rats, and in mice successful discrimination was found after short ITIs (10 min and 1h) but not after longer ITIs (2, 3, and 6h). However, the impact of ITI length during development has gained less attention. In one study the length of ITI was investigated during the ontogeny of Sprague Dawley rats (Reger et al., 2009). Weanling rats (pd 20 – 23) could recognize a previously encountered object after 15 min and 1h, however, after 24h, only older animals (pd 29 – 40 and > pd 50) were able to display a preference for a novel object. Although this study employed age points and ITIs varying from the present

project, similarly to the present results, younger animals had difficulties discriminating objects encountered before when the ITI was longer.

Many protocols and testing procedures exist for the OBJR test and it is important to reduce the risk of possible confounding factors *a priori*, like object affordances (see Ennaceur, 2010), strain differences, and the length of the ITI. The present data show that pd 45 old male Wistar rats can discriminate the novel object after 20 min, however, performance is even better if the ITI is shorter. In adult animals, ITIs of 10 and 20 min yielded similar percentage discrimination performance and discrimination indices. Therefore, an ITI of 15 min was chosen for the subsequent testing paradigm.

Locomotor Behavior during Development

The development of locomotor behavior in an open field arena during repeated testing every ten days revealed a higher distance traveled of animals during late adolescence and early adulthood (from pd 59 – 99) compared to when the same animals were tested on pd 129. Repeated testing in a separate group of animals in adulthood showed no variation in locomotor activity.

Altered locomotor activity at various age points has been observed before in mice and rats (Spear et al., 1980, Darmani et al., 1996, Ricceri et al., 2000, Erickson et al., 2014). In some studies an increased activity was observed earlier during development than in the present results, i.e. in early adolescence. For example, one study showed increased locomotor activity during a 20 min open field test in mice (Darmani et al., 1996). Locomotor activity increased gradually from pd 7 (over pd 14, 18, 22, and 28) to pd 35, and afterwards decreased as measured on pd 42, 63, 120, and 180. However, this study investigated mice (vs. rats as observed here) immediately after an injection procedure, which may have had a stressful effect. In another study, locomotor activity of Sprague Dawley rats during a 10 min measurement in a hole poke open field displayed higher numbers of matrix crossings in animals tested on pd 35 and 36 compared to earlier (pd 23 and 24) and later (pd 47 and 48) ages (Spear et al., 1980). In contrast to an observed higher activity in early adolescence, an increased distance traveled was observed in a recent study in early adult animals (at 3 months of age) compared to younger and older ones tested (Erickson et al., 2014), comparable to the results of the present thesis. In this study the locomotor behavior of Long Evans rats has been investigated on several time points in late adolescence and adulthood (Erickson et al., 2014) where animals were tested repeatedly with 1.5, 3, 7, and 13 months of age (corresponding to approx. pd 50, 84 – 90, 196 – 210, and pd 364 – 390). Animals were tested for 10 min in an open field arena and the results resemble the ones observed in this project during the 15 min open field test. Unfortunately no earlier time points than pd 50 were analyzed in the study by Erickson et al. (2014). Similarly to the present results, higher locomotor activity in an open

field test in mice was also observed on pd 90 compared to earlier test points on pd 18, 28 and 46 (Ricceri et al., 2000).

The increased locomotor activity during late adolescence displayed in the present experiment may be due to an increased exploratory drive during this age period. It has been observed before that adolescent rodents display higher exploration of novel environments and increased novelty seeking (Spear, 2000, Laviola et al., 2003). This probably reflects an age-related migration of young animals in order to find new living environments and avoid inbreeding (Spear, 2000). Although the animals in the present project had been placed in the open field every ten days starting on pd 29, this increased locomotor acitivity was only observed starting from pd 59 indicating a developmental alteration. Furthermore, the test-free time period of seven days between experiments (from the last OBJRecency test to the next open field exposure) appears to be sufficient to avoid a habituation effect to the open field environment (usually resulting in decreased locomotor activity; see Ricceri et al., 2000, Heyser and Ferris, 2013). It rather appears like the open field may be perceived as a novel environment again by the test animals when tested every ten days. However, repeated testing in a separate group of adult animals did not display alterations in locomotor activity, further indicating that the increase in adolescent animals appears to be a developmental effect.

Additionally, a longer test-free period may explain the finding of a still increased locomotor activity on pd 99 which is already considered early adulthood. Animals tested on pd 99 were not placed in the open field arena for 18 days giving them a longer test-free period, thus possibly eliciting a higher novelty exploration response again than the younger animals with only seven days of test-free period.

Altogether, altered adolescent behavior in rats includes locomotor activity and in the present part of the project this behavior was found to be increased during late adolescence and early adulthood.

Basal Development of Object Recognition and Object Recency

Object Recognition Test

Here, the ability of animals to discriminate a novel from a familiar object displayed a developmental variation from adolescence to adulthood. In the OBJR test animals already showed percentage object discrimination levels on pd 30 that were similar to levels of adult animals (on pd 130) after a 15 min ITI. However, the performance was significantly lower on pd 40 and tended to be lower on pd 50 compared to the same animals tested as adults on pd 130. After pd 50,

performance levels increased again until displaying stable levels in adulthood. Furthermore, the discrimination index revealed a trend for a lower level on pd 40 compared to adult animals.

The reduced level of object discrimination on pd 40 observed in this project did not depend on the repeated testing employed, since adult control animals tested repeatedly in the same paradigm did not show any variation of percentage object discrimination or discrimination index.

Additionally, the exploration time during P1 of adult animals tested repeatedly did not differ whereas the animals tested throughout development displayed a higher object exploration time on every test point from pd 30 – 80 compared to pd 130. This observation implies that the decrease in object discrimination on pd 40 is not due to a decreased exploration of the objects at this test point. The adolescent animals rather seem to explore the objects more intensively than in adulthood. This would reflect an increased novelty seeking behavior which is commonly observed during adolescence (Adriani et al., 1998, Laviola et al., 2003) and is partially similar to observations in several studies investigating the ontogeny of object recognition in rodents (Reger et al., 2009, Cyrenne and Brown, 2011, Heyser and Ferris, 2013). Although in one study a higher object exploration time was observed in rats on pd 90 compared to pd 42 (hence later than found in this project), the animals still displayed a higher object exploration time on pd 42 than animals on pd 35 and pd 21 (Heyser and Ferris, 2013). Another study found that mice displayed the highest object exploration on pd 46 compared to earlier (pd 18 and pd 28) or later age points (pd 90; Ricceri et al., 2000), similarly to the present findings.

Alternatively, an increased object exploration of adolescent animals may also be necessary in order to sufficiently encode the objects. For example, Reger et al. (2009) hypothesized that younger rats required “more time for exploration to acquire comparable levels of object interaction and memory performance”. However, the animals are not instructed to remember the presented objects actively and do not know that they will be tested again for exploration of a novel and a familiar object in a subsequent session. Therefore, the increased time exploring novel surroundings appears to be an unconscious process and is more likely driven by the higher novelty-seeking behavior during adolescence.

Although it has been debated if this test is suitable for rodents at very young ages (Ricceri et al., 2000, Anderson et al., 2004), refinement of some test conditions (e.g. ITI) produced reliable performance levels of animals as early as pd 20 – 23 (Reger et al., 2009, Heyser and Ferris, 2013). It is important to choose testing conditions carefully (e.g. illumination, ITI), and thereby reduce possible confounding factors to avoid low discrimination performance of animals due to non-mnemonic related issues (e.g anxiety or arousal). In the present study animals were already able to discriminate a novel from a familiar object on pd 30, suggesting that the testing conditions were suitable to measure recognition memory in animals at this age. Interestingly, after displaying the

ability to discriminate a novel from a familiar object on pd 30, the performance on pd 40, approximately the beginning of puberty in male rats (Korenbrot et al., 1977, Schneider, 2013), was decreased compared to adult performance levels. Only few studies have investigated the ontogeny of object discrimination in rodents, so far yielding contradicting results (Ricceri et al., 2000, Anderson et al., 2004, Reger et al., 2009, Cyrenne and Brown, 2011, Heyser and Ferris, 2013). One study found an increased preference for a novel object in male Lister Hooded rats on pd 40 compared to earlier (pd 28) and later ages (pd 80) (Cyrenne and Brown, 2011), whereas other studies found a similar performance across adolescence (e.g. in Sprague Dawley rats on pd 21, 35, 42, and 90; (Heyser and Ferris, 2013) and on pd 20 – 23, 29 – 40, and > pd 50; (Reger et al., 2009), as well as in CD-1 mice on pd 28, 46, and 90; (Ricceri et al., 2000)).

Most of these studies focused on various additional factors, thus almost all of them used different groups of animals for every age point (e.g. Ricceri et al., 2000, Cyrenne and Brown, 2011, Heyser and Ferris, 2013) or used only some of the test subjects repeatedly (Reger et al., 2009). For instance, Reger et al. (2009) additionally investigated the influence of ITI length on different ages of Sprague Dawley rats (as mentioned above). Moreover, the test points employed comprised animals of broad age ranges (e.g. pd 29 – 40) and early adult animals that might still be considered adolescent on pd 50. In contrast to this, testing of the same subject repeatedly (as in the present project) has the advantage to minimize possible differences between separate cohorts of animals (see Palm et al.).

One study testing mice repeatedly in an OBJR test found strain dependent differences across development when testing animals on the following test points: 4, 6, 8, and 10 – 12 weeks of age (corresponding to approx. pd 28, 42, 56, and 70 - 84; Molenhuis et al., 2014). In a comprehensive characterization of four mouse strains they found that the reference strain of C57 mice displayed object recognition memory from 6 weeks of age whereas two other strains (129Sv and BTBR) showed a delayed onset of discrimination capacity only from 8 weeks of age. Puberty onset in mice starts earlier than in rats and complete BPS has been observed around pd 26 and pd 30 but appears to be strain-dependent (Schneider, 2013). Furthermore, another strain, AJ mice, did not display reliable object recognition memory at any of the tested age points (Molenhuis et al., 2014). However, in addition to species differences (mice vs. rats), the OBJR test was conducted differently than in the present project. The test was part of an extensive screen involving the placement of the animals from one arena to a novel one in which they encountered two objects, one of which had been presented for at least 48h before in their home cage for habituation. It is therefore difficult to compare the results with the ones in the present part of this project but the study indicates that there is a development of OBJR memory abilities and earlier age points may differ from later ones.

In their ontogenetic study, Cyrenne and Brown (2011) additionally focused on possible sex differences in Lister Hooded rats. Interestingly, an increased preference for the novel object over a familiar one on pd 40 was only observed in male rats (compared to successful but lower preference on pd 28 and pd 80). This appears to be in contrast with the present results, however, in their study the authors employed shorter ITIs (2 min) and longer presentation intervals (5 min) in which the animals were allowed to explore the objects (Cyrenne and Brown, 2011). Furthermore, a different rat strain was observed (Lister Hooded vs. Wistar rats). Females, in contrast, displayed no novel object preference on pd 40, but did so when tested earlier (on pd 28) and later (on pd 80). The authors interpreted their results by means of sex differences in the context of sexual selection pressure, which would favor riskier strategies in males than in females. However, it has been observed that in various species, puberty in females starts before and sexual maturity is reached earlier than in males (Spear, 2000). It is therefore very important to keep the possibly resulting gender differences in mind. Males would have to have been tested a few days later than females in order to directly compare the developmental status. Additionally, only three time points during development were investigated in the study by Cyrenne and Brown (2011) and these were spaced further apart than the ones investigated in the present project. Thus, several differences between this study and the present project make direct comparison difficult and a possible non-linear performance in the OBJR memory of Lister Hooded rats may have been missed.

In human studies a non-linear development of cognitive abilities has been observed for various measurements (e.g. Carey et al., 1980, Anderson et al., 2001, Waber et al., 2007). It is believed that periodic functional reorganization processes lead to spurts and plateaus in cognitive development which is probably also subject to individual development (Elman, 2005, Waber et al., 2012). The organizational abilities of children between 7 and 13 years of age showed a regression in organizational strategies used for recalling and drawing a complex figure in 12 – 13 year-olds (Anderson et al., 2001). Younger children (11 years of age) displayed better organizational abilities, employing conceptual strategies to a greater extend than older children (12 – 13 year-olds), who used fragmented or piecemeal approaches more often. Unfortunately, no older subjects were investigated in this study and both male and female children were pooled in each age group. In another study, face recognition abilities were investigated in children and adolescents from 6 – 16 years of age (Carey et al., 1980). Interestingly, a marked improvement was observed until 10 years of age followed by a period of decline or no improvement in performance until further improvements were observed from 14 – 16 years of age. Although both boys and girls were analyzed in this study no effects for sex of the subjects were found. This remarkable developmental pattern resembles the observations in the present project, where

animals were first able to discriminate a novel object from a familiar one at young age, but then this ability temporarily decreased and picked up again at older ages.

Interestingly, the decreased performance on pd 40 was found at a time when numerous developmental alterations take place, *inter alia* in neurotransmitter systems. Both the ECS and the DA system display highest receptor densities at this age and both transmitter systems are involved in cognitive processes (Rodriguez de Fonseca et al., 1993, Andersen et al., 2000, Myhrer, 2003, Wotjak, 2005). A possible explanation may be that alterations in these neurotransmitter systems could impair successful performance in the OBJR test at this particular age point. Application of ECS agonists are known to impair OBJR memory (Schneider and Koch, 2003, O'Shea et al., 2004) thus, an enhanced eCB tone during adolescence may decrease performance in this test. Hence, adolescent animals would hypothetically display a higher eCB tone or a higher ECS activity compared to adult animals. Similarly, acute administration of a D1 agonist impaired recognition memory after 15 min (Hotte et al., 2005). Moreover, these neurotransmitter systems have been shown to interact indirectly (reviewed in van der Stelt and Di Marzo, 2003, Fernandez-Ruiz et al., 2010) and therefore a combination of ongoing alterations in both may underlie the present findings. In the mesocorticolimbic pathway, for example, medium spiny neurons from the NAc exert a tonic inhibition on DA releasing cells in the VTA (van der Stelt and Di Marzo, 2003). These medium spiny neurons in the NAc are in turn controlled by glutamatergic afferents from the PFC that also contain CB1Rs. Upon activation of these CB1Rs the excitatory transmission is reduced (Robbe et al., 2001, Pistis et al., 2002, Robbe et al., 2002) and the medium spiny neurons exert less inhibition on the DA releasing VTA neurons. Thus, an overall increased DA release from the VTA to the PFC and the NAc is the result of the CB1R activation. This process may be enhanced during adolescence when increasing levels of CB1R and DA receptors are expressed.

Taken into account that both cannabinoid agonist application and D1 agonist administration were shown to impair OBJR performance, increased receptor densities at this age may imply increased activity in one or both of these neurotransmitter systems and thereby underlie the reduced decrease in OBJR performance.

Taken together, the longitudinal development of object recognition over the adolescent period displayed first of all the ability of pd 30 old animals to discriminate a novel from a familiar object after a 15 min ITI. This is followed by a decrease in performance on the approximate onset of puberty on pd 40 and a subsequent improvement until performance levels of adult ages (on pd 130). Thus, this particular aspect of cognition, namely the ability to recognize a novel object presented shortly before, demonstrates a non-linear development over the course of the rats' adolescence.

Object Recency Test

In the OBJRecency test, the percentage recency discrimination during development revealed a lower level on pd 31 compared to adulthood tested on pd 131. Thereafter, performance did not differ significantly from adult levels and displayed similar values over the complete developmental period. Additionally, no variation was observed upon repeated testing in adult animals. An increased object exploration during P1 was observed from pd 41 – 71 in the adolescent group of animals but not in the adult control group after repeated testing.

In contrast to the OBJR test with a 15 min ITI, the OBJRecency test consisted of 3 sessions separated by two 45 min ITIs, yielding a total test duration of over 90 min for each animal. This rather long time span puts a high temporal load on the mnemonic capacities of the animals and the prolonged time interval between test sessions may be too long for pd 30 old animals to remember at P3 having explored the objects from P2 more recently than those from P1. As mentioned before, younger animals can have difficulties in OBJR tests with longer time intervals (Reger et al., 2009) and in turn younger animals perform better when the ITI length is shorter as was observed in the preliminary study of this project.

The prior experience of the animals to the previously encountered open field and OBJR test could have resulted in a habituation effect, thus decreasing the object exploration time. However, rather a contrary effect could be observed since the exploration time during P1 of the OBJRecency test appears generally higher than during P1 of the OBJR test. Although on pd 31 the exploration time was not significantly different from when the animals were tested in adulthood (pd 81, 101, and 131), they still displayed a rather high exploration time. One possible explanation for a higher exploration in adolescence may be that younger animals need more time exploring a novel object for sufficient encoding and retrieval abilities (as mentioned for the OBJR test). Additionally, the previously conducted OBJR test may have elicited a general arousal state in the animals that resulted in an overall higher exploration activity during the OBJRececy test on the day following the OBJR test. Therefore, the previously conducted OBJR test may have influenced performance on the OBJRecency test.

Apparently, no rodent studies have so far investigated the development of OBJRecency discrimination abilities. However, in a human study of associative episodic memory in children and adolescents, the developmental trajectory of temporal memory processes has recently been investigated (Guillery-Girard et al., 2013). In this test children were asked to encode a sequence of animals and were subsequently asked to match them with their corresponding placement. Interestingly, the authors observed a marked increase in performance from 9 – 10 years of age followed by a plateau afterwards (measured at 11 – 12, 14 – 15, and 20 – 23 years of age). This test setting relied on several factors and processes like recency, location, and sequential memory.

Furthermore, the children were instructed to encode, remember, and later actively retrieve the sequence of animals. Debriefing after the test revealed that many adolescents employed language to encode a script in order to remember which animal followed after which one. Of course, this test setting for humans varies tremendously from the present OBJRecency test, as rats were presented with a test situation without any instructions or the knowledge of a possible subsequent test situation. Moreover, the rats' performance was deducted from their natural exploratory behavior; they did not (actively) have to recall the objects in the correct order. Additionally, in the rodent test one cannot be certain if the animal remembers a specific episode of having an object encountered before or if it has some sense of familiarity about it. Nevertheless, it is interesting that in both tests investigating temporal memory abilities first an increase at early ages before adolescence is found and this is then followed by no further variation until adulthood.

In the study by Guillery-Girard et al. (2013), the increase of associative memory was related to a decrease in gray matter volume in a network of cerebral structures including the dorsolateral and ventrolateral parts of the PFC, temporal regions and the Hip. Therefore, the developmental alterations of these structures appear to be linked to the observed behavioral performance. However, these regions were described to "subserve episodic memory efficiency as a whole" (Guillery-Girard et al., 2013), thus also including additional aspects of episodic memory like spatial and factual information measured in their paradigm. Finer relationships between the temporal component and brain structures could not be detected in this study. Lesion studies implicated the mPFC and the perirhinal cortex in the ability of rodents to successfully perform an OBJRecency test (Mitchell and Laiacona, 1998, Hannesson et al., 2004, Barker et al., 2007). Therefore maturational processes in these brain regions may contribute to the observed behavioral findings. Particularly the PFC displays a prolonged development (Paus et al., 1999, Sowell et al., 1999) and immaturities in this region on pd 31 may account for deficits in OBJRecency performance.

Altogether, the abilities of adolescent rats to perform an OBJRecency test appear to develop later than recognition abilities for an OBJR test with shorter ITIs. However, during the adolescence and early adulthood the ability to discriminate a more recently encountered object from an earlier encountered one remains approximately stable when tested every ten days. Therefore, recognition memory focusing especially on temporal aspects, i.e. recency of encountered objects, does not display the same developmental trajectory as object recognition memory. A delayed onset of these abilities was shown here with no significant variation of performance until adulthood.

Basal Development of Prepulse Inhibition of the Acoustic Startle Reflex

The longitudinal analysis of the development of PPI of the ASR and baseline ASR revealed a gradual increase for both measurements until adult ages. Both the percentage PPI and the startle amplitude of adolescent and young adult animals (from pd 30 – 80) were significantly lower than on pd 130, when the same animals were tested in adulthood. This difference was, however, not present anymore when the same animals were tested on pd 100 and pd 120, implicating that both the PPI and the ASR had already reached adult levels on these time points. Additionally, repeated testing in adulthood did not show any variation of PPI and ASR in a control group of adult animals (> pd 100), implicating that repeated testing every ten days does neither influence percentage PPI nor the startle amplitude *per se*.

The acoustic startle response is a reflex and, although it can be modulated by pleasant or unpleasant stimuli and is subject to individual variability (Koch, 1999), the reaction to a loud startling noise is a reliable measurement often used in animal research. Additionally, this reflex and its inhibition by a preceding weaker prepulse can be investigated in humans and has a high translational value (Braff et al., 2001). The startle reaction can be measured in rats as early as pd 18 (Engel et al., 2000, Martinez et al., 2000) although, it has been shown that the onset of hearing abilities in rats predates this age with auditory functioning starting at about the age of pd 12 – 14 and adult thresholds being reached on pd 22 (Geal-Dor et al., 1993). Yet, little is known about the basal development of the startle amplitude and the percentage PPI during adolescence in rats. Most studies interested in PPI during development employ a pharmacological interference prenatally or early in development and subsequently measure ASR and PPI in test animals and controls on distinct age points (e.g. Lipska et al., 1995, Engel et al., 2000, Martinez et al., 2000, Romero et al., 2010). However, the basal ontogeny of untreated animals has seldom been analyzed. In one study the ontogeny of the PPI in Sprague Dawley rats was investigated and animals were tested on pd 18, 20, 22, 25, 29, and 40 (Engel et al., 2000). In this study an increase of the PPI of the ASR was observed until pd 40 which was the last test point analyzed. The authors stated that adult levels of PPI had been reached by then. However, the adult levels, to which pd 40 PPI performance was compared to, were obtained from animals aged pd 58, thus from animals still categorized as late adolescent (Schneider, 2013). Furthermore, the percentage PPI still continued to increase further up until pd 100 in the present project. Similarly, PPI levels have been found to increase from pd 35 – 70 in control animals in a study investigating the influence of prenatal LPS treatment on the offspring of Wistar rats (Romero et al., 2010). In this study, both male and female rats displayed an increase of PPI through these age points. Although, the test points investigated in this study were spread further apart than in the present project

(pd 28, 35, 70, 170, and 400) so that the development between pd 35 and pd 70 was not analyzed in detail. Nevertheless, the continued increase of PPI fits the present results.

Interestingly, in human studies the modulation of the startle amplitude was first reported to have reached mature levels by the age of 8 years in children (compared to adults of 18 - 22 years of age; Ornitz et al., 1986, Ornitz et al., 1991). However, another study investigating the development of PPI during aging revealed an inverted U-shaped curve for PPI in adult subjects (Ellwanger et al., 2003). In this study, four age groups were analyzed for PPI and startle reaction: college (approx. 21 years of age), young (approx. 29 years), middle (approx. 41 years), and old (approx. 74 years). The mean startle magnitude decreased with age but PPI was highest in middle or young aged participants. Hence, the authors emphasized the importance of similarly aged groups when comparing PPI of adults. This is certainly also true for animal studies, particularly in developmental studies which compare “adult” animals that are often tested on various age points which are often still late adolescent ones.

Altogether, the present project shows the gradual increase of PPI and ASR in developing Wistar rats and reveals that both values increase up until pd 100 in the animals under basal conditions. It is therefore important to consider the age of test subjects, for example when comparing control groups between studies. Furthermore, this precognitive measurement shows a rather linear increase up until adult levels compared to the non-linear development of the OBJR discrimination performance.

Pharmacological Influence of SR141716 on Behavior during Development

Previous studies showed that the ECS is involved in OBJR memory (Schneider et al., 2008, Campolongo et al., 2013, Galanopoulos et al., 2014). Therefore, developmental alterations in the ECS during adolescence may be involved in the reduced object discrimination observed in the OBJR test on pd 40. Studies indicate an increased level of CB1R during the approximate onset of puberty (Rodriguez de Fonseca et al., 1993, Klugmann et al., 2011b) implicating a possible upregulation of the ECS around that time. Therefore, during this time in development, the animals may be more sensitive to the effects of the selective CB1R antagonist/inverse agonist SR than on earlier or later age points. In this part of the project the previously observed decrease in the object discrimination abilities of animals on pd 40 was observed again in vehicle treated animals and this deficit was ameliorated by a low does of SR (0.3 mg/kg i.p.). This effect was specifically observed on pd 40 but did not affect performance on earlier (pd 30) or later (pd 130) age points.

Apparently, no studies have so far investigated the effects of SR on basal cognitive abilities during development. However, one recent study observed impairing effects of a low dose of WIN (0.3 mg/kg) on OBJR memory in rats (aged 3 months) that were ameliorated by a very low dose

of SR (0.03mg/kg) (Galanopoulos et al., 2014). Although this was observed for longer ITIs than employed in the present project (both for short-term and long-term intervals of the OBJR test: 1h and 24h ITI respectively), these observations appear to be similar to the present project, in which a low dose of SR specifically improved the age-dependently decreased object discrimination abilities. Additionally, no correlation of discrimination performance and altered object exploration during P2 was observed in the study by Galanopoulos et al. (2014). Furthermore, injections of SR and vehicle (without WIN) did not display any effects on OBJR (Galanopoulos et al., 2014), similar to the present lack of an altered performance on earlier or later age points. Observations in operant studies revealed no improved memory performance when SR was administered alone (Brodkin and Moerschbaecher, 1997: 1mg/kg , Mallet and Beninger, 1998: 0 - 2 mg/kg). In contrast, SR alone has been shown to enhance memory in a radial arm maze and in a SOCR test (Terranova et al., 1996, Lichtman, 2000). However, in addition to employing different behavioral tests, these latter studies used higher doses of SR (3 mg/kg) making a direct comparison to the present project even more difficult.

Strain differences regarding the effects of low doses of SR have been observed before (Brand et al., 2012). In this study, Wistar and Fischer rats were compared in paradigms for reward sensitivity and additionally, ECS alterations were investigated. The Wistar rats displayed higher reward sensitivity for SCM and this was paralleled with higher CB1R and FAAH levels in the Hip compared to Fischer rats, indicating a basic higher eCB tone. Additionally, Wistar rats were more sensitive to the effects of SR regarding their SCM intake. Already a low dose of 0.3 mg/kg SR reduced the SCM intake in Wistar rats significantly whereas only higher doses (0.6 and 1.2 mg/kg) reduced the SCM intake in Fischer rats. Although this study did not investigate cognitive abilities, several important considerations have to be taken into account. Wistar and Fischer rats displayed differences in several ECS components (CB1R and FAAH levels), similar to the hypothesized differences between adolescent and adult rats (see above as discussed for OBJR memory; Rodriguez de Fonseca et al., 1993, Klugmann et al., 2011b). Additionally, the impact of a low dose of SR (0.3 mg/kg) was more effective in animals with a higher eCB tone. This may be comparable to the present observations in adolescent rats since a higher ECS during this time period was observed previously (Rodriguez de Fonseca et al., 1993, Klugmann et al., 2011b).

Alternatively, developmental alterations in other neurotransmitter systems may account for the observed ameliorated OBJR performance since the ECS modulates other neurotransmitter systems, e.g. indirectly the DA system (as discussed above for the findings in the OBJR test; van der Stelt and Di Marzo, 2003). In the context of the mesocorticolimbic pathway, inhibiting the ECS with a low dose of SR could restore the effects of potentially increased neurotransmitter

levels or activity in adolescence to adult levels. Thus, a low dose of SR could counteract the CB1R–mediated reduction of excitation of glutamatergic afferents from the PFC to the NAc. As mentioned above (in the context of the basal OBJR memory abilities), an enhanced ECS during adolescence may confer a stronger reduction of excitation, yielding a net increase of DA to the NAc and the PFC. Complementary, restoring the adult-like levels of inhibition of the medium spiny neurons from the NAc to the VTA would also restore adult-like DA release levels from the VTA to the PFC and NAc and thereby explain the ameliorated OBJR memory abilities of pd 40 old animals. Overall, in adolescent animals the DA release into the PFC and the NAc could be reduced to a level comparable to adult animals by a low dose of SR, enabling them to display similar OBJR performance. This would particularly affect adolescent animals since they display higher CB1R and DA receptor levels (Rodriguez de Fonseca et al., 1993, Andersen et al., 2000) whereby older animals with lower receptor levels may be less sensitive to low doses of SR. Therefore, it is possible that by influencing the ECS, and thereby indirectly the DA system, SR differently modulates recognition memory performance specifically on pd 40.

In the present part of the project, the object exploration time during P1 of animals on pd 30 was reduced by SR without affecting the object exploration at other age points. Interestingly, this reduction in exploration time on pd 30 did not impair the animals' ability to discriminate a novel from a familiar object. Although locomotor activity was not directly measured in this part of the project, it is possible that this low dose of SR reduced locomotor activity specifically in younger animals. Studies investigating the effects of SR alone on locomotor activity found contradicting results. For example, while some studies observed a reduction in locomotor activity (Jarbe et al., 2002, Jarbe et al., 2006), another study observed increased locomotor activity in an open field test (Costa and Colleoni, 1999). However, all of these results were found after considerably higher doses of SR (5.6 and 3 mg/kg SR respectively) were administered in Sprague Dawley rats. Thus, the low dose SR employed here was unlikely to induce locomotor effects in the adult animals and in fact did not influence exploration times of animals on pd 40 and pd 130. The youngest animals may have been more sensitive to the effects of SR on locomotor activity as they displayed a lower exploration time during P1, however, this did not influence their ability to recognize a novel from a familiar object.

Alternatively, if younger animals needed more time encoding the encountered objects (see Reger et al., 2009), the low dose of SR may have enabled them to perform successfully in this test even with a lower exploration time. However, the animals are not instructed to actively encode the objects for later retrieval, therefore if this was indeed the effect of the low SR treatment, it was an unconscious process.

Furthermore, the low dose of SR in the present project was ineffective in altering the startle amplitude or PPI performance on any of the tested age points. Similar to the present results, SR did not alter PPI or ASR in control Wistar rats in a study in which possible effects of the drug in SHR rats were investigated (Levin et al., 2014). Additionally, in another study investigating the ameliorating effects of SR on a PCP induced PPI deficit, SR administered alone did not influence PPI or ASR in Sprague-Dawley rats (Ballmaier et al., 2007). These results fit the present findings although in both studies higher doses of SR were used (0.75, 1.5, and 3 mg/kg) compared to the present study.

It is possible that the previously conducted OBJR test may have influenced the PPI of the ASR. If this was the case, the animals may have displayed a higher arousal state during pulse alone trials. However, this was not detected in ASR amplitude and is therefore less likely. Another issue may be the elapsed time between SR administration until the test for PPI and ASR which was conducted directly after the OBJR test. However, care was taken that all animals completed the tests within less than 2h after drug administration. Previous studies investigating the time-course of functional effects of SR administration showed that SR displayed a rather long duration of action (e.g. 15 h; McLaughlin et al., 2003). One recent study argued for a shorter duration of action, however, in their study the functional *in vivo* half-life was still around 2h (Jarbe et al., 2010), thus during the presently analyzed time-window SR should have not yet been completely washed-out of the animals' system. The previously mentioned studies investigating the influence of SR on PPI conducted the test 30 min after drug administration (Ballmaier et al., 2007, Brand et al., 2012, Levin et al., 2014) whereas in the present experimental design first the OBJR test was conducted 30 min after drug administration followed by the PPI test approx. 60 min after drug injection.

Further reasons may be involved for the differential findings of the influence of SR on the OBJR test and the PPI of the ASR. Although both tests analyze cognitive abilities, each paradigm investigates different aspects of cognition (i.e. pre-attentive filtering mechanisms vs. short-term recognition memory). Moreover, each test involves differently involved sensory modalities (acoustic vs. visuo-tactile) that are processed distinctly in the brain. The present results imply that these two aspects of cognition may be differently influenced by prior administration of SR but further experiments carefully eliminating any possible confounding factors should be conducted to confirm the lack of effect of the low dose of SR on PPI of the ASR during development.

Taken together, SR appears to affect the behavior of younger (pd 30 and pd 40) animals differently than older ones depending on the test paradigm employed. The altered ECS during adolescence may render the pd 40 old animals sensitive to effects of low doses of SR that are ineffective in younger and adult rats as observed in the OBJR test.

Molecular Analysis

Myelination

Ongoing myelination throughout adolescence and early adulthood has been observed in human studies (Sowell et al., 1999, Lenroot and Giedd, 2006) but the development of myelination in the adolescent rodent brain has been less well investigated. Measurements taken in this experiment on different time points during adolescence revealed a brain region dependent increase of myelination. In the CPu lower levels of myelination were found for adolescent animals (on pd 30, 50, and 60) compared to adult ones (pd 130). Interestingly, levels on pd 40 were not different from adult ones, thus already displaying comparably high levels of myelination. In the NAc no significant difference from adolescence to adulthood was observed. In the Hip all adolescent time points displayed lower myelination levels than adult animals, indicating an increase of myelination after pd 60 until adult levels were reached

Human studies investigating the development of myelination found links between increasing white matter and cognitive functions (Bava et al., 2010, Yeatman et al., 2012, Peters et al., 2014). For example, white matter connections between several brain regions are critical for proficient reading (Yeatman et al., 2012). In a MRI study possible associations between white matter maturation and reading skill development in children aged 7 – 15 years were investigated (Yeatman et al., 2012). In this study fractional anisotropy was measured in two important white matter tracts for reading abilities (the inferior longitudinal fasciculus and the arcuate fasciculus). Fractional anisotropy (i.e. directionality of diffusion) is influenced both by myelination and synaptic pruning (Yeatman et al., 2012) and high fractional anisotropy reflects highly myelinated fibers (Cascio et al., 2007, Bava et al., 2010). In their study the authors observed that children with above-average reading skills displayed initially a low fractional anisotropy followed by an increase of the latter. Complementary, below-average readers initially displayed high fractional anisotropy and a decrease over time. Furthermore, correlations of cognitive data and white matter changes were found in another MRI study comprising 16 – 20 year-olds (Bava et al., 2010). Increased myelination was observed (16 months after being tested for the first time in the same test subjects) and associated with higher performances in tests of complex attention, working memory, and verbal fluency.

Only few studies have investigated the development of myelination in rats so far (Jacobson, 1963, Norton and Poduslo, 1973, Meier et al., 2004, Mengler et al., 2014). Early studies described the progressive intensities of myelination semi-quantitatively (“faint, light, medium and dark”, Jacobson, 1963) or investigated myelin changes in brain myelin composition by isolating myelin components (Norton and Poduslo, 1973). However, more recent studies employed similar

methods as in the present part of the project and are partially in accordance with the present findings. One study investigating brain changes in volume and myelination during brain maturation focused on the cortex and the (dorsal) striatum (Mengler et al., 2014). In their analysis they combined longitudinal MRI scans of male Wistar rats with histological evaluations by black gold staining for myelin and cresyl violet staining for cell density levels. Although they investigated different time points histologically than in the current project (3 weeks, 3, and 6 months which corresponds approx. to pd 21, 90, and 180), their results correspond to the present findings for the CPu because the myelination was found to increase significantly from 3 weeks to 3 months of age. At 3 weeks of age only 5% of myelination was found which is still lower than the 26% measured in the present project on pd 30. However, at 3 months of age about 28% of the striatum was myelinated in their study (with no further increase from 3 – 6 months of age) and this corresponds to the approx. 30% found presently on pd 130. Interestingly, in the current project the myelination on pd 40 was already about as high as in adults, followed by lower levels on pd 50 and 60 and again higher levels in adulthood on pd 130. Thus, a non-linear development appears to take place in the CPu. Rather than interpreting this as a possible peak in myelination and a subsequent decrease thereafter (with a following increase to adult levels), other explanations may account for this observation. Myelination was quantified as percentage of the whole area and therefore changes in gray matter are likely to contribute to changes in relative myelination in this brain region. Concurrently, Mengler et al. (2014) found a pronounced increase in striatal volume between 3 weeks and 2 months of age (approx. pd 60) as measured by MRI. Furthermore, cell density decreased between 3 weeks and 3 months of age (Mengler et al., 2014). Therefore, additional processes of maturation and reorganization appear to take place in the CPu, possibly including synaptic overproduction and pruning. It is therefore likely that instead of a decrease in myelination taking place after pd 40, it is other processes which increase gray matter in this brain region and thus the relative amount of white matter, hence myelin appears lower.

Another study investigated the development of myelination particularly in the Hip (Meier et al., 2004). Myelin was analyzed from pd 17 and increased myelination until pd 25 was found employing a black gold staining method. Unfortunately, no further time points were investigated because the myelination levels on pd 25 “showed no remarkable difference compared with the adult” (Meier et al., 2004). However, the study did not specify the age of their adult control Wistar rats and instead stated that their weight ranged from 200 – 300g which is a large variation in bodyweight for a control group of animals. Therefore, it is unclear how old those controls had been at the time of analysis and if they may have still been adolescent. Wistar rats with a body weight of 200g are usually about 10 weeks old and a body weight of 300g corresponds to approx. 15 weeks (Harlan Laboratories, 2011). The results of the present project indicate that myelination

in the Hip increases profoundly between pd 60 and pd 130. It is therefore quite likely that the adults in the afore-mentioned study were investigated during adolescence.

In the current project the myelination in the mPFC was not quantifiable. Interestingly, Mengler et al. (2014) also found barely detectable myelinated fibers in the cortex at 3 weeks of age (about 1%). At 3 months they measured still only about 15% of myelinated fibers. This suggests a slowly increasing myelination over a prolonged period in the cortex. Thus, the age points investigated in the current experiment were presumably in a period when the relative myelination of the mPFC was too low to be quantified.

The present results imply an ongoing myelination process during adolescence in the Wistar rat that varies in different brain regions involved in cognition. Although myelination in the CPu did show an overall increase, this increase was not linear and the measured relative amount of white matter was possibly influenced by additional developmental processes. While no particular differences were detected on the presently investigated time points in the NAc, the Hip revealed an overall increase from all adolescent time points to early adulthood.

The last analyzed time point of the present investigation was pd 130 based on the plateau reached in the behavioral performance of the analyzed cognitive tests (i.e. OBJR; OBJRecency, PPI, and ASR) at that age although, it is possible that alterations of myelination are still ongoing in rats after this age point. Human studies indicate that peaks in white matter volume are reached between the twenties to mid-forties depending on the investigated tract (Hasan et al., 2010, Westlye et al., 2010, Kochunov et al., 2012, Lebel et al., 2012) and Mengler et al. (2014) still found increases of myelination from 3 – 6 months of age in the rat. However, as these time points are relatively far spaced (and the last measured time point here was in between of the time points analyzed by Mengler et al. (2014), the highest amount of myelination may be found somewhere in between these age points. Further analysis comprising both earlier and more detailed time points after adolescence and in young adulthood (between pd 60, pd 130, and pd 180) may delineate a more thorough picture of the development of myelination in the rat.

The present findings of the development in myelination over the course of adolescence in various brain regions involved in cognition shows an overall increase in myelination. This increase is brain region dependent and can be non-linear as in the CPu or gradually increasing as in the Hip. The behavioral performance in the OBJR test revealed a non-linear developmental pattern as well but the performance in other tests displayed a gradual increase (PPI of the ASR) or a delayed but less varied (OBJRecency) pattern. Increasing myelination during development is associated with WM improvements (Bava et al., 2010) and faster information exchange (Brenhouse and Andersen, 2011) and thus the increasing myelination observed in the present project probably also subserves increased performance observed in the cognitive tests. Conversely, deficits in

subcortical myelination have been associated with impairments of OBJR in rats (Wu et al., 2008) and myelin loss in the mPFC, CPu and Hip were associated with PPI deficits in mice (Xu et al., 2010). Because several brain regions are most likely to function in a coordinated network together in the investigated behavioral tests (Warburton and Brown, 2010) or are involved in the modulation of a certain cognitive aspect e.g. in the PPI of the ASR (Swerdlow et al., 2001), one can probably not simply conclude from the myelination pattern observed here in one brain region to a particular behavioral finding. It seems more likely that the myelination pattern in different brain regions develops differently throughout the adolescent time period as do various behavioral cognitive abilities. The myelination patterns (together with other neurodevelopmental alterations during adolescence) may contribute to the various behavioral performance patterns in cognitive tests until adult levels are reached. However, further and more detailed analyses are needed to establish the exact connections of myelination patterns linked to distinct behavioral output behaviors.

Staining and Western Blot Analysis of the Cannabinoid Receptor 1

CB1R staining in brain slices of animals aged pd 40 and pd 130 are in accordance with the staining pattern shown before (Egertova and Elphick, 2000). In the study by Egertová and Elphick (2000), staining in the mPFC was specifically found in layers II-III and layer VI, and this pattern was also detected in the stained sections in the current project for both age points. Similar staining patterns as in the study by Egertová and Elphick (2000) were also found in the CPu and additionally in the Hip, where intense staining was detected in the Stratum pyramidale, most intensely in the CA1-3 region but also in the DG on both age points. However, no significant difference between the age points could be obtained by quantification. In contrast, western blot analysis for CB1R in the current project revealed differences between early and adult age points in several brain regions. In the mPFC and in the NAc adult animals displayed a higher CB1R content than animals on earlier age points. In the CPu no differences were detected while in the Hip adolescent animals displayed higher levels of CB1R protein expression than adult ones.

The discrepancies between the results of the two methods could be due to several reasons. On the one hand, the samples were processed differently for each method (perfused and sectioned vs. dissected and homogenized) and different antibodies were used. On the other hand, staining of the CB1R reveals its distribution on the brain sections and this method may be more suitable for demonstrating its particular localization than its quantification. For western blot analysis whole regions of the brain were dissected whereas the stained sections only represent a small part of the designated brain area. Thus, the quantification of the staining in the sections may be more sensitive to larger differences than those detected here. Additionally, a lack of significant

differences could be due to the limited number of animals available for quantification in the section-based approach. Conversely, in western blot analysis the protein content of the CB1R in a certain brain region can be quantified but the cellular localization of it cannot be determined.

The expected molecular mass of CB1R is 52 kDA (Matsuda et al., 1990) but a prominent band of approx. 46 kDA was revealed by western blot analysis. The slightly lower band detected may represent the N-terminally unglycolsylated protein which is expected at about 46 kDA (Shire et al., 1995, Egertova and Elphick, 2000). Furthermore, a lower protein band of approx. 45 kDA for the CB1R has been observed before (Sim-Selley et al., 2006). Alternatively, the detected lower protein band may be due to possible alternative splice variants or differences in the amino acid sequence of the CB1R protein between species because the employed antibody was specifically raised against the mouse and human peptide sequence and its reactivity for homologues in the rat had not yet been tested (ImmunoGenes AG). Therefore, the stable protein band of about 46 kDA was used for quantification of the western blot analysis.

Previous studies showed partially controversial findings of CB1R levels during development. Either an increase of CB1R levels until adult levels was observed (Belue et al., 1995, Berrendero et al., 1999, Mato et al., 2003, Verdurand et al., 2011) or a peak during early adolescence followed by a reduction until adult levels (Rodriguez de Fonseca et al., 1993). For example, one study found CB1R binding levels peaked on pd 30 and pd 40 in male and female rats in limbic regions, the striatum and the mesencephalon (Rodriguez de Fonseca et al., 1993). Another study found higher CB1R binding levels in the frontal cortex, (further caudally located) cortex, Hip and cerebellum in young adult rats (pd 70 – 72) compared to adolescent ones (pd 35 - 37; Verdurand et al., 2011). However, studies during adolescence are limited as most of them focused on earlier developmental periods and omitted adolescence (Mato et al., 2003). Additionally, in some of these studies animals that may be still considered as adolescent (Schneider, 2013) are employed as an adult reference point (Belue et al., 1995: pd 60, Berrendero et al., 1999: > pd 56) similarly to conflicting results of behavioral findings (as discussed for the OBJR and PPI tests), thus complicating a direct comparison. Moreover, most studies investigated either radioactive ligand binding (e.g. Rodriguez de Fonseca et al., 1993, Belue et al., 1995, Berrendero et al., 1999, Mato et al., 2003, Verdurand et al., 2011) or *in situ* mRNA measurements (Heng et al., 2011, Long et al., 2012, Van Waes et al., 2012) instead of protein levels. Analyses of mRNA revealed a different pattern from CB1R levels and showed a general decrease until adult ages. One study found the highest levels of mRNA expression on pd 1 compared to pd 5 and 56 in various brain regions of Wistar rats including the cerebral cortex and the Hip (Berrendero et al., 1999). Others detected similar patterns of decreasing mRNA expression from pd 25 to pd 40 and 70 in the prefrontal cortex, limbic areas and the striatum of Sprague Dawley rats (Heng et al., 2011, Van Waes et al.,

2012). Furthermore, similar results were found in the human dorsolateral PFC where highest mRNA expression was detected in neonates and toddlers (Long et al., 2012). However, mRNA measurements do not necessarily predict the corresponding protein levels (Pascal et al., 2008, Bedse et al., 2014). Particularly for the CB1R discrepancies between protein levels and mRNA measurements may be explained by a differential distribution of these components. The CB1R is mainly expressed on synaptic terminals whereas the mRNA is synthesized in the cell body (Bedse et al., 2014). Furthermore, post-translational modifications and different half-lives of the components may also account for the detected variations (Pascal et al., 2008).

One study investigated the CB1R expression by fluorescent immunosorbent assay during adolescence (pd 29, 38, 50; Ellgren et al., 2008). In the NAc shell an age-dependent increase was measured, whereas in the NAc core, densities were decreased from pd 29 compared to pd 50. Similar to the present results, no differences were found in the CPu. But in contrast to the present findings a decrease was found in the PFC (from pd 29 to pd 38 in the study by Ellgren et al. (2008)). Unfortunately no earlier and later time points were investigated. Additionally, the animals had been injected with THC or vehicle (the older ones repeatedly) which may have posed a stress effect and makes the comparison of the vehicle group to the measurements in this experiment here difficult. Therefore comparison with the basal levels of the untreated animals of the present project should be considered cautiously. The higher CB1R levels in the Hip on pd 30 and pd 40 compared to adulthood measured in the present project are in accordance with the analyzed peak during this time in a study employing radioactive ligand binding (Rodriguez de Fonseca et al., 1993) although, no earlier time point was investigated in the present project. This finding may also explain the increased sensitivity of the adolescent animals to a low dose of SR as discussed before. During this time an elevated ECS activity with increased levels of CB1R protein content would render the animal more susceptible to pharmacological influences of a CB1R antagonist/inverse agonist, e.g. SR. Unfortunately, although the study by Rodríguez de Fonseca (1993) investigated many age points in both male and female animals, the brains were dissected into broader areas (into limbic regions, the striatum and the mesencephalon).

In the present project, no elevated levels during adolescence compared to adulthood were detected in other brain regions (mPFC, NAc, CPu). These results are in contrast to earlier findings in the (ventral) striatum and PFC where elevated CB1R levels were found in adolescent animals (pd 40) compared to adult ones (pd 100) animals (Klugmann et al., 2011b). Differences between the two studies include different rat lines (Wistar han hsd vs. rcc), different antibodies employed (both primary and secondary) and further methodological variations that may be accountable for the discrepancies.

Altogether, the western blot analysis showed regionally dependent differences in CB1R protein content over development that is only partially in line with findings from previous studies. In fact, these results add to the somewhat ambiguous picture in the present field of research. The increased CB1R levels in the Hip during early adolescence fit the hypothesized increased ECS at this age that may also explain the behaviorally observed decreased OBJR performance and ameliorated recognition memory after a low dose of SR. However, the role of the Hip in OBJR memory is still controversial (see Warburton and Brown, 2010). Several studies found that the Hip is not involved in OBJR *per se* but is rather needed in recognition memory that involves contextual cues like location or temporal information (Mumby et al., 2002, Barker and Warburton, 2011) and may therefore rather be connected to the observed delayed performance in the OBJRecency test. This would additionally rely on the interaction with other brain regions like the mPFC (Warburton and Brown, 2010). The findings that the Hip is less important in the OBJR test mainly rely on lesion studies and this kind of manipulation usually destroys a brain region (and sometimes adjacent tissue) to a great extend, thus direct comparison to the present developmental alterations has to be considered cautiously. Other studies showed an involvement of the mPFC and the striatum in OBJR memory as demonstrated by upregulation of the immediate early gene c-fos (Rinaldi et al., 2010, Barbosa et al., 2013). This may rather implicate functional interactions between multiple brain regions in the OBJR test. Furthermore, although in the present version of the OBJR test additional contextual cues like location information were minimized, it is difficult to deduce how the animals perceive the objects and the test situation (Ennaceur, 2010), thus contextual cues may be important and this would also argue for the involvement of multiple brain regions in the OBJR.

If a brain region involved in a cognitive process cannot entirely functionally operate (either if it is damaged or not fully developed yet) there is the possibility that other regions may compensate its function to a certain degree. For the Hip suggestions have been made according to which a mal-functioning one is worse than a non-existent one (Warburton and Brown, 2010). This may also contribute to the observed behavioral differential performance patterns in various cognitive tests. Furthermore, it has been shown that during working memory tests in children a shift from using more ventrally to more dorsally located regions of the PFC takes place over the course of development (Catts et al., 2013). Thus, different brain regions may become differentially involved in cognitive operations over the course of development. As the PFC is developing rather late (Fuster, 2001), its influence in cognitive tests may increase as the animal matures.

While the ECS in distinct brain regions is differentially developing throughout adolescence, the functional interactions between these regions most likely underlie the behavioral performance observed in the presently employed cognitive tests. Thus, the performance variations observed in

the behavioral tests may reflect *inter alia* the differential maturation of the ECS during adolescence in different brain regions. Conversely, adult like behavioral performance levels in cognitive tests are reached after developmental alterations in this (and possibly also additional) neurotransmitter system(s) have reached mature levels.

Conclusion

The main finding of the present project was that various behavioral tests of cognition display different developmental trajectories in the adolescent rat. This included a non-linear development of OBJR memory. After successful discrimination of novel from familiar objects in the OBJR test on pd 30 a performance decrease was observed on pd 40, followed by a gradual increase to adult levels (until pd 130). The successful performance in an OBJRecency test developed later than OBJR memory but remained stable across development. ASR and PPI developed gradually until adult levels were reached. Some of these behavioral alterations appear to be linked to the developing ECS since the influence of a low dose of the CB1R antagonist/inverse agonist SR specifically ameliorated the age-dependent dip in performance of OBJR on pd 40. Additionally, increased levels of CB1R in the Hip of adolescent animals were found in western blot analysis. Therefore, an enhanced ECS during adolescence may render the animals more sensitive to pharmacological agents affecting eCB tone and/or receptors. Furthermore, the development of myelination, which is associated with developing cognitive abilities, was shown to increase in a brain region-dependent way.

Neurodevelopmental processes during adolescence including the maturation of neurotransmitter systems and changes in gray and white matter take place with varying time-courses and patterns in distinct brain regions. The present molecular analyses showed that the CB1R levels and the relative myelination in brain regions involved in cognition display non-linear increases (e.g. the myelination in the CPu), gradual increases (e.g. the myelination in the Hip, the CB1R levels in the mPFC) or decreases (e.g. the CB1R levels in the Hip). These processes most likely underlie and contribute to the behavioral performances in the analyzed cognitive tests and may explain the variable developmental patterns in different aspects of cognition. However, due to the interconnection of brain regions and their functional interaction it is hardly possible to infer from the influence of one mechanism to a particular aspect of behavioral output from the present findings. While some brain regions develop rather late (e.g. the PFC) others develop earlier (e.g. subcortical regions) and this may create an imbalance during adolescence (Casey et al., 2008) which may result in the presently observed variable behavioral output in the cognitive tests. Altogether, successful mature cognitive performance necessitates a mature and interconnected network of brain regions including mature molecular components.

4.3 Project III: Long-term Effects of Chronic Pubertal WIN Treatment on Cognition

The present study revealed long-term effects of a chronic pubertal WIN treatment in adult male W[rcc] rats on cognitive skills. In an object recognition test both the percentage object discrimination and the discrimination index were decreased in WIN treated animals compared to vehicle treated controls. No lasting effects were observed for sensorimotor gating abilities or startle amplitude as revealed by PPI or ASR tests. In an ASST both WIN and vehicle treated animals learned to discriminate different rewarding sets and displayed similar set shifting abilities for both intradimensional shifts and an extradimensional shift. However, WIN treated animals displayed impaired reversal learning abilities based on the higher number of trials needed to reach the criterion during the reversal stage of the test. Western blot analysis for CB1R revealed a significantly increased protein level in the mPFC of WIN treated animals in adulthood. No differences in protein levels were observed in the CPu, the Hip, or for the degrading enzymes FAAH and MAGL in these brain regions.

Object Recognition

Several studies have shown long-term effects of a chronic pubertal cannabinoid treatment on the object recognition test (Schneider and Koch, 2003, O'Shea et al., 2004, O'Shea et al., 2006, Schneider and Koch, 2007, Schneider et al., 2008, Abush and Akirav, 2012, Renard et al., 2013). Particularly, chronic WIN or CP 55,940 treatments during adolescence have been shown to impair object recognition memory in adulthood (O'Shea et al., 2004, Schneider and Koch, 2007, Schneider et al., 2008). In the present project both the object discrimination and the discrimination index were significantly reduced in animals chronically treated with WIN during puberty and tested in adulthood compared to vehicle treated controls. The exploration time during P1 did not differ between the groups; therefore differences in exploratory behavior or locomotor activity are likely not accountable for the observed recognition memory deficit.

These results add to the previously mentioned observations and furthermore show that the W[rcc] rat line displays similar deficits after chronic pubertal WIN treatment as previously observed in W[hsd] animals. Rat line and strain differences for various behavioral paradigms are of great interest (see project I (4.1) and Rex et al., 1999, Brand et al., 2012) and only little attention has been directed to this topic in studies of long-term cannabinoid treatment. Recently, one study investigated the differential effects of chronic CP 55,940 treatment in Wistar and Lister Hooded rats during adolescence and adulthood (Renard et al., 2013). They found an impaired OBJR

memory for both Wistar and Lister Hooded rats (treated for 3 weeks starting from pd 29) after ITIs of 30 and 120 min. Although the present project was not intended to compare the two lines of Wistar Han rats directly it is important to validate that similar object recognition deficits persist in these two lines after the same chronic pubertal WIN treatment.

Various studies implicated different brain regions in the successful performance of OBJR memory (Dere et al., 2007, Rinaldi et al., 2010, Warburton and Brown, 2010, Barbosa et al., 2013). Lesion studies suggest that for OBJR memory that does not involve contextual information like changed location of an object or temporal information, the Hip and mPFC are not involved (Warburton and Brown, 2010). However, it is difficult to compare the present pharmacological manipulation with the extensive damage of a lesion study. Studies of immediate early genes implicate the mPFC and the striatum in OBJR memory in rats and mice (Rinaldi et al., 2010, Barbosa et al., 2013) and this indicates the involvement of multiple brain regions that are connected during OBJR test performance.

Interestingly, increased levels of the CB1R were found in the mPFC by western blot analysis in the present project. This may indicate a possible elevation of the ECS activity or sensitivity as a long-term effect of the pubertal WIN treatment in this brain region. The ECS is modulating a number of neurotransmitter systems directly and indirectly (see Kano et al., 2009) and may therefore influence OBJR memory via one or multiple of these systems. In the cortex, intense CB1R staining is found in layers II/III and V/VI (Hill et al., 2007, El Khoury et al., 2012). CB1Rs are mainly expressed by GABAergic interneurons and to a lesser extent by pyramidal neurons (Marsicano and Lutz, 1999). Studies investigating the localization of CB1Rs in the differential cortical layers as well as data from electrophysiology suggest that in layers II/III the receptors are mainly expressed by GABAergic interneurons (Katona et al., 1999, Trettel and Levine, 2002, Fortin and Levine, 2007) while in layer V they are suggested on glutamatergic terminals (Fortin and Levine, 2007). Layer V is the main output layer of the cortex while layers II/III are intercortically projecting layers (Fortin and Levine, 2007). Activation of the CB1Rs is supposed to elicit opposite effects in cortical network activity: In GABAergic terminals (where GABA release is suppressed upon CB1R activation) a disinhibition would result in a net increase of glutamate and in the pyramidal neurons CB1R activation would decrease glutamatergic activity (Fortin and Levine, 2007, El Khoury et al., 2012).

Although from the present results it is not possible to further elucidate where exactly the CB1Rs have been increased in the mPFC, their increase particularly in this brain area and not in other regions may result in an imbalance of network activity between this and connected regions. However, these connections are probably involved in successful cognition and therefore altered CB1R levels in the mPFC most likely underlie the cognitive deficits observed here. Furthermore,

the reduction in neuronal activity in the PFC has been associated with an imbalance of additional neurotransmitter systems like the DAergic system and brain regions like the NAc (Del Arco and Mora, 2008, El Khoury et al., 2012).

A hypothetical model has been described for the effects of adolescent cannabinoid exposure leading to prefrontal dysfunction (Caballero and Tseng, 2012). According to this model, cannabinoid exposure leads to functional maturational deficits of the prefrontal GABAergic interneurons that are developmentally regulated (Caballero and Tseng, 2012). This would lead to a reduced inhibitory tone of cortical pyramidal cells and consequently to a decreased synchronization of the prefrontal network. Additionally, it has been shown that coordinated network oscillations between the Hip and the mPFC are disrupted by the cannabinoid agonist CP 55940 which also decreases accuracy in performance of a working memory test (Kucewicz et al., 2011). Altogether, the increased levels of CB1Rs in the mPFC of adult animals appear to underlie the cognitive deficits observed after pubertal WIN treatment.

Prepulse Inhibition of the Acoustic Startle Reflex

No differences were observed between WIN and vehicle treated animals for PPI and ASR after chronic pubertal WIN treatment. Previously, a PPI deficit after chronic WIN treatment (from pd 40 – 65) was observed in the W[hsd] rat line (Schneider and Koch, 2003) or for earlier treatment periods (pd 15 - 40; Schneider et al., 2005). However, in addition to another rat line, the PPI in those former studies was conducted in a different apparatus (TSE vs. SR-LAB) using lower startle impulses (100dB vs. 115dB employed in the present study). Therefore, the lack of effect of the pubertal WIN treatment on PPI and ASR amplitude may be attributable to the possible differences in the two lines of Wistar Han rats and experimental settings. As previously discussed, strain and supplier differences have been found regarding the PPI of the ASR (Rigdon, 1990, Swerdlow et al., 2000). Additionally, different rat lines within the same rat strain revealed significant differences in basal PPI levels in project I (see 4.1).

Attentional Set Shifting

In the present project a chronic pubertal WIN treatment induced impairments specifically in the reversal stage of an ASST while leaving initial learning of the task as well as intra- and extra-dimensional shifting abilities intact. Intake of the food reward analyzed in a free intake paradigm before the ASST did not differ significantly between vehicle treated and WIN treated animals. Therefore, differences in reward perception are unlikely to have influenced the cognitive differences observed.

Several studies have investigated the effects of cannabinoids on the performance in complex cognitive tasks including attentional set shifting and reversal learning (Egerton et al., 2005a, Hill et al., 2006, Harte and Dow-Edwards, 2010, Sokolic et al., 2011, Wright et al., 2013). Mainly acute effects have been investigated and less attention has been directed to long-term effects of chronic treatments. For example, one study found impaired reversal learning and intradimensional set shifting abilities in rats after acute administration of 1 mg/kg Δ^9-THC (Egerton et al., 2005a). Similarly, in rhesus monkeys impaired reversal learning was observed after acute Δ^9-THC (0.1 – 0.5 mg/kg i.m.) administration (Wright et al., 2013). One study investigated the effects of a chronic intermittent Δ^9-THC treatment in rats (1 mg/kg i.p. once per week for 4 weeks; Allison, 2004). In this study deficits were observed for IDS, EDS, and the first reversal learning stage. However, animals were already tested 30 min after the last injection, which may not be enough time to exclude any possible acute effects of the treatment (Allison, 2004). As far as the author knows, the present project appears to be the first investigation of long-term effects of a chronic pubertal cannabinoid treatment on behavioral flexibility abilities measured in an ASST. The results showed that the abilities of adult rats to learn several discriminations (Pre, SD, CD) were intact both after chronic pubertal WIN and vehicle treatment. Additionally, transferring an acquired rule to a novel situation was also intact as observed in similar IDS performance levels. Moreover, shifting the previously learned rule when reward contingencies had changed was also similar in both groups as similar EDS performance levels were observed. The higher number of trials to criterion during this stage indicates that the EDS was more challenging than the others stages (see Barense et al., 2002, Colacicco et al., 2002) since subjects had formed attentional sets at this point and rats successfully learned the cognitive task.

In the reversal stage both groups needed more trials to complete the rule learning than in the previous CD stage although, this increase was less pronounced in the vehicle group compared to a previous study conducted with identical settings in our lab (Klugmann et al., 2011a). This may be due to, for example, differences between batches of animals (see Palm et al., 2011a) since also the trials needed to complete the pretraining stage differed between the present project and the previous study (Klugmann et al., 2011a). Nevertheless, in the reversal stage WIN treated animals displayed significantly more errors compared to vehicle treated animals. This strongly implies a lack of ability to inhibit a previously learned response while employing a new rule when reward contingencies have been changed (Egerton et al., 2005a, Bissonette et al., 2013). Thus, this reversal learning impairment is implicated as a long-term effect of a chronic pubertal WIN treatment on complex cognitive abilities.

Interestingly, similar observations were made in a previous study, after a viral-mediated upregulation of the CB1R in the mPFC of adult rats (Klugmann et al., 2011a). In this study both

the abilities to learn and to shift attention in IDS and EDS stages remained intact. But animals with increased levels of CB1R in the mPFC had difficulties reversing a previously learned rule compared to control animals. These behavioral observations resemble those of the present project after chronic pubertal WIN treatment. It is therefore highly likely that the pubertal WIN treatment may have altered the ECS in adulthood, possibly by increasing CB1R levels as a long-term treatment effect, providing similar behavioral outcomes in the reversal learning ability of the ASST.

Although, in lesion studies the OFC was shown to be critical for reversal learning (McAlonan and Brown, 2003, Kim and Ragozzino, 2005), other studies have suggested the involvement of additional brain areas in reversal learning (Joel et al., 1997, Li and Shao, 1998, Oualian and Gisquet-Verrier, 2010, Klugmann et al., 2011a). For example, one study showed that lesions restricted to either the PL or the IL region of the mPFC induced reversal learning deficits in a T-maze task (Li and Shao, 1998). More recently, similar observations were found in the reversal stage of a strategy shifting task in IL and/or PL cortices-lesioned rats compared to sham-lesioned controls (Oualian and Gisquet-Verrier, 2010). Lesioned animals displayed higher preservative errors for rule shifting and reversals. In this study the authors suggested that these brain regions are specifically involved in the resolution of a response conflict, i.e. either testing for and choosing a previously irrelevant strategy or selecting and maintaining that strategy (rather than inhibiting a previously valid response). However, in the ASST employed in this project it is not possible to differentiate whether the animals were not able to inhibit a previously learned rule or to look for, select, and maintain a novel strategy during the reversal stage of the test. It is possible that, during this stage, the combination of both, the inhibition of the previously learned rule and the novel strategy selection, is dependent on the integrity of the mPFC (and possibly additional brain areas). This appears to be compromised by the chronic pubertal WIN treatment leading to the increased number of trials needed to complete the reversal learning stage in the adult animals. Because no impairments in IDS and EDS were observed in the present project, the WIN treated animals appear to be able to look for, select, and maintain a new strategy in the present paradigm, therefore strengthening the possibility of deficits in inhibition of a previously learned rule (or a combination of inhibition and novel rule selection). However, one has to consider that, in addition to using a strategy set shifting paradigm in a Y-maze (instead of the presently employed digging test), in the study by Oualian and Gisquet-Verrier (2010) the ibotenic acid lesions were conducted in Sprague-Dawley rats, thus making a direct comparison difficult.

Further evidence suggesting the involvement of additional brain areas in the ASST performance comes from molecular analysis. Acute Δ^9-THC treatment was observed to alter immediate early gene expression of c-fos and ngfi-b in the dorsolateral striatum and frontal cortical regions

(including the PL and the IL frontal cortical regions; Egerton et al., 2005a). Furthermore, associations between this altered mRNA expression and reversal learning in the NAc and the dorsolateral striatum were found in this study.

Behavioral evidence for the involvement of cortico-striatal interactions in behavioral flexibility was also observed, again in strategy set shifting tests (reviewed by Floresco et al., 2009). According to a hypothetical neural circuitry, different components of a network between the PFC, the NAc, and the mediodorsal thalamus contribute to behavioral flexibility during strategy set shifting (Block et al., 2007, Floresco et al., 2009). Inputs from the PFC to the NAc appear to facilitate the maintenance of a novel behavioral strategy whereas DAergic inputs to the PFC are involved in disengaging from a previously employed strategy. Thus, disconnections between the PFC and the NAc induced an increase in preservative errors which, according to the authors, may be “attributed to disruptions of the transfer of updated information from the PFC regarding the success or failure of current strategies to the NAc for maintenance of a novel discrimination strategy“ (Block et al., 2007).

This is comparable to the present observations of a deficit in reversal learning in the ASST where animals appear to have difficulties in disengaging from a previously rewarded strategy, while maintaining a new strategy. Therefore, the functional network between the PFC and the NAc appears to be required for successful completion of the test.

Complementary, alterations of neurotransmitter systems (e.g. the DA system) in these areas may cause deficits in one or more aspects of the test. DAergic transmission in the NAc is increased by natural rewards and the mesocorticolimbic pathway is important in acquiring behavior reinforced by natural rewards (Spanagel and Weiss, 1999). An involvement of the ECS in indirectly modulating the DA system has previously been observed (reviewed by van der Stelt and Di Marzo, 2003). Additionally, previous studies found that repeated administration of Δ^9-THC or WIN decreased DA turnover in the rat PFC which persisted up to two weeks following treatment cessation (Jentsch et al., 1998, Verrico et al., 2003) and this may underlie the impaired reversal learning abilities. A reduction in DA transporter levels in the striatum was found in one study after a methamphetamine treatment which also induced reversal learning impairments (Izquierdo et al., 2010). However, modulation of the DA system by the ECS was not measured in the present project and a possible involvement in the behavioral observations is therefore highly theoretical.

Overall, the present project revealed that a chronic pubertal WIN treatment impaired reversal learning of animals tested in an ASST in adulthood therefore, implicating long-term cognitive effects of this treatment. This chronic pubertal WIN treatment has been suggested as an animal model for aspects of schizophrenia (Leweke and Schneider, 2011) and patients suffering from this disorder displayed more preservative errors and responses in the Wisconsin Card Sorting test (an

analogous test of the presently employed ASST; Van der Does and Van den Bosch, 1992, Everett et al., 2001, Hartman et al., 2003). Additionally, heavy marijuana use in humans is associated with deficits in behavioral flexibility measured in the Wisconsin Card Sorting Test (Bolla et al., 2002, Lane et al., 2007). Moreover, particularly prefrontal regions (the dorsolateral PFC and the anterior cingulate cortex) have been shown to display higher CB1R levels in schizophrenic patients (Dean et al., 2001, Zavitsanou et al., 2004) and similarly, higher CB1R levels may be the long-term effect of the presently administered pubertal WIN treatment. Altogether, these observations are in line with the present results and suggest long-term effects on behavioral flexibility skills (particularly reversal learning) after an interference with the developing ECS.

Western Blots of Cannabinoid Receptor 1, Fatty Acid Amino Hydrolase and Monacylglycerol Lipase

Western blot analysis for CB1R, FAAH, and MAGL were conducted in adulthood (> pd 100) after a chronic pubertal WIN treatment and brain regions implicated in cognitive skills (mPFC, CPu, Hip) were investigated. In the mPFC a significant increase of CB1R was found in WIN treated animals compared to vehicle treated controls. No differences of CB1R expression levels were measured in the CPu, the Hip, or for the eCB degrading enzymes FAAH and MAGL in any of the three brain regions investigated.

Effects of repeated cannabinoid administration on CB1R expression have been investigated in several studies (Romero et al., 1998, Sim-Selley and Martin, 2002, Breivogel et al., 2003, Sim-Selley et al., 2006). These studies mainly found a CB1R downregulation or desensitization after injection (or cannulation as in the study of Breivogel et al., 2003) of Δ^9-THC or WIN in adult rats or mice. However, these studies employed adult animals and focused on time points soon after treatment. For example, 24h after the last injection of a Δ^9-THC treatment (lasting 1, 3, 7, or 14 days; Romero et al., 1998), 1, 3, 7, and 14 days after the final injection of a treatment of Δ^9-THC or WIN (lasting 15 days; Sim-Selley and Martin, 2002, Sim-Selley et al., 2006), and 25h after a Δ^9-THC infusion treatment (lasting 4 days; Breivogel et al., 2003). Thus, it appears that a short-term consequence of repeated cannabinoid treatment in adulthood is a downregulation of the CB1R. However, several studies also implicated that this short-term effect is only transient (Sim-Selley et al., 2006, Hirvonen et al., 2012). In one study, in which mice received either incremental doses of Δ^9-THC or WIN for 15 days, receptor levels returned to untreated levels in the striatum and globus pallidus after 7 days and in the Hip after 14 days of treatment cessation (Sim-Selley et al., 2006). Additionally, a PET study found a reversible and regionally dependent downregulation of the CB1R in human chronic cannabis smokers (approx. 28 – 32 years old Hirvonen et al.,

2012). After about 4 weeks of continuously monitored abstinence these levels returned to control levels further indicating that this downregulation appears to be a transient effect.

Several other studies have investigated the effects of a chronic adolescent cannabinoid treatment on the CB1R levels in rodents (Rubino et al., 2008, Dalton and Zavitsanou, 2010, Chadwick et al., 2011, Winsauer et al., 2011, Behan et al., 2012, Winsauer et al., 2012). However, similarly to the studies mentioned above, samples were often taken 24h after the last injection, revealing CB1R downregulation as a short-term effect of a chronic cannabinoid agonist, e.g. 25, 50, or 100 µg/kg HU210 i.p. for 4 or 14 days (Dalton and Zavitsanou, 2010) or a Δ^9-THC treatment in adolescence with escalating doses of 2.5 – 10 mg/kg i.p. from pd 35 – 45; (Rubino et al., 2008). The latter study also investigated long-term effects of a chronic Δ^9-THC treatment in samples taken between pd 75 and pd 80. Decreased CB1R levels were observed only in the amygdala of male rats. Differences between this and the present study are the drug used, dosage, and treatment time as well as strain differences. Another study found no effect in the PFC and Hip of adult male mice (sample preparation on pd 150 – 160) after an adolescent Δ^9-THC treatment (8 mg/kg s.c. from pd 32 - 52; Behan et al., 2012). Other studies found further contradicting results after adolescent Δ^9-THC treatment (5.6 mg/kg i.p. from pd 35 – 75) of female Long-Evans rats (Winsauer et al., 2011, Winsauer et al., 2012). In their first study these authors found an increase of CB1R in adulthood (measured approx. on pd 200) in the Hip (Winsauer et al., 2011) whereas, in a later study, no alterations were found in the Hip although reduced levels were measured in the striatum (where no alterations had been observed previously after Δ^9-THC treatment; Winsauer et al., 2012). However, in their second study the western blot analysis appears to have been conducted a bit earlier (around pd 180) than in the first one. Several other differences to the present results further complicate direct comparison. Although they checked for estrus stage to control for possible effects of circulating endogenous hormones, these studies investigated females. Moreover, in addition to treatment differences (drug and doses employed, age of administration, and rat strain), the control animals were sham-operated and injected again with Δ^9-THC during a progressive ratio learning task after pd 76. Thus the current literature investigating the effects of cannabinoid administration during adolescence on CB1R expression is somewhat limited and controversial. The present results of an increased CB1R expression in the mPFC in adulthood after more than 35 days of a drug-free period suggests a long-term effect of the chronic pubertal WIN treatment. This effect may be a developmental compensation for disturbances of the ECS during this critical period of neurodevelopment. Interestingly, a recent study investigating effects of a repeated stress paradigm on CB1R levels observed region- and age-dependent effects in adolescent and adult rats (Lee and Hill, 2013). In this study CB1R levels in the PFC were increased 24h after stress both in adolescents and in adult animals. Moreover, after a recovery

period of 40 days, CB1R levels in the PFC of the animals after stress treatment in adulthood returned to normal levels whereas a sustained downregulation in the PFC of animals after stress treatment in adolescence was observed. This effect was attributed to the prolonged development of the PFC which could have been disturbed by the treatment specifically in the adolescent time period. This study shows that limited treatments during adolescence can cause long-term effects on CB1R levels that are different from the effects of the same treatment in adulthood.

This would be in line with a possible explanation of the present results. Accordingly, the presently employed pubertal WIN treatment increases the CB1R levels specifically in the mPFC due to its prolonged development and may also account for the region-specifically observed effect here compared to the CPu and the Hip where no alterations were detected. Additionally, this increase may only be found at a particular time window of cannabinoid administration during adolescence due to the enhanced vulnerability at this time period of continuing developmental processes and constitute a compensatory mechanism for disturbing the developing ECS. Investigating the time-course of these long-term CB1R alterations more thoroughly by including more time points may show similar patterns in other brain areas. Regionally dependent alterations of the CB1R are in accordance with results of a previous time-course study, however, this study did not investigate long-term effect (as mentioned above; Romero et al., 1998). Altogether, the long-term effects of a chronic pubertal WIN treatment are increased CB1R levels in the mPFC of adult rats which may be a compensatory mechanism for the disturbances of the ECS during development.

Conclusion

The present project revealed long-term deficits of a chronic pubertal WIN treatment on cognitive abilities in adult animals. Decreased OBJR memory performance has been observed before in the W[hsd] rat line (Schneider and Koch, 2007, Schneider et al., 2008) and could here be confirmed for the W[rcc] rat line. No effects could be found for sensorimotor gating abilities and startle amplitude in the PPI of the ASR. In an ASST specifically the reversal learning ability was impaired while other learning abilities remained intact, thus indicating deficits particularly in the complex behavioral flexibility domain of reversing a previously learned rule. This is in accordance with previously found similar behavioral observations in the ASST after viral-mediated upregulation of the CB1R (Klugmann et al., 2011a). In this previous study a long-lasting upregulation of the CB1R was specifically found in the mPFC (Klugmann et al., 2011a). Similarly, molecular analysis revealed increased levels of CB1R in the mPFC in the current project. Thus, the long-term effects of the pubertal WIN treatment resulted in a distinct elevation of the CB1Rs in the mPFC of adult animals possibly increasing the ECS activity or sensitivity in this region. This most likely underlies the deficits in OBJR memory and reversal learning abilities

in the ASST since the ECS is modulating neurotransmitter systems involved in cognition (Lopez-Moreno et al., 2008, Kano et al., 2009, El Khoury et al., 2012). Furthermore, the mPFC is connected to multiple brain regions that most likely act in a coordinated way during various cognitive tests and imbalance in signaling between these regions is associated with cognitive deficits (Kucewicz et al., 2011, El Khoury et al., 2012).

Overall, the long-term molecular alterations in the mPFC after chronic pubertal WIN treatment may demonstrate a compensatory mechanism for disturbances of the ECS during the vulnerable developmental period of adolescence which are probably region specific in their manifestation (Schneider, 2008).

4.4 Project IV: Long-term Effects of Chronic Pubertal MPH Treatment on Cognition

In this project the long-term effects of a chronic MPH treatment with 2 mg/kg during puberty (pd 40 – 55) were investigated in adulthood (> pd 80) 25 days after treatment cessation in male Wistar rats. Tests for locomotor activity and anxiety related behavior did not show any behavioral differences between MPH and saline treated animals. There were no differences found either in the intake of liquids of differing palatability or in cognitive tests between MPH and saline treated animals.

Open Field

MPH increases locomotor activity upon acute administration (e.g. Yang et al., 2011, Jones and Dafny, 2013). However, the effect of chronic administration has been less well investigated. One study showed that MPH (2 mg/kg i.p. for 28 days starting either at pd 25 or pd 60) was the major modulator (in addition to light cycle) in locomotor behavior (Gomes et al., 2009). However, in their experimental design animals were tested 2 h after the last injection not ruling out any possible acute effects of the MPH treatment. In this project, no effect of 2 mg/kg MPH during adolescence was found in adult animals. Similar to the present results, one study showed that Wistar rats treated with 1, 2, or 10 mg/kg MPH i.p. for 28 days (pd 25 – 53) showed no increased locomotor activity in adulthood (pd 67) after a 14 days long wash-out period (Valvassori et al., 2007).

Anxiety-Related Behavior

Several studies have investigated the possible long-term effects of a chronic MPH treatment but the results vary considerably for anxiety related behavior in rats. On the one hand, an increased anxiety-like behavior was observed (for a treatment paradigm of 2 mg/kg MPH administered i.p. twice daily from pd 20 – 35 and testing 6 or 8 weeks after treatment Bolanos et al., 2003, Bolanos et al., 2008, Wiley et al., 2009). On the other hand, an anxiolytic-like effect was observed after a 5 mg/kg MPH i.p. treatment twice daily from pd 7 – 35 (Gray et al., 2007). But also no effect could be observed for 2 mg/kg MPH i.p. from pd 27 – 53 and testing on from pd 92 – 94 (Britton and Bethancourt, 2009) or a 21 day long lasting s.c. administration of MPH (2, 4, or 8 mg/kg) via osmotic minipumps (Gill et al., 2013). In addition, an EPM test in mice revealed no differences in anxiety related behavior after a 7 day long treatment of 2.5, 5, 10, 20, 40, or 80 mg/kg MPH s.c. twice daily from pd 26 – 32 (McFadyen et al., 2002).

These contradictory results are often attributed to differences in either the employed behavioral tests or treatment paradigms. One study even found contradicting results within the same treatment conditions (Crawford et al., 2013) where the same dose of MPH (5 mg/kg i.p. from pd 11 – 20) showed an increased anxiety-like phenotype in an EPM test whereas a novelty CPP suggested a reduced anxiety-like behavior in the same animals. The authors stated that the novelty CPP is generally very similar to the EMT test and this usually reveals similar results as the EPM. However, as anxiety is a multidimensional emotional behavior, different test paradigms can lead to various outcomes (Ramos, 2008).

In this project no significant difference was found in the anxiety-related behavior between animals treated chronically with MPH during puberty and saline control animals neither in the EMT nor the EPM test, which is similar to the results found in previous studies (Bethancourt et al., 2009, Gill et al., 2013). These studies employed similar doses of MPH and analyzed the behavior of rats on the EPM. Particularly Britton and Bethancourt (2009) investigated the behavior of Wistar rats after a similar time of treatment (pd 27 – 53) and tested the animals in adulthood (pd 92 – 94 for the EPM) which is a similar regimen as in this project. For 2 mg/kg there was no difference in behavior which is in line with the present results, apart from a trend for a higher open arm entry rate of MPH treated animals observed in the present project. This trend might be explained by a higher arousal state of the animals in the novel environment of the EPM. In the EMT Britton and Bethancourt (2009) found a decreased time spent in the lit compartment of 2 mg/kg treated animals but other measures (latency, risk assessments and transitions) did not differ.

Intake of Liquids of Variable Palatability

The consumption of SCM was investigated in only one other study (Eckerman et al., 1991). In this study a decrease after a 15 min intake paradigm was found after an acute injection of MPH. But they employed a repeated drinking paradigm and used eight months old animals. In addition, the injected MPH concentrations were much higher than in this project (2.5 – 17.5 mg/kg). The consumption of sucrose however, was investigated during a ten days MPH treatment in food restricted male rats (Bello and Hajnal, 2006, Gill et al., 2013) and a reduced intake at a dose of 1.5 mg/kg MPH was observed. Nonetheless, in this paradigm adult rats were used and the intake was measured 3 days after treatment onset, therefore probably analyzing also acute effects of MPH.

Other studies showed that chronic administration of MPH (2 mg/kg i.p. twice daily from pd 20 for 16 days or 15 days respectively) can reduce the preference for a sucrose solution (Bolanos et al., 2003, Bolanos et al., 2008). In contrast, another study showed that, in a lever press task for sucrose pellet intake, chronic MPH treatment increased break points in treated animals compared

to controls (Crawford et al., 2007). This task however did not investigate the free choice intake of palatable food but used a conditioning paradigm. Similar to the results of the SCM intake in this project, one recent study found no differences between MPH treated and control animals for sucrose solution intake or preference in a free choice two-bottle paradigm (Crawford et al., 2013).
There is an ongoing discussion whether the treatment with MPH might increase the risk of drug addiction (Lambert and Hartsough, 1998) or rather reduce the risk of substance abuse disorders (Biederman et al., 1999). Interestingly, only very few studies have investigated whether the treatment of MPH influences ethanol consumption or preference in animal models so far. One study found that adolescent MPH treatment (2 mg/kg i.p. twice daily for 16 days from pd 23 – 38) increases EtOH consumption only in female and not male SHR rats (Vendruscolo et al., 2008). Similar to the present project no difference in EtOH preference was found. However, SHR rats are used as an animal model of ADHD which makes the comparison to the Wistar rats in this project difficult. Additionally, another study did not find a changed consumption of EtOH in SHR or Wistar-Kyoto rat (i.e. the control strain from which SHR were derived) after animals had been treated with MPH from pd 21 – 35 and tested after pd 50 (Soeters et al., 2008). In contrast, after an acute treatment with MPH in adult mice a decrease in EtOH consumption was found (Griffin et al., 2010). However, in addition to the species differences (mice vs. rats) the acute administration of MPH makes a comparison of the present project with this study difficult. Altogether there appear to be contradicting effects of MPH treatment on EtOH intake and preference as found in the literature. In the present project, no differences in the preference of a 6 or a 10 % EtOH solution was observed.

Cognitive Tests

Sensorimotor gating like PPI of the ASR can be modulated by dopaminergic agents (Swerdlow et al., 1995). For example the DA agonist apomorphine can disrupt PPI (Mansbach et al., 1988, Geyer et al., 1990). A recent study found reduced PPI in ADHD patients (Schulz-Juergensen et al., 2014) and this has also been observed previously in ADHD patients with a comorbidity for Tourette's syndrome (Castellanos et al., 1996). However, another study reported no reduced PPI in ADHD (Ornitz et al., 1999). MPH as a treatment for ADHD seems to have an effect on PPI if the subjects were instructed to focus their attention on the prepulse (Hawk et al., 2003, Ashare et al., 2010). Both, the study done by Ashare et al. (2010) and Schulz-Juergensen et al. (2014) furthermore found that MPH increased PPI in ADHD patients with a low baseline PPI. Regarding the ASR, one study found that MPH restores the normal attenuation and potentiation of startle modulation in response to pleasant and unpleasant visual stimuli respectively in ADHD patients

(Conzelmann et al., 2011). Before treatment these patients had displayed deficient responses to pleasant stimuli.

Only few rodent studies have investigated the effect of MPH on PPI and ASR so far. Interestingly, only acute doses of MPH were analyzed and mainly an increase of startle and an impairment of PPI was found for rats (Drolet et al., 2002, Conti et al., 2006, McLaughlin et al., 2011). The doses applied were all higher than in the current project (5, 10 and 20 mg/kg) and testing was conducted shortly after injection. In contrast, one study found an increased PPI after administration of 4 mg/kg MPH s.c. (Palsson et al., 2011). It is difficult to compare the impact of the chronic MPH treatment in this study to the results of the studies mentioned above. Here no long-term effect of a chronic MPH treatment on ASR and PPI was observed in adult rats. From a translational point it could also mean that the group of animals tested here was too heterogeneous to show any effects of MPH (as in the human studies without the division of high and low baseline PPI). Most human studies focus on ADHD patients in contrast to healthy individuals. In addition, effects of MPH in humans could only be observed in specific subgroups e.g. individuals with a low baseline PPI or those who were instructed to focus their attention to the prepulse. From the present results in adult Wistar rats the chronic treatment of a low dose of MPH from pd 40 – 55 does not seem to have a long lasting impact on ASR and PPI performance.

Several studies investigated the effect of MPH treatment on OBJR performance, some of which found no effect while others found an impairment in performance (Heyser et al., 2004, LeBlanc-Duchin and Taukulis, 2007, Bethancourt et al., 2009, Gomes et al., 2009, LeBlanc-Duchin and Taukulis, 2009, Pires et al., 2010). High doses generally appear to impair memory for objects whereas low doses do not have such an impact (for review see Britton, 2011). For example, one study found a disruption of novel object discrimination after a 7 days treatment (from pd 15 – 21 or pd 28 – 34) of MPH for 5 mg/kg but not for 2 mg/kg in Sprague-Dawley rats (Heyser et al., 2004). However, this treatment was injected i.p. twice daily and testing occurred 30 min after the last injection, not ruling out any possible acute effects of the drug. An impairment of OBJR performance was also observed by another study after MPH treatment of rats starting from pd 35 – 39 for 21 days (3 or 5 mg/kg administered orally twice daily with drug free days on weekends; LeBlanc-Duchin and Taukulis, 2007). In this study the animals were tested 14, 28, and 42 days after treatment and the impairment persisted up until the last testing point. Interestingly, in the same study, animals treated with 2 mg/kg for only 11 days did not show any impairments of OBJR memory. This is very similar to the present results, although the ITI was longer (3h compared to the 15 min in the present project) and MPH was administered orally. Furthermore, similarly to the results of the present study, no impairments of OBJR performance were found by another study (Bethancourt et al., 2009) although, rats were treated orally twice daily from

pd 27 – 71 with 2 and 5 mg/kg MPH and performance was analyzed during the dark phase of the light/dark cycle 18 days after treatment cessation.

A more recent study in female rats revealed that the chronic effect of MPH (2 mg/kg i.p. from pd 25 – 38) in SHR rats improved OBJR performance, but at the same time impaired discrimination abilities in control Wistar rats (Pires et al., 2010). Here, animals were tested four weeks after the treatment had ended (at 9 – 10 weeks of age). However, in that study only female animals were observed.

The social aspect of MPH effects has received less attention so far. One study observed that MPH suppressed social play behavior without abolishing general social interest (Vanderschuren et al., 2008). In contrast, another study found no effect on social play behavior (Bolanos et al., 2003). Both studies did not investigate social memory. One more study also investigated MPH influence on social interaction and found no effect (Leblanc-Duchin and Taukulis, 2004). As far as the author is aware this is the first time social memory has been investigated after chronic pubertal treatment with MPH. Here, no differences between chronically MPH and saline treated animals in the ability to recognize a conspecific rat could be observed. Altogether, the current results imply that long-term MPH treatment during this specific time period of adolescence does not appear to have a specific effect on the abilities of rats to recognize an object or a social partner.

General Conclusion

The time window of MPH treatment, onset, and duration varies considerably between studies, starting as early as pd 7 (Gray et al., 2007) and pd 11 (Crawford et al., 2013) or around and shortly after weaning (pd 20: Bolanos et al. (2003), pd 25: Britton et al. (2007), pd 27: Britton and Bethancourt (2009)). Treatment duration varies from 7 days (Heyser et al., 2004) to 15/16 days (Britton et al. (2007) and Bolanos et al. (2003) respectively) but also longer periods like 4 or 7 weeks have been employed (Gray et al., 2007, Britton and Bethancourt, 2009). Some studies administer the drug twice a day (Bolanos et al., 2003, Britton et al., 2007) , others only once a day (Adriani et al., 2006). Moreover, in some studies the weekends are drug-free days (Britton and Bethancourt, 2009) whereas others continue treatment on every day (Bolanos et al., 2003).

In general, most studies investigated treatment during the juvenile period or early adolescence whereas prolonged treatment until early adulthood is only rarely investigated.

However, from a translational perspective and in the context of neuroenhancement the age group of MPH users includes high-school students that take MPH to increase their performance in school. Furthermore, these adolescents often take MPH while during this age period most of them start to drink alcohol. This was why in the current project the time between the approximate onset

of puberty (pd 40) up until almost early adulthood (pd 55) in males was of particular interest and the influence of MPH on EtOH intake was investigated.

Additionally, the time of testing often varies and animals are tested shortly after treatment cessation (24h: McFadyen et al., 2002) or directly after the last treatment (Gill et al., 2013) when acute effects of MPH can not be ruled out. Our testing time had started after the animals reached adulthood at > pd 80 after a drug free period of 25 days therefore, any potential acute effects are unlikely. However, the time that elapsed after treatment cessation and the start of behavioral testing might explain the lack of effects in the current project. It can also imply that the specific time window between pd 40 and pd 55 employed here does not yield any lasting effects of the drug treatment.

Also the route of drug administration varies across studies including i.p. injection (e.g. Adriani et al., 2006, Crawford et al., 2013) as used in this project or oral administration (e.g. Britton and Bethancourt, 2009) but also osmotic minipumps are being employed (e.g. Gill et al., 2013). Moreover, different doses of MPH are employed often ranging from 2 – 5 mg/kg.

Differences in employed strains of rats also make it difficult to compare studies. Often Wistar rats are investigated (this project, Adriani et al., 2006, Britton and Bethancourt, 2009) but also Sprague Dawley rats are used (Bolanos et al., 2003, Crawford et al., 2013, Gill et al., 2013). The importance to mind possible differences between rat strains has been mentioned before (see project I; Rex et al., 1999, Brand et al., 2012), even within the same rat strain but in different lines or obtained from different suppliers (Langer et al., 2011, Palm et al., 2011b, Goepfrich et al., 2013).

Altogether, in the current project a chronic, 2 mg/kg MPH treatment of male Wistar rats from pd 40 – 55 injected i.p. once a day and with the beginning of behavioral testing after pd 80, did not induce any persistent behavioral effects. Nevertheless, the lack of enduring effects of a MPH treatment are no evidence for the safety of prolonged MPH use (Bethancourt et al., 2009). More research is needed to determine if there are developmental periods more or less risky to use psychostimulant medication**.**

4.5 Conclusion

In the present thesis various behavioral and molecular characteristics of basal cognitive abilities, the influence of the ECS and the development throughout adolescence and in adulthood in male Wistar rats were studied.

First of all, the comparison of three Wistar Han rat lines revealed several differences in cognitive abilities and therefore added to the increasing evidence of behavioral differences even within the same strain but different lines of rats.

The second project showed differing trajectories of the development of cognitive abilities throughout adolescence that included non-linear, gradual and delayed patterns for various cognitive tests. These behavioral findings appear to be connected to underlying molecular changes in the ECS and myelination during development. Particularly the deficit in OBJR memory was ameliorated by the CB1R antagonist / inverse agonist SR indicating an involvement of the developing ECS at the approximate onset of puberty in memory processing. Furthermore, differing levels of the CB1R in various brain regions throughout development suggested that these molecular processes subserve behavioral performances in different cognitive paradigms. Similarly, increasing myelination contributes to increased cognitive performance and was shown to develop differentially in various brain regions. This indicates that the differential development of brain regions and transmitter systems involved in cognition proceeds in variable ways and most likely contributes to the observed behavioral patterns. Because several brain regions are working in a coordinated network together during cognitive tasks the variable development of these regions may create an imbalance at specific developmental time points and may therefore add to the observed behavioral trajectories.

The third project showed that the interference with the developing ECS can lead to long-term impairing effects on cognition. These observations support the animal model of chronic pubertal WIN treatment at the specific vulnerable time period of puberty. Furthermore, the here found increased CB1R levels in the mPFC, most likely underlie the cognitive deficits observed and may demonstrate a compensatory mechanism of the disturbance of the ECS at this vulnerable developmental time period.

The presently employed chronic pubertal MPH treatment did not reveal any long-term effects in various behavioral tests, thus indicating that this particular treatment regimen may not have impairing effects after a drug-free period in adulthood.

Altogether, this thesis revealed a differential development of basal cognitive abilities in the male Wistar rat, some of which are likely due to underlying alterations in the developing ECS. Furthermore, adolescence is very prone to the influence of pharmacological agents interfering

with this system. Thus, this vulnerable period of neurodevelopment is a time period where manipulations of the ECS can have lasting effects on cognitive abilities into adulthood.

5. List of Abbreviations

(m)PFC	(medial) prefrontal cortex
(m)sec	(milli)second
(RM) ANOVA	(repeated measurement) analysis of variance
2-AG	2-Arachidonoylglycerol
AC	adenylat cyclase
ACh	acetylcholine
ADHD	attention deficit hyperactivity disorder
AEA	anandamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASR	acoustic startle reflex
ASST	attentional set shift test
BPS	balano-preputial separation
BW	body weight
CA	cornu ammonis
cAMP / ATP	cyclic adenosin monophosphate / adenosin triphosphate
CB1R /CB2R	cannabinoid receptor 1 / 2
CD	compound discrimination
cm	centimeter
COMT	catechol-o- methyl transferase
COX-2	cyclooxygenase 2
CP	casein pellet
CPu	caudate putamen
D1/2R	dopamine receptor 1/2
DA	dopamine
DAB	2,4 diaminobutyric acid
DAG	diacylglycerol
dB	decibel
DG	dentate gyrus
DSI / DSE	depolarization-induced suppression of inhibition / excitation
DSM	Diagnostic and Statistical Manual of Mental Disorders
eCB	endocannabinoid
ECS	endocannabinoid system
EDS	extradimensional shift
EDTA	ethylenediaminetetraacetic acid
EMT	light/dart emergence test
EPM	elevated plus maze
ERK	extracellular signal-regulated kinase
EtOH	ethanol
FAAH	fatty acid amino hydrolase
FAK	focal adhesion kinase
FAN	factor associated with neutral sphingomyelinase activation
GABA	ɤ-aminobutyric acid
GPR	G-protein coupled receptor
h	hour
HCl	hydrochloride acid
Hip	Hippocampus
i.p.	intraperitoneal
IDS	intradimensional shift
IEG	immediate early gene
IL	infralimbic
IQ	intelligence quotient
ITI	intertrial interval

JNK	c-jun N-terminal kinase
K^+_A	A-type potassium channels
kDA	kilodalton
K_{ir}	inwardly rectifying potassium channels
LOX	lipoxygenase
LTM	long-term memory
LTP	long-term potentiation
lx	Lux
mA	milliampere
MAGL	monoaclyglycerol lipase
MANOVA	multivariate analysis of variance
min	minute
ml	milliliter
MPH	methylphenidate
MRI	magnetic resonance imaging
NAc	Nucleus accumbens
NaCl	natrium chloride
NAPE	N-arachidonoyl-phosphatidylethanolamine
NAT	N-Acetyltransferase
NGS	normal goat serum
NIH	National Institute of Health
NMDA	N-Methyl-D-Aspartate
OBJR	object recognition
OBJRecency	object recency
OD	optical density
OFC	orbifrontal cortex
p38	p38 mitogen-activated protein kinase
P1 / 2 / 3	presentation 1 / 2 / 3
PBS (-T)	phosphate buffered saline (-Tween)
PCP	phencyclidine
pd	postnatal day
PE	phosphatidylethanolamine
PFA	paraform aldehyde
PI	phophatidylinositol
PKA / C	protein kinase A / C
PL	prelimbic
PLC / PLD	phospholipase C / D
PnC	nucleus reticularis pontis caudalis
PPI	prepulse inhibition
Pre	pretraining
PVC	polyvinylchloride
PVDF	polyvinylidene difluoride
RDoC	Research Domain Criteria
rep	repetition
rev	reversal
ROI	region of interest
RT	room temperature
S.E.M.	standard error of the mean
SCM	sweetened condensed milk
SD	simple discrimination
SDS	Sodium dodecyl sulfate
SHR	spontaneously hypersensitive rats
SM	sphingomyelin

Smase	sphingomyelinase
SOCR	social recognition
SPL	sound pressure level
SR	SR 141716A
STD / LTD	short-term depression / long-term depression
STM	short-term memory
TBS (-T)	tris-buffered saline (-Tween)
TRPV1	transient receptor potential vanilloid 1
V	Volt
W[hsd]	HsdHan:WIST
W[Jan]	RjHan:WI
W[rcc]	RccHan:WIST
WCST	Wisconsin Card Sorting Test
WIN	WIN 55,212-2
WM	working memory
Δ9-THC	Δ9-tetrahydrocannabinol

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“Science says our bond is made with chemistry
Oxytocin’s all it’s fashioned of
I just know that you’re the only vole for me
And chemicals don’t mean I’m not in love”

Monty Harper

“Bottom line is even if you see 'em coming, you're not ready for the big moments. No one asks for their life to change, not really. But it does. So what, are we helpless? Puppets? No. The big moments are gonna come, can't help that. It's what you do afterwards that counts. That's when you find out who you are.”

Whistler, BtVS, #2.21

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