

On the Effects of Sensory Noise in ADHD

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Cover illustration:

Background noise in the Prefrontal Cortex: Difference of Gaussians of the two
theoretically identical Gaussian functions;

(for(i = 0; i < 50; i + +); run ("Gaussian Blur...", "sigma=3"))

and

run ("Gaussian Blur...", "sigma= $\sqrt{(3^2 \times 50)}$ ")

Illustrated by Daniel Eckernäs

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"It's bigger on the inside!"

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ABSTRACT

Attention deficit hyperactivity disorder (ADHD) is one of the most common neurodevelopmental disorders amongst children of the developed world. The main symptoms of the disorder include hyperactivity, inattention and impulsivity. Psychotropic stimulants are considered the first line treatment option. Although effective, they are associated with negative side effects. A recently proposed non-pharmacological intervention for ADHD is loud (>70 dBA) acoustic white noise, a random signal with equal intensities across all included frequencies. Acoustic white noise has demonstrated positive effects on cognitive performance in children with ADHD.

The aim of this thesis is to investigate possible neurobiological effects of sensory noise in experimental pre-clinical and clinical test paradigms and to evaluate possible mechanisms of action behind the positive effects of acoustic white noise in ADHD.

The pre-clinical studies were conducted using the spontaneously hypertensive (SH) rat, currently the best validated animal model of ADHD. Skilled reach in the Montoya staircase test and gross motor skill acquisition on the rotarod were assessed. Further, spontaneous motor behavior was evaluated in an open field activity box. The effect of acoustic white noise on neuronal brain activity was investigated using immunohistochemistry. Results indicate that the SH rat develops skilled reach more slowly and has lower plateau performance in rotarod running compared to a control strain. Additionally, the SH rat displays less habituation to an open field chamber and has significantly higher locomotion and rearing activity. Acoustic white noise exposure during training increased the skilled reach acquisition and performance on the rotarod to the same level as a control strain. Acoustic white noise had no attenuating effects on the increased

locomotor activity or rearing activity of the SH rat. Compared to a control strain the expression of the two neuronal activity/plasticity markers Δ FosB and Ca^{2+} /Calmodulin dependent protein kinase II (CaMKII) tended to be lower in several brain areas in the SH rat model of ADHD. Similarly (but not identically) to methylphenidate (MPH), acoustic white noise reduced the observed differences in neuronal activity/plasticity marker expression.

Possible beneficial effects of stochastic vestibular stimulation (SVS) on cognitive function were assessed in an ADHD population in a clinical trial. However, SVS did not benefit cognitive function in ADHD in any meaningful way.

Effects of acoustic white noise on acquisition of skill and neural brain activity were similar to the effects of MPH in SH rats. Unlike previously demonstrated effects of loud acoustic white noise, SVS did not improve situational cognitive function in ADHD. The increased performance in ADHD during acoustic white noise can probably be attributed to informational masking mechanisms, and possibly to altered cortical arousal.

Keywords: ADHD, attention, sensory noise, motor learning, behavior, immunohistochemistry, image analysis

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SAMMANFATTNING PÅ SVENSKA

ADHD är en av de vanligaste neuropsykiatriska funktionsnedsättningarna som drabbar barn i västvärlden. ADHD utmärks av att personen har problem med att kontrollera impulser och att upprätthålla uppmärksamheten. Patienter med ADHD gör ofta slarvfel, har svårt att vänta på sin tur och avbryter ofta andra. Det är även vanligt att de har svårigheter att sitta stilla och det uppfattas ofta som att de går på "högvarv". Orsaken till ADHD är fortfarande inte helt känd, men flera observationer tyder på att det finns för lite av signalsubstansen dopamin (DA) i hjärnan. Detta tror man är en följd av att personer med ADHD har onormalt många eller onormalt aktiva dopamintransportörer (DAT) i hjärnan. DAT har till uppgift är att pumpa tillbaka DA från nervcellernas synapser efter att det frisatts. Centralt stimulerande läkemedel som blockerar funktionen av DAT är en mycket effektiv behandling av symptomen vid ADHD. Men även om dagens behandlingar är effektiva så finns det negativa aspekter så som biverkningar samt att de ibland är mindre lämpliga för dem med fler psykiatriska diagnoser. Därför finns det behov av nya behandlingsformer, både farmakologiska och icke-farmakologiska.

En behandling som nyligen har visat positiva effekter på den kognitiva förmågan hos barn med ADHD är starkt (>70 dBA) akustiskt vitt brus, ett ljud som innehåller samma intensitet inom alla frekvenser (tänk myrornas krig på TV). Den positiva effekten av detta brus tycks enbart finnas hos personer som har dålig koncentrationsförmåga. Stimulering av ett annat sensoriskt system, balanssystemet, med hjälp av elektriskt brus (stokastisk vestibulär stimulering - SVS) har tidigare visat positiva effekter på balansförmåga och förbättrad kognitiv funktion vid neurodegenerativa sjukdomar, men har aldrig prövats vid ADHD. Till skillnad från starkt akustiskt brus kan SVS administreras omärkbart och utan att det stör andra sinnesintryck.

Den här avhandlingen ämnar huvudsakligen utforska potentiella effekter av sensoriskt brus i både kliniska och pre-kliniska studier samt undersöka hur hjärnans aktiveringsmönster påverkas av akustiskt vitt brus. Dessutom utvärderar vi potentiella mekanismer bakom dessa effekter.

I den första studien (delarbete I) undersöker vi om akustiskt vitt brus har positiva effekter på inlärning även i en djurmodell av ADHD, den spontant hypertensiva

(SH) råttan. Djurens spontana rörelseaktivitet samt deras fin- och grovmotoriska prestationsförmåga utvärderades. SH-råttor hade signifikant högre spontan rörelseaktivitet och det tog längre tid för dem att vänja sig vid den nya omgivningen än för kontrollråttor. Det tog längre tid för dem att utveckla finmotorisk skicklighet och de hade sämre grovmotorisk förmåga. Träning under akustiskt vitt brus ökade SH-råttans prestation både finmotoriskt och grovmotoriskt till samma nivå som kontrollråttorna. Brus hade dock ingen effekt på det spontana rörelseaktivitetsmönstret.

I den andra studien (delarbete II) utvärderades effekten av akustiskt vitt brus på neuronal aktivering i hjärnan hos råttor. Hjärnor från råttor som exponerats för akustiskt vitt brus en timme om dagen i fem dagar undersöktes gällande uttryck av Δ FosB och Ca^{2+} /kalmodulin-beroende proteinkinase II (CaMKII), vilka är markörer för nervcellssignalering i hjärnan. Dessa markörer uttrycktes i mindre grad hos SH-råttan i flera områden i hjärnan som är viktiga för beteende och kognitiv förmåga vid ADHD. Exponering för akustiskt vitt brus återställde detta underuttryck i flera regioner till nivåer liknande kontrollråttorna.

I den tredje studien (delarbete III) undersökte vi om ett sensoriskt brus av annan modalitet (SVS) hade positiva effekter i personer med ADHD. En fördel med SVS är att till skillnad från akustiskt brus är att man utföra så kallade dubbel-blinda studier (vare sig försöksperson eller undersökare vet om det är aktiv behandling). Detta går att göra eftersom SVS inte känns/erfars av försökspersonen. Studiedeltagare med ADHD-diagnos utförde tre inlärningstester under antingen aktiv SVS eller utan SVS. Resultatet var att inlärningsförmågan för personer med ADHD ej påverkades av SVS.

LIST OF PAPERS

The thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. Göran Söderlund*, **Daniel Eckernäs***, Olof Holmblad and Filip Bergquist. *Acoustic noise improves motor learning in spontaneously hypertensive rats, a rat model of attention deficit hyperactivity*. Behavioural Brain Research 280 (2015) 84–91 <https://doi.org/10.1016/j.bbr.2014.11.032>
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- II. **Daniel Eckernäs**, Fredrik Hieronymus, Thomas Carlsson and Filip Bergquist. *Acoustic white noise ameliorates reduced regional brain expression of CaMKII and Δ FosB in the spontaneously hypertensive rat model of ADHD*. Submitted.
- III. **Daniel Eckernäs***, Ghazaleh Samoudi*, Göran Söderlund, Fredrik Hieronymus, Peik Gustafsson, Sebastian Lundström, Pia Tallberg, Aikaterini Trantou, Christopher Gillberg and Filip Bergquist. *Vestibular near threshold stochastic electric stimulation does not improve cognitive performance in ADHD - A pilot study*. Submitted.

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ABBREVIATIONS

ADHD	Attention Deficit Hyperactivity Disorder
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
DA	Dopamine
DAT	Dopamine Transporter
dB(A)	A-weighted decibel
DL-PFC	Dorsolateral Prefrontal Cortex
DLS	Dorsolateral Striatum
MPH	Methylphenidate
nAc	Nucleus Accumbens
PFC	Prefrontal Cortex
SH	Spontaneously Hypertensive rat
SVS	Stochastic galvanic Vestibular Stimulation
TMN	Tuberomammillary Nucleus
WKY	Wistar Kyoto rat

1 INTRODUCTION

1.1 Attention Deficit Hyperactivity Disorder

1.1.1 Definition and pathophysiology

Attention deficit hyperactivity disorder (ADHD) is a common neurodevelopmental disorder, with an estimated prevalence of 5-7% in children in the developed world (Thomas et al., 2015). The disorder is associated with considerable negative individual outcomes relating to educational attainment (Hinshaw, 1992; Fergusson and Horwood, 1995; Barry et al., 2002), anti-social behavior (Satterfield et al., 1994; McKay and Halperin, 2001) and significant psychiatric comorbidity (Steinhausen et al., 2006). The main symptoms of the disorder include hyperactivity, inattention and impulsivity (American Psychiatric Association, 2013). Moreover, the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, describes three main subtypes of the disorder; predominantly hyperactive, predominantly inattentive and a combined subtype. Although mainly a disorder of childhood it is not uncommon that symptoms persist late into adolescence (Biederman et al., 1996; Dopheide and Pliszka, 2009) and even into adulthood (Biederman et al., 1993; Dopheide and Pliszka, 2009). As an additional burden, it is not uncommon that individuals afflicted with the disorder experience reduced fine motor skills, for example reflected in reduced quality of handwriting (Flapper et al., 2006).

The causes of ADHD are not fully understood and a multitude of factors are believed to contribute to the development of the disorder. Hereditary factors are suggested by an increased risk of developing the disorder if a parent or a sibling is afflicted (Biederman et al., 1990; 1992; Faraone et al., 2000). By comparing the likelihood of identical twins to develop the disorder as compared to other siblings the degree to which genes explain the variability of the disorder can be estimated. Results from several twin studies suggest the heritability figure to be around 60-90% (Thapar et al., 1995; Nadder et al., 1998; Thapar et al., 2000; Rietveld et al., 2003; Martin et al., 2018). Furthermore, there are support from epidemiological studies that environmental factors such as prenatal tobacco, caffeine and narcotics exposure increase the risk of developing the disorder (see Froehlich et al., 2011 for a review).

Although the etiology of ADHD is not clear, converging evidence have indicated abnormalities in the catecholaminergic systems of the brain in the ADHD population. In an imaging study conducted by Dougherty and colleagues (1999), a 70% increase of dopamine transporter (DAT) density was reported in patients with ADHD compared to healthy controls. These findings were later replicated by Krause and colleagues (2000) using a different ligand. These results suggest that an increased DAT activity is an important factor in the etiology of ADHD (Madras et al., 2005). This is further supported by the fact that psychotropic stimulants, such as methylphenidate (MPH), can effectively ameliorate ADHD symptoms and that this effect is dependent on blocking DAT (Spencer et al., 2000). Other imaging studies have indicated reduced dopaminergic nigrostriatal transmission in patients with ADHD (Ernst et al., 1999) and results from several gene association studies further support a dopaminergic hypothesis (See Faraone et al., 2005 for a detailed review).

A salient feature in ADHD is a general distractibility where attentional focus easily gets shifted from an ongoing task. This is believed to be caused by an inability to filter out and suppress external stimuli that are not relevant to the task at hand (Aboitiz et al., 2014). This feature has been attributed to impaired cognitive and behavioral control mechanisms caused by insufficient dopamine (DA) signaling in cortico-striatal pathways that regulate goal directed behavior (Clark et al., 1987; Swanson et al., 2007). Others have suggested the default mode network, first established by Raichle and colleagues (2001), to play a role in the distractibility of ADHD. The default mode network works in opposition to the task-positive network which is active during tasks demanding high attention (Fox et al., 2005). The default mode network and the task positive network are anticorrelated towards each other, meaning that when one is active the activity in the other is suppressed and vice versa. The interplay between the default mode network and the task positive network are believed to be off balance in people with ADHD (Sonuga-Barke and Castellanos, 2007) and it has been suggested that the task positive network cannot sufficiently suppress the activity in the default mode network during tasks, thus increasing distractibility (Fassbender et al., 2009).

1.1.2 Treatment options for ADHD

Reduced activity of the catecholaminergic systems of the brain is believed to play a major role in the pathophysiology of ADHD. Stimulants, substances that enhance dopaminergic transmission, were demonstrated to be beneficial as early as 1937 (Bradley, 1937). Stimulants are still a first line treatment which effectively ameliorates both cognitive symptoms and hyperactivity (Spencer et al., 1996; Bilder et al., 2016) in children and adolescents, as well as in adults (Barbarese et al., 2006; Santosh et al., 2011). Therefore, pharmacological treatment remains central in the management of moderate to severe ADHD. There are also behavioral therapies for the management of ADHD symptoms (Kutcher et al., 2004). Although behavioral therapies alone can be effective in some patients, the best improvement is seen if they are given in combination with stimulant medication (Catala-Lopez et al., 2017; Knouse et al., 2017).

Other pharmacotherapeutic alternatives than the above mentioned are the norepinephrine-reuptake inhibitors, which are formally not classified as stimulants but share the norepinephrine re-uptake properties of classic stimulants like amphetamine and MPH. They are considered safe and effective for treating behavioral problems (Garnock-Jones and Keating, 2009) but it can take up to 24 weeks to reach optimal efficacy and approximately 30% of patients do not respond (Young et al., 2011).

While considered the first line treatment, stimulants have downsides relating to side-effects like poor appetite, insomnia, stomach-aches and headaches (Barkley et al., 1990), risk for abuse (Clemow and Walker, 2014). Also, long-term use of stimulants has been linked to growth suppression (Spencer et al., 1996; Swanson et al., 2017). Furthermore, although stimulant treatment effectively improves cognitive performances in ADHD (Bilder et al., 2016) it is not evident that they enhance learning processes (Molina et al., 2009). Given these circumstances, current drug therapies has limitations that also make them less than ideal for those with mild symptoms or significant comorbidity (Shier et al., 2013). Therefore, new or improved interventions, both pharmacological and non-pharmacological are desirable.

1.2 Sensory noise

1.2.1 Acoustic white noise

Acoustic white noise is a stochastic auditory signal that contains equally distributed intensities over a wide frequency range which gives it a constant power spectrum, i.e. all given frequencies contributes equally to the energy of the signal. The notion that acoustic white noise could be a useful tool for the management of ADHD symptoms originates from a study by Söderlund and colleagues (2007). Söderlund wanted to test the hypothesis that children with low attention are more easily distracted when they perform cognitively demanding tasks. In the study, forty-two boys aged 9-14 years participated, twenty-one diagnosed with ADHD and twenty-one matched controls. A 2x2 cross-over design was used and, in randomized order, participants undertook a verbal episodic recall task once in ambient silence and once with a distractor present. As a distractor Söderlund chose 80 A-weighted decibel (dB(A)) acoustic white noise. The hypothesis was that the low attentive group would perform worse than the normally attentive group during quiet conditions, but also that they would be more easily disturbed by the acoustic white noise and hence perform even worse under this condition. As expected, children with low attentional rating performed worse than children with normally rated attention during quiet conditions, however, when exposed to the white noise distractor the results were not as predicted. There was a significant interaction between noise and group ($F(1,33) = 5.73, p = 0.023$), but noise seemed to benefit children with low baseline attention whereas it impaired the performance for normally attentive children. These findings have since been replicated in several published studies (Söderlund et al., 2010; Helps et al., 2014; Söderlund et al., 2016; Söderlund and Jobs, 2016).

1.2.2 Stochastic vestibular stimulation – Vestibular noise

Stochastic galvanic vestibular stimulation (SVS) is a random patterned electrical stimulation of the vestibular system of the brain. The randomness of the signal is similar to the randomness of auditory white noise. The peripheral vestibular organs are bilateral structures which form part of the inner ear found in the posterior portion of the temporal bone. The vestibular organs respond to rotational movements and linear accelerations of the head. Signals from the vestibular system contribute to an individual's sense of balance and spatial

orientation. Studies have shown that SVS at levels near the threshold of vestibular activation improves postural control in healthy volunteers (Mulavara et al., 2011) as well as in patients with Parkinson's disease (Pal et al., 2009; Samoudi et al., 2015).

The vestibular nuclei are functionally connected to the limbic system and regions of the neocortex concerned with learning and memory (see Fukushima, 1997; Smith, 1997 for review). Evidence from animal studies has shown that electrical stimulation of the vestibular nuclei facilitates the release of acetylcholine in the hippocampus (Horii et al., 1994; Horii et al., 1995). Furthermore, electrical stimulation of the peripheral vestibular system induces field potentials in the CA1 and CA2 regions of the hippocampus in guinea-pigs (Cuthbert et al., 2000). Vestibular dysfunction in rats and guinea-pigs has been shown to induce long lasting deficits in spatial orientation and memory (Horn et al., 1981; Chapuis et al., 1992), and rats with bilateral vestibular lesion show signs of persisting hyperactivity (Goddard et al., 2008). It is therefore not unwarranted to propose that increased vestibular stimulation could improve cognitive function in humans, perhaps especially so in those exhibiting hyperactivity and impaired attention (e.g. ADHD).

1.2.3 Acoustic white noise in ADHD - possible mechanisms of action

Stochastic resonance in the CNS

Stochastic resonance is a phenomenon that can be observed in all nonlinear systems with threshold effects. By threshold effects we mean the requirement to surpass a certain level of activity or a level of a measurable to activate the system or propagate a signal. A classic example of a nonlinear system with a threshold effect is a neuron, where the membrane potential fluctuates continuously and when it passes a threshold, sodium channels open to elicit an action potential, an all-or-none event. A weak input signal that would not normally lead to an action potential may do so if there is an appropriate amount of noise that occasionally brings the potential over the action potential threshold (see Moss et al., 2004 for review). The same principle can be applied to the detection of a sensory stimulus in the periphery, like touch or sound. Neural activity is conducted under a considerable amount of background noise (Bialek and Rieke, 1992). This noise is

however an essential part of the communication between neurons and it has been proposed that an appropriate amount of noise is crucial for the CNS to operate optimally (Li et al., 2006; McDonnell et al., 2007; Ghosh et al., 2008).

The stochastic resonance phenomenon inspired Sikström and Söderlund (2007) to propose the moderate brain arousal model as an explanation to why children with ADHD are aided by the addition of external noise. Kroener and coworkers (2009) postulate that optimal DA concentrations in the PFC modulates neural responses to increase the signal-to-noise ratio to support cognitive function.

In accordance, Sikström and Söderlund proposed that the CNS response to external stimuli is conditioned by the current DA related gain and levels of internal neural noise in combination with any external noise. This means that a system lacking DA could benefit from increased signal-to-noise ratio by i) addition of additional DA or ii) by adding external noise into the system (it is assumed that internal neuronal noise cannot be changed). In this fashion the activity of the CNS could be improved by external noise through mechanisms involving stochastic resonance. Such effects are conditional on that the origin of the problem is not excessive noise in the system.

Cortical arousal

The moderate brain arousal model proposed by Sikström and Söderlund (2007) is also based on the optimum stimulation theory presented by Zentall and Zentall (1983). They proposed that people with ADHD suffers from a state of suboptimal cortical arousal as a result of aberrant neurotransmission or inadequate central stimulation. According to this theory hyperactivity and increased verbalization in people with ADHD are byproducts that reflect compensatory strategies to increase cortical arousal. This is supported by the fact that stimulant medications used for the treatment of ADHD also are potent mediators of cortical arousal and alertness (see Wood et al., 2014 for a review). In the context of cortical arousal, it can be proposed that acoustic white noise would increase the reduced cortical arousal in persons with ADHD to a more optimal level and thereby increase attention and alertness.

Masking

As mentioned earlier, one salient feature of ADHD is a general distractibility due to the inability to filter out and suppress irrelevant stimuli (Aboitiz et al., 2014). When performing a task, they are more susceptible to distractions that shift their attention elsewhere. Informational masking, as defined by Pollack (1975) is the “threshold change in statistical structure resulting from the presence of a neighboring signal of the same amplitude” i.e. by adding a noise of the same or higher amplitude as an irrelevant stimuli, the threshold to detect this stimuli will increase masking its presence. Loud acoustic white noise is a signal bearing no information, it is homogeneous and continuous and it is possible, if loud enough, that it masks the occurrence of external irrelevant stimuli that would otherwise have shifted the attention away from the task at hand in people with ADHD.

1.3 Animal models of ADHD

1.3.1 Model validity

When choosing an animal model to study a specific disorder it is important to assess the validity of the model. Ideally, an appropriate animal model should display i) face validity, i.e. the trait studied in an animal should appear to be similar to some fundamental behavioral characteristic of the disorder in humans. It should also display ii) construct validity: the model needs to adhere to the theoretical rationale of the disorder, i.e. the etiology and cause of the abnormalities seen in the clinical case needs to be reflected in the animal model (e.g., a model of acute myocardial infarction should involve ischemic heart injury) Lastly, it needs to exhibit iii) predictive validity, that is a model should correctly respond to various interventions effective in the human state, while not responding to those that are ineffective (e.g., a model of depression should respond to antidepressants but not to, say, ACE-inhibitors) and should ideally make the researcher able to make predictions about the disorder that were previously not known (Sarter et al., 1992). Naturally, all these criteria might not be possible to fulfill - it may be hard to have a model displaying good construct validity, for example, if the pathophysiology of a disorder is insufficiently understood.

1.3.2 Animal models of ADHD

Dopamine-transporter knockout mouse

The DAT knockout mouse model lacks the DAT gene. While this is the opposite to what is believed to be the etiology of ADHD, the model paradoxically displays some of the symptoms of the disorder such as spatial memory deficits and hyperactivity (Gainetdinov et al., 2001). Since the animals lack the DAT, clearance of DA in the synapses is slow. This results in a five-fold increase of extracellular DA in the striatum (Gainetdinov et al., 1999), which possibly contributes to the observed hyperactivity of the strain. Interestingly, exposure to MPH nevertheless significantly reduces hyperactivity in the DAT knockout mouse (Takamatsu et al., 2015). Furthermore, Takamatsu report that systemically administered MPH increased extracellular DA levels in the PFC but not in the striatum of the DAT knockout mouse. This is possibly explained by the relatively high concentration of norepinephrine transporter compared to DAT in the PFC (Moll et al., 2000) as well as the fact that MPH also exerts its effect via norepinephrine transporter-inhibition and that the norepinephrine transporter also can reuptake extracellular DA (Carboni et al., 1990), indeed, in several important cortical areas such as the PFC DAT exhibits low or no expression and the instead facilitates DA uptake (Moron et al., 2002). The DAT knockout mouse model of ADHD displays some face validity and maybe some predictive validity regarding the nature of hyperactivity. However, since there are no indications of individuals with ADHD displaying impaired DAT functionality, in fact rather the opposite, the construct validity of the model is low.

Coloboma mutant mouse

The Coloboma mutant mouse is deficient in the SNAP-25 gene encoding for a t-SNARE protein important for the fusion of the neurotransmitter vesicles with the presynaptic membrane that result in the release of neurotransmitters (Theiler and Varnum, 1981). The model displays behavioral deficits such as hyperactivity and some impulsive traits in a delayed reinforcement task (Bruno et al., 2007). Hyperactivity is reduced by amphetamine (Wilson, 2000) but not by MPH (Hess et al., 1996). Reports of increased noradrenergic activity (Jones et al., 2001) as well as amelioration of hyperactive symptoms following treatment with antiadrenergic drugs (Bruno and Hess, 2006) suggest that the hyperactive phenotype in the Coloboma mutant mouse can be attributable to a hyperactive

noradrenergic system. The DA metabolites DOPAC and HVA have been shown to be decreased in the striatum of the Coloboma mouse, suggesting a hypofunctional dopaminergic system. The model has some face validity relating to hyperactivity and impulsiveness, but does not model ADHD symptoms completely. However, SNAP-25 polymorphism has been associated with ADHD, and coupled with reports of reduced DA transmission which lend some predictive and construct validity to the model.

The spontaneously hypertensive rat

The spontaneously hypertensive (SH) rat was originally developed as a model for hypertension by inbreeding rats from the Wistar-Kyoto strain (WKY; Okamoto and Aoki, 1963). Later, the SH rat was recognized to show elevated spontaneous motor activity (Moser et al., 1988) and suggested as an animal model for hyperactivity. The model was studied extensively by Sagvolden and colleagues who established it as one of the best animal models of ADHD (Sagvolden et al., 1992a; 1998; Sagvolden, 2000). At a young age (up to 12 weeks) the SH rat display several major symptoms of ADHD such as hyperactivity, impulsivity and attentional and learning deficits, i.e. the model has good face validity (Moser et al., 1988; Wyss et al., 1992; Sagvolden, 2000). Like people with ADHD (Dickstein et al., 2006) several reports indicate that the SH rats have a dysfunctional fronto-striatal system, expressed e.g. as impaired DA release in the prefrontal cortex (PFC), caudate putamen and nAc (Myers et al., 1981; Deutch and Roth, 1990; Jones et al., 1995; Russell et al., 1995, 1998). SH rats also display increased densities of DA D₁ and D₅ receptors in the nAc and neostriatum (Carey et al., 1998), as well as reduced expression of DA D₄ receptors in the PFC (Li et al., 2007). Furthermore, it displays elevated levels of noradrenergic reuptake in the PFC, cerebellum, hypothalamus and pons-medulla (Myers et al., 1981) as well as reports of a down regulation of beta-adrenoceptors in the nAc (de Villiers et al., 1995). Considering these findings, the SH rat displays not only good face validity, but also construct validity. Regarding predictive validity, stimulant medication has been demonstrated to have positive effects on behavioral and hyperactive symptoms of the SH rat (Myers et al., 1982; Kantak et al., 2008). With this in mind, the SH rat is the best validated animal model of ADHD available today.

Since the WKY and SH rat both were derived from the same prenatal Wistar stock, the WKY has long been considered the best control strain for the SH rat

(Sagvolden et al., 2009). However, in recent years, concerns have been raised regarding the validity of using WKY as a control strain (Alsop, 2007). Diana (2002) reported no differences in cognitive decline in aged SH rats compared to WKY and compared to Sprague-Dawley, both strains display cognitive deficits across all age groups. Sontag and colleagues (2013) reported no difference regarding impairments of spatial working memory and reference memory in either strain. Furthermore, the WKY strain has been reported to have an abnormally low spontaneous motor activity in comparison to both outbred Wistar strains and SH rats (Pare, 1989; Sagvolden et al., 1993) as well as decreased activity in the forced swim test. It was consequently suggested that WKY rats have a depression-like phenotype (Lahmame et al., 1997), and the relevance of comparing rats with a depression-like phenotype is questionable in the same way as using depressed subjects as a control group for patients with ADHD would be.

Taking these concerns into account and as a way to avoid exaggerated genetic effects we chose to use the out-bred Wistar rat as controls in Paper I and II of this thesis.

1.4 Methods to evaluate brain activation in human and/or rodent models

1.4.1 *In vivo* studies of brain activity patterns

Brain activity patterns in humans can be studied *in vivo* with imaging techniques and electrophysiology. Functional magnetic resonance imaging measures brain activity by detecting changes in blood oxygenation and blood flow in areas of the brain following brain activity. Active neurons require more oxygen and the apparatus picks up these fluctuations indicating what areas of the brain are active during different mental processes. Positron emission tomography detects a short-lived radioactive material injected into the bloodstream of the subject before the scan. When the radioactive material decays it emits a positron that is detected by the apparatus. To achieve good results from functional magnetic resonance imaging and positron emission tomography requires the subject to lie perfectly still during the scanning process. This is, for obvious reasons, a hard task to accomplish in awake and non-restrained animals, so in animal research the methods are limited to anesthetized conditions. Furthermore, the spatial resolution in these techniques is low (1-2mm) which limits the size of the

structure studied. Therefore, these techniques are not commonly used in rodents and finds its main use in the clinics.

Electroencephalography records the electrical activity of the brain via electrodes placed on the scalp, and is routinely used as a diagnostic tool in the clinic. The output represents the voltage fluctuations from a large number of neurons firing in the brain. Magnetoencephalography measures, similarly to electroencephalography, the activity of a large number of neurons firing. However, instead of measuring voltage fluctuations it measures changes in the magnetic fields produced by the neurons action potentials. Temporal resolution in these techniques is very good, however the spatial resolution is limited and there is no efficient way to measure electroencephalography or magnetoencephalography in behaving rodents non-invasively and for this reason surgical implications on behavior would have to be considered.

1.4.2 Activity induced gene expression

One way to study neural processes is to *post mortem* look at the expression of various proteins and markers that are part of or a consequence of neural signaling. By subjecting an animal to a condition and then fixate their brain, preserving it in its pre-mortem state, the neural activity pattern which was induced by the condition can be measured by looking at such markers. This approach has many advantages compared to the above-mentioned *in vivo* techniques. Primarily, gene expression enables the researcher, down to a cellular level, to identify specific neurons and cells that display recent activity. On the other hand, the temporal resolution is limited to the time of brain fixation, i.e. it does not allow us to detect changes over time. With knowledge of the temporal activation window of specific genes the time from intervention to brain fixation can be adapted to detect the expression of these genes. The markers investigated in Paper II are briefly described below.

Immediate Early Genes

Immediate early genes are cellular genes which are rapidly and transiently expressed following stimulation of a resting cell by an external signal (Fowler et al., 2011). The transcription factor FosB and its truncated splice variant Δ FosB, is together with c-Fos, Fra1, and Fra2 some of the earliest discovered and characterized immediate early genes and are upregulated in neurons a few hours

after increased neuronal activity (Sagar et al., 1988). Δ FosB accumulates following repeated exposures to an activating stimulus like addictive drugs, stress or natural rewards (Nestler et al., 2001; Perrotti et al., 2004) and can therefore be used as an activity marker reflecting recent neuronal activity in the brain (Bahrami and Drablos, 2016).

Ca²⁺/calmodulin-dependent protein kinase II

Ca²⁺/Calmodulin dependent protein kinase II (CaMKII) is a highly abundant Ca²⁺ activated enzyme in the mammalian brain. CaMKII is most prominently expressed in synapses and the postsynaptic density, and constitutes approximately 1-2% of the total protein found in the brain (Erondu and Kennedy, 1985). Strong evidence suggest that CaMKII mediates synaptic plasticity that plays a pivotal role in inducing long term potentiation, a form of molecular process that strengthens synapses based on recent activity and plays a central role in facilitating learning and memory (Lisman et al., 2012). Glutamate mediated activation of postsynaptic NMDA- and AMPA- receptors during the induction of long term potentiation facilitate an influx of Ca²⁺ into the cell. CaMKII detects this rise in Ca²⁺ levels and initiates a biochemical cascade that potentiates synaptic transmission (Lisman et al., 2012). For a detailed review of CaMKII's role in neural plasticity, see (Lisman et al., 2002).

Immunohistochemistry

To be able to analyze the expression of these markers they first need to be visualized. A common technique used for this is immunohistochemistry. Briefly, immunohistochemistry exploits the principle that antibodies specifically bind to antigens in biological tissue. The antibodies bound to antigens can be can be visualized in different manners. Images of the expression of the specific structure or molecule stained for can then be acquired via microscopy. Images are then analyzed and the expression of the target molecule can be measured, giving an indication on e.g. neural activity patterns or protein expression in specific brain regions.

1.4.3 Behavioral tests

Behavioral testing aims to measure behavioral response to different situations and during different conditions. By subjecting an animal to a specific condition

(i.e., a drug) and measuring and analyzing the subsequent behavioral response we can get an indirect indication on how this condition affected the brain. Learning and memory are some of the most commonly studied endpoints in behavioral testing and there are a great variety of paradigms designed to examine a wide range of brain structures involved in these processes. Behavioral tests can easily be performed in both clinical and pre-clinical settings. In Paper I we used two different paradigms designed to measure fine and gross motor skill acquisition in rats, as well as behavioral measurements of open field spontaneous motor activity.

The Montoya staircase test

The Montoya staircase test is a skilled reach paradigm that enables an assessment of reaching and grasping of the forelimbs in rodents (Figure 1A). The procedure has previously been described in detail (Montoya et al., 1991). In short, the design of the apparatus is an aluminum box with a plexiglas extension containing a platform where the rats can freely enter. The extension is narrow enough not to allow the animal to turn around while inside. A staircase in which food pellets can be placed in seven ascending steps is inserted bilaterally below the platform. Each staircase can only be reached with the ipsilateral forelimb.

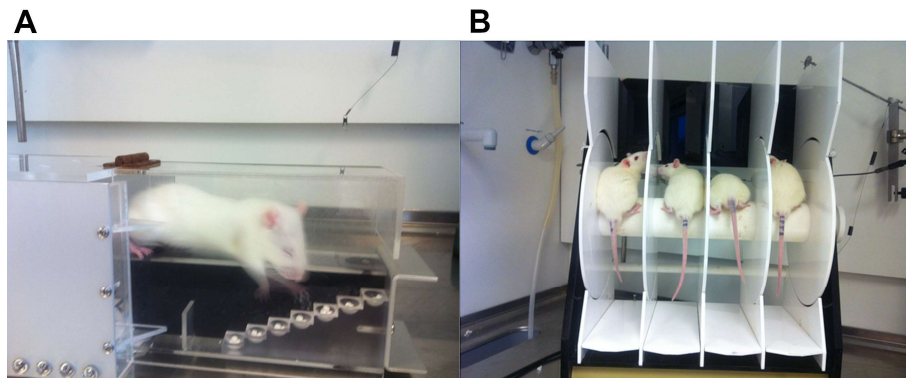


Figure 1. (A) *The Montoya staircase fine motor learning task. Animals are placed inside the box and are left to forage for sugar pellets placed in two parallel seven step staircases.* (B) *The Rotarod gross motor learning task. Rats are trained to stay on the rotor as it accelerates from four to forty r.p.m. over five minutes. Latency to fall is automatically recorded by a lever when the animals fall off the rotor.*

The Rotarod test

The Rotarod (Figure 1B) test is one of the oldest tests for evaluating deficiencies in gross motor skill in rodents. Originally developed to measure effects of drugs on animal behavior (Dunham and Miya, 1957), the Rotarod test has more recently been modified with an accelerating rotor and is deemed to be a highly sensitive measure for assessing motor deficiencies after brain injury (Hamm et al., 1994; Rozas et al., 1997). In addition to this, the Rotarod test has also been suggested as a valid paradigm for studying the learning of gross motor skills (Buitrago et al., 2004).

Open field motor activity

The concept of measuring behavior of rodents in an open area was first described in 1932 by Hall et al. (1932). The apparatus is typically a box measuring approximately 50 x 50 cm with walls high enough to prevent the animal from escaping. The novelty of the new environment may first elicit a freezing response from the animal indicating anxiety (Denenberg, 1969). Behavioral indices commonly recorded in the open field test include locomotor activity, time spent in corners and rearing activity. In the initial novelty phase of being placed in the chamber an animal will explore the new environment, thus locomotor activity will be high during early measurements. During the exploratory phase the animal commonly performs several rearing activities, where it stands up on its hind legs to get a better view of the environment. Normally, an animal will avoid open spaces, since in their natural environment this would leave them exposed to predators. This is true also for the open field box, and after the initial exploratory phase the animal will usually find a "safe" corner and largely remain here for the remainder of the test. In summary, a normal animal will typically have high locomotion and rearing activity and little time spent in corners during the initial part of the test. During the later part, the animal will spend more time in corners, move around and rear less, as expressed by low locomotion counts and few rearing activities.

1.5 Image analysis

1.5.1 Background

A digital image can be described as a numerical representation of a two-dimensional signal defined by the mathematical function $F(x, y)$ where x and y represent the horizontal and vertical co-ordinates within the dimension of the image. Every digital image is built up of small square elements known as pixels and the function $F(x, y)$ gives the value of the pixel corresponding to the spatial location defined by x and y . The value a pixel can attain depends on the number of bits per pixel in the image. A pixel value of 0 always represents black color, while the value that represents white can vary. The simplest form is a 1-bit image, also known as a binary image, in which a color can be described by either 0 or 1 where 0 represents black and 1 represents white. If we increase the number of bits used to describe the pixel we exponentially increase the number of color combinations possible. In a 2-bit image each pixel is described by two bits, e.g. 00, 01, 10 and 11, giving us four possible combinations. By using the formula $(2)^{bpp}$ we can calculate the number of possible color variations expressed at a given number of bits per pixel.

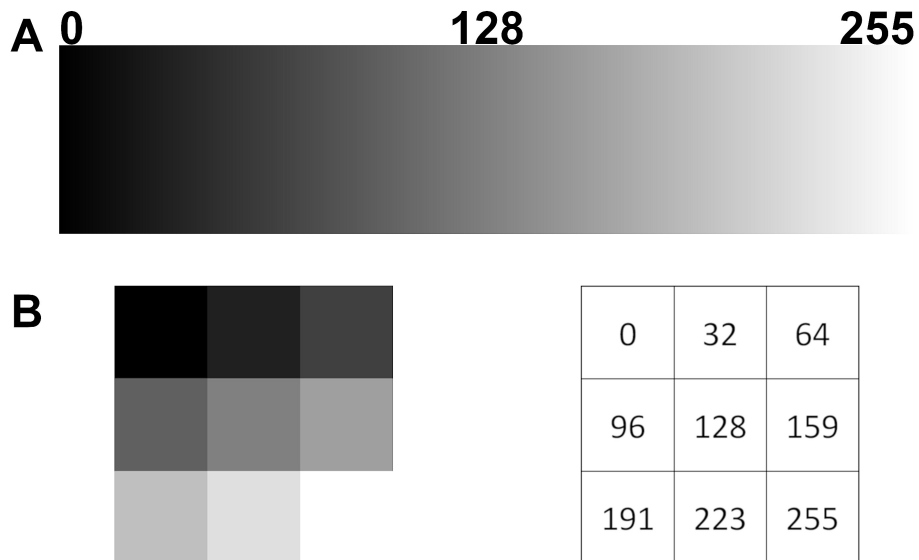


Figure 2. (A) Color spectrum of the 256 different colors represented in an 8-bit grayscale image where 0 represents black, 127-128 represents pure gray and 255 represents white. (B) On the left is a representation of an 8-bit grayscale image and on the right is the same image described numerically as the grayscale value of the pixel from the function $F(x,y)$.

One of the most common image formats is 8-bit grayscale. Given the formula mentioned earlier, an 8-bit color format gives us $(2)^8 = 256$ different color variations where 0 is black, 255 is white and halfway in-between 127-128 represents pure gray (Figure 2A). Given the function $F(x,y)$ an image can be described in numerical form where (F) is the grayscale value of the pixel in the (x,y) position of a two-dimensional array (Figure 2B).

1.5.2 Spatial filtering

Image processing can be seen as a system where the input and output signal is an image. What effects the processing will have on the output signal are determined by what operations are applied to the system. When applying multiple operations to an image, the order they are applied in will affect the final output image and have to be considered.

Filtering is a commonly used pre-processing step used in most image analysis. A filter consists of a matrix of numbers known as a kernel and when applied to an image (a process known as convolution) the kernel moves from each value of $F(x, y)$ by placing the center square of the kernel over the pixel and multiplying the value of that pixel and all other overlapping pixels with the corresponding value in the kernel. The sum of all these will be the new value for $F(x, y)$ after the filter has been applied (Figure 3).

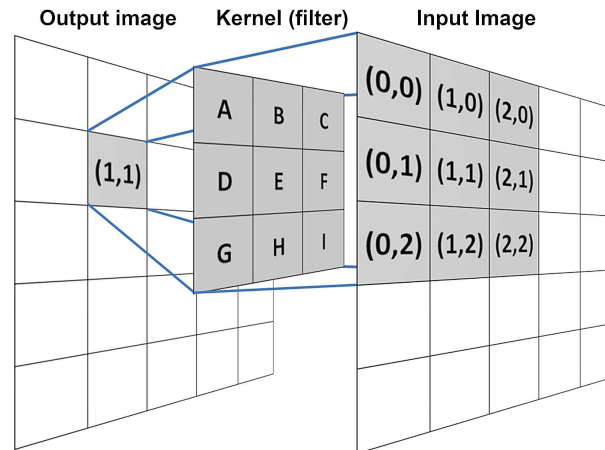


Figure 3. When applying a filter to an image the center square of the kernel is placed on the pixel and values of pixels in the original image is multiplied with the value of the overlapping kernel, e.g. $F(0, 0)$ is multiplied by A, $F(1, 0)$ by B etc. The sum of all these multiplications is assigned as the new pixel value and the process continues for all the pixels in the image.

Varying the values in the kernels will produce different effects such as sharpening, detection of edges, blurring and noise reduction while changing the size of the kernel will increase the intensity of the filter. Figure 4 illustrates an example of a filter used for edge detection. The sum of the values in the kernel equal zero with high weighting on the center square and negative weighting on the surrounding squares. This will result in pixels in the original image that have neighboring pixels with lower values will become brighter while the pixels that

have neighbors with more similar or higher values will become darker, revealing edges in an image.

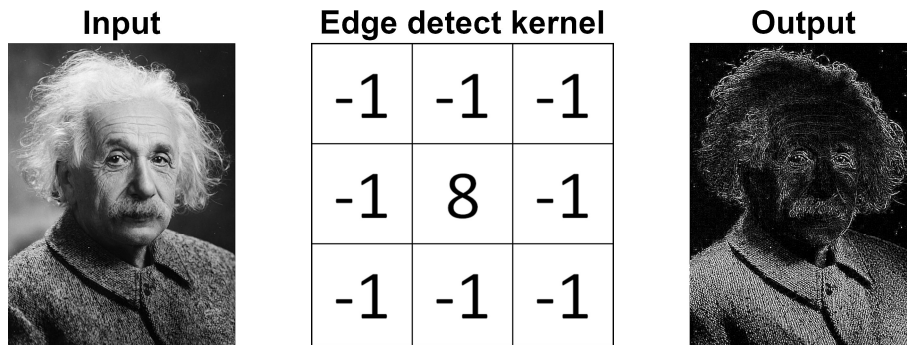


Figure 4. Example of a filter used for detecting edges in an image. In the output image pixels that don't have a sharp difference between neighboring pixels will become darker while pixels that do will become brighter revealing only the edges of the image. Image adapted with kind permission from the Hebrew University of Jerusalem, represented by Greenlight, Branded Entertainment Network®.

Gaussian Blur

The Gaussian filter is a weighted filter, meaning that all the values in the kernel sums up to one. This weighting can be found in lots of filters and is used to avoid adding more information to the original image after the filter has been applied. But what makes a Gaussian filter special is that the values in the kernel correspond to the values of a Gaussian curve. Furthermore, the Gaussian filter is symmetrical, making it possible to separate the kernel into a row vector and a column vector with equal values (Figure 5). Independently applying the vector to the x and y axis will produce the same results as applying the Gaussian filter to an image. Because of the Gaussian distribution in the kernel, pixels directly neighboring will have a higher impact on the (F) value of pixels in the output image, while further away pixels will have decreasing influence. Therefore, a Gaussian filter will blur the image removing noise while preserving edges.

$$\frac{1}{256} \times \begin{bmatrix} 1 & 4 & 6 & 4 & 1 \\ 4 & 16 & 24 & 16 & 4 \\ 6 & 24 & 36 & 24 & 6 \\ 4 & 16 & 24 & 16 & 4 \\ 1 & 4 & 6 & 4 & 1 \end{bmatrix} = \frac{1}{256} \times \begin{bmatrix} 1 \\ 4 \\ 6 \\ 4 \\ 1 \end{bmatrix} \times [1 \quad 4 \quad 6 \quad 4 \quad 1]$$

Figure 5. A Gaussian filter follows a Gaussian distribution with the highest weighting on the value in the middle of the kernel. When applied, the pixels directly neighboring will have a higher impact on the (F) value of the pixel in the output image, while pixels further away will have decreasing influence. This produces a blur that preserves edges and removes noise in an image.

1.5.3 Image calculations

Image calculations are performed by arithmetic or logical operations between two images. Of particular interest for the work in this thesis are the subtract and minimum operations used in Paper II. Subtract ($\text{Img} = (\text{Img1} - \text{Img2})$) is an arithmetic operation that subtracts the $F(x, y)$ value in the second image from the $F(x, y)$ value in the first image. Applying this operation between an image and a version of that image blurred with a Gaussian filter will subtract the background making the cells in the image easier to threshold by increasing the contrast between activated cells and background staining.

The minimum operation ($\text{Img} = \min(\text{Img1}, \text{Img2})$) is of logical nature and when applied between two images, transfers the lower $F(x, y)$ value in both images to the output image. When a minimum operation is applied to an image and a version of that image blurred with a Gaussian filter, the output image will retain all the stained cells while noise and inconsistencies in the background staining will be removed. This is a result of how Gaussian filters works, when applied, every pixel in the image will be balanced according to the values of their neighboring pixels. Pixels with a low (F) value will become brighter and pixels with a high (F) value will become darker. When the minimum operation is applied the darker pixels of the activated cells will be retained while the background will be taken from the blurred image (which has become darker after application of the Gaussian filter). The result is an image with the activated cells unchanged against a more homogenous background reducing the occurrence of noise and artefacts.

2 AIM

2.1 The general aim

The overall aim of this thesis was to investigate possible effects of sensory noise on brain function in ADHD, both in pre-clinical and clinical settings, as well as to assess the effects of acoustic white noise on neuronal brain activity patterns in rodents. Further, we aimed to evaluate possible mechanisms of action behind the positive effects of acoustic white noise in ADHD.

2.2 Aim of individual papers

- I *“Acoustic noise improves motor learning in spontaneously hypertensive rats, a rat model of attention deficit hyperactivity disorder”* aimed to investigate if acoustic white noise benefit also improves learning in an animal model of ADHD, the SH rat. This forms a foundation for the validity of investigating the mechanism of acoustic white noise in the SH rodent model of ADHD.
- II *“Acoustic white noise ameliorates reduced regional brain expression of CaMKII and Δ FosB in the spontaneously hypertensive rat model of ADHD”* aimed to investigate how acoustic white noise alters the brain activity in SH rats and in corresponding control brains by immunohistochemically staining for the neural activity markers CaMKII and Δ FosB.
- III *“Vestibular near threshold stochastic electric stimulation does not improve cognitive performance in ADHD - A pilot study”* aimed to investigate if stochastic noise of a different sensory modality than sound also is beneficial for persons with ADHD. Additionally, as the SVS stimulus is not really perceived, it allows testing of potential beneficial effects while eliminating informational masking effects as well as allowing the study to be conducted while blind to the stimulation protocol. A secondary aim was therefore to assess if a non-masking stimulus could induce noise benefit in ADHD.

3 METHODS

3.1 Animal studies

3.1.1 Animals

A total of 149 male rats were used in the animal experiments. The animals used were the SH rat (n = 77; SH/NCrl, Charles River, Germany), Wistar SCA rat (n = 48; WIS/SCA, Scanbur AB, Sweden), Wistar Han (n = 16; CrI:WI (Han), Charles River, Germany) and Wistar Han (n = 8; RccHan:WIST, Harlan Laboratories, United Kingdom). In Paper I, 55 SH rats and 48 Wistar SCA were used for behavioral testing. In Paper II, brains from 24 Wistar Han and 22 SH rats were immunohistochemically investigated regarding expression of the neural activity markers Δ FosB and CaMKII. The animals were four weeks of age at arrival and were housed four per cage (55 × 35 × 20 cm) with *ad libitum* access to food and water. The animals were kept on a 12/12 h light/dark cycle. All experiments were conducted during the bright part of the cycle.

3.1.2 Definition of acoustic white noise

The white noise sound file used in Paper I and Paper II contained equally distributed frequencies that spanned between 0 – 8 kHz. The noise was played back in a continuous loop through strategically placed loudspeakers (SBA1600/00, Philips, Amsterdam, Netherlands). In Paper I, the loudspeaker was mounted on top of the Montoya apparatus or behind the rotarod apparatus at head level of the animals. In Paper II, the speaker was placed face down over the ventilation mesh on top of the cages. Before testing began, the volume was adjusted to provide 75 dB(A) of noise at a height corresponding to rat head level, the volume being regularly checked with a sound level meter. The amount of background sound present in a quiet testing environment is referred to as ambient silence in this thesis and was measured to 38-dB(A) on average. During rotarod training (Paper I), the background sound varied between 50 and 68-dB(A) depending on the speed of the rotarod.

3.1.3 Study design (Paper I & II)

In Paper I, animals were divided into eight different treatment groups and trained in the Montoya staircase test or on the rotarod during either 75-dB(A) acoustic

white noise (SH n = 15; Wistar n = 12) or ambient silence (SH n = 16; Wistar n = 16) conditions. In parallel experiments using the same learning paradigm, animals were trained 30 minutes after receiving either 4mg/kg MPH (SH n = 12; Wistar n = 8) or an equal volume NaCl (0.9%) (SH n = 12; Wistar n = 12). Animals were trained in batches of 8 or 16 animals as they arrived and, in most cases, Wistar and SH were trained in parallel to ensure as similar conditions as possible.

In Paper II, animals were divided into the following treatment groups: animals kept in ambient silence (SH n = 8; Wistar n = 12), animals exposed to 75-dB(A) acoustic white noise (SH n = 8; Wistar n = 12) and SH rats kept in ambient silence after receiving an intraperitoneal injection of 4mg/kg MPH (n = 6). The animals were exposed to their respective treatment condition for one hour each day for five consecutive days. During the treatment period the cages were covered with a piece of dark cloth to reduce visual stimuli.

3.1.4 Behavioral assessments

Montoya Staircase (Paper I)

As per Montoya's recommendation (1991), two days before testing started and throughout testing procedures the animals were food deprived over night to maximize food seeking behavior. Three sugar pellets (45mg; BioServ, Frenchtown, NJ, USA) were placed in a small well at each of the seven levels of the two staircases. The animal was placed in the apparatus, which was in turn covered by a sound- and light-attenuating polyurethane box. The animal was left in the apparatus for 15 minutes before removal and the number of pellets consumed and dropped was counted. Each rat was trained for a total of 10 days and the main outcomes were the number of pellets consumed each day as well as the observed success rate of pellet retrieval.

Rotarod (Paper I)

Animals were trained to stay on a rotating cylinder of a Rotarod device (LE-8500, Panlab S.L.U., Spain) accelerating from 4 – 40 RPM over five minutes. Each animal completed four trials each day for 10 consecutive days. The latency to fall was automatically recorded by way of a lever activated by the force of an animal falling from the rotor. Animals falling off the rotor during the first 30 seconds were returned to the rotor, and the test continued. If the same animal were to fall

off the rotor a second time or if the animal fell after the first 30 seconds the trial was terminated. If an animal managed to stay on the rotor for more than 6 minutes the test was terminated and the trial result was noted as maximum performance (360 seconds). The main outcome of the test was latency to fall off the rotor. The average time the animals managed to stay on the rotor of the four daily trials recorded was used as a data-point for each day.

Open field motor activity (Paper I)

Locomotor activity, rearing activity and corner time was assessed in injection naïve SH and Wistar rats that had already completed the 10 days of rotarod and Montoya training. The apparatus used was a standard open field activity box (48 × 48 cm) with light beams that registered animal movements in 5-min bins. The animals were placed in the middle of the open field activity box and left there for 60 minutes under dimmed light conditions. Each rat was tested on two consecutive days with either silent or noisy conditions applied in a random order.

3.1.5 Perfusion and fixation (Paper II)

As described in Paper II, 48 hours after the animals received the final treatment, they were sacrificed for immunohistochemical analysis of the brain. The animal was put in to deep anesthesia with an excess of sodium pentobarbital (120 mg/kg) and was then perfused trans-cardially with 20-50 ml physiological saline (\approx 1 minute, until runoff liquid was clear) immediately followed by 200 ml freshly made ice-cold 4% paraformaldehyde solution in 0.1 M phosphate buffer (PB), pH 7.4, for 7 minutes. The brain was removed and post-fixed in 4% paraformaldehyde in PB, pH 7.4 overnight in 4 °C before being transferred to a 25% sucrose solution. All brains were sectioned into 35 μ m thick slices using a cryostat (Leica CM1950, Leica Biosystems, Heidelberg, Germany), divided in to 8 series and stored in a cryo-protectant solution at -20 °C until staining.

3.1.6 Immunostaining protocol (Paper II)

As described in Paper II, the free-floating sections were first washed 3 x 10 minutes in PBS. Heat induced epitope retrieval was performed by submerging the sections in heated sodium citrate buffer (90 °C; 10 nM) containing 0.05% Tween-20, pH 6.0, and placed in a 90 °C water bath for 6 minutes. Further, to block endogenous peroxidase activity free floating sections were quenched in PBS containing 3% H₂O₂ and 10% methanol during gentle agitation. Sections were

thereafter pre-incubated in 5% normal horse or goat serum (Vector laboratories, Burlingame, CA) containing 0.25% Triton-X in PBS, followed by overnight incubation with primary antibodies against either CaMKII α (mouse, 1: 2000; ab22609; 6G9; Abcam, Cambridge, UK) or Δ FosB (Rabbit, 1:5000; SC-48X; Santa Cruz Biotechnology, Dallas, Tx). On the second day, sections were incubated using an appropriate biotinylated secondary antibody (1:250 horse anti-mouse BA2001 for CaMKII α and 1:250 goat anti rabbit BA1000 for Δ FosB; Vector Laboratories,) for one hour followed by one-hour incubation in avidin-biotin peroxidase in PBS (ABC Elite Kit, Vector Laboratories). Finally, the staining was visualized by the chromogen 3, 3'-diaminobenzidine in PBS containing H₂O₂ (DAB Peroxidase substrate kit, Vector Laboratories). Sections were left in 3, 3'-diaminobenzidine for 5 minutes or until satisfactory background staining was achieved, the staining process was then stopped with an excess of PBS and the sections were washed 3x10 minutes in PBS. To achieve satisfactory results, the CaMKII α staining had to be re-stained over 5 minutes in 3, 3'-diaminobenzidine directly following the washing step. Sections were mounted on poly-L-lysine coated glass slides (Histobond, Marienfeld, Lauda-Königshofen, Germany), dried over 72 hours, washed in dH₂O, dehydrated in ethanol baths with gradually increasing ethanol percentage (70%, 90%, 95% and 99.5%, respectively), cleared in xylene and cover-slipped with DPX mounting medium for microscopy (Merck Millipore, Darmstadt, Germany).

3.1.7 Image acquisition and workflow for quantification of staining (Paper II)

Image acquisition was performed using a light microscope (Nikon Eclipse 90i; Nikon Instruments inc., Shinagawa, Tokyo, Japan) and a CCD camera (Nikon DS-Fil-U2; Nikon Instruments inc., Shinagawa, Tokyo, Japan). The microscope imaging software used was NIS Elements D (V 4.40; Nikon Instruments inc., Shinagawa, Tokyo, Japan). Images were analyzed using Fiji version 1.51s for Windows (Schindelin et al., 2012).

A standardized work flow for cell counting was established in the Fiji software. Removal of irrelevant background noise was performed by applying 50 iterations of Gaussian blur (sigma = 3) to the original image (ImgA; Figure 6A). A new image (ImgB; Figure 6B) was created from ImgA and its blurred counterpart using the algorithm $\text{ImgB} = \min(\text{ImgA}, \text{ImgA blurred})$. Subtraction of the background was performed by applying 100 iterations of Gaussian blur (sigma = 4) to ImgB and a new image (ImgC; Figure 6C) was created by using the subtraction algorithm $\text{ImgC} = (\text{ImgB} - \text{ImgB blurred})$. ImgC was converted to 8-bit color depth and a local threshold value was determined by the Phansalkar algorithm (radius = 15, Parameter_1 = 0.19, Parameter_2 = 0.9; Figure 6D; (Neerad et al., 2011)). To separate cells that appeared joined together after thresholding, a watershed operation was performed. The region of interest was selected and cells was counted using "Analyze Particles" (size = 20-200; circularity 0.5-1.0; Figure 6E).

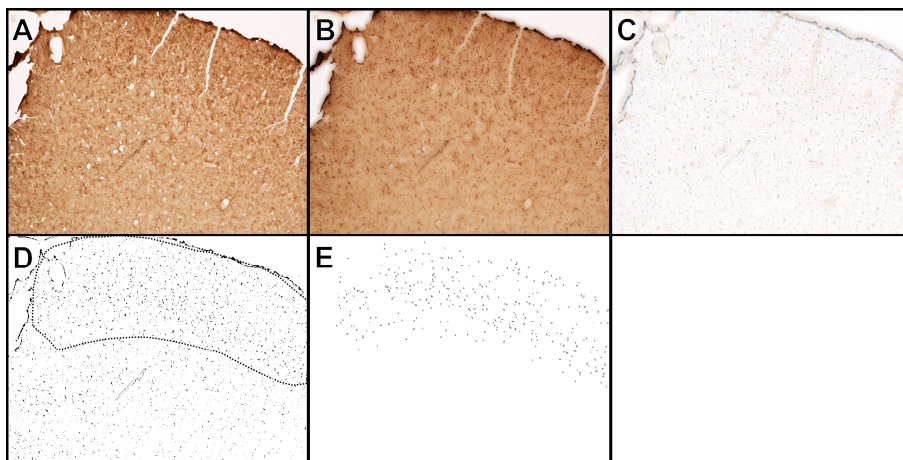


Figure 6. Image analysis work-flow. The acquired photographed original image (A; *ImgA*), was processed using noise removal by application of a minimum algorithm to *ImgA* and its blurred counterpart (B; *ImgB*). Following removal of background staining using a subtract algorithm on *ImgB* and its blurred counterpart (C; *ImgC*), a Local threshold determined by Phansalkar algorithm was applied to *ImgC* (D). Region of interest was finally outlined (black dotted line; D) and particles were analyzed, giving the counted cells within the region of interest (E). Image adapted with minor changes from Paper II, "Acoustic white noise ameliorates reduced regional brain expression of CaMKII and Δ FosB in the spontaneously hypertensive rat model of ADHD". Submitted.

To compensate for different background intensities of the Δ FosB staining, threshold values in each individual image were established by sampling the mean grayscale intensity of Δ FosB activated cells. The background noise was removed by applying the algorithm $ImgB = \min(ImgA, ImgA \text{ blurred})$, and five visually identified Δ FosB activated cells were selected, and the mean grayscale pixel values of these cells were measured. A threshold value was set to cover all pixels ranging from zero to ten grayscale units brighter than the mean pixel value measured for the activated Δ FosB cells. This was to assure that pixels at the edges of each active cell, which tend to be brighter, were included in the analysis. After thresholding, the image was converted to binary, a watershed operation was performed and cells were counted using "Analyze Particles" (size = 20-200; circularity 0.5-1.0).

Mean neuronal staining intensity calculations in the tuberomammillary nucleus (TMN) were also performed using Fiji software. The unprocessed image was converted to 8-bit, and the colors were inverted to negative. The background intensity was determined by selecting three regions bordering to, but not including, TMN. The mean intensity of these selections was used as a reference point for background staining. The difference between TMN intensity and background staining was used in the statistical analysis.

3.2 Clinical study

3.2.1 Participants (Paper III)

In the clinical study 24 participants, 16 male and 8 female, median age 16.5 years with a definite ADHD diagnosis according to DSM-5 (American Psychiatric Association, 2013) were included. The participants were recruited from Gillberg Neuropsychiatry Centre in Gothenburg, Sweden, the Child and Adolescent Psychiatric unit in Lund, Sweden and the Adult General Psychiatric unit in Mölndal, Sweden.

3.2.2 Study design (Paper III)

Cognitive evaluations were performed on two separate occasions where participants received either SVS or shamSVS stimulation in a randomized order. On the first day participants were randomized to a stimulation protocol (X or Y) balanced towards age and gender using an open source web-based minimization

process (Qminim; <http://rct.mui.ac.ir/q/>). One stimulation protocol contained active SVS while the other delivered no active SVS current. If allocated to the active SVS stimulation protocol on the first day, on their second visit the participant received sham treatment, and vice versa. The content of both stimulation protocols was unknown to participants as well as examiners. The participants underwent three cognitive tests: Ray's auditory verbal learning test, the span-board spatial working memory test and the Flower-trail fine motor learning test.

3.2.3 SVS protocol (Paper III)

Oval electrodes (4 × 6 cm; Axelgaard Manufacturing, CA, USA) were placed over the mastoid processes behind both ears after thorough cleaning using Nuprep® skin prep gel (Weaver and Company, USA). Before testing commenced, the individual galvanic vestibular stimulation threshold was determined by applying sinusoidal bipolar currents (1 Hz). When stimulation is presented in this manner, alternating activation of the left and right side of the vestibular system is achieved, producing a swaying sensation. A total of 12 different amplitudes ranging between ±0.0 mA to ±0.7 mA (peak to peak) were delivered in a pseudo-randomized order. Each amplitude was presented in 15s intervals followed by a 20s 0-current pause. Participants were asked to sit down with their lower back relaxed and their eyes closed and report any rocking or swaying sensations. The lowest amplitude that produced swaying noticeable to the examiner or where the participant reported a self-perceived rocking sensation was set as the maximum allowed current in the individual's SVS protocol.

The active SVS protocol was a bipolar stochastic vestibular signal (0-30 Hz) created using a Gaussian white noise pattern generator and filtered using a 10th order low-pass Butterworth filter. The current never exceeded the individually determined threshold amplitude and for 90% of the time the current was lower than 45% of the threshold amplitude.

Ray Auditory Verbal Learning Test (Paper III)

Participants were asked to listen to and memorize a pre-recorded list of 15 common Swedish words presented with a 1s inter-stimulus interval. Directly after each trial, they were asked to repeat as many words as they could, in any order. This was repeated five times with the same list being read before each

recall. After the fifth trial, a distractor list was read. The distractor list contained 15 new words which the participants were asked to memorize and repeat in the same manner. In a seventh trial, directly after the distractor trial, the participants were asked to recite as many words as they could from the initial list without it being read to them again prior to recall.

Span-board spatial learning test (Paper III)

The participants were asked to memorize the location and sequence of markers that appeared randomly on a 4x4 square board on a computer screen. The test started with two markers in sequence and for every two correct attempts, one more marker was added to the next sequence. The test continued until the participant made an error in two consecutive sequences on that particular level.

Flower trail fine motor learning test (Paper III)

The test entailed drawing a continuous line within the borders of a “flower” pattern. The participants were informed that each trial was timed and that drawing outside the line was counted as an error. A total of 15 trials were completed in this manner. Due to a strong retained learning effect (Göran Söderlund, personal communication) the flower-trail test was only performed once and was therefore not part of the crossover design.

3.3 Statistical analysis

All statistical analyses in Paper I were performed in GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, USA). In Paper II and III all statistical analyses were performed using SPSS for Windows version 22 (IBM, Chicago, IL, USA) and SAS for Windows, version 9.4 (SAS Institute, Cary, NC, USA). The threshold of significance was set to $p \leq 0.05$

3.3.1 Paper I

Results from the Montoya staircase test and Rotarod running were analyzed using two-way repeated measure analysis of variance with Bonferroni multiple comparisons. A Mann-Whitney U-test was used to determine if the ratio of animals that managed to learn the task differed between treatment groups. Nonlinear regression was used to analyze results from open field motor activity.

3.3.2 Paper II

We used linear mixed models to analyse cell counts and grayscale intensity measures. The model used for both the whole-brain subsample and the full sample included fixed factors for strain (Wistar or SH) and treatment (silence and noise), and the interaction between strain and noise. Only descriptive statistics are given for the expression of CaMKII and Δ FosB in MPH treated rats because of the lack of a natural control group.

Within-subject correlations between measurements from left and right hemispheres were modelled using a compound symmetry covariance matrix, and the Kenward-Roger approximation was used to estimate denominator degrees of freedom. Measurements from different areas were analyzed independently and separate analyses were run for CaMKII and Δ FosB, respectively. Post hoc comparisons between SHS and WIN were not deemed relevant and are therefore not shown.

3.3.3 Paper III

Based on previous studies we estimated an effect size of about 3-5%. Based on this effect size we calculated that we needed 30 (± 10) participants to obtain statistically significant results.

We used linear mixed models to analyze the results from the Ray's auditory verbal learning test. Data consisted of repeated measures both within and between visits, so the model included fixed factors for treatment (SVS/shamSVS), visit (one or two), trial (one to seven) and the interaction between trial and treatment. Random slopes for subject, interaction between subject and trial as well as the three-way interaction between subject, visit and treatment were included in the model.

The effect of SVS on span-board performance was analyzed using three-way analysis of variance with fixed factors for subject, visit and treatment (Grieve and Senn, 1998). The outcomes analyzed were the number of correctly indicated markers, the number of correct sequences and the maximum number of markers in a correct sequence.

In the flower-trail test we assessed whether SVS had an effect on trial drawing time using linear mixed models with predictors for age (years), sex and treatment. Number of errors committed (line crossings) was analyzed in a similar manner including trial drawing as an additional covariate. The correlated nature of the repeated measurements was modelled using an unstructured covariance matrix and the Kenward-Roger approximation was used to estimate denominator degrees of freedom.

3.4 Ethics

Animal studies (Paper I and II) were conducted in accordance with Swedish animal welfare legislation and the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental designs were approved by the Gothenburg Animal Research Ethics committee. The clinical study (Paper III) was approved by the regional Ethical Review Board in Gothenburg, Sweden, and conducted in accordance with the ethical principles stated in the Declaration of Helsinki. Informed written consent was obtained prior to study inclusion and any study related activity.

Ethical reflections

All animal studies were conducted with the three R's in mind. Replacement of research animals in experiments focused on behavioral evaluations is hard to achieve. As of today, the complex processes in the CNS that facilitates e.g. behavioral responses and learning processes simply cannot be recreated in cell lines or computer simulations. However, we can make sure that we reduce the number of animals that we use in each study.

In Paper I, measures from the Montoya staircase, rotarod and open field was made in the same animals, significantly reducing the total number needed in the study. Pain and stress affect the animals negatively, which impairs reliability and reproducibility.

When conducting a clinical study, as in Paper III, there are additional ethical considerations that need to be addressed. Participants enrolled in the study of their own free will and was given sufficient information about the experimental procedure to be able to consider the risks and benefits associated with

participation in the study. They could then make an informed decision if he or she still want to proceed. Since a minor cannot provide a legally binding consent it is up to the parent or legal guardian to do so on his or her behalf. All potential participants were sent home a brochure detailing the study design and necessary information regarding the procedure, allowing the parents sit down with their child and together agree upon if the child wanted to enroll in the study or not. If consent was given, the parent was allowed to stay in the room during the testing procedure if they or the child wanted them to do so.

4 RESULTS AND DISCUSSION

4.1 Animal studies (Paper I & II)

4.1.1 Open field behavior (Paper I)

During ambient silence the initial exploratory behavior of SH and Wistar rats did not differ between strains, however SH rats habituated more slowly to the open field chamber over time. In the last 30 min in the chamber, Wistar rats displayed significantly less locomotor activity than the SH rat. Wistar rats spent the majority of the last 30 minutes in a corner whereas SH rats did not develop a corner preference at all. SH rats displayed significantly more rearing activity in the later part of the test than Wistar rats. These findings are in agreement with low anxiety level in the SH rat reported in the elevated plus maze (Goto et al., 1993) and with previous work where habituation to spontaneous activity was studied in a similar fashion (Sagvolden et al., 1992b; Qian et al., 2010; Umehara et al., 2013).

Exposure to acoustic white noise during open field activity did not change the activity pattern in SH or Wistar rats, except for a slightly higher corner time for Wistar rats compared to during ambient silence conditions.

4.1.2 Motor learning (Paper I)

In Paper I, prior to the experiment strict exclusion/inclusion criteria for animals trained in the Montoya staircase and on the Rotarod were decided. The Montoya staircase test involves a great deal of reinforcement learning. When learning the task, it is crucial that the animals receive positive feedback in the form of sucrose pellets to become motivated to forage further. We therefore decided that animals that only retrieved one or no pellets during the entire training period would be excluded from the statistical analysis, as described in Table 1. Similarly, in the Rotarod test, animals that during the training period learned to stay on the rotor for at least 100s were included, however if the animal showed declining performance they were excluded from the statistical analysis. We argue that these criteria were justifiable because the main outcome of the study was the animal's ability to learn these tasks and by including the "avoiders" the results would have been misleading. The number of animals in the different treatment

groups that managed to learn the task according to these criteria are summarized in Table 1.

Table 1 The ratio of animals having learned the tasks per treatment group.

Learning ratios	Montoya	Rotarod
SH silence	14/16 ^a	8/8
SH noise	14/15 ^b	5/7
SH MPH	4/12	7/8
SH NaCl	8/12	6/8
Wistar silence	7/16	6/8
Wistar noise	4/12	-
Wistar MPH	7/8	5/8
Wistar NaCl	4/12	5/8

a Significantly fewer excluded SH than Wistar trained in ambient silence.

b Significantly fewer excluded SH than Wistar trained in acoustic white noise.

In the Montoya staircase test, during ambient silence conditions significantly less SH rats than Wistar rats were excluded from the statistical analysis. In animals that learned the task, the rate at which SH and Wistar rats developed skilled reach differed between the groups during ambient silence conditions. Although retrieval scores were similar during the first and last day of training, SH rats needed 2.2 more training days to reach 50% of their maximum performance. These findings are in agreement with previous reports of deficits in skilled reach performance in the SH rat demonstrated by Qian et al. (2010). However, our results are somewhat different from Qian and colleagues, who reported a lower end point performance of SH rats compared to Wistar. This can possibly be attributed to differences in the skilled reach paradigms used, different outcome measures or that Qian and co-workers used an inbred control strain. One finding of particular interest is that SH rats, although slower to learn the task, had a significantly higher proportion of learners who fulfilled the inclusion criteria. A possible explanation is that the Montoya test involves an initial exploratory phase where the rat must voluntarily explore the staircase to locate the sucrose pellets and by doing so receive a positive feedback. A trend observed while training the animals was that if no successful pellet retrieval had been made during the first five days, the probability that the animal would retrieve two or more pellets by the end of the last training session was very low. We therefore argue that the increased exploratory behavior of the SH rat to be a contributing factor to the higher proportion of learners rather than reflecting a

greater capacity of SH rats for learning. This claim is supported by a report from Sontag and colleagues (2013) in which they argue that the increased locomotor activity of the SH rat is a confounding factor in spatial memory tasks.

SH rats exposed to 75 dB(A) white noise during training demonstrated faster acquisition of skilled reach than SH rats trained during ambient silence. This was evident by a significant interaction noise \times training day as well as a left shift in time to 50% of maximum performance. Skilled reach in SH rats exposed to white noise developed as fast as for Wistar trained during ambient silence. MPH did not alter learning of the skilled reach task of SH rats in comparison to vehicle treated animals. A possible explanation for the lack of effect of MPH on skilled reach learning is that one of the adverse effects of MPH is decreased appetite. Motivated food seeking behavior is an important aspect when learning tasks involving food as reinforcement. Therefore, when studying effects of treatments on food reinforced acquisition of fine motor skill it is problematic if these treatments also attenuate appetite.

Rotarod performance was significantly lower in SH rats compared to Wistar rats trained during ambient silence. Although training proceeded for ten days, the highest improvement was seen during the first three days and plateau performance was achieved after approximately four to five days of training. These results can be contrasted to those of Qian and colleagues (2010) that reported no difference in Rotarod running skill between SH and Wistar rats. However, the Rotarod paradigm used by Qian was somewhat different from ours. Qian trained the animals on the Rotarod for ten trials in only one day compared to our paradigm of four trials per day over ten days. Buitrago and colleagues (2004) report that improvements in Rotarod performance can be seen both within trials and between training days, however the first trial on a given training day did typically not reach the performance of the last trial of the preceding training day. It is plausible that the day-long interval between training sessions and the relatively small trial repetitions per training day constituted less-than-ideal conditions for the SH rats ability to learn optimally, especially when considering deficits in learning and working memory displayed by the SH strain (Nakamura-Palacios et al., 1996; Gattu et al., 1997). One might argue that the hyperactive nature of the SH rat would prove useful on the Rotarod, however, Buitrago (2004) propose that the increased performance seen over several days of Rotarod

training cannot be attributed to an enhanced locomotor activity but rather by the rat acquiring new motor strategies by learning to modify gait patterns during training.

SH rats that were trained on the Rotarod during white noise conditions performed significantly better than the SH rats trained in ambient silence. This was evident by a main effect of noise. MPH medication improved the performance of the SH rat to the level of Wistar controls. The MPH Rotarod improvement was strain specific and supports the validity of the SH rat model of ADHD in relation to known effects of MPH on motor function in children with ADHD.

4.1.3 Neural activity (Paper II)

Micro-array studies of the SH rat have revealed that the expression of the plasticity marker CaMKII is significantly decreased in the medial PFC, ventral striatum, dorsal striatum, hippocampus, vermis, and ventral mesencephalon (DasBanerjee et al., 2008). Furthermore, the expression of CaMKII and components of the Fos family of immediate early genes have been reported to be decreased in the nAc of SH rats (Papa et al., 1996; Papa et al., 1997, 1998), a decrease that may be reversed by MPH treatment (Sadile, 2000). In paper II, we investigated how acoustic white noise altered neuronal activity in the brains of SH and Wistar rats using immunohistochemical staining of CaMKII and Δ FosB.

The overall result of this study is that the expression of the neuronal activity/plasticity markers Δ FosB and CaMKII tends to be lower in several brain areas in the SH rat model of ADHD compared to Wistar rats and that this difference can be reduced by acoustic white noise in a fashion that resembles, but is not identical to the effects of MPH. Consequently, it appears that repeated acoustic white noise can normalize plasticity markers in some brain areas known to influence cognition and behavior in the SH rat model of ADHD (Russell, 2000, 2002, 2003).

In the dorsolateral PFC (DL-PFC), there were no differences in CaMKII expression between the strains, but in the analysis of Δ FosB positive cells we found a main effect of strain and of noise but no strain \times noise interaction. Post hoc tests further revealed that SH rats had significantly fewer positive Δ FosB cells in the DL-PFC than Wistar rats in the ambient silence condition. Acoustic white noise exposure

increased Δ FosB expression in the SH rat but had no effect in the Wistar rat. The DL-PFC receives, among other, sensory inputs from the auditory sensory system and is important for top-down control of motor behaviors (Miller and Cohen, 2001). Reduced activation of the DL-PFC is congruent with the ADHD phenotype of the SH rat, as well as with known alterations of brain activity and structure in children and adolescents with ADHD (Dickstein et al., 2006; Cao et al., 2013; Dimatelis et al., 2015). It is therefore intriguing that this study shows that both MPH and acoustic white noise can ameliorate the reduction in DL-PFC Δ FosB activity in SH rats as it suggests similar effect pathways of the two interventions. Furthermore, the effect of acoustic white noise is strain specific, or conditioned by a reduced baseline expression of Δ FosB.

In the nAc, similarly to the DL-PFC we found no difference in CaMKII expression between the two strains. However, there was a clear difference in the number of positive Δ FosB cells between the different treatment conditions. We found a significant strain \times noise interaction and post-hoc tests indicate that this interaction was explained by lower Δ FosB in the nAc of SH rats than in Wistar rats exposed to the ambient silence condition, and that noise exposure increased the activity in the SH rat but had no significant effect in the Wistar animals. The nAc respond to reward feedback and forms parts of the corticostriatal reward system and is important for habit learning. The nAc is anatomically connected to PFC with more prominent projections to the medial part rather than the lateral part of the PFC (Tzschentke and Schmidt, 2000), which was the area that displayed altered Δ FosB activity in Paper II. It is therefore interesting that we observed similar changes in Δ FosB activity in the nAc and the DL-PFC following acoustic white noise and MPH exposure and it may indicate a functional connection between these areas. As a part of the mesolimbic pathway, the nAc is rich in dopaminergic terminals (Calipari et al., 2016). The caudal part of nAc shell receives direct norepinephrine innervation from the locus coeruleus (Berridge et al., 1997) while noradrenergic innervation of the rostral part of the nAc is scarce (Park et al., 2010). Acute administration of MPH increases DA and norepinephrine levels in both the PFC and the striatum of SH rats, as reviewed in Heal and colleagues (2008), but chronic administration may have more specific catecholamine-enhancing effects in the PFC than in the nAc (Koda et al., 2010).

It should be noted that previous studies have demonstrated reduced expression of CaMKII regionally in the shell but not in the core of the nAc of SH rats (Papa et al., 1996; Papa et al., 1998), but since we performed no separate analyses of subdivisions of the nAc in Paper II, we cannot confirm these earlier findings.

In the Dorsolateral striatum (DLS), we found no quantifiable staining of CaMKII. The analysis of Δ FosB positive cells revealed a main effect of strain, but not of noise and there was no interaction. Post hoc tests indicated that the strain effect was explained by significantly lower Δ FosB positive cells in the DLS of SH rats compared to Wistar rats in the ambient silence condition. Exposure to acoustic white noise did not increase Δ FosB expression in the SH rat or the Wistar rat. The DL-PFC is more strongly connected to the DLS than to the nAc (Averbeck et al., 2014; Haber, 2016). It was therefore expected to find a correlation between effects of strain and treatment in the DLS and the DL-PFC. In the DLS, however, acoustic white noise did not increase Δ FosB positive cells in SH rats as it did in the DL-PFC. An increase in Δ FosB positive cells in the DLS was nevertheless found following MPH treatment. This suggests that MPH treatment compared to acoustic noise activates the DLS to a larger proportion than the DL-PFC. One possible explanation for this may be that the origin of the effect of acoustic white noise is external and could possibly project from sources more functionally connected to the DL-PFC than the DLS, whereas MPH exerts its effect directly in areas of the brain with high availability of dopaminergic and noradrenergic terminals. Previous studies from our group have demonstrated no increase of extracellular DA following acoustic white noise exposure (Palsson et al., 2011) suggesting that noise mediates its neural activating effects via other pathways and mechanisms than dopaminergic ones, an obvious candidate for further investigation would be the noradrenergic system.

There was no Δ FosB staining in cell bodies in the TMN, however a difference in CaMKII fiber expression was observed between conditions. The analysis revealed a significant main effect of strain and of noise, but no interaction. SH rats exposed to the ambient silence condition had significantly lower expression of CaMKII positive fibers than Wistar rats. The TMN is the sole source of the histamine pathways that regulate arousal and wakefulness in the brain (Haas and Panula, 2003; Blandina et al., 2012). Exposure to the acoustic white noise condition increased CaMKII expression in both strains. The main afferent

projections to the TMN include neurons from the infralimbic cortex (ventromedial PFC), hypothalamus and the basal forebrain, and to a lesser extent from the brain stem (Ericson et al., 1991). As there is a considerable convergence of afferents from many parts of the brain we can only speculate to what the observed difference in CaMKII positive fibers indicates in the TMN of SH rats. It is nevertheless possible that it represents altered regulation of histaminergic neurons. This assumption, if correct, fits well into the optimum stimulation theory by Zentall and Zentall (1983) as well as with the moderate brain arousal model proposed by Sikström and Söderlund (2007). In the TMN, the synthesis of histamine has been shown to be mediated through CaMKII phosphorylation and subsequent activation of histidine carboxylase, the enzyme responsible for histamine synthesis (Torrent et al., 2005; Moreno-Delgado et al., 2006). The increased CaMKII expression observed in both strains following acoustic white noise exposure could therefore be interpreted in terms of altered arousal through increased histamine production. On the contrary, both the fact that white noise applications are popular sleeping aids and observations regarding the role of acoustic white noise for arousal and sleep (Stanchina et al., 2005; Messineo et al., 2017) suggest that acoustic white noise will increase the threshold needed to elicit an arousal event, possibly by masking other acoustic stimuli. Since our data does not allow us to measure histamine concentrations, further research is needed to determine what outcome increased CaMKII expression in the TMN would have on histamine synthesis and cortical arousal.

4.2 Clinical study (Paper III)

In this double-blind randomized crossover pilot study, we evaluated the effects of near threshold SVS on word-recall, span-board spatial memory and fine motor learning and skill in 24 persons with ADHD. The rationale for studying weak near threshold SVS was the ability to perform a blinded crossover trial where both the participant and the examiner could be blind to the stimulation protocol, which would have been impossible to accomplish using acoustic white noise or higher stimulation levels. Furthermore, as the SVS stimulus is not really perceived it allows testing potential beneficial effects of sensory noise while eliminating the informational masking mechanism discussed earlier.

In the Ray auditory verbal learning test, SVS did not influence final recall scores compared to sham-SVS. An analysis employing a linear mixed model did not reveal any main effect of SVS and there was no interaction between trial and treatment.

SVS did not have a significant effect on the primary outcome, the total number of correct markers, of the span-boards spatial memory task. On the secondary outcome variables, SVS did not influence the number of correct sequences carried out, however, during the SVS condition the number of markers in a sequence completed without errors was significantly higher than during sham-SVS.

In the flower trail test, drawing time was positively associated with age but not affected by gender or SVS. The number of errors committed was inversely related to trial drawing time and age, i.e., accuracy was sacrificed to achieve faster drawing speed, and younger participants committed more errors. Women committed fewer errors than men, but there was no effect of SVS.

SVS did not in any meaningful way increase performance in any of the tests in this double-blind randomized multicenter trial. The only measure that was significantly increased was the secondary outcome variable, the maximum number of markers in a sequence completed without error, in the span-board test. Even though this may indicate some effects of SVS, especially since the other measures of the span-board task followed the same pattern. However, the effects were much smaller in comparison to previously reported differences in visuospatial memory of individuals with ADHD compared to healthy controls (Narimoto et al., 2018) as well as for previously reported effects of acoustic white noise (Söderlund et al., 2016; Söderlund and Jobs, 2016).

Negative results can be challenging to interpret. In a clinical study e.g., a negative outcome can indicate that the intervention is ineffective, that the doses used were too small or be a consequence of poor statistical power. Although near threshold SVS is a weak stimulation where the current is kept lower than noticeable for 90% of the time, several studies have demonstrated positive effects on both balance/motor functions and on cognitive performance of near thresholds SVS using stimulation protocols at levels similar to the one used in Paper III of this thesis (Wilkinson et al., 2008; Mulavara et al., 2011; Wilkinson et

al., 2012; Kim et al., 2013; Bloomberg et al., 2015; Goel et al., 2015; Lee et al., 2015; Mulavara et al., 2015; Samoudi et al., 2015). Consequently, the weak or absent effect cannot reasonably be attributed to that the stimulation intensity was too low to activate the vestibular system at all. It is however possible that higher activity of the vestibular system would be necessary to achieve noise benefit in ADHD. The obvious advantage of using an unperceivable stimulus would however be lost at higher stimulation levels, and there would be no obvious advantage of vestibular stimulation over acoustic stimulation other than the academic interest in determining if a different sensory modality than sound is effective. An important consideration is whether the current study was underpowered. The pre-study power analysis indicated that a population of 20-40 participants would be sufficient, depending on outcome variable. Inclusion turned out to be slow, so unfortunately it was not possible to reach the higher level of that interval. Our study population was nevertheless larger than previous studies that have demonstrated significant effects of acoustic white noise (Baijot et al., 2016; Söderlund and Jobs, 2016). In the span-board task, the 95% confidence interval for mean change in performance during SVS was -1.67 to 10.3, or a relative change of -4.4% to 27.4%. In the first trial of Ray's auditory verbal learning test the outcome 95% confidence interval was -1.2 to 0.12 (-21.6% to 2,1%) and in the last trial it was -1.1 to 1.2 (-11.1% to 12.0%). A previous report demonstrates a performance improvement of 50% in the span-board spatial test during acoustic white noise in patients with non-medicated ADHD. From the same study, improvements during acoustic white noise reached about 13% in first time free recall (Söderlund et al., 2016), which corresponds to trial 1 in Ray's auditory verbal learning test. It is possible that we would have found significant effects in Paper III with a larger study population. However, it is also likely that if SVS improves cognitive tests in ADHD the effects are smaller than those of loud acoustic noise and it is questionable if they would motivate the practical obstacles of using skin electrodes during learning activities.

If masking of irrelevant stimuli is the important mechanism of the beneficial effects of acoustic white noise observed in ADHD it could explain why the effects of SVS were weak or absent in this study, as the stimulus is not perceived. According to the moderate brain arousal model proposed by Sikström and Söderlund (2007) adding an external sensory noise to a nervous system that is not functioning optimally could help improve its function through the

phenomenon of SR. However, the results acquired in Paper III of this thesis do not support the notion that near threshold sensory noise improves function in ADHD.

5 CONCLUDING REMARKS

In Paper I we have demonstrated that the performance enhancing effects of acoustic white noise that have been shown in children with ADHD are also observed during the learning of a skilled reach task and rotarod running in the SH rat model of ADHD. To the best of our knowledge this is the first time that beneficial effects of acoustic white noise have been demonstrated on the acquisition of fine and gross motor skills in the SH rat. Just like in children with ADHD the noise benefit was specific to the animals with the ADHD-like phenotype and this finding further strengthens the predictive validity of the SH rat as an animal model of ADHD.

Paper II confirmed that the SH rat compared to a Wistar control display reduced expression of the neuronal activity marker Δ FosB in the DL-PFC, nAc and the DLS. Furthermore, the plasticity marker CaMKII was reduced in the DL-PFC, nAc and the TMN. Acoustic white noise appears to induce changes in brain activity in some brain areas (DL-PFC, TMN and nAc), but not in DLS. The effect of acoustic white noise in the PFC in particular suggests that it could influence top-down control of behavior, but presumably via other mechanisms than MPH. Following acoustic white noise exposure, the TMN expression of CaMKII was increased in both strains. CaMKII have previously been credited to mediate synthesis of histamine and this could possibly indicate that the beneficial effects of acoustic white noise in ADHD is attributed to an increased arousal. However, our data does not allow us to assess histamine transmission so we have no way of determining if this is true or not.

In Paper III we found no positive effects of near threshold SVS on the cognitive performance of persons with ADHD. This does not preclude the possibility that other sensory modalities can be used for noise benefit in ADHD, but the hypothesis that even a small increase in sensory noise would be beneficial was not supported, and we were therefore also found no direct support for a stochastic resonance mechanism of sensory noise in ADHD. Since the SVS stimulus is not perceived, it remains possible that that informational masking is a key mechanism for the cognitive enhancing effects of acoustic white noise.

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