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ROLE OF THE DOPAMINE SYSTEM IN MOTOR SKILL LEARNING: IMPLICATIONS FOR NEURODEVELOPMENTAL DISORDERS

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Cover illustration: A chemical formula of dopamine as a key, with the symbolic neuron as a keyhole. Original from Corey-Lee, modified by Yu Qian.

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Role of the Dopamine System in Motor Skill Learning: Implications for Neurodevelopmental Disorders

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Dopamine (DA), released by midbrain neurons, is critical for motor performance, motor skill learning, and corticostriatal synaptic plasticity. Dysregulation of DAergic signaling in corticostriatal circuitry has also been implicated in several highly heritable neurodevelopmental disorders, such as attention/deficit hyperactivity disorder (ADHD), which are often associated with deficits in fine motor skills. However, the cellular and molecular pathways mediating the effects of DA are still poorly understood. In the present thesis, we used a skilled reaching task to investigate potential DAergic mechanisms contributing to the acquisition and performance of fine motor skills (i.e., skilled reaching and grasping).

To explore the influence of natural genetic variation in the DA system in motor skill learning, we took advantage of two inbred strains of mice (i.e., BALB/c and C57BL/6) that differ markedly in the number of midbrain DA neurons. We demonstrate significant variation in skilled reaching behavior in these two strains. Specifically, variations in the rate of motor learning correlated with divergent DA-related gene expression (e.g., DA D1 receptors and DARPP-32) in frontal cortex and striatum. These results implicate genetically driven variation in frontostriatal DAergic neurotransmission as a key contributor to individual differences in fine motor skill.

To identify brain activity patterns associated with different phases of motor skill learning, we studied the induction of the plasticity-related gene *Arc* (also known as *Arg3.1*), and also investigated learning-induced changes in the DA system. In the early phase of motor skill learning, *Arc* mRNA was significantly induced in the corticostriatal circuitry, including the medial prefrontal cortex (mPFC), cingulate cortex, primary motor cortex, and striatum. In the late phase, however, a shift in the expression pattern of *Arc* was evident—with a significant decrease in *Arc* mRNA in most regions examined (except in the mPFC and striatum). There were also significant changes in the expression of DA D1 receptors and their intracellular target DARPP-32 in the striatum (but not cortical regions) during the early, but not late, phase of motor skill learning. Analysis of the phosphorylation state of dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32) and its downstream target cAMP response element-binding protein (CREB) in the striatum indicated increased levels of phospho-Thr34-DARPP-32 and phospho-Ser133-CREB during the early, but not late, phase of motor skill learning. These findings implicate the cAMP/PKA/DARPP-32 signaling pathway in the acquisition of novel motor skills, and also demonstrate a dynamic shift in the contribution of corticostriatal circuitry during different phases of motor skill learning.

Finally, we explored whether spontaneously hypertensive rats (SHRs), the most commonly used genetic animal model of ADHD, is valid for investigating fine motor skill problems displayed by the majority of children with ADHD. Although SHRs could learn the skilled reaching task, their performance is significantly poorer than that of control rats in the most sensitive measure of skilled performance (i.e., success on the first attempt). However, gross motor coordination appears to be normal in SHRs, suggesting that the SHR strain displays specific deficits only in fine motor skills. Moreover, DARPP-32 was significantly higher expressed in corticostriatal circuitry of SHR compared to controls. Our results support the notion that the SHR strain is a useful animal model system to investigate potential molecular mechanisms underlying fine motor skill problems in ADHD.

The present thesis gives evidence supporting the notion that normal genetic variation in the DAergic system might contribute substantially to variability in the acquisition of motor skills in humans. More specifically, the results suggest the involvement of the D1R/cAMP/DARPP-32 signaling pathway in those neurodevelopmental disorders that are associated with fine motor skill deficits.

LIST OF SCIENTIFIC PAPERS

- I. **Qian Y**, Chen M, Forssberg H, Diaz Heijtz R. Genetic variation in dopamine-related gene expression influences motor skill learning in mice. *Genes Brain Behav.* 2013 Aug;12(6):604-14.
- II. **Qian Y**, Forssberg H, Diaz Heijtz R. Different phases of motor skill learning in rats are accompanied by distinct patterns of modifications in the cAMP/PKA/DARPP-32 signaling pathway. *Submitted manuscript*
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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
Arc	Activity-regulated cytoskeleton-associated protein
ADHD	Attention deficit hyperactivity disorder
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	Autism spectrum disorder
BDNF	Brain-derived neurotropic factor
CDK5	Cyclin-dependent kinase 5
CG	Cingulate cortex
COMT	Catechol-O-methyltransferase
CP	Cerebral palsy
CREB	cAMP response element-binding protein
DA	Dopamine
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa
DAT	Dopamine transporter
DCD	Developmental coordination disorder
DDC	Dopa decarboxylase
DMS	Dorsal medial striatum
ECL	Electrogenerated chemiluminescence
HSP90	Heat shock protein 90
L-DOPA	L-3, 4-dihydroxyphenylalanine
LTD	Long-term depression
LTP	Long-term potentiation
M1	Primary motor cortex
mPFC	Medial prefrontal cortex
NMDA	N-methyl-D-aspartate
OFC	Orbitofrontal cortex
PET	Positron emission tomography
PFC	Prefrontal cortex
PKA	Protein kinase A
PP-1	Protein phosphatase 1
PVDF	Polyvinylidene difluoride

qRT-PCR	Quantitative real-time polymerase chain reaction
SHR	Spontaneously hypertensive rats
SNe	Substantia nigra pars compacta
SNP	Single nucleotide polymorphism
STR	Striatum
TH	Tyrosine hydroxylase
TMS	transcranial magnetic stimulation
VLS	Ventrolateral striatum
VMS	Ventromedial striatum
VTA	Ventral tegmental area
WIS	Wistar
WKY	Wistar Kyoto

1 INTRODUCTION

Neurodevelopmental disorders are common (about 10% of all children) and include a large number of diagnostic categories, e.g., cerebral palsy (CP), autism spectrum disorder (ASD), attention-deficit/hyperactive disorder (ADHD), intellectual disability, specific language impairments, and developmental coordination disorder (DCD). Each neurodevelopmental disorder has a distinct pattern of cognitive, emotional and motor deficits. However, several single functional deficits such as poor motor coordination are shared across the different diagnostic categories. CP is the most common childhood-onset motor disability with a prevalence of 2.2 per 1000 children (Gordon et al., 2013). It is due to a non-progressive injury to the developing brain that has a life-long impact on movements, posture and muscle tone. DCD is more common but less severe with an impairment in the acquisition and execution of coordinated motor skills, which has not been attributed to general intellectual, sensory or motor neurological impairment (Kirby et al., 2014). In addition to DCD, several other neurodevelopmental disorders also show poor motor performance besides their cardinal symptoms (e.g., inattention, impulsivity, hyperactivity and impairments in social interaction), and often co-occur with this disorder. A large proportion of children with ADHD have less developed fine-motor skills and poor motor coordination (Eliasson et al., 2004; Meyer and Sagvolden, 2006). Atypical movement patterns are also common in ASD (Chukoskie et al., 2013). The wide range of motor problems in the various neurodevelopmental disorders include difficulties in both learning and performing a variety of motor tasks (e.g., different sport activities) as well as deficits in fine motor skills (e.g. reaching and grasping small objects, handwriting, tying shoe laces).

Improvement of cognitive and motor functions through training is an emerging field that may have a large impact on future health care. Motor training and motor learning have recently been emphasized as important components of rehabilitation programs for various movement disorders [e.g., CP and stroke; see (Krishnan, 2006; Novak et al., 2013) for review]. Several studies have demonstrated that children and adolescents with CP can improve their hand functioning by intense training of the impaired hand (Bonnier et al., 2006; Gordon et al., 2011). Understanding the cellular and molecular mechanisms underlying motor skill learning is of fundamental neurobiological importance and is a necessary requirement for the development of new strategies for optimizing motor function in rehabilitation programs.

1.1 Cortical plasticity associated with motor skill learning

Research during the last decades has greatly advanced our understanding of brain plasticity, i.e., how neuronal circuits can be modified by experience, drugs, learning and in response to brain lesions. Cortical plasticity has been demonstrated in response to a variety of experimental manipulations [for a review, see (Kolb et al., 2012)]. Here, our main focus is on the structural and functional brain plasticity associated with motor skill learning.

Motor skill learning can be described as “a task-specific modification of spatial and temporal organization of muscle synergies resulting in smooth and accurate movement sequences” (Hammond, 2002). Several brain structures, including motor cortical regions of the frontal cortex, basal ganglia, and cerebellum, are critical for the acquisition and consolidation of motor skills [for a review, see (Dayan and Cohen, 2011)]. However, most monkeys, human and rodent studies investigating plasticity after motor skill learning have focused on the primary motor cortex. These studies have revealed that the mammalian motor cortex is highly dynamic and capable of circuit reorganization driven by the acquisition of new motor skills. A number of classic studies (first in non-human primates and later in humans) have demonstrated that motor training changes the cortical maps of the primary motor cortex, e.g., topographic cortical areas projecting to the trained digit expand at the expense of areas projecting to non-trained digits [see (Sanes and Donoghue, 2000; Nudo, 2003) for review]. Similar results have been demonstrated in experiments in rodents using a skilled reaching task. Motor training induces an expansion of distal forelimb (wrist/digit) movement representations within the primary motor cortex (M1) that is consistent with the development of coordinated wrist and digit movements acquired during the task (Kleim et al., 1998). Importantly, this cortical reorganization is not simply due to increased limb use (e.g., rats trained in unskilled conditions do not exhibit expansion of distal forelimb movement representation [for a review, see (Monfils et al., 2005)]).

Studies investigating the temporal relationship between map plasticity and skilled learning in rats have revealed that despite significant improvements in reaching accuracy taken place already after 3 and 7 days of training, motor cortex reorganization does not occur until after 10 days of training (Kleim et al., 2004). Kleim and collaborators have speculated that transient changes in map topography may occur during the initial training experience but require sufficient repetition of the movement to be observable after the termination of training. Consistent with this notion is the observation that when rats are removed from skilled reach training during the initial phase of training (when significant improvements in reaching

accuracy are observed), no changes in map reorganization are observed 30 days later (Hogg et al., 2001). In contrast, animals that are trained for longer periods in the skilled reaching task exhibit changes in map topography and retained the acquired skill 30 days after termination of the motor training, indicating that map reorganization may represent the consolidation of motor skill. Previous studies showed that learning-induced motor cortical plasticity depends on intact signaling of modulatory neurotransmitters (e.g., acetylcholine). Studies by Tuszynski and colleagues have shown that either lesions of the nucleus basalis or local depletion of cholinergic afferents to M1 impair motor skill acquisition and abolish motor cortical plasticity associated with motor skill learning (Conner et al., 2003; Conner et al., 2010). In addition, neurotrophic factors such as brain-derived neurotrophic factor (BDNF) have been implicated in modulating dendritic morphology and motor map topography (Kleim et al., 2003).

Electrophysiological studies in rats have demonstrated that motor training increases the synaptic strength within intracortical connections in layer II/II within the forelimb area of M1 (Riout-Pedotti et al., 1998). These training-induced changes in the cortical microcircuit limit the capacity to induce long-term potentiation (LTP), but enhance the capacity for long-term depression (LTD) (Riout-Pedotti et al., 2000). These observations suggest that activity-dependent mechanisms similar to that involved in LTP induction might support motor cortex reorganization during motor skill learning (Martin and Morris, 2001). Motor skill learning also induces a variety of structural alterations (e.g., increase in synaptogenesis) within the same regions of M1 that undergo functional reorganization (Kleim et al., 2004). Recent advances in imaging techniques and genetic tools in mice have allowed *in vivo* imaging of synaptic plasticity in the mouse motor cortex (Zuo et al., 2013). A study by Xu and colleagues demonstrated that training in a skilled reaching task leads to rapid formation (within an hour) of postsynaptic dendritic spines on the output neurons in M1 contralateral to the trained forelimb, but not the ipsilateral cortex (Xu et al., 2009). Moreover, they showed that motor learning selectively stabilizes learning-induced new spines and destabilizes pre-existing spines, suggesting that stabilized synapses are the foundation of long-lasting motor memories. In addition, evidence also indicates structural changes in white matter microstructure after extensive motor training (Sampaio-Baptista et al., 2013). Although the specific cellular mechanisms underlying motor cortical plasticity are yet to be fully characterized, the synthesis of new proteins within the motor cortex seems to be necessary (Kleim et al., 2003). Recent studies exploring the motor-cortical transcriptome across different phases of motor skill learning have found that most of the transcriptional changes occurred immediately before motor task improvement (i.e., day 5), but not after performance reached its peak (i.e., day 12) (Cheung et al., 2013). Many modulated genes are implicated in synaptic plasticity,

synaptogenesis, and cytoskeletal dynamics. Collectively, the above findings suggest that motor skill learning induces a set of neuronal signals that in turn regulate the expression of synaptic-related genes within M1. With repetitive training proteins are translated to modify the cortical microcircuitry, resulting in the reorganization of motor cortical maps and long-term consolidation of motor memories.

1.2 The dopamine system

Dopamine (DA) is one of the most powerful neuromodulators used by the mammalian brain to regulate circuit function and plasticity [for a review, see (Tritsch and Sabatini, 2012)]. It plays a critical role in the control of movement, motivation, arousal, cognition and reward. The midbrain DA neurons in the substantia nigra pars compacta (SNc; A9 cell group) and ventral tegmental area (VTA; A10 cell group) contain the vast majority of DA neurons in the brain and the main DAergic innervation to basal ganglia and forebrain [for a review, see (Bjorklund and Dunnett, 2007)]. The SNc contains not only neurons projecting to the striatum along the nigrostriatal pathway, but also neurons that innervate cortical and limbic areas. The DA neurons of the VTA project to limbic and cortical areas along mesolimbic and mesocortical pathways, and also to the ventral striatum and the ventro-medial part of the head of the caudate-putamen in rodents. In addition, there is another DA cell group (A8; forming a dorsal and caudal extension of the A9 cell group) that also projects to both striatum, limbic and cortical areas (see **Fig. 1**).

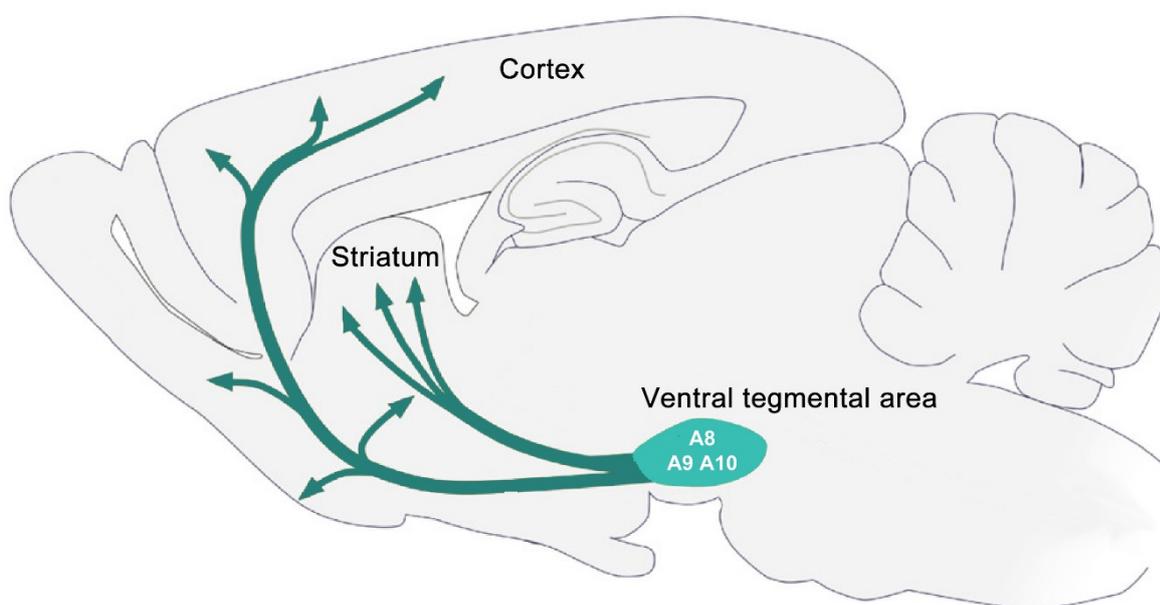


Figure 1. Main projections of the midbrain dopamine system in the adult rodent brain.

DA is synthesized from the amino acid L-tyrosine. The initial step involves the conversion of L-tyrosine into L-DOPA (L-3, 4-dihydroxyphenylalanine), which is catalyzed by the enzyme tyrosine hydroxylase (TH; the rate-limiting step in the synthesis of DA). Once formed, L-DOPA (also known as the drug levodopa) is rapidly decarboxylated to DA by dopa decarboxylase (DDC). Dopamine nerve terminals contain high-affinity dopamine-uptake sites (i.e., dopamine transporter; DAT), which are important in controlling DA neurotransmission by driving the reuptake of extracellular DA into presynaptic neurons (Vaughan and Foster, 2013) (see **Fig. 2**). Several neurodevelopmental disorders, including ADHD are associated with abnormal DA levels, implicating DAT as a factor in their pathophysiology. For example, stimulant medications (e.g., methylphenidate) used to treat ADHD target DAT and enhance DAergic signaling by blocking transmitter reuptake. Moreover, molecular positron-emission

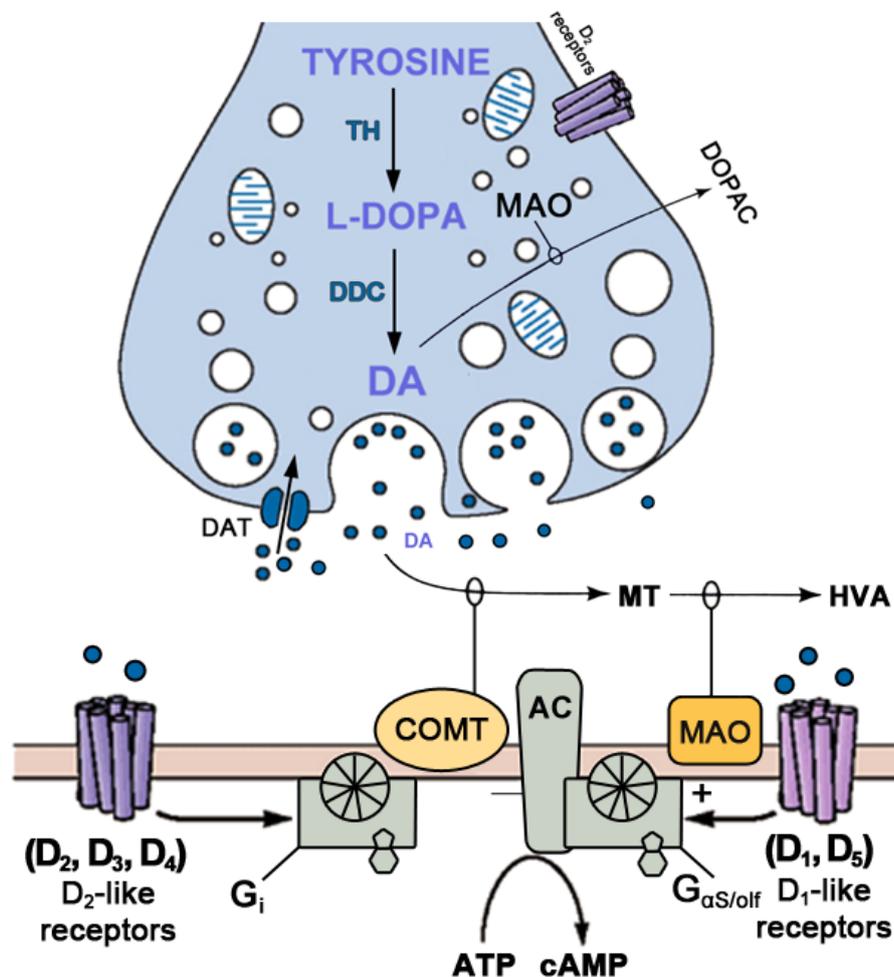


Figure 2. Dopamine synthesis and its receptors and metabolism. The pathway of dopamine synthesis proceeds from tyrosine via tyrosine hydroxylase (TH) catalysis to levodopa (L-DOPA), and subsequent decarboxylation by dopa decarboxylase (DDC) to dopamine. Activation of the D₁-like family receptors (D₁ and D₅) is coupled to the G protein G_{αs/olf}, which subsequently activates adenylyl cyclase, increasing the intracellular concentration of the second messenger cyclic adenosine monophosphate (cAMP). Activation of the D₂-like family receptors (D₂, D₃ and D₄) is coupled to the G protein G_{αi}, which directly inhibits the formation of cAMP by inhibiting the enzyme adenylyl cyclase. Dopamine is metabolized to DOPAC by presynaptic monoamine oxidase (MAO) after reuptake by the dopamine transporter (DAT). Released dopamine is also converted to homovanillic acid (HVA) at the postsynaptic site through the sequential action of catechol-*O*-methyltransferase (COMT) and MAO.

tomography (PET) studies have demonstrated alterations in DA synthesis and DA transporter density in adolescents and adults with ADHD (Forssberg et al., 2006; Swanson et al., 2007).

DA modulates corticostriatal circuitry through the activation of two distinct families of receptors referred to as the D1-like (D1R and D5R) and D2-like (D2R, D3R and D4R) [for a review, see (Missale et al., 1998)]. Dopamine receptors are members of a large superfamily of neurotransmitter and hormone receptors that are coupled to their specific second messenger pathways via guanine-nucleotide-binding regulatory proteins (G proteins). D1-like receptors are coupled to G_{α_s} -like proteins; thereby stimulating adenylyl cyclase activity and the production of cAMP, whereas D2-like receptors are linked to $G_{i/o}$ proteins and exert an inhibitory influence on adenylyl cyclase. A major route of these intracellular pathways is conveyed by the DA and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) [see (Greengard, 2001; Girault, 2012) for review]. DARPP-32 is highly enriched in medium spiny neurons of the striatum and glutamatergic pyramidal neurons of the frontal cortex (albeit at lower levels) (Ouimet et al., 1984; Ouimet et al., 1992; Perez and Lewis, 1992; Kuroiwa et al., 2012). The function of DARPP-32 depends on its relative state of phosphorylation at multiple regulatory sites (e.g., Thr-34, Thr-75, and Ser-97) that are regulated by calcium and cAMP signaling pathways (Walaas et al., 2011; Girault, 2012). For example, when DARPP-32 is phosphorylated at the Thr-34 site by cAMP-dependent protein kinase (e.g., by DA acting via D1-like receptors), it is converted into a potent inhibitor of the multifunctional serine/threonine protein phosphatase-1 (PP1). PP1 controls the phosphorylation state and hence the physiological activity of a number of downstream targets such as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptors, kinases (e.g., calcium/calmodulin-dependent protein kinase II) and transcription factors (e.g., cAMP response element-binding protein; CREB) that are necessary for synaptic plasticity and learning.

1.3 Dopamine and motor skill learning

DA, a key modulator of plasticity in corticostriatal circuitry, is critically involved in motor skill learning [for a review, see (Wickens et al., 2007)]. Studies in rodents have shown that motor skill learning of a rotarod task is impaired by moderate disruption of DA release in the dorsal striatum by the neurotoxin 6-OHDA (Ogura et al., 2005) and by downregulation of synaptotagmin I (a protein involved in neurotransmitter release) in the nigrostriatal terminals (Akita et al., 2006). Others have reported region-specific changes in neuronal activity within the dorsal striatum during different phases of motor learning (Yin et al., 2009). In humans,

studies using molecular PET imaging have found changes in striatal dopamine release during the initial acquisition of a motor skill (Kawashima et al., 2012). Recently, Beeler and colleagues took advantage of PITx3-deficient mice, in which DA is dramatically reduced in the dorsal striatum, to investigate whether motor deficits arising from DA denervation could be dissociated from performance deficits, by transiently manipulating DA levels during different phases of motor skill learning with L-DOPA (Beeler et al., 2010). These authors showed that PITx3-deficient mice exhibit profound impairments in motor learning on the rotarod that can be rescued with L-DOPA. However, after cessation with L-DOPA treatment, the PITx3-deficient mice exhibit a gradual decline in performance, suggesting that DA is critical not only for motor performance but also for the acquisition and maintenance of learned skills.

Recent studies in rats have revealed that the integrity of the mesocortical DAergic pathway in M1 is also essential for successful motor skill learning and the associated synaptic plasticity in this region (Molina-Luna et al., 2009; Hosp et al., 2011). Elimination of DAergic terminals and /or blockade of DA receptors in M1 impaired motor skill acquisition, but not execution of a previously acquired motor skill. DA fibers innervating M1 originates in the VTA and the medial portion of the SNc. A recent study in mice demonstrated that DA innervates the deep layers of M1 targeting preferentially the forelimb representation area of M1 (Vitrac et al., 2014). Studies have demonstrated that DAergic signaling modulates the M1 circuitry at multiple levels e.g., increasing cortical excitability and enhancing the stability of motor maps [for a review, see (Hosp and Luft, 2013)]. Moreover, DA is required for the formation of LTP, a potential mechanism involved in the formation of motor memories. Collectively, the above findings indicate that corticostriatal circuits require an optimal level of DA neurotransmission to facilitate motor skill learning. However, we still lack a comprehensive understanding of the cellular and molecular pathways mediating effects of DA on motor learning and associated synaptic plasticity.

1.4 Genetic contributions to motor skill learning

There is considerable interindividual variation in the capacity of healthy individuals to learn new fine motor skills. Large individual variations in treatment outcome have also been observed in children with motor disabilities. For example, studies of constraint-induced movement therapy in children with unilateral cerebral palsy have shown large variations in treatment outcome, which can not be explained by factors such as corticomotor projection pattern and brain lesion characteristics (Islam et al., 2014). These observations raise the

question of the relative importance of genetic and environmental influences on motor skill learning.

Twin studies suggest that heritability is a main factor responsible for interindividual differences in motor learning (Williams and Gross, 1980; Fox et al., 1996; Missitzi et al., 2013). Recent studies have provided evidence that common genetic variation (e.g., single nucleotide polymorphism; SNP) can influence motor learning and motor cortex plasticity in humans [for a review, see (Cheeran et al., 2009)]. One of the best-characterized examples is the neurotrophin BDNF, which not only regulates neuronal survival, proliferation and synaptic growth in the developing brain, but also synaptic changes associated with learning and memory [see (Cohen-Cory et al., 2010; Cowansage et al., 2010) for review]. Moreover, treatment with exogenous BDNF is associated with better motor recovery after small cortical ischemia (Schabitz et al., 2004).

The BDNF gene contains a functional SNP (rs6265) at position 196 at exon 2, in which a G to A substitution results in an amino acid switch from valine to methionine at codon 66 (val⁶⁶met). Studies have shown that this polymorphism, which is located in the 5' pro-BDNF sequence, does not affect the mature protein, but rather the intracellular trafficking of pro-BDNF and, thus, experience-dependent BDNF release (Egan et al., 2003; Chen et al., 2004). Approximately 30% of individuals in the United States are either heterozygous (Val/Met) or homozygous (Met/Met) for the BDNF val⁶⁶met polymorphism (Shimizu et al., 2004). In humans, the BDNF val⁶⁶met polymorphism has been associated with abnormal cortical and hippocampal morphology (Pezawas et al., 2004; Szeszko et al., 2005; Bueller et al., 2006; Ho et al., 2006; Frodl et al., 2007). In addition, this polymorphism has been associated with hippocampal-dependent memory impairments. For example, Met allele carriers have been shown to have poorer performance on episodic memory tasks, which involve recalling places and events (Egan et al., 2003; Hariri et al., 2003; Goldberg et al., 2008).

Animal studies have also identified BDNF as one of the key regulators of synaptic plasticity and memory [for a review, see (Lu et al., 2008)]. Moreover, BDNF is elevated in the motor cortex in response to motor training (Klintsova et al., 2004). In a recent study, Kleim and colleagues demonstrated an influence of the BDNF val⁶⁶met polymorphism in the motor cortex of humans (Kleim et al., 2006). These authors used transcranial magnetic stimulation (TMS) to study the motor cortex representational map for a hand muscle before and after short-term motor practice on three fine-motor tasks: maximum finger tapping rate, nine-hole pegboard and pinch-grip strength. Previous TMS experiments in healthy individuals have demonstrated that motor training can induce profound physiological plasticity within the primary motor

cortex, including reorganization of movement representation (Classen et al., 1998) and enhance corticospinal output (Pascual-Leone et al., 1995). Although individuals in the different BDNF genotypes showed similar organization of motor maps at baseline, Met allele carriers (i.e., Val/Met or Met/Met) showed reduced training-dependent increases in the amplitude of motor-evoked potentials and motor reorganization. Interestingly, the polymorphism-related differences seen with short-term motor cortical plasticity are not found with more intense training (McHughen et al., 2011). In another study, McHughen and colleagues investigated the effects of the Val⁶⁶Met polymorphism in relation to short-term plasticity and learning in a skilled fine motor task using their right index finger and a simulated functional driving task (McHughen et al., 2010). Individuals with the polymorphism demonstrated greater error during short-term learning and retention on the simulated driving task. In addition, they had smaller fMRI activation volumes following skilled training as compared to individuals without the polymorphism. Taken together, these studies indicate that genetic variation in the BDNF gene may alter the capacity for short-term learning and plasticity in the healthy human motor cortex.

A recent study provides evidence that genetic variation in the DA system influences motor learning and its modulation by L-DOPA (Pearson-Fuhrhop et al., 2013). Specifically, these authors investigated the collective influence of five polymorphisms with established effects on dopamine i.e., DA receptors D1, D2 and D3, DAT and COMT (see **Fig. 2**). Similar to prior studies, dopamine genotype was not associated with differences at baseline, but instead through interaction with training. For example, individuals with higher scores corresponding to higher dopaminergic neurotransmission showed greater motor learning. However, while training with L-DOPA treatment, enhancements associated with increased dopamine occur in an inverted U-shaped manner. Individuals with lower gene scores (i.e., lower endogenous dopaminergic neurotransmission) showed the greatest learning improvement; these individuals moved towards an optimal level. In contrast, learning was disrupted when L-DOPA was given to individuals with high endogenous dopamine neurotransmission. Since these individuals are higher on the curve at baseline the addition of L-DOPA shifted them past the optimal level. Given the importance of DA in motor control and synaptic plasticity, these findings may have clinical implications for children with motor disabilities. Future research in this field should take into account the influence of common variants in genes regulating DA neurotransmission (e.g., DAT, DARPP-32, D1R, D2R, and COMT) and plasticity (e.g., BDNF) in the context of rehabilitation programs for these children. We envision that as rehabilitation programs become more advanced, genetics will likely play an important role in the determination of treatment strategies.

2 AIMS OF THE THESIS

The overarching aim of this thesis is to improve our understanding of DAergic molecular mechanisms contributing to motor skill learning with special focus on fine motor skills (e.g., skilled reaching and grasping).

2.1 Specific aims of the thesis

- To investigate the influence of natural genetic variation in the DA system in motor skill learning.
- To identify brain activity patterns associated with different phases of motor skill learning and to investigate learning-induced changes in the DA system (from gene expression to biochemical modifications of intracellular pathways).
- To determine whether an existing genetic rodent model of ADHD is valid for investigating not only locomotor hyperactivity, but also deficits in fine motor skills commonly observed in children with ADHD.

3 METHODS

3.1 Animals

The following animals were used throughout the different studies:

- In paper I, we used 10-week-old male C57BL/6 and BALB/c mice (Charles River, Sulzfeld, Germany).
- In paper II, we used 5-week-old male outbred Wistar rats (Charles River, Sulzfeld, Germany).
- In paper III, we used 5-week-old male Spontaneously Hypertensive Rats (SHR/NCrl; Charles River, Sulzfeld, Germany) and Wistar/Furth/Sca rats (WIS; Scanbur AB, Sollentuna, Sweden).

Animals arrived at the animal facilities and were acclimatized for at least 5 days before the start of experiments and housed in groups in standard plastic cages (Type IV Makrolon[®] and Type III Makrolon[®] for rats and mice, respectively) under controlled conditions of light: dark cycle (12:12 h, lights on at 07.00 h). Food and water were available ad libitum. Animals involved in the skilled reaching task were housed in pairs in the same type of cage. Each set of paired rats was separated by a clear Plexiglas partition (containing small holes, 10 mm in diameter) that divided the home cage in half in order to monitor the daily food intake of each individual rat.

All behavioral testing was performed between 09:00 and 16:00 h under low illumination in order to reduce stress. In all experiments, animals were experimentally naïve in order to avoid possible carryover effects between tests. Prior to any behavioral procedure, animals were brought in their home cages to the experimental testing room and allowed to habituate for at least 1 hour before the testing sessions were started in order to minimize stress caused by environmental changes. All experimental procedures were conducted according to a protocol approved by the Ethical Committee on Animal Research, Stockholm North, and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Since a detailed description of the materials and methods section is present in each paper, the methodology will only be briefly presented here.

3.2 Behavioural tests

3.2.1 Open-field / locomotor activity test

The open field test is commonly used to measure general locomotor activity and exploration in rodents, as well as anxiety-like behavior by including other parameters such as time spent in the center of the open field and levels of defecation and urination (Prut and Belzung, 2003). In the present thesis, we used the open field test to investigate the exploratory activity and habituation profile of our animals after repeated exposure to the same environment (i.e., the open field box). Typically, when rodents are exposed to a novel environment they display high levels of exploratory behavior (i.e., novelty phase; **Fig. 3A**). However, when animals are repeatedly placed into the same open field, as well as after a prolonged exposure to an open field box within a single session, a progressive reduction occurs in exploratory behavior as the novel environment becomes increasingly familiar (i.e., the habituation phase).

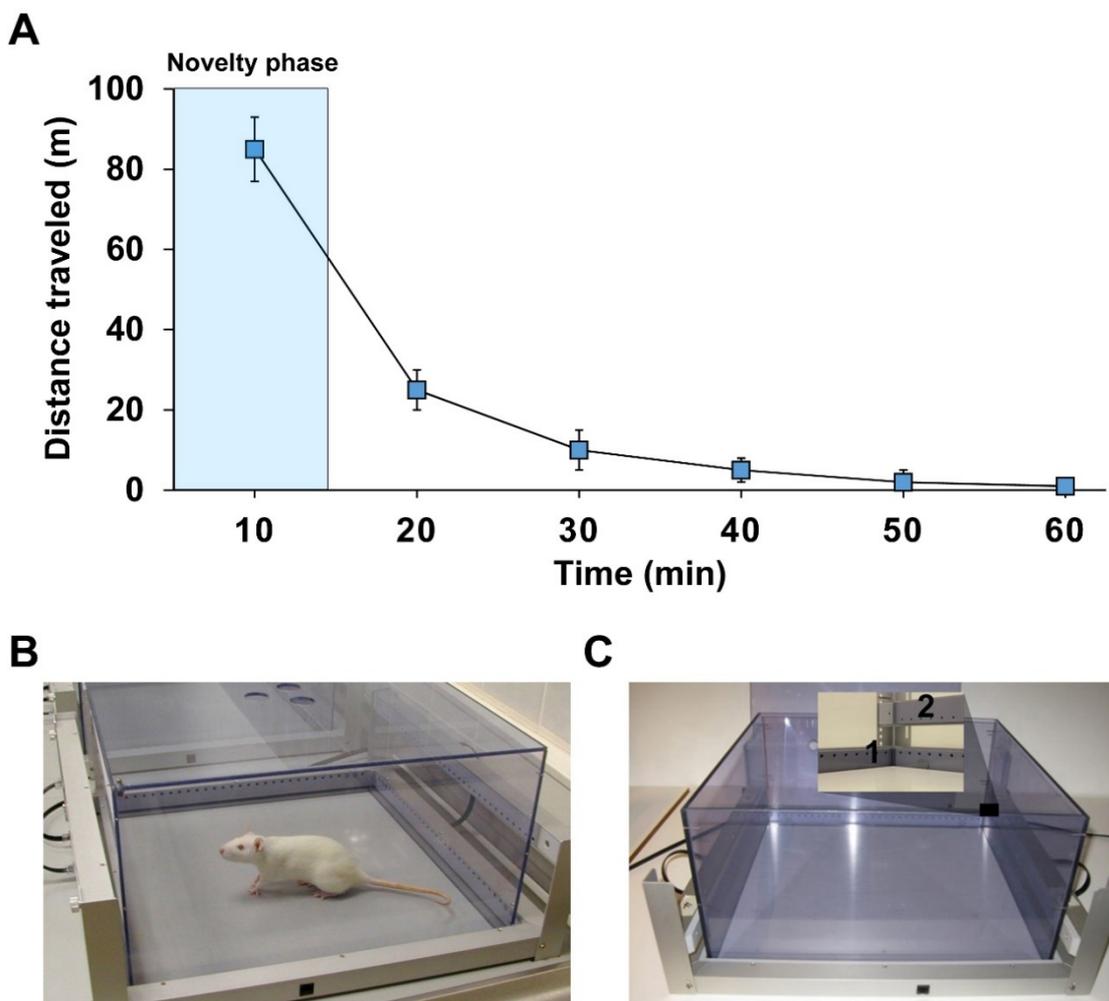


Figure 3. ActiMot activity detection system from TSE. (A.) Average distance traveled in meters measured in 10-min time bins across a 60-min session in an open-field box. (B.) A rat in the ActiMot box. (C.) Structure of detection system. The base frame (1) consists of two pairs of infrared beam strips arranged in the same plane used to detect horizontal activity. An additional pair of Infrared barrier strips (2) above the frame base is used to detect vertical activity. The open field box is 48 × 48 cm.

The open field test was performed using a computerized multi-box ActiMot Detection System (TSE, Bad Homburg, Germany). This system uses photocells sensitive to infrared light to detect movements of the animal (**Fig. 3B-C**). For spontaneous motor activity two parameters were analyzed: (1) locomotor activity, recorded as distance travelled in meters, and (2) rearing activity, measured by counting the number of times an animal stands on its hind legs.

3.2.2 Rotarod test

The rotarod test is widely used to evaluate motor coordination and balance in rodents. Modified versions of this test have also been used to assess motor skill learning (Ogura et al., 2005). In the present thesis, the rotarod test was performed using an accelerating Rota-Rod® (UGO Basile, Comerio, Italy). One day before testing, animals were acclimated to the rotarod by being placed on the cylinder rotating at a fixed speed (i.e., 4 rpm) for two 90-second periods, 2 hours apart. On the testing day, animals were placed on the rotating cylinder, and the time each animal was able to maintain its balance was recorded. The speed of the rotarod accelerated from 4 to 40 rpm over 5 min. Performance on the rotarod was measured by latencies to falling off the rotating cylinder, during 10 trials over 1 day, with a 5-min inter-trial interval.

3.2.3 Skilled reaching task

The skilled reaching task is a well-established paradigm used to evaluate skilled forelimb movements in rodents. In this task, rodents are trained to reach for a small food pellet through a narrow opening, and to grasp and retrieve the pellet with a single forelimb, under a regime of

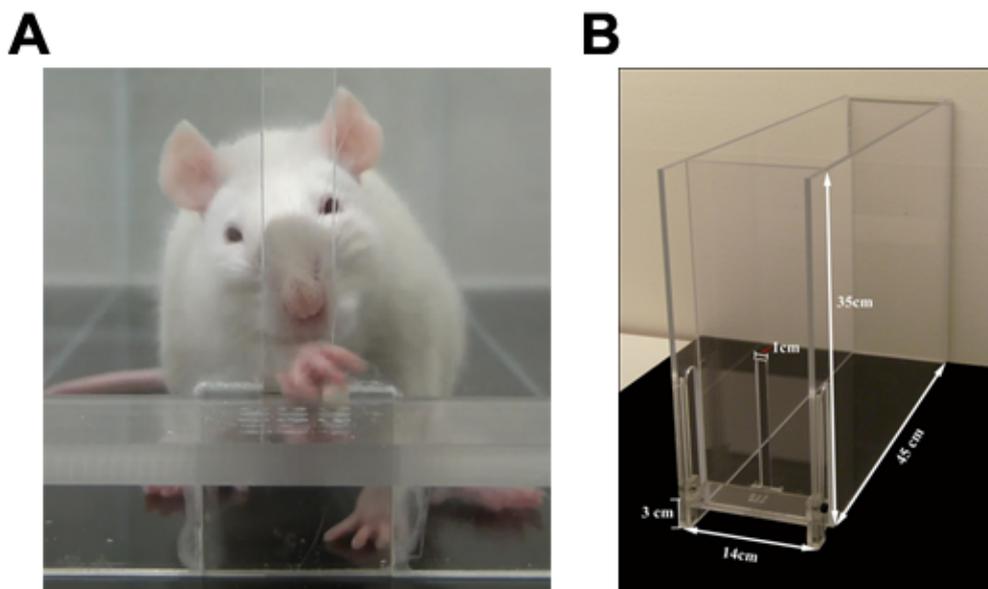


Figure 4. The single pellet reaching task. (A.) A rat performing the reaching task. (B.) The single pellet reaching apparatus.

mild food deprivation (see **Fig 4**). This action involves a number of movements that include adjusting body posture, aiming, moving the forelimb over the food pellet, using digits to grasp the food, withdrawing the food back through the slot, and finally putting the food into the mouth (Whishaw and Pellis, 1990). Reaching behavior was analyzed by measuring: (1) total number of success (total number of pellets obtained regardless of the number of forelimb advances), (2) first attempt success (number of pellets obtained on the first advance of the forelimb), and (3) total number of attempts (total number of forelimb advances towards the food pellet). For further details see individual papers.

3.3 Molecular studies

3.3.1 *In situ hybridization*

In situ hybridization is a well-established technique for localizing and detecting mRNA expression in morphologically preserved tissue sections. The present thesis used riboprobes which give a strong signal and a low background. For *Drd1*, *Drd2*, *DAT* and *TH*, we used common mouse-specific riboprobes that have been previously described (see **Paper I**). For *DARPP-32* and *Arc/Arg3.1*, new riboprobes were prepared by amplifying a conserved region of these genes from a mouse/rat brain cDNA library. The amplified cDNA fragments were subcloned into a pCR1II-TOPO vector (Invitrogen, Lidingö, Sweden), and confirmed by nucleotide sequencing. Linearized plasmids were used to synthesize [³⁵S] UTP-labeled riboprobes. In-vitro transcription was carried out using the MAXIscript™ SP6/T7 kit (Applied Biosystems, Uppsala, Sweden) and [³⁵S] UTP (NEG039H; Perkin Elmer, Upplands Väsby, Sweden) according to the manufacturer's instructions. The transcripts were purified using NucAway™ spin columns (Applied Biosystems, Uppsala, Sweden). Fixation, pre-hybridization, hybridization and washes were performed as previously described (Diaz Heijtz et al., 2010).

3.3.2 *Quantitative real-time polymerase chain reaction*

Quantitative real-time PCR (qRT-PCR) is a molecular biology technique widely used to amplify and simultaneously quantify the expression of a target gene. This technique follows the general concept of the PCR. Its key characteristic is that the amplified DNA is detected as the reaction progresses in “real time”. Two common detection methods are: (1) DNA-binding dyes (e.g., SYBR® Green), and (2) dye-labeled, sequence specific oligonucleotide primers or probes (e.g., TaqMan). In this thesis, we used SYBR® Green, which is a common fluorescent dye that binds to double-stranded DNA. This DNA-dye-complex absorbs blue light ($\lambda_{max} =$

497 nm) and emits green light ($\lambda_{\max} = 520$ nm). The main advantage of using SYBR[®] Green is that there is no requirement for the incorporation of a fluorescent reporter system into the primer design. This simplifies primer design and reduces experimental costs, particularly when testing multiple genes.

Briefly, total RNA from various brain tissue samples were isolated using the RNeasy[®] Mini Kit (Qiagen) and quantified by spectrophotometry using a NanoDrop[®] ND-2000 spectrophotometer (NanoDrop Technologies, USA). Quantification was performed using the iQ5 real time PCR detection system with SYBR green super mix (Bio-Rad). Primers for specific genes of interest were custom designed using Primer3 software (for more details, see **Table I**).

3.3.3 *Western blotting*

Western blotting is a widely used analytical technique for detection and analysis of proteins in tissue homogenates. There are three main steps in this technique: 1) separation of proteins by size using gel electrophoresis (under denaturing conditions), (2) transfer to a polyvinylidene difluoride (PVDF) membrane, and (3) detecting target protein using antibodies specific to target protein (Liu et al., 2014).

Briefly, animals were sacrificed by decapitation and their brains were rapidly removed. The tissues were dissected out within 20s on an ice-cold surface and frozen immediately on dry ice and kept at -80°C until used. Samples were homogenized in 700 μl of 1% SDS, 50mM NaF, and boiled for 10 min as previously described (Santini et al., 2007). Aliquots (10 μl) of the homogenate were used for protein determination using the Bradford Protein Assay. Equal amounts of protein (5-10 μg) for each sample were loaded onto 12% polyacrylamide gels. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were immunoblotted-using polyclonal antibodies against phospho-Thr34-DARPP-32, phospho-Thr75-DARPP-32, phospho-Ser97-DARPP-32, and phospho-Ser133-CREB. Antibodies against the total DARPP-32 and CREB that are not phosphorylated state specific were also used to estimate the total amount of these proteins. Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies, and the ECL. Protein band detection was carried out using the CCD camera based computer system and quantitated using the NIH Image J software. Rabbit polyclonal antibody against heat shock protein 90 (HSP90) served as a loading control. At the end of the experiment, nitrocellulose membranes were also stained with Coomassie Blue staining in order to verify equal loading of proteins.

Table I. List of primer sequences

Target gene	Primers	Sequence 5' to 3'	Acc. No.	T (°C)
Drd1	Forward	ccttcgatgtgtttgtgtgg	NM_012546	60
	Reverse	ggcatgagggatcaggtaaa		
Drd5	Forward	accaagacacggctctccac	BC049663	60
	Reverse	cacagtcaagctcccagaca		
Drd2L	Forward	aactgtaccacacctgagga	NM_012547	55
	Reverse	gttgctatgtagaccgtg		
Drd2s	Forward	caccactcaaggatgctgcccg	NM_012547	50
	Reverse	gttgctatgtagaccgtg		
Drd3	Forward	ggggtgactgtcctggctcta	NM_007877	60
	Reverse	aagccaggtctgatgctgat		
Drd4	Forward	aaccgagactatgtggctactcg	NM_007878.2	60
	Reverse	ccagtaaagcagtagcatgagc		
DARPP-32	Forward	ggcagagcaacacaaagtga	AY601872	60
	Reverse	ttcagccaaagcaaacacag		
DAT	Forward	ctgcctggtgctggtcatt	AF109391	60
	Reverse	atccacaccaccttcctga		
TH	Forward	gccaaggacaagctcaggaac	NM_009377	60
	Reverse	atcaatggccagggtgtacg		
TBP	Forward	ctcagttacaggtggcagca	NM_001004198	50-60
	Reverse	cagcacagagcaagcaactc		

4 RESULTS & DISCUSSION

4.1 Genetic variation in the mouse brain dopamine system influences motor skill learning

Recent studies have provided evidence that common genetic variation in the DA system can influence motor learning and motor plasticity in humans (see **Introduction**). In rodents, substantial genetic variation is observed in the mesotelencephalic DA systems and parallel differences in response to DAergic drugs and spontaneous motor activity have been identified (Fink and Reis, 1981; Reis et al., 1983). In **paper I**, we took advantage of two inbred strains of mice (BALB/c and C57BL/6) that differ markedly in the number of midbrain DA neurons in order to investigate the influence of such naturally occurring genetic variation on the acquisition and performance of fine motor skills. In the first sets of experiments, we compared the expression of various DAergic markers in the midbrain, frontal cortex and striatum of naïve BALB/c and C57BL/6 mice. In the midbrain, we found that naïve BALB/c mice express higher levels of presynaptic DA markers (e.g., TH, DAT, D2RS, D2RL, and D3R; see **Fig. 5**). These observations are consistent with previous studies demonstrating that BALB/c mice have more midbrain DA neurons than mice of the C57BL/6 strain (Vadasz et al., 2007). Moreover, these two strains of mice exhibit substantial variation in the expression of various postsynaptic DAergic markers in the frontal cortex (including M1) and striatum. Thus, BALB/c mice have higher expression levels of DARPP-32, D3R and D5R in the frontal cortex, but lower levels of D5R. In the striatum, BALB/c mice have lower levels of D3R and D5R. Other studies have found that BALB/c mice have higher DA turnover in these brain areas (Browne et al., 2011). Taken together, these findings suggest an alteration in DA neurotransmission within the frontostriatal circuitry of BALB/c mice involved in motor control and cognitive functions.

Next, we trained a new set of naïve BALB/c and C57BL/6 mice in a skilled reaching task for 10 days. We found that these two strains of mice also exhibit differences in the acquisition and performance of fine motor skills. Although BALB/c mice were able to learn the skilled forelimb-reaching task, their performance was significantly poorer than the C57BL/6 mice (**Fig. 6A**), and they also had a lower learning rate (**Fig. 6B**).

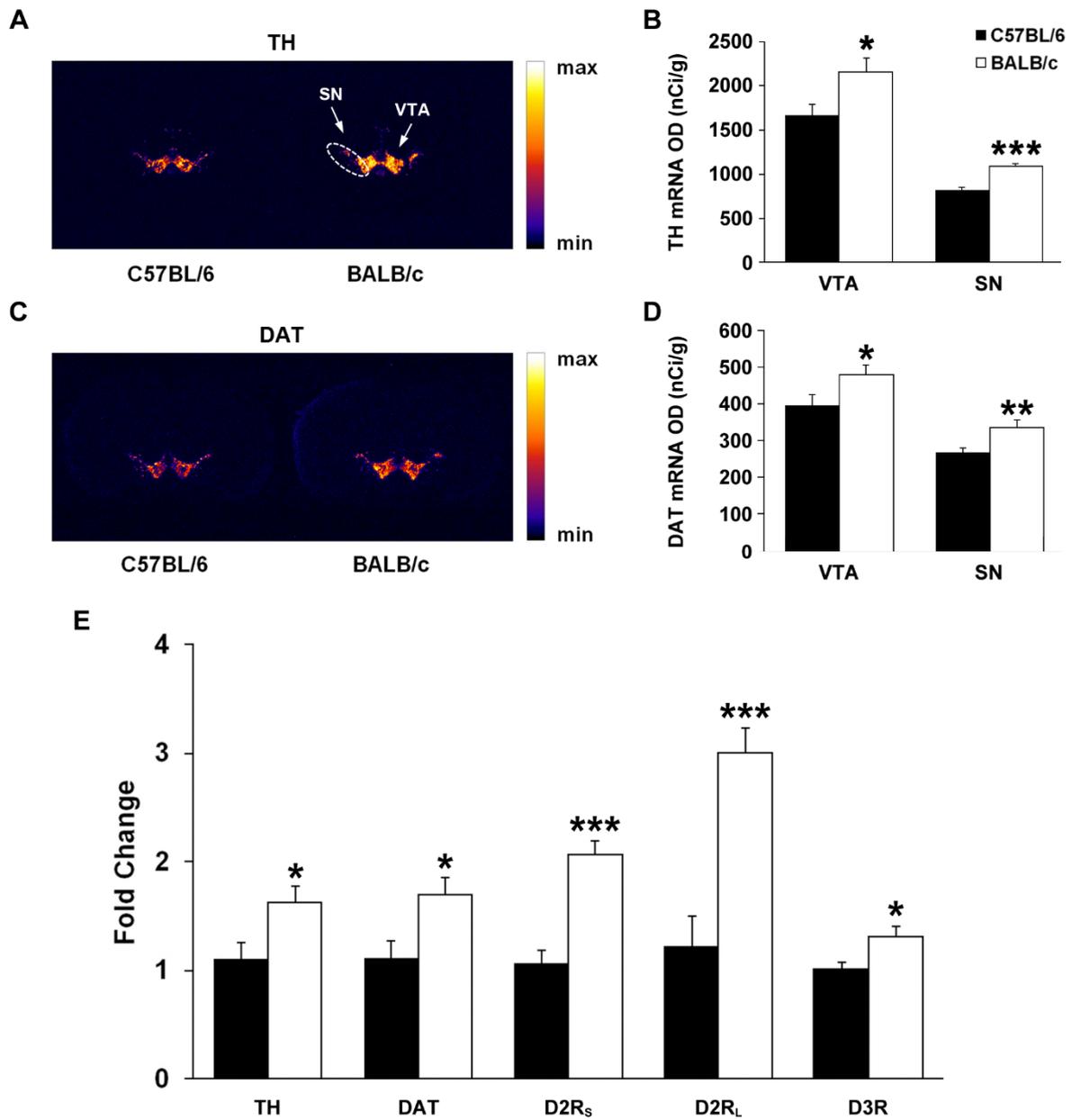


Figure 5. BALB/c mice show altered expression of dopaminergic markers in the ventral midbrain region. Representative autoradiographs showing the mRNA expression levels of (A) TH and (C) DAT at the level of the midbrain region of naïve adult C57BL/6 and BALB/c mice. The pseudo-coloring indicates signal intensity ranging from low (black/purple) to high (yellow/white). Data in bar graphs show mean optical density (OD) values for (B) TH and (D) DAT mRNAs in VTA and SN. The mRNA expression levels of TH, DAT, D2R_s, D2R_L and D3R were examined by means of qRT-PCR. Expression levels of each gene examined were normalized to TATA-binding protein levels and expressed relative to the C57BL/6 group. The results (B, D and E) are presented as means ± SEM; n = 8 per group. Asterisks denote where BALB/c mice differ significantly (*P<0.05, **P<0.01, ***P<0.001) from C57BL/6 mice.

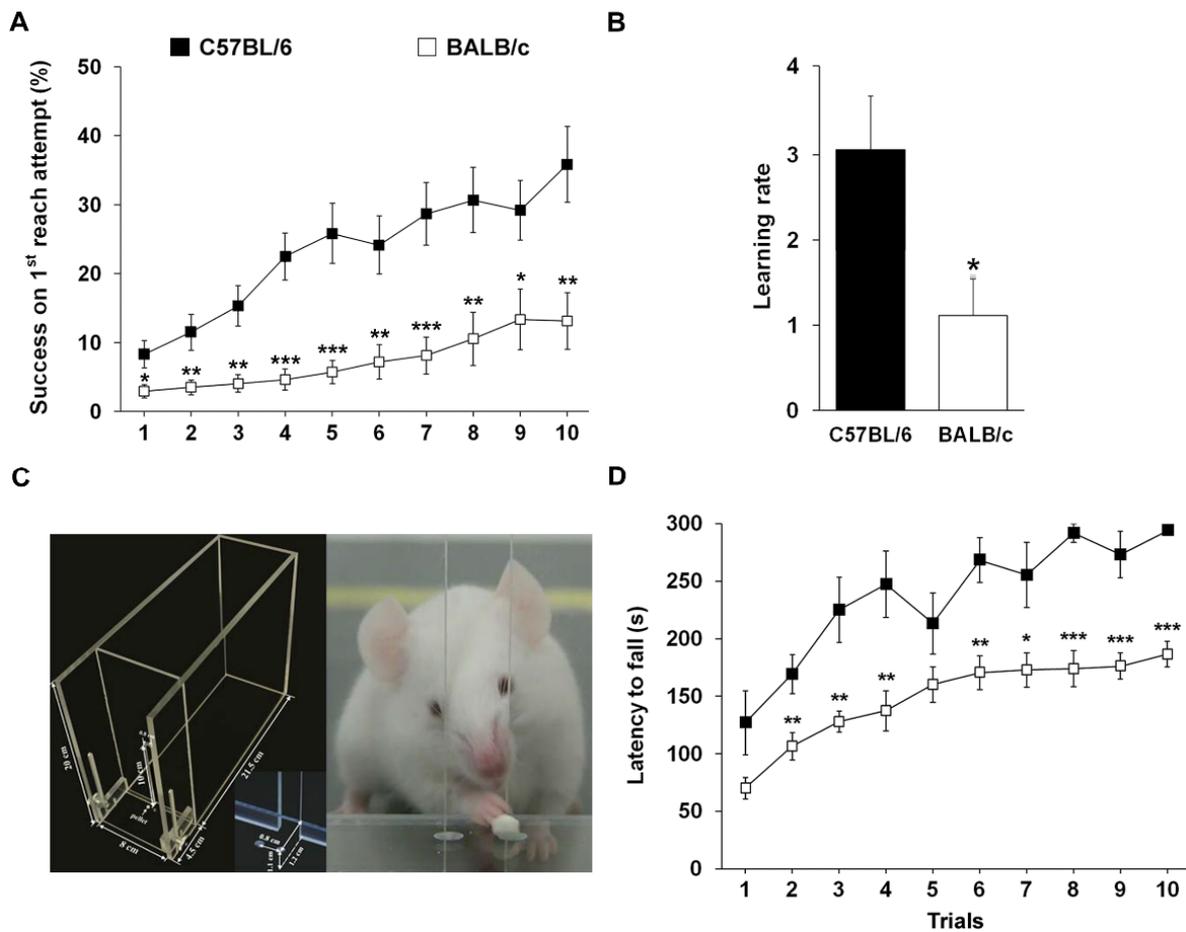


Figure 6. Deficits in skilled reaching behavior and gross motor coordination in BALB/c mice. (A) Success on the first reach attempt, in percentage; $n=18-20$ per group, (B) Bar charts show the learning rate for success on the first reach attempt, (C) a BALB/c mouse performing the single pellet skilled reaching task. (D) The latency to fall off from an accelerating rotarod is presented; $n=8$ per group. The results are presented as means \pm SEM. Asterisks denote where BALB/c mice differ significantly (* $P<0.05$, ** $P<0.01$, *** $P<0.001$) from C57BL/6 mice.

Interestingly, we found that an improvement of skilled reaching behavior (as reflected by the rate of learning) was negatively correlated with the expression levels of DARPP-32 in the frontal cortex and striatum contralateral to the trained forelimb. The rate of motor learning was also negatively correlated with the levels of both DARPP-32 and D1R mRNAs in the striatum (Fig. 7). However, in naïve mice, the expression of neither D1R nor DARPP-32 mRNAs in the striatum differs significantly between BALB/c and C57BL/6 mice. One possible explanation is that extensive motor training may alter the expression of D1R and DARPP-32 and that the mechanisms mediating this type of plasticity may be aberrant in poor learners. In future studies, it will be interesting to explore this possibility by including an active control group (see **Paper II**).

DARPP-32 is a signaling “hub” that plays a critical role in the integration of information arriving at dopaminoceptive neurons via a variety of neurotransmitters and neuromodulators (see **Introduction**). The function of DARPP-32 depends on its relative state of phosphorylation

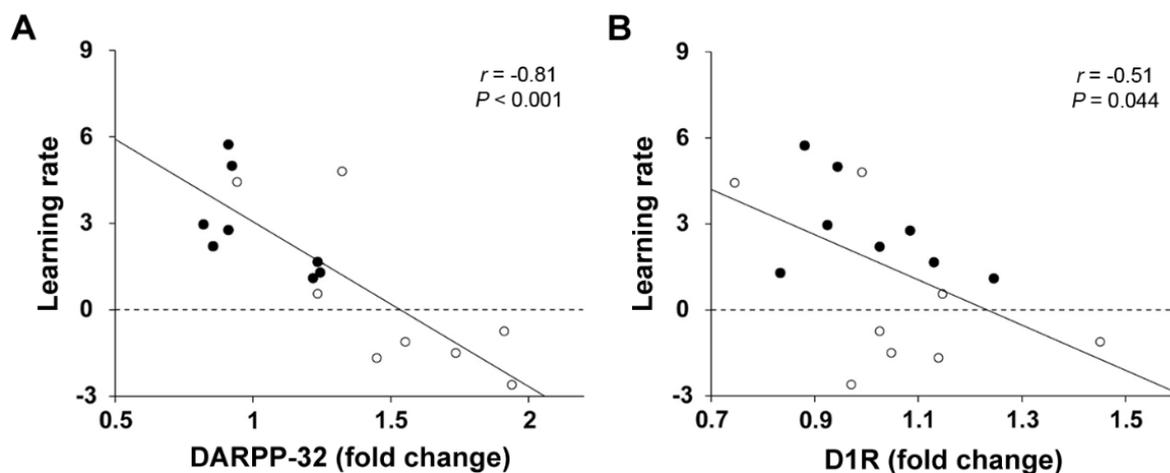


Figure 7. Correlation between learning rate for total success and expression of dopamine-related markers in the striatum. Circles represent C57BL/6 (black) and BALB/c (white) mice.

at multiple regulatory sites, including Thr34, Thr75, and Ser97. Phosphorylation of DARPP-32 at the Thr34 regulatory site results in the potent inhibition of a PP-1 that controls the phosphorylation state of several downstream proteins necessary for synaptic plasticity and learning (e.g., CREB). Overall, our findings suggest that DARPP-32 plays an important role in the acquisition of new fine motor skills and that its overexpression in frontostriatal circuitry could lead to deficits in fine motor skills.

The laboratory of Brodtkin has demonstrated that BALB/c mice display several behavioral traits relevant to autism, including low levels of sociability and high levels of anxiety (Brodtkin, 2007). Other studies have demonstrated that BALB/c mice, similar to children with ADHD, respond to low doses of amphetamine with hypolocomotion, whereas C57BL/6 mice do not (Helmeste and Seeman, 1982). However, there has been conflicting data in terms of the exploratory behavior of BALB/c mice: with some authors finding BALB/c mice to be more anxious and hypoactive in the open-field test compared with C57BL/6 mice (Moy et al., 2007), while others reporting that BALB/c mice are more active compared with several strains, including C57BL/6 mice (Isles et al., 2004). We therefore decided to further characterize the behavior of BALB/c mice with respect to motor activity and gross motor coordination. In a new set of experiments, we compared the exploratory activity and habituation profile of BALB/c and C57BL/6 mice after repeated exposure to a novel environment (i.e., open-field box; see **Fig 8**). Our results revealed that BALB/c mice failed to habituate to the novel environment. They displayed similar levels of motor activity throughout the entire testing period (i.e., three consecutive days). However, BALB/c mice showed decreased levels of motor

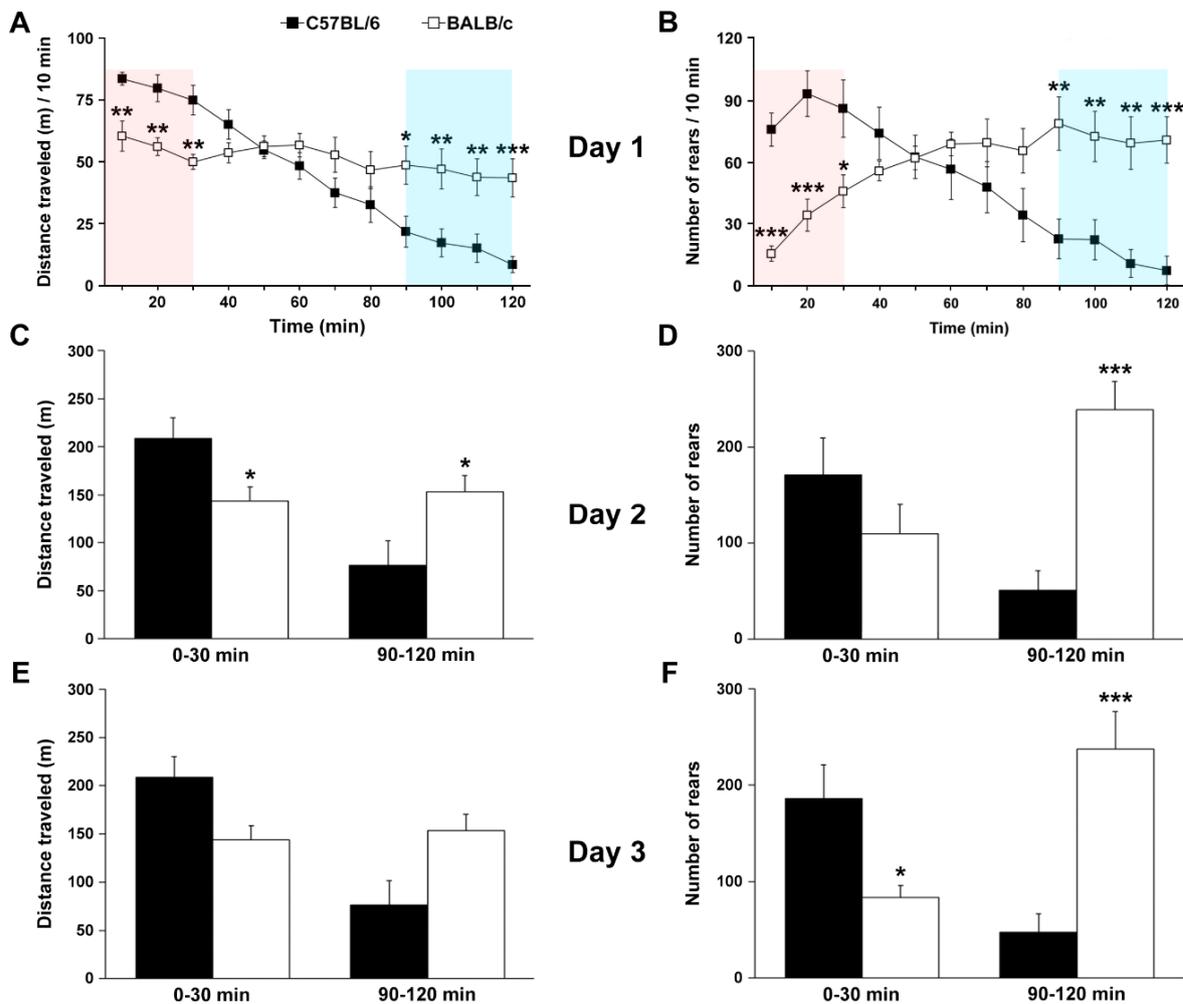


Figure 8. BALB/c mice display impaired habituation to a novel environment. Naïve BALB/c and C57BL/6 mice were placed into an activity/open field box for 120 min on day one and then re-exposed to the same testing box for two consecutive days. Average distance traveled (meters; A, C and E) and number of rears (B, D and F) measured during the 0- to 30- min and 90- to 120- min intervals of testing across three consecutive days (i.e., Days 1-3). The results are presented as means \pm SEM; n=8 per group. Asterisks denote where BALB/c mice differ significantly (* P <0.05, ** P <0.01, *** P <0.001) from C57BL/6 mice.

activity during their initial response to the open-field exposure, which is consistent with the anxiety-like trait of the BALB/c strain. It is worth mentioning that hyperactivity in children with ADHD is absent in novel situations and it develops gradually over time. Consistent with previous findings of other sub-strains of BALB/c mice (i.e., BALB/cByJ) we found that BALB/c display poorer motor coordination and balance in the rotarod test when compared with C57BL/6 mice (see **Fig 6D**). Taken together, our results indicate that the BALB/c strain may be a useful animal model to explore molecular mechanisms underlying deficits in gross and fine motor skills, as well as hyperactivity, often observed in children with ADHD.

4.2 Enhanced phosphorylation of DARPP-32 at the Thr34 and its downstream target CREB is associated with the early, but not late, phase of motor skill learning in rats

The neurotransmitter DA is known to play an important role in motor learning and synaptic plasticity (Costa, 2007). However, the cellular and molecular pathways mediating its effects are still poorly understood. In **Paper I**, we found a negative correlation between motor learning rate and the expression levels of DARPP-32 in the frontal cortex and striatum (see above), thus implicating DARPP-32 as one potential target for the actions of DA. Given the fact that the function of DARPP-32 depends on its relative state of phosphorylation we assessed whether different phases of motor skill learning could be accompanied by distinct patterns in the phosphorylation state of DARPP-32. To test this possibility, in **Paper II**, we examined the expression of activity-regulated cytoskeleton-associated protein (Arc), a cellular marker of learning-related synaptic plasticity, within the corticostriatal network during different phases of motor skill learning (using the same skilled reaching task described above), and changes in the phosphorylation of DARPP-32 and its downstream target cAMP-response element-binding protein (CREB) in rats.

Performance of rats on the skilled reaching task used in **Paper II** is characterized by an initial phase when skilled reach accuracy improves rapidly over the first five days of training, followed by a later slow phase of learning when performance improvement reaches nearly asymptotic levels [(Kleim et al., 2004); see also **Fig. 9**]. Based on this information, we defined days 3 and 12 as the early and late phases of motor skill learning, respectively. Animals were therefore trained for 3 or 12 consecutive days before being evaluated for molecular and biochemical changes during these two phases.

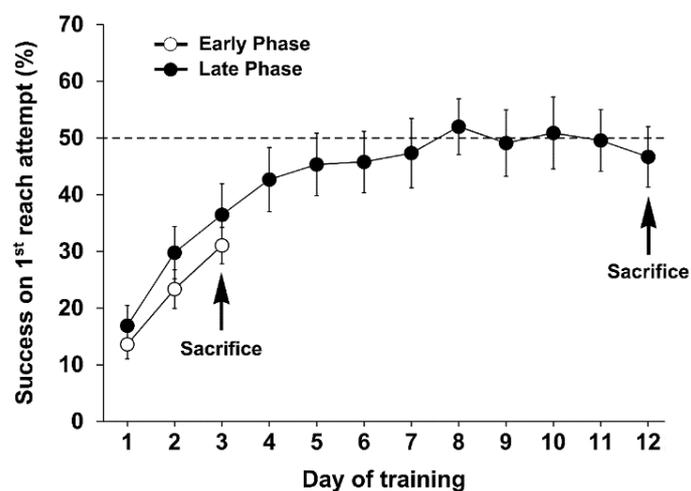


Figure 9. The performance of Wistar rats in the single pellet skilled reaching task. The success on the first reach in percentage is presented. One group of rats, the Early Phase (EP), was trained in the skilled reaching task for 3 days. A second group, the Late Phase (LP), was trained for 12 days (i.e., until asymptotic levels of reaching performance were achieved).

In the first set of experiments, we studied the expression of Arc mRNA in various brain regions during the early (i.e., after 3 days of motor training) and late (i.e., after 12 days of motor training) phases of motor skill learning. We found that Arc mRNA is induced in corticostriatal circuitry, including the medial prefrontal cortex (mPFC; functionally homologous to the dorsolateral PFC in humans), cingulate cortex (CG), M1, and striatum during the early phase of motor skill learning (see **Fig. 10**). These results are consistent with human neuroimaging studies demonstrating recruitment of similar cortical regions (e.g., M1 and dorsolateral prefrontal cortex) during the initial stages of motor skill learning [for a review, see (Dayan and Cohen, 2011)]. In the late phase, however, expression of Arc is decreased significantly in most regions examined, except in the mPFC and striatum. Our findings demonstrate a dynamic shift in the contribution of corticostriatal circuitry during different phases of motor skill. In contrast, a very recent study using a similar skilled reaching task found induction of Arc mRNA expression only in M1 contralateral to the trained forelimb (Hosp et al., 2013). These discrepancies are likely due to the fact that these authors only evaluated Arc mRNA expression at a single time-point (i.e., on day 2 of training) and had a more intense training than we used in **Paper II** (i.e., 100 trials on day 1 and 50 trials on day 2 vs. 30 trials per day in our study).

We then explored the possibility of training-induced changes in the expression of DA markers since previous human PET studies indicated that training of working memory is associated with changes of cortical DA D1R (McNab et al., 2009). In the same set of samples (where we evaluated Arc mRNA expression), we found significant changes in the expression of DA D1R (see **Fig 11A**) and their intracellular target DARPP-32 in the striatum (but not cortical regions) during the early, but not late, phase of motor skill learning. In contrast, we failed to detect alterations in the expression of DA D2R. The observed changes in D1R and DARPP-32 mRNA expression levels are not a result of age-related differences between rats sacrificed 9 days apart as we failed to detect a difference between early- and late-phase home cage controls (see **Fig 11B**), thus indicating that expression of D1R, but not D2R, is sensitive to training.

In a new set of experiments, we further investigated the potential involvement of DARPP-32 in motor skill learning. For this purpose, we investigated the state of phosphorylation of DARPP-32 at the Thr34, Thr75, and Ser97 regulatory sites in the striatum contralateral to the trained forelimb. Western blot analysis revealed that the early, but not late, phase of motor skill learning is associated with enhanced phosphorylation of DARPP-32, specifically at the Thr34 site (see **Fig. 12A**). Previous studies have demonstrated that activation of D1R results in PKA-

catalyzed phosphorylation of DARPP-32 at this site [for a review, see (Svenningsson et al., 2004)]. Together with previous studies demonstrating that DARPP-32 is a crucial step for striatal synaptic plasticity (Calabresi et al., 2000), our results indicate that the striatal DA D1R/cAMP/PKA/DARPP-32 pathway is important for acquisition of new fine motor skills.

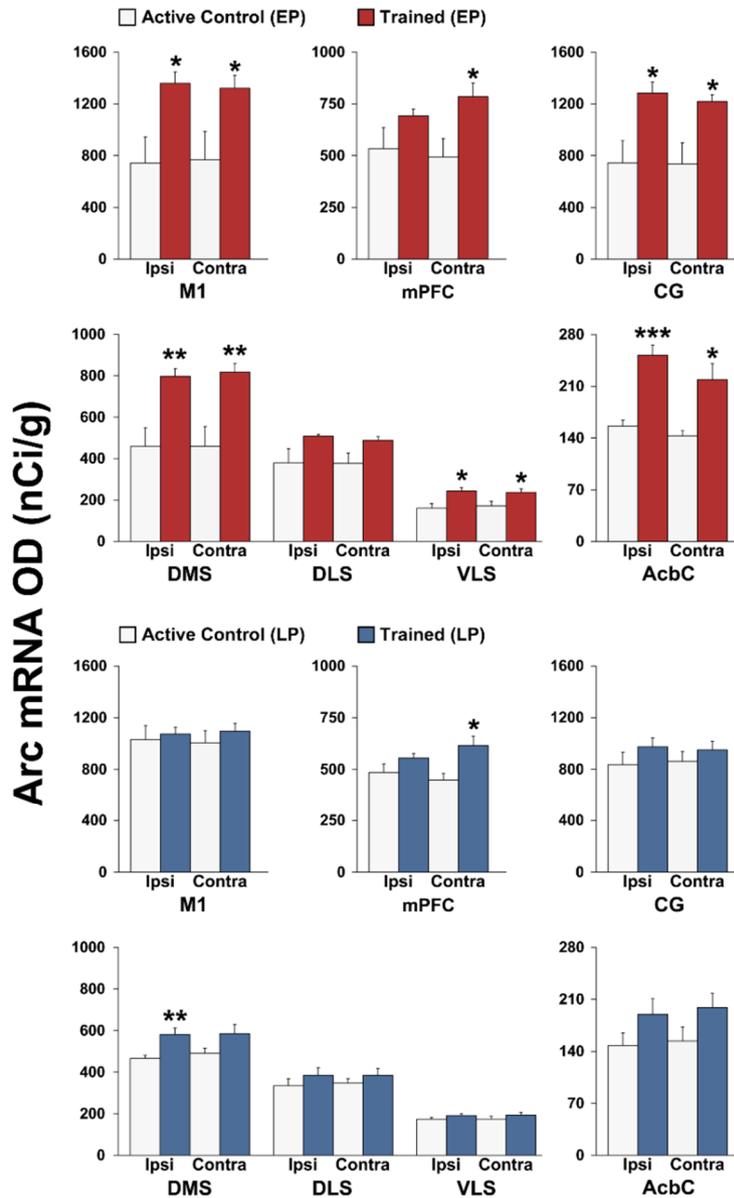


Figure 10. Arc mRNA expression is regulated differently in cortical and striatal regions during different phases of motor skill learning. (A) Regional analysis of Arc mRNA expression in various cortical regions of (top) Early Phase (EP) Active Controls (white bars) vs. EP Trained rats (red bars), and (bottom) Late Phase (LP) Active Controls (white bars) vs. LP trained rats (blue bars). Data in bar graphs show mean optical density (OD) values for Arc mRNA in M1, mPFC, CG, DMS, DLS, VLS, and AcbC. Labeled areas correspond to the following brain regions: medial prefrontal cortex (mPFC), cingulate cortex (CG), primary motor cortex (M1), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), dorsal lateral striatum (DLS), dorsal medial striatum (DMS), and ventrolateral striatum (VLS). The results are presented as means \pm SEM; $n = 5$ per group. Asterisks indicate where trained rats differ significantly (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$) from their respective active control rats.

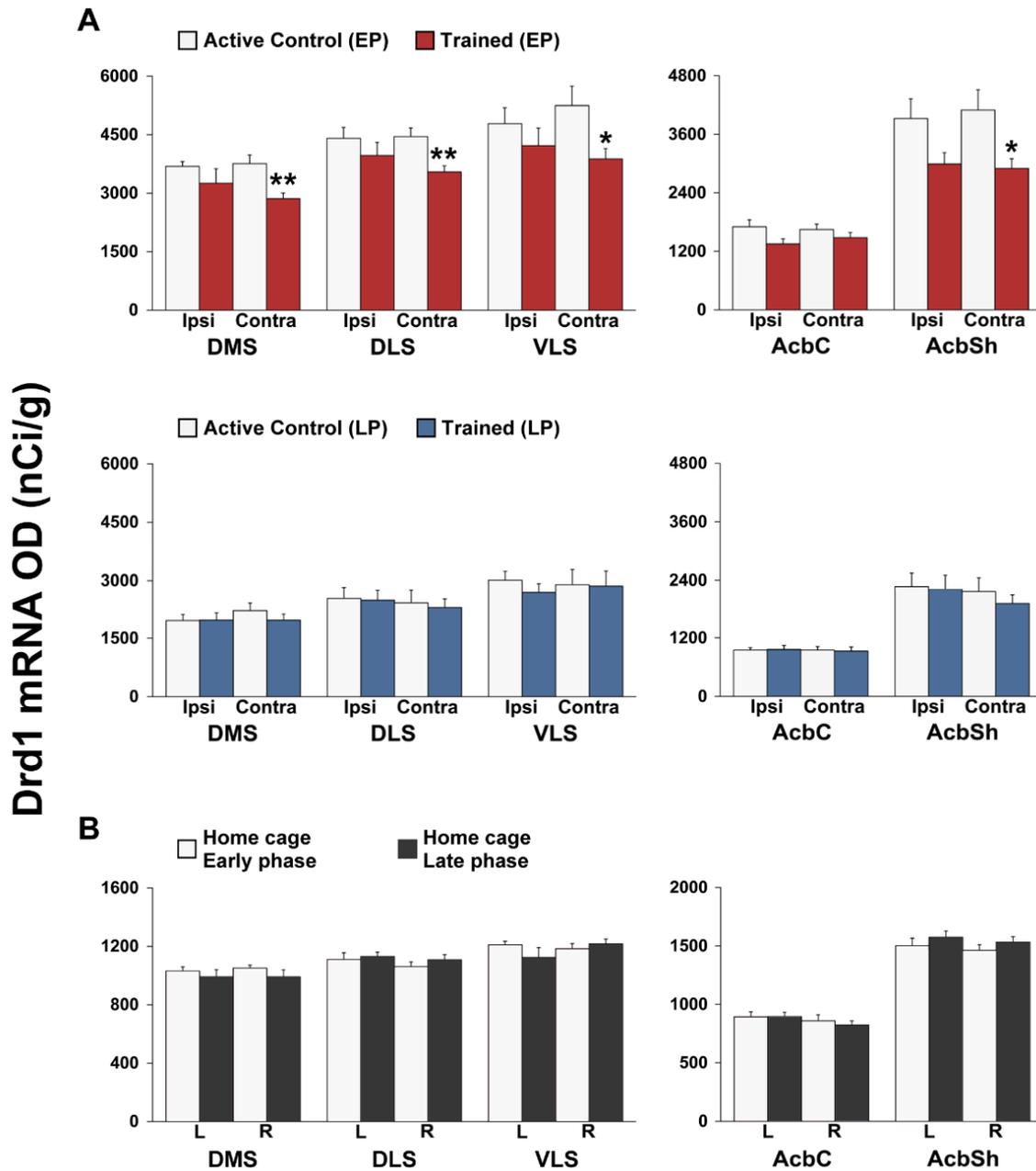


Figure 11. Decreased Drd1 mRNA expression during the early, but not late, phase of motor skill learning. (A) A regional analysis of Drd1 mRNA expression with sub-regions of striatum of (top) Early Phase (EP) Active Controls (white bars) vs. EP Trained rats (red bars), and (bottom) Late Phase (LP) Active Controls (white bars) vs. LP trained rats (blue bars). Data in bar graphs show mean optical density (OD) values for Drd1 mRNA in DMS, DLS, VLS, and AcbC. (B) A regional analysis of Drd1 mRNA expression with sub-regions of striatum of home cage Early Phase (red bars) and home cage Late Phase (blue bars). The results are presented as means \pm SEM; $n = 5$ per group. Asterisks indicate where trained rats differ significantly ($*P < 0.05$ and $**P < 0.01$) from their respective active control rats. For more details see Figure 10.

Given that CREB is a downstream target of DARPP-32 that has also been implicated in the molecular cascade that mediates the formation of long-lasting memories and corticostriatal synaptic plasticity (Pittenger et al., 2006), we investigated potential changes in CREB phosphorylation after different phases of motor skill learning. Consistent with a role of CREB in memory formation, we found an increase in phosphorylation of CREB on Ser133 during the early phase of motor skill learning (see **Fig. 12B**). Although we failed to detect at the group level differences in phospho-Ser133-CREB during the late phase of motor learning, we did find a positive correlation between individual skilled-reaching performance during this phase and levels of phospho-Ser133-CREB, with a significant increase in phosphorylation in the striatum of “good learners” (i.e., animals that displayed a clear learning curve and had an average or above average level of performance during the last 5 days of training) relative to controls. The absence of any correlations with the levels of PKA-mediated phosphorylation at the Thr34 site suggests that other signaling cascades (e.g., calcium signaling pathway) may be controlling the state of CREB phosphorylation during the late phase of motor skill learning. It is worth mentioning here that we failed to find any type of correlations between individual skilled-reaching performance during the early phase of motor skill learning and levels of phospho-DARPP-32 (i.e., Thr34, Thr75 and Ser97) or phospho-Ser133-CREB.

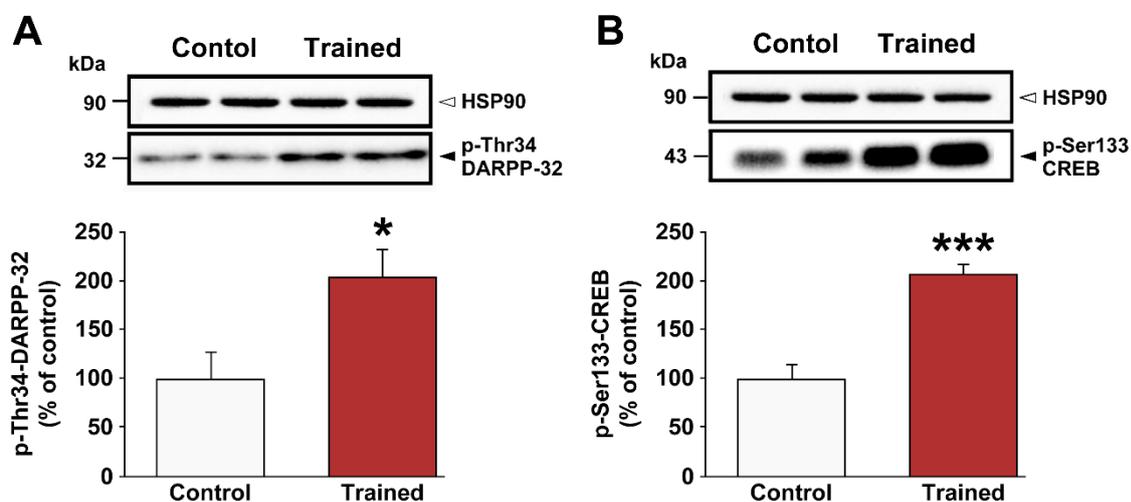


Figure 12. Enhanced DARPP-32 and CREB phosphorylation in the striatum is associated with early phase of motor skill learning. Levels of phospho-Thr34-DARPP-32 (A) and phospho-Ser133-CREB were evaluated in the striatum contralateral to the trained forelimb of active controls (white bars) and trained (red bars) rats after 3 days of motor skill learning by western blot. The results are presented as means \pm SEM ($n = 6-8$ per group). Asterisks indicate where trained rats differ significantly ($*P < 0.05$ and $***P < 0.001$) from active control rats. Heat Shock Protein 90 (HSP90) served as the loading control

Interestingly, we also found a strong positive correlation between individual skilled reaching performance during the late phase of motor learning and levels of phospho-Thr75-DARPP-32 (see **Fig. 13**), but not with levels of phospho-Thr34-DARPP-32 or phospho-Ser97-DARPP-32 (data not shown). In this case, “poor learners” (i.e. animals that failed to display a learning curve and had a level of performance 50% lower than the average) had lower levels of phospho-Thr75-DARPP-32 when compared to the active control group. It is known that cyclin-dependent kinase 5 (Cdk5) phosphorylates DARPP-32 at the Thr75 site, converting it into a PKA inhibitor (Nishi et al., 2000). However, we failed to detect changes in the expression of Cdk5 in poor learners (unpublished results). We therefore postulate that there might be an imbalance in other neurotransmitter systems involved in phosphorylating Cdk5 or its regulatory factors in poor learners since we also failed to observe enhanced PKA mediated phosphorylation of DARPP-32 at the Thr34 site during the late phase of motor skill learning. In future studies, it will be interesting to investigate the phosphorylation state of Cdk5 and other downstream targets of DARPP-32 (e.g., glutamate receptors) during different phases of motor skill learning.

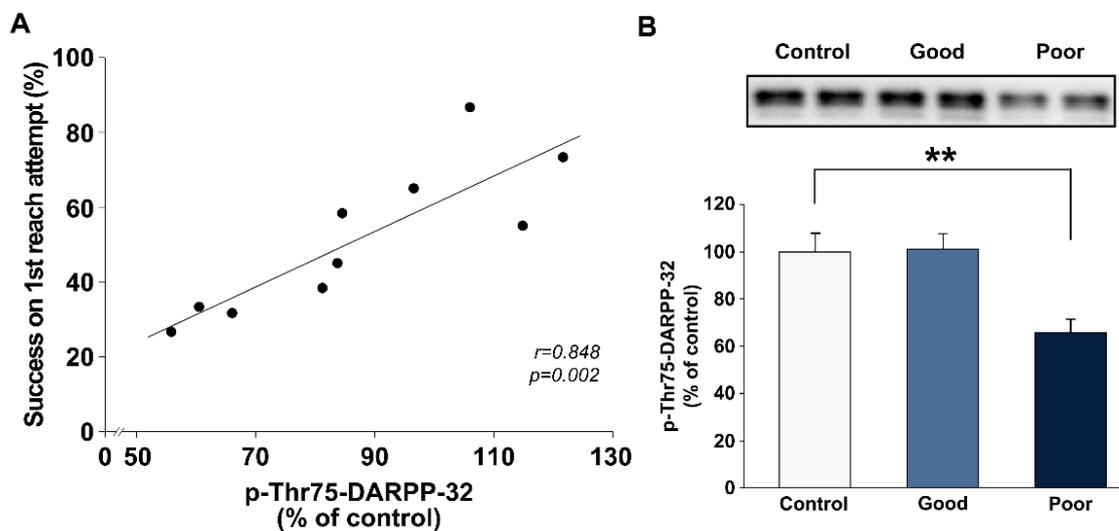


Figure 13. Correlations between the skilled-reaching performance during the late phase of motor skill learning and levels of phospho-Thr75-DARPP-32. Simple regression analysis indicating a significant correlation between the success on the first reach attempt, in percent, during the late phase of motor skill learning and levels of phospho-Thr75-DARPP-32 (A). Filled circles represent values from individual rats from the late phase (LP) group. LP trained rats were divided into good and poor learners according to their individual learning curve and endpoint performance (B). The top row shows representative Western blot autoradiograms for phospho-Thr75-DARPP-32. Data in bar graphs represent the levels of phospho-Thr75-DARPP-32 in controls (n = 6), good learners (n = 6), and poor learners (n = 4) expressed as means \pm SEM. The asterisks indicate where poor or good learners differ significantly (**P < 0.01) from active controls.

4.3 Deficits in fine motor skills in a genetic animal model of ADHD

ADHD is a life-long disorder, which begins in early childhood and is characterized by difficulties with attention, motor hyperactivity, and impulsivity [for a review, see (Biederman and Faraone, 2005)]. It is estimated to affect 3-9% of school-aged children and approximately 4% of adults worldwide. Besides the cardinal symptoms of ADHD, up to 50% of children with ADHD experience motor problems e.g., difficulties in both learning and performing motor skills (Eliasson et al., 2004; Meyer and Sagvolden, 2006). Despite the fact that poor motor abilities in children with ADHD are associated with lower academic performance and social functioning, these specific problems have received little attention in both clinical and experimental research.

In **Paper III**, we investigated whether SHRs, a heuristically useful genetic animal model of ADHD [for a review, see (Sagvolden et al., 2009)], were valid for investigating deficits in fine motor skills. For this purpose, we subjected young adolescent SHRs to a battery of tests for motor activity, gross motor coordination and skilled reaching. The Wistar (WIS) strain was used as control since this strain is more active than the SHR progenitor strain (i.e., Wistar Kyoto; WKY). Importantly, both the SHR and WIS rats were kept as inbred strains.

In the first set of experiments, we compared the exploratory and habituation profile of SHRs and WIS rats after repeated exposure to a novel environment. We found that both SHRs and WIS rats displayed similar locomotor activity during the initial exploratory phase of open field

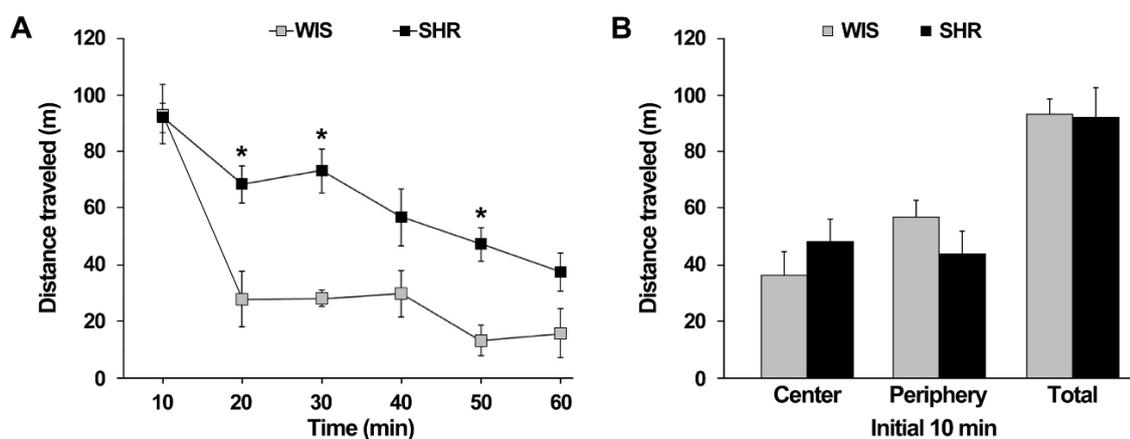


Figure 14. SHRs show increased locomotor activity. Naïve SHR and WIS rats were exposed to an activity/open field box and their spontaneous motor activity was recorded for 1 hour. (A) Distance traveled (meters) as a function of time during the 60 min open field test. (B) Bars show cumulative distance traveled (meters) per zone and in the entire box (total) during the initial 10-min open field test session. All data (A, and B) are represented as mean \pm S.M.E. (N = 5 animals per group). *P < 0.05 when compared to WIS rats.

exposure (**Fig. 14A**), and comparable exploratory activity in different areas of the open-field (center vs. periphery; **Fig. 14B**).

Instead, SHRs failed to habituate to the novel environment. This behavioral trait of the SHR strain was consistent over three consecutive days of testing (**Fig. 15**). Thus, similar to children with ADHD, SHRs display locomotor hyperactivity in a familiar, but not in a novel environment.

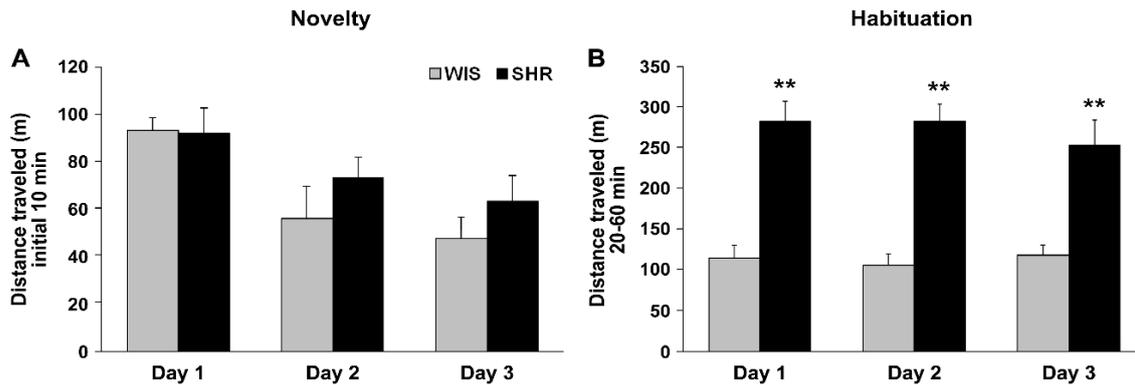


Figure 15. SHRs display impaired habituation to both novel and familiar environments. Naïve SHR and WIS rats were exposed to an activity/open field box for 1 h on day one and were re-exposed to the same testing box for two consecutive days. Average distance traveled (meters) during the initial 10 min (A) and during the 20-60 min (B) interval of testing. All data are represented as mean \pm S.M.E. (N = 5 animals per group). **P < 0.001 when compared to WIS rats.

In another set of experiments, we investigated potential motor skill deficits of SHRs using a skilled reaching task. It is worth mentioning that different measures evaluated in this motor task provide progressively increasing sensitivity in terms of measuring skilled performance, i.e., success on the first forelimb attempt more than overall success (i.e., regardless of the number of forelimb advances). Although SHRs can learn this task (as reflected by their overall successes), their performance is significantly poorer than that of WIS rats in the most sensitive measure of skilled performance (i.e., success on the first attempt). However, gross motor coordination (as evaluated on the rotarod test) appears to be normal in SHRs, suggesting that the SHR strain displays specific deficits in fine motor skills. The lower performance of SHRs does not appear to be due to abnormality or absence of a reach sequence (advance-grasp-withdrawal-release; unpublished observations). We noticed that SHRs make faster forelimb movements than WIS rats. Interestingly, previous studies have demonstrated that children with ADHD perform jerky arm movements and demonstrate a reduced capacity to select a movement speed appropriate to the accuracy demands of the task (Eliasson et al., 2004). It will be relevant in future studies to investigate potential alterations in the kinematics of forelimb

movements during skilled reaching in the SHR strain to confirm our observations (Foroud and Whishaw, 2006).

The mechanisms mediating motor dysfunctions in adolescent SHRs are still unclear. Some characteristics may be explained by alterations in pre- and/or postsynaptic DAergic markers (e.g., alterations in DAT levels) observed in fronto-striatal circuitry of SHRs [for a review, see (Roessner et al., 2010)]. Despite the considerable appeal of the SHR model, several authors have questioned its validity and the use of the WKY strain as an adequate control (Bull et al., 2000; van den Bergh et al., 2006). In an effort to clarify some of these issues, Sagvolden and collaborators carried out a whole genome Single Nucleotide Polymorphism (SNP) array analysis to investigate the total amount of genomic divergence among the SHR/NCrl, WKY/NCrl, WKY/NHsd, SD/NTac, and WH/HanTac strains. These studies revealed the WKY/NHsd is the most appropriate control for SHR/NCrl [for a review, see (Sagvolden et al., 2009)]. Additional studies from the same group provided further genetic and behavioral evidence supporting the use of the WKY/NCrl strain as a model for the inattentive subtype of ADHD (Sagvolden et al., 2008).

Given our recent results implicating DARPP-32 in the acquisition of new fine motor skills (see **Papers I-II**), we assessed potential alterations in the expression of DARPP-32 mRNA in the corticostriatal circuitry of SHR. We also included the WKY/NHsd and the WKY/NCr strains, as control and a putative model of the inattentive subtype of ADHD, respectively. We found that naïve SHRs have higher expression levels of DARPP-32 in the striatum (i.e., ventrolateral region), nucleus accumbens, cingulate cortex, and M1 compared to naïve WKY rats from both Harlan and Charles Rivers (see **Fig. 16**). Together with previous results obtained in **Papers I-II**, the present data suggest that overexpression of DARPP-32 in corticostriatal circuitry of SHR could be responsible for deficits in fine motor skills observe in this strain.

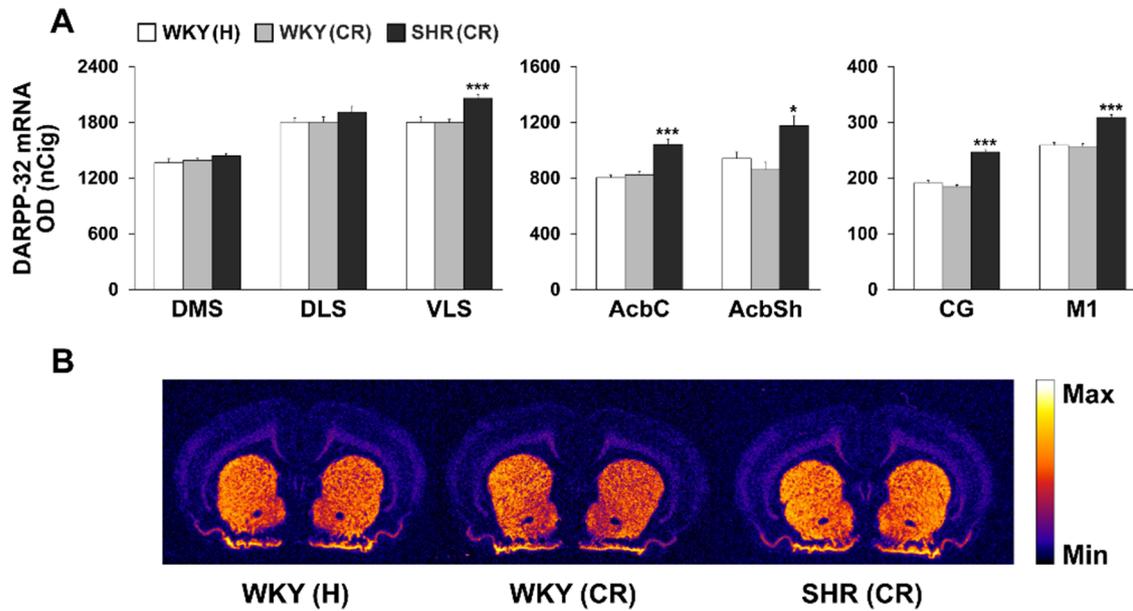


Figure 16. SHRs show increased expression of DARPP-32 mRNA. (A) Regional analysis of DARPP-32 mRNA expression within sub-regions of the striatum and various cortical regions. Data in bar graphs show mean optical density (OD) values for DARPP-32 mRNA in DMS, DLS, VLS, AcbC, AcbSh, CG, and M1. (B) Representative autoradiographs showing the mRNA expression levels of DARPP-32 at the level of the striatum. The pseudo-coloring indicates signal intensity ranging from low (black/purple) to high (yellow/white). The results are presented as means \pm SEM; $n = 6$ per group. Asterisks indicate where SHRs differ significantly ($*P < 0.05$ and $***P < 0.001$) from WKY/NHsd rats. For more details see Figure 10.

5 CONCLUSIONS

Paper I: Genetic variation in dopamine-related gene expression influences motor skill learning in mice

- The influence of naturally occurring genetic variation in the DA system on the acquisition and performance of fine motor skills were examined in mice.
- The results provide evidence supporting the notion that normal genetic variation in the DAergic system might contribute substantially to variability in the acquisition of motor skills in humans. More specifically, the results suggest the involvement of the D1R/cAMP/DARPP-32 signaling pathway in abnormal development of fine motor skills.

Paper II: Different phases of motor skill learning in rats are accompanied by distinct patterns of modifications in the cAMP/PKA/DARPP-32 signaling pathway

- Brain activity patterns associated with different phases of motor skill learning and learning-induced changes in the DA system were investigated in rats using a skilled reaching task.
- These findings implicate the cAMP/PKA/DARPP-32 signaling pathway in the acquisition of novel motor skills, and also demonstrate a dynamic shift in the contribution of corticostriatal circuitry during different phases of motor skill learning.

Paper III: Deficits in fine motor skills in a genetic animal model of ADHD

- This study evaluated whether an existing genetic animal model of ADHD is valid for investigating not only locomotor hyperactivity, but also more complex motor coordination problems displayed by the majority of children with ADHD.
- The results support the notion that the SHR strain is a useful animal model system to investigate potential molecular mechanisms underlying fine motor skill problems in children with ADHD. In addition, the molecule DARPP-32 was identified as a potential target mediating motor dysfunctions in adolescent SHRs.

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